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# DESCRIPTION

## Description

**[0001]** The present invention relates to the field of vaccination, and vaccines for the prevention and/or treatment of colorectal cancer (CRC).

**[0002]** Colorectal cancer (CRC) Globally, CRC is a common and lethal disease and the third most commonly diagnosed cancer, third in males and second in females, with more than 1.36 million new cases and about 694,000 deaths occurring in 2012. The risk of developing CRC is influenced by human, environmental and genetic factors (Cancer, I.A.f.R.o. GLOBACAN 2012. 2012 [cited 2015 July 5]; Available from: [http://globocan.iarc.fr/Pages/burden\\_sel.aspx](http://globocan.iarc.fr/Pages/burden_sel.aspx)). Although the CRC incidence is more predominant in man than in woman, it is more significantly impacted by the age and life style. Indeed over 90% of patients diagnosed with CRC are 50 years old or more (Prevention, C.f.D.C.a. What Are the Risk Factors for Colorectal Cancer? 2015 [cited 2015 July 5]) while 2/3 of the cases occurred in developed countries. There is evidence that consumption of meat in general and more specifically red meat or consumption of alcoholic beverages that are leading to general obesity, are significantly increasing the risks of CRC (Stewart, B. and C. Wild, World Cancer Report 2014, B. Stewart and C. Wild, Editors. 2014, International Agency for Research on Cancer: Geneva). Two other factors are significantly impacting the risks of developing colorectal cancer: hereditary and inflammatory bowel disease. Familial adenomatous polyposis (FAP) is for example significantly increasing the risks of CRC for less than 50 years old people (Burt, R.W., J.A. DiSario, and L. Cannon-Albright, Genetics of colon cancer: impact of inheritance on colon cancer risk. *Annu Rev Med*, 1995. 46: p. 371-9). FAP, like MAP (MUTYH-associated polyposis) (Sieber, O.M., et al., Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. *N Engl J Med*, 2003. 348(9): p. 791-9) or Lynch syndrome (Lynch, H.T., et al., Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenterology*, 1993. 104(5): p. 1535-49), is associated with gene mutations that predispose patients to inheritance of multiple colonic adenomas (Dennis J Ahnen, D.M., FA. Colorectal cancer: Epidemiology, risk factors, and protective factors. 2015 [cited 2015 July 9]; Available from: <http://www.uptodate.com/contents/colorectal-cancer-epidemiology-risk-factors-and-protective-factors>). The influence of diseases such as ulcerative colitis or Crohn's disease in developing a CRC is well documented. Ulcerative colitis impact is for example linked to a 3 to 15-fold increase in risk (Ekbom, A., et al., Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med*, 1990. 323(18): p. 1228-33). Although there are less data for Crohn's disease, similar relative risks are associated with this disease. For patients having family history in terms of CRC, adenomatous polyposis or inflammatory bowel disease, early screening is strongly recommended (Jemal, A., et al., Global cancer statistics. *CA Cancer J Clin*, 2011. 61(2): p. 69-90).

**[0003]** Like in many cancer, the CRC patient's survival time depends on disease development stage. Patients with early stages (Stage 0, I and II) have good prognosis (more than 75%). Stages III have a more heterogeneous survival rate (from 90% down to 50%) depending on the tumor invasion on peripheral tissues. Finally, only stage IV is showing a fast and significant effect on the patient survival time: Only about 10% of patients are surviving more than 60 months after diagnosis.

**[0004]** Existing treatments are generally based on a surgery followed or not by well-established chemotherapy, radiotherapy and/or targeted therapy (Moertel, C.G., Chemotherapy for colorectal cancer. *N Engl J Med*, 1994. 330(16): p. 1136-42; Meyerhardt, J.A. and R.J. Mayer, Systemic therapy for colorectal cancer. *N Engl J Med*, 2005. 352(5): p. 476-87). Depending on the disease progression stage, existing treatments allow a 30% to 95% survival rate two years after diagnosis. All early stage CRC treatment strategies are initiated with surgery, combined or not with additional regimen. The decision on the follow-up therapy regimen depends on the disease progression stage identified during the preliminary screening results.

**[0005]** For advanced CRC, the use of chemotherapy or targeted therapy as first line treatment is generally recommended. The most commonly used regimens are FOLFOX, CapeOX or FOLFIRI. These treatments may be used in combination with one of the following recommended biological drugs targeting VEGF (Genentech bevacizumab/Avastin® or Regeneron-Sanofi afibbercept/Zaltrap®) or EGFR (Merck-Serono cetuximab/Erbbitux®). Other targeted therapies could be proposed as stand-alone first line treatment. Amgen's EGFR monoclonal antibody (panitumumab/Vectibix®) and, more recently, Bayer's small molecule kinase inhibitor Regorafenib (Stivarga®) have demonstrated their capacity to increase overall CRC patients' survival time. If the disease already importantly spread to other organs, the VEGF monoclonal antibody Ramucirumab (Cyramza®) from Eli Lilly may be used. For stage IV, radiation may be used to relieve symptoms such as pain.

**[0006]** Although CRC chemotherapy is well established (Moertel, C.G., Chemotherapy for colorectal cancer. *N Engl J Med*, 1994. 330(16): p. 1136-42) the momentum towards more effective and less subject to secondary effects strategies importantly evolved in the past decades, namely for stage IV CRC (Gallagher, D.J. and N. Kemeny, Metastatic colorectal cancer: from improved survival to potential cure. *Oncology*, 2010. 78(3-4): p. 237-48). The first trials were directed towards complementary adjuvant therapy. However after many years, the value of postoperative 5-FU based therapy remains controversial, in particular for patients with stage II CRC (Meyerhardt, J.A. and R.J. Mayer, Systemic therapy for colorectal cancer. *N Engl J Med*, 2005. 352(5): p. 476-87).

**[0007]** In that context, immunotherapies have been carefully evaluated. The immune system can recognize and to some extent eliminate tumor cells, however, this anti-tumor response is often of low amplitude and inefficient. Boosting this weak anti-tumor response with therapeutic vaccination has been a long sought goal for cancer therapy. Modulating the immune system to

enhance immune responses has thus become a promising therapeutic approach in oncology as it can be combined with standard of care treatments.

**[0008]** Promising preclinical data and advances in clinical trials, including the recent FDA approval of the Sipuleucel-T vaccine and of the anti-CTLA-4 antibody, show that active immunization is a safe and feasible treatment modality for certain cancer types. Induction of tumor-specific cytotoxic T lymphocytes (CTLs) mediated immune responses has been reported using different approaches including modified tumor cell vaccines, peptide vaccines, recombinant viral vectors, DNA, protein, or dendritic cell vaccines. However, the anti-tumoral immunity mediated by CTLs only occasionally correlates with tumor regression and only a few projects have reached the phase III clinical stage.

**[0009]** Overall, cancer vaccines showed very limited clinical efficacy so far. Indeed, at the end of 2011, amongst the 300 hundred ongoing cancer vaccine clinical trials, only 19 phase III trials were reported (*globaldata*, 2012). Amongst them, there are NeuVax, a peptide vaccine for breast cancer, Stimuvax, a liposome based vaccine for Non-Small Cell Lung Carcinoma (NSCLC) and breast cancer, TG4010, a vaccinia-based vaccine for NSCLC and GSK1572932A, an adjuvanted liposome for NSCLC. These four cancer vaccines are based on different technologies and have in common that they are targeting one single antigen.

**[0010]** Therapeutic cancer vaccines can be divided into two principal categories: personalized (autologous) and standardized vaccines, and further classified depending on the technology platform. Current personalized vaccines include tumor lysate vaccines as well as dendritic cells based vaccine (hereinafter cell based). For the latter, antigen loading can occur either with a pulse using tumor lysates, or transfection with RNA extracted from the tumors. In this case, the antigens are tumor specific or associated, but are not clearly defined. Dendritic cells can also be loaded with defined antigens either with peptide pulse or using a protein such as the Prostatic Acid Phosphatase (PAP) used to engineer the Provenge® vaccine. However, the manufacturing process of these cell-based therapies is time-consuming and labor-intensive while quality standards are difficult to reach and maintain. Immunomonitoring creates further complications. Moreover, the majority of the autologous cancer vaccines do not allow the identities or quantities of antigens used to be controlled, unlike defined and standardized vaccines.

**[0011]** In contrast to cell-based therapy (APCs, T cells, CARs, lysates), subunits vaccines (protein or peptides) allow the development of a standardized vaccine with an easier production and significantly better batch to batch reproducibility that can be administrated to a broad range of patients. Furthermore, the antigens are fully defined allowing for better immune-monitoring and reducing the risk of unwanted effects of vaccine component.

**[0012]** The different approaches which were evaluated in pre-clinical and clinical development include short peptide vaccines (Slingluff CL, Jr. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? *Cancer journal* 2011;17(5):343-50), long-peptide vaccines (Melfi CJ, van der Burg SH. Immunotherapy of

established (pre)malignant disease by synthetic long peptide vaccines. *Nature reviews Cancer* 2008;8(5):351-60) and proteins. In contrast to long peptide and protein vaccines, short peptide vaccines have a very short half-life and can have negative consequences on the immune response.

**[0013]** For the protein-based vaccines, the results of targeting MAGE-A3 with a recombinant fusion protein-based vaccine have been enthusiastically awaited after promising phase II data in metastatic melanoma (Kruit WH, Suciu S, Dreno B, Mortier L, Robert C, Chiarion-Sileni V, et al. Selection of immunostimulant AS15 for active immunization with MAGE-A3 protein: results of a randomized phase II study of the European Organisation for Research and Treatment of Cancer Melanoma Group in Metastatic Melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013;31 (19):2413-20) and non-small cell lung cancer (NSCLC)(Vansteenkiste J, Zielinski M, Linder A, Dahabreh J, Gonzalez EE, Malinowski W, et al. Adjuvant MAGE-A3 immunotherapy in resected non-small-cell lung cancer: phase II randomized study results. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013;31(19):2396-403). However, in 2013 the phase III DERMA trial in melanoma (NCT00796445) did not meet its first co-primary endpoint, followed in 2014 with a stop of the phase III MAGRIT study in NSCL (NCT00480025). Despite these very disappointing clinical results, protein based vaccines undeniably present many advantages.

**[0014]** Also in the context of colorectal cancer scientific progresses in tumor immunology led to a better understanding of anti-tumor immune response through cellular and humoral pathways (Smith, C.L.,et al., Immunotherapy of colorectal cancer. *Br Med Bull*, 2002. 64: p. 181-2001; Kido, S., et al., Immunotherapy for colorectal cancer. *World J Gastroenterol*, 2013. 19(46): p. 8531-42) and helped better identifying tumor antigens. This progresses opened new perspective for immunotherapies in CRC. Passive immunotherapies such as antibodies were proved as the first targeted therapies for CRC treatment. Thanks to their capacity in being able to interact with tumor growth pathway through the epidermal growth factor receptor (EGFR) or to inhibit the vascular endothelial growth factor (VEGF), antibodies such as cetuximab, panitumumab and bevacizumab received FDA approval for CRC treatment respectively in 2004, 2006 and 2009.

**[0015]** Adoptive cell transfer (ACT) therapy clinical trials for CRC have unfortunately returned poor results so far. Indeed the limited patient population for both non-engineered and engineered T-cells treatments seems being a major hurdle. Similarly, secondary effect observed on patients treated with T-cells fused with chimeric antigen receptors (CARs), failed to demonstrate ACT as a safe and efficient treatment (Kido, S., et al., Immunotherapy for colorectal cancer. *World J Gastroenterol*, 2013. 19(46): p. 8531-42; Xiang, B., et al., Colorectal cancer immunotherapy. *Discov Med*, 2013. 15(84): p. 301-8). Thus added to usual ACT drawbacks in terms of feasibility and cost and immune response memory seems blocking ACT option for the time being.

**[0016]** More recently, the positive clinical trial phase III results on patient survival for afibbercept, allowed this anti-VEGF fusion protein to be approved by the FDA for metastatic

colorectal cancer (mCRC) (Clarke, J.M. and H.I. Hurwitz, Ziv-aflibercept: binding to more than VEGF-A--does more matter? *Nat Rev Clin Oncol*, 2013. 10(1): p. 10-1). This targeted therapy approval paves the way for non-antibody immunotherapies. So far, no active immunotherapies and no immuno-modulators have been approved for CRC. Structurally, the vast majority of the molecules tested for CRC are either small molecules, in general kinase inhibitors, or antibodies (respectively 52% and 28%).

**[0017]** In general, a therapeutic cancer vaccine is administrated to cancer patients to strengthen the capability of their immune system to recognize and kill the tumor cells. The main goal of a therapeutic cancer vaccine is to generate killer T cells (also called cytotoxic T lymphocytes) specific for the tumor cells. To this end and to achieve a potent immune response, the vaccine must contain molecules called antigens that are also present in the tumor and that need to be delivered to Antigen Presenting Cells (APCs), especially dendritic cells (DCs), to allow cancer immunity to be initiated. The DCs process these tumor antigens into small peptides that are presented on cell surface expressed MHC class I or MHC class II molecules to T cells. Peptides that are then recognized by T cells and thereby induce their stimulation are called epitopes. Presentation by MHC class I and MHC class II molecules allows activation of two classes of T cells, CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) and CD4<sup>+</sup> helper T (T<sub>h</sub>) cells, respectively. In addition, to become fully activated, beside antigen recognition T cells require a second signal, the co-stimulatory signal, which is antigen non-specific and is provided by the interaction between co-stimulatory molecules expressed on the surface of APCs and the T cell. Therefore two major requirements for an efficient therapeutic cancer vaccine are the specificity of the tumor antigens and the ability to deliver them efficiently to DCs.

**[0018]** Taken together, induction of a tumor specific immune response thus requires three main steps: (i) an antigen must be delivered to dendritic cells, which will process it into epitopes, (ii) dendritic cells should receive a suitable activation signal, and (iii) activated tumor antigenloaded dendritic cells must generate T-cell mediated immune responses in the lymphoid organs.

**[0019]** Since tumor cells can escape the immune system by down-regulating expression of individual antigens (passive immune escape), multi-epitopic antigen delivery provides an advantage. Indeed, protein based vaccines allow multi-epitopic antigen delivery to antigen presenting cells (APCs) such as dendritic cells (DCs) without the limitation of restriction to a single MHC allele. Another strength is long-lasting epitope presentation recently described in dendritic cells loaded with proteins (van Montfoort N, Camps MG, Khan S, Filippov DV, Weterings JJ, Griffith JM, et al. Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proceedings of the National Academy of Sciences of the United States of America* 2009;106(16):6730-5). Furthermore, proteins require uptake and processing by DCs to achieve MHC restricted presentation of their constituent epitopes. This reduces the risk of inducing peripheral tolerance as has been shown after vaccination with short peptides that do not have such stringent processing requirements (Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced

tumor growth through specific T-cell tolerance induction. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93(15):7855-60).

**[0020]** However, most soluble proteins are generally degraded in endolysosomes and are poorly cross-presented on MHC class I molecules and are therefore poorly immunogenic for CD8<sup>+</sup> T cell responses (Rosalia RA, Quakkelaar ED, Redeker A, Khan S, Camps M, Drijfhout JW, et al. Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *European journal of immunology* 2013;43(10):2554-65). Moreover, although mature DCs are more potent than immature DCs in priming and eliciting T-cell responses (Apetoh L, Locher C, Ghiringhelli F, Kroemer G, Zitvogel L. Harnessing dendritic cells in cancer. *Semin Immunol.* 2011; 23:42-49), they lose the ability to efficiently take up exogenous antigens, particularly for MHC class II restricted antigens (Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998; 392:245-252). As a result, peptide-pulsed DCs as vaccines have several limitations. For example, peptide degradation, rapid MHC class I turnover, and the disassociation of peptide from MHC class I molecules during the preparation and injection of DC/peptides may result in short half-lives of MHC class I/peptide complexes on the DC surface, leading to weak T-cell responses.

**[0021]** To improve the efficacy of protein-based vaccine delivery, the use of cell penetrating peptides for intracellular delivery of cancer peptides into DCs has been proposed (Wang RF, Wang HY. Enhancement of antitumor immunity by prolonging antigen presentation on dendritic cells. *Nat Biotechnol.* 2002; 20:149-156). Cell penetrating peptides (CPPs) are peptides of 8 to 40 residues that have the ability to cross the cell membrane and enter into most cell types (Copolovici DM, Langel K, Eriste E, Langel U. Cell-penetrating peptides: design, synthesis, and applications. *ACS nano* 2014;8(3):1972-94, Milletti F. Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 2012). Alternatively, they are also called protein transduction domain (PTDs) reflecting their origin as occurring in natural proteins. Several potent CPPs have been identified from proteins, including the Tat protein of human immunodeficiency virus, the VP22 protein of herpes simplex virus, and fibroblast growth factor (Berry CC. Intracellular delivery of nanoparticles via the HIV-1 tat peptide. *Nanomedicine.* 2008; 3:357-365; Deshayes S, Morris MC, Divita G, Heitz F. Cell-penetrating peptides: Tools for intracellular delivery of therapeutics. *Cell Mol Life Sci.* 2005; 62:1839-1849; Edenhofer F. Protein transduction revisited: Novel insights into the mechanism underlying intracellular delivery of proteins. *Curr Pharm Des.* 2008; 14:3628-3636; Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv Drug Deliv Rev.* 2005; 57:637-651; Torchilin VP. Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. *Annu Rev Biomed Eng.* 2006; 8:343-375). It was found that T-cell activity elicited by DC/TAT-TRP2 was 3- to 10-fold higher than that induced by DC/TRP2 (Wang HY, Fu T, Wang G, Gang Z, Donna MPL, Yang JC, Restifo NP, Hwu P, Wang RF. Induction of CD4+ T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells. *J Clin Invest.* 2002a; 109:1463-1470).

**[0022]** Moreover, subunits vaccines (peptides or proteins) are poorly immunogenic. Therefore

in the context of therapeutic cancer vaccine, a potent adjuvant is mandatory to be added to the vaccine in order to increase the level of co-stimulatory molecules on DCs and therefore augment the immune system's response to the target antigens. Adjuvants accomplish this task by mimicking conserved microbial components that are naturally recognized by the immune system. They include, lipopolysaccharide (LPS), components of bacterial cell walls, and nucleic acids such as double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), and unmethylated CpG dinucleotide-containing DNA. Their presence together with the vaccine can greatly increase the innate immune response to the antigen. Furthermore, this adjuvant should promote an adaptive immune response with CTLs and type polarized Th1 rather than a humoral immune response resulting in antibody production. Different adjuvants have been evaluated, with a limited number having gained regulatory approval for human use. These include Alum, MPL (monophosphoryl lipid A) and ASO<sub>4</sub>(Alum and MPL) in the US, and MF59 (oil-in-water emulsion), ASO<sub>4</sub>, liposomes in Europe (Lim, Y.T., Vaccine adjuvant materials for cancer immunotherapy and control of infectious disease. *Clin Exp Vaccine Res*, 2015. 4(1): p. 54-8).

**[0023]** Recently, Toll Like Receptor (TLR) ligands are emerging as promising class of adjuvants (Baxevanis, C.N., I.F. Voutsas, and O.E. Tsitsilis, Toll-like receptor agonists: current status and future perspective on their utility as adjuvants in improving anticancer vaccination strategies. *Immunotherapy*, 2013. 5(5): p. 497-511). A significant development of cancer vaccine studies was thus to include various TLR agonists to vaccine formulations, including TLR-3 (poly I:C), TLR-4 (monophosphoryl lipid A; MPL), TLR-5 (flagellin), TLR-7 (imiquimod), and TLR-9 (CpG) (Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol Rev*. 2011; 239:178-196). The types of signaling and cytokines produced by immune cells after TLR stimulation control CD4+ T-cell differentiation into Th1, Th2, Th17, and Treg cells. Stimulation of immune cells such as DCs and T cells by most TLR-based adjuvants produces proinflammatory cytokines and promotes Th1 and CD8+ T responses (Manicassamy S, Pulendran B. Modulation of adaptive immunity with Toll-like receptors. *Semin Immunol*. 2009; 21:185-193).

**[0024]** Conjugating the vaccine to a TLR ligand is an attractive approach that offers several advantages over non-conjugated vaccines including (i) preferential uptake by the immune cells expressing the TLR, (ii) higher immune response and (iii) reduced risk of inducing peripheral tolerance. Indeed, all the antigen presenting cells loaded with the antigen will be simultaneously activated. Different groups explored this approach with various TLR ligands being mainly linked chemically to the peptide or protein vaccine (Zom GG, Khan S, Filippov DV, Ossendorp F. TLR ligand-peptide conjugate vaccines: toward clinical application. *Adv Immunol*. 2012;114:177-201). As the chemical linkage to peptide is easily performed, the most highly investigated TLR ligands for conjugate vaccine are the TLR2 agonist Pam2Cys and Pam3Cys (Fujita, Y. and H. Taguchi, Overview and outlook of Toll-like receptor ligand-antigen conjugate vaccines. *Ther Deliv*, 2012. 3(6): p. 749-60).

**[0025]** US 2012/231030 A1 relates to isolated polypeptides comprising: (i) a protein transduction domain consisting of ZEBRA or a fragment thereof that retains the capacity of

internalization, (ii) at least one CD4<sup>+</sup> epitope; and (iii) at least one CD8<sup>+</sup> epitope. It also relates to antigen presenting cells loaded with said polypeptides, and the use thereof in immunotherapy including prevention and/or treatment of cancers.

**[0026]** Derouazi et al., Cancer Research, 75(15), 2015 (pp. 3020-3027) studies novel cell-penetrating peptide-based vaccines inducing robust CD4<sup>+</sup> and CD8<sup>+</sup> T cell-mediated antitumor immunity. The recombinant protein is fused with Z12, a novel cell-penetrating peptide that promotes efficient protein loading into the antigen-processing machinery of dendritic cells.

**[0027]** EP 2 476 440 A1 is directed to the therapeutic compositions for the treatment of HPV-induced diseases. It also refers to immunogenic conjugates comprising an immunogenic region of human papilloma virus E7 protein and the fibronectin EDA region, as well to compositions comprising said conjugates. In addition, EP 2 476 440 A1 is directed to dendritic cells obtained by stimulation with said conjugates and compositions.

**[0028]** WO 2015/073632 A1 describes annexin II variant compositions, in particular polypeptide fragments of annexin II, which may be covalently coupled to other components, e.g. antigens, targeting moieties, stabilizing moieties or second adjuvants. They may be used for immunotherapy.

**[0029]** However, to date the majority of cancer vaccines trials have shown limited efficacy. One explanation is the lack of a therapy that can simultaneously (i) stimulate multi-epitopic cytotoxic T cell-mediated immunity, (ii) induce T<sub>h</sub> cells and (iii) promote immunological memory. These three parameters are essential to generate potent, long lasting anti-tumor immunity. Indeed, CTLs specific for different epitopes will allow destruction of more cancer cells within a heterogeneous tumor mass and avoid the outgrowth of antigen-loss variants (tumor immune escape). T<sub>h</sub> cells are involved in the maintenance of long-lasting cellular immunity and tumor infiltration by T<sub>h</sub> cells is also an essential step for the recruitment and function of CD8<sup>+</sup> CTLs. Immunological memory is essential to protect against tumor relapse.

**[0030]** In view of the above, it is the object of the present invention to overcome the drawbacks of current cancer vaccines outlined above and to provide a novel complex for colorectal cancer immunotherapy applications representing a more potent vaccine, having improved antitumor activity for use in the prevention and/or treatment of colorectal cancer.

**[0031]** This object is achieved by means of the subject-matter set out below and in the appended claims.

**[0032]** It is to be understood that the terminology used herein is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

**[0033]** In the following, the elements of the present invention will be described.

**[0034]** Throughout this specification and the claims which follow, unless the context requires otherwise, the term "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated member, integer or step but not the exclusion of any other non-stated member, integer or step. The term "consist of" is a particular embodiment of the term "comprise", wherein any other non-stated member, integer or step is excluded. In the context of the present invention, the term "comprise" encompasses the term "consist of". The term "comprising" thus encompasses "including" as well as "consisting" e.g. a composition "comprising" X may consist exclusively of X or may include something additional e.g., X + Y.

**[0035]** The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

**[0036]** The word "substantially" does not exclude "completely" e.g., a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

**[0037]** The term "about" in relation to a numerical value x means x + 10%.

***Complexes according to the present invention***

**[0038]** In a first aspect the present invention provides a complex comprising:

1. a) a cell penetrating peptide;
2. b) at least one antigen or antigenic epitope; and
3. c) at least one TLR peptide agonist,

wherein the components a) - c), i.e. the cell penetrating peptide, the at least one antigen or antigenic epitope and the at least one TLR peptide agonist, are covalently linked,

for use in the prevention and/or treatment of colorectal cancer,

wherein the complex comprises an epitope of ASCL2,

wherein the TLR peptide agonist is a TLR2 peptide agonist and/or a TLR4 peptide agonist, and

wherein the complex is a polypeptide or a protein, in particular a recombinant polypeptide or a recombinant protein.

**[0039]** Such a complex for use according to the present invention provides simultaneous (i) stimulation of multi-epitopic cytotoxic T cell-mediated immunity, (ii) induction of  $T_h$  cells and (iii) promotion of immunological memory. Thereby, a complex for use according to the present invention provides a potent vaccine, in particular having improved anti-tumor activity.

**[0040]** The complex for use according to the present invention is a polypeptide or a protein, in particular a recombinant polypeptide or a recombinant protein, preferably a recombinant fusion protein or a recombinant fusion polypeptide. The term "recombinant" as used herein means that it (here: the polypeptide or the protein) does not occur naturally. Accordingly, the complex for use according to the present invention, which is a recombinant polypeptide or a recombinant protein, typically comprises components a) to c), wherein components a) to c) are of different origins, i.e. do not naturally occur in this combination.

**[0041]** In the context of the present invention, i.e. throughout the present application, the terms "peptide", "polypeptide", "protein" and variations of these terms refer to peptide, oligopeptide, oligomer or protein including fusion protein, respectively, comprising at least two amino acids joined to each other preferably by a normal peptide bond, or, alternatively, by a modified peptide bond, such as for example in the cases of isosteric peptides. A peptide, polypeptide or protein can be composed of L-amino acids and/or D-amino acids. Preferably, a peptide, polypeptide or protein is either (entirely) composed of L-amino acids or (entirely) of D-amino acids, thereby forming "retro-inverso peptide sequences". The term "retro-inverso (peptide) sequences" refers to an isomer of a linear peptide sequence in which the direction of the sequence is reversed and the chirality of each amino acid residue is inverted (see e.g. Jameson et al., *Nature*, 368,744-746 (1994); Brady et al., *Nature*, 368,692-693 (1994)). In particular, the terms "peptide", "polypeptide", "protein" also include "peptidomimetics" which are defined as peptide analogs containing non-peptidic structural elements, which peptides are capable of mimicking or antagonizing the biological action(s) of a natural parent peptide. A peptidomimetic lacks classical peptide characteristics such as enzymatically scissile peptide bonds. In particular, a peptide, polypeptide or protein can comprise amino acids other than the 20 amino acids defined by the genetic code in addition to these amino acids, or it can be composed of amino acids other than the 20 amino acids defined by the genetic code. In particular, a peptide, polypeptide or protein in the context of the present invention can equally be composed of amino acids modified by natural processes, such as post-translational maturation processes or by chemical processes, which are well known to a person skilled in the art. Such modifications are fully detailed in the literature. These modifications can appear anywhere in the polypeptide: in the peptide skeleton, in the amino acid chain or even at the carboxy- or amino-terminal ends. In particular, a peptide or polypeptide can be branched following an ubiquitination or be cyclic with or without branching. This type of modification can be the result of natural or synthetic post-translational processes that are well known to a person skilled in the art. The terms "peptide", "polypeptide", "protein" in the context of the present invention in particular also include modified peptides, polypeptides and proteins. For

example, peptide, polypeptide or protein modifications can include acetylation, acylation, ADP-ribosylation, amidation, covalent fixation of a nucleotide or of a nucleotide derivative, covalent fixation of a lipid or of a lipidic derivative, the covalent fixation of a phosphatidylinositol, covalent or non-covalent crosslinking, cyclization, disulfide bond formation, demethylation, glycosylation including pegylation, hydroxylation, iodization, methylation, myristylation, oxidation, proteolytic processes, phosphorylation, prenylation, racemization, senesoylation, sulfatation, amino acid addition such as arginylation or ubiquitination. Such modifications are fully detailed in the literature (Proteins Structure and Molecular Properties (1993) 2nd Ed., T. E. Creighton, New York ; Post-translational Covalent Modifications of Proteins (1983) B. C. Johnson, Ed., Academic Press, New York ; Seifter et al. (1990) Analysis for protein modifications and nonprotein cofactors, Meth. Enzymol. 182: 626-646 and Rattan et al., (1992) Protein Synthesis: Post-translational Modifications and Aging, Ann NY Acad Sci, 663: 48-62). Accordingly, the terms "peptide", "polypeptide", "protein" preferably include for example lipopeptides, lipoproteins, glycopeptides, glycoproteins and the like.

**[0042]** However, in a particularly preferred embodiment, the complex for use according to the present invention is a "classical" polypeptide or protein, whereby a "classical" polypeptide or protein is typically composed of amino acids selected from the 20 amino acids defined by the genetic code, linked to each other by a normal peptide bond.

**[0043]** As the complex for use according to the present invention is a polypeptide or a protein, it is preferred that it comprises at least 50, at least 60, at least 70, preferably at least 80, at least 90, more preferably at least 100, at least 110, even more preferably at least 120, at least 130, particularly preferably at least 140, or most preferably at least 150 amino acid residues.

**Component a) - Cell penetrating peptide**

**[0044]** The CPP allows for efficient delivery, i.e. transport and loading, in particular of at least one antigen or antigenic epitope, into the antigen presenting cells (APCs), in particular into the dendritic cells (DCs) and thus to the dendritic cells' antigen processing machinery.

**[0045]** The term "cell penetrating peptides" ("CPPs") is generally used to designate short peptides that are able to transport different types of cargo molecules across plasma membrane, and, thus, facilitate cellular uptake of various molecular cargoes (from nanosize particles to small chemical molecules and large fragments of DNA). "Cellular internalization" of the cargo molecule linked to the cell penetrating peptide generally means transport of the cargo molecule across the plasma membrane and thus entry of the cargo molecule into the cell. Depending on the particular case, the cargo molecule can, then, be released in the cytoplasm, directed to an intracellular organelle, or further presented at the cell surface. Cell penetrating ability, or internalization, of the cell penetrating peptide or complex comprising said cell penetrating peptide, according to the invention can be checked by standard methods known to one skilled in the art, including flow cytometry or fluorescence microscopy of live and fixed cells, immunocytochemistry of cells transduced with said peptide or complex, and

Western blot.

**[0046]** Cell penetrating peptides typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or have a sequence that contains an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. These two types of structures are referred to as polycationic or amphipathic, respectively. Cell-Penetrating peptides are of different sizes, amino acid sequences, and charges but all CPPs have a common characteristic that is the ability to translocate the plasma membrane and facilitate the delivery of various molecular cargoes to the cytoplasm or to an organelle of a cell. At present, the theories of CPP translocation distinguish three main entry mechanisms: direct penetration in the membrane, endocytosismediated entry, and translocation through the formation of a transitory structure. CPP transduction is an area of ongoing research. Cell-penetrating peptides have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer and virus inhibitors, as well as contrast agents for cell labeling and imaging.

**[0047]** Typically, cell penetrating peptides (CPPs) are peptides of 8 to 50 residues that have the ability to cross the cell membrane and enter into most cell types. Alternatively, they are also called protein transduction domain (PTDs) reflecting their origin as occurring in natural proteins. Frankel and Pabo simultaneously to Green and Lowenstein described the ability of the transactivating transcriptional activator from the human immunodeficiency virus 1 (HIV-TAT) to penetrate into cells (Frankel, A.D. and C.O. Pabo, Cellular uptake of the tat protein from human immunodeficiency virus. *Cell*, 1988. 55(6): p. 1189-93). In 1991, transduction into neural cells of the Antennapedia homeodomain (DNA-binding domain) from *Drosophila melanogaster* was described (Joliot, A., et al., Antennapedia homeobox peptide regulates neural morphogenesis. *Proc Natl Acad Sci USA*, 1991. 88(5): p. 1864-8). In 1994, the first 16-mer peptide CPP called Penetratin, having the amino acid sequence RQIKIYFQNRRMKWKK (SEQ ID NO: 1) was characterized from the third helix of the homeodomain of Antennapedia (Derossi, D., et al., The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem*, 1994. 269(14): p. 10444-50), followed in 1998 by the identification of the minimal domain of TAT, having the amino acid sequence YGRKKRRQRRR (SEQ ID NO: 2) required for protein transduction (Vives, E., P. Brodin, and B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem*, 1997. 272(25): p. 16010-7). Over the past two decades, dozens of peptides were described from different origins including viral proteins, e.g. VP22 (Elliott, G. and P. O'Hare, Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell*, 1997. 88(2): p. 223-33) and ZEBRA (Rothe, R., et al., Characterization of the cell-penetrating properties of the Epstein-Barr virus ZEBRA trans-activator. *J Biol Chem*, 2010. 285(26): p. 20224-33), or from venoms, e.g. melittin (Dempsey, C.E., The actions of melittin on membranes. *Biochim Biophys Acta*, 1990. 1031(2): p. 143-61), mastoporan (Konno, K., et al., Structure and biological activities of eumenine mastoparan-AF (EMP-AF), a new mast cell degranulating peptide in the venom of the solitary wasp (*Anterhynchium flavomarginatum micado*). *Toxicon*, 2000. 38(11): p. 1505-15), maurocalcin

(Esteve, E., et al., Transduction of the scorpion toxin maurocalcine into cells. Evidence that the toxin crosses the plasma membrane. *J Biol Chem*, 2005. 280(13): p. 12833-9), crotamine (Nascimento, F.D., et al., Crotamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. *J Biol Chem*, 2007. 282(29): p. 21349-60) or buforin (Kobayashi, S., et al., Membrane translocation mechanism of the antimicrobial peptide buforin 2. *Biochemistry*, 2004. 43(49): p. 15610-6). Synthetic CPPs were also designed including the poly-arginine (R8, R9, R10 and R12) (Futaki, S., et al., Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem*, 2001. 276(8): p. 5836-40) or transportan (Pooga, M., et al., Cell penetration by transportan. *FASEB J*, 1998. 12(1): p. 67-77). Any of the above described CPPs may be used as cell penetrating peptide, i.e. as component a), in the complex for use according to the present invention. In particular, the component a), i.e. the CPP, in the complex for use according to the present invention may comprise the minimal domain of TAT, having the amino acid sequence YGRKKRRQRRR (SEQ ID NO: 2). In particular, the component a), i.e. the CPP, in the complex for use according to the present invention may comprise Penetratin having the amino acid sequence RQIKIYFQNRRMKWKK (SEQ ID NO: 1).

**[0048]** Various CPPs, which can be used as cell penetrating peptide, i.e. as component a), in the complex for use according to the present invention, are also disclosed in the review: Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012. In other words, the CPPs disclosed in Milletti, F., 2012, Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60 can be used as cell penetrating peptide, i.e. as component a), in the complex for use according to the present invention. This includes in particular cationic CPPs, amphipatic CPPs, and hydrophobic CPPs as well as CPPs derived from heparan-, RNA- and DNA-binding proteins (cf. Table 1 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012), CPPs derived from signal peptides (cf. Table 2 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012), CPPs derived from antimicrobial peptides (cf. Table 3 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012), CPPs derived from viral proteins (cf. Table 4 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012), CPPs derived from various natural proteins (cf. Table 5 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012), and Designed CPPs and CPPs derived from peptide libraries (cf. Table 6 of Milletti, F., Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov Today* 17 (15-16): 850-60, 2012).

**[0049]** Preferably, the cell penetrating peptide, which is comprised by the complex for use according to the present invention,

1. i) has a length of the amino acid sequence of said peptide of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total; and/or

2. ii) has an amino acid sequence comprising a fragment of the minimal domain of ZEBRA, said minimal domain extending from residue 170 to residue 220 of the ZEBRA amino acid sequence according to SEQ ID NO: 3, wherein, optionally, 1, 2, 3, 4, or 5 amino acids have been substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, or a sequence variant of such a fragment.

**[0050]** Thereby, it is preferred that the cell penetrating peptide, which is comprised by the complex for use according to the present invention,

1. i) has a length of the amino acid sequence of said peptide of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total; and
2. ii) has an amino acid sequence comprising a fragment of the minimal domain of ZEBRA, said minimal domain extending from residue 170 to residue 220 of the ZEBRA amino acid sequence according to SEQ ID NO: 3, wherein, optionally, 1, 2, 3, 4, or 5 amino acids have been substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, or a sequence variant of such a fragment.

**[0051]** Such preferred CPPs are disclosed in WO 2014/041505.

**[0052]** The term "ZEBRA" (also known as Zta, Z, EB1, or BZLF1) generally means the basic-leucine zipper (bZIP) transcriptional activator of the Epstein-Barr virus (EBV). The minimal domain of ZEBRA, which exhibits cell penetrating properties, has been identified as spanning from residue 170 to residue 220 of ZEBRA. The amino acid sequence of ZEBRA is disclosed under NCBI accession number YP\_401673 and comprises 245 amino acids represented in SEQ ID NO: 3:

MMDPNSTEDVKFTPDPYQVPFVQAFDQATRVYQDLGGPSQAPLPCVLWPVLPEPLPQGQL  
...TAYHV\$TAPTCWSFAPQPAPENAYQAYAAPQLFPVSDITQNQQTNQACGEAPQPGDNST...

VQTAAAVVFACPGANQGQQQLADIGVPQPAPVAAPARRTRKPQQPESLECDSELEIKRYKNR  
VASRKCRAKFKQLLQHYREVAAKSSENDRLRLLKQMCPSDLVDSIIPRTPDVLHEDLLNF  
(SEQ ID NO: 3 - ZEBRA amino acid sequence (natural sequence from Epstein - Barr virus (EBV)) (YP\_401673))

**[0053]** Recently, a CPP derived from the viral protein ZEBRA was described to transduce protein cargoes across biological membranes by both (i) direct translocation and (ii) lipid raftmediated endocytosis (Rothe R, Liguori L, Villegas-Mendez A, Marques B, Grunwald D, Drouet E, et al. Characterization of the cell-penetrating properties of the Epstein-Barr virus ZEBRA trans-activator. The Journal of biological chemistry 2010;285(26):20224-33). The present inventors assume that these two mechanisms of entry should promote both MHC class

I and II restricted presentation of cargo antigens to CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively. Accordingly, such a CPP can deliver multi-epitopic peptides to dendritic cells (DCs), and subsequently to promote CTL and Th cell activation and anti-tumor function. Such a CPP can thus efficiently deliver the complex for use according to the present invention to antigen presenting cells (APCs) and lead to multi-epitopic MHC class I and II restricted presentation.

**[0054]** In the context of the present invention, the term "MHC class I" designates one of the two primary classes of the Major Histocompatibility Complex molecules. The MHC class I (also noted "MHC I") molecules are found on every nucleated cell of the body. The function of MHC class I is to display an epitope to cytotoxic cells (CTLs). In humans, MHC class I molecules consist of two polypeptide chains,  $\alpha$ - and  $\beta$ 2-microglobulin (b2m). Only the  $\alpha$  chain is polymorphic and encoded by a HLA gene, while the b2m subunit is not polymorphic and encoded by the Beta-2 microglobulin gene. In the context of the present invention, the term "MHC class II" designates the other primary class of the Major Histocompatibility Complex molecules. The MHC class II (also noted "MHC II") molecules are found only on a few specialized cell types, including macrophages, dendritic cells and B cells, all of which are dedicated antigen-presenting cells (APCs).

**[0055]** Preferably, the sequence variant of a fragment of the minimal domain of ZEBRA as described above shares, in particular over the whole length, at least 70%, at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% amino acid sequence identity with the fragment of the minimal domain of ZEBRA as described above without abrogating the cell penetrating ability of the cell penetrating peptide. In particular, a "fragment" of the minimal domain of ZEBRA as defined above is preferably to be understood as a truncated sequence thereof, i.e. an amino acid sequence, which is N-terminally, C-terminally and/or intrasequentially truncated compared to the amino acid sequence of the native sequence. Moreover, such a "fragment" of the minimal domain of ZEBRA has preferably a length of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total.

**[0056]** Accordingly, the term "sequence variant" as used in the context of the present invention, i.e. throughout the present application, refers to any alteration in a reference sequence. The term "sequence variant" includes nucleotide sequence variants and amino acid sequence variants. Preferably, a reference sequence is any of the sequences listed in the "Table of Sequences and SEQ ID Numbers" (Sequence listing), i.e. SEQ ID NO: 1 to SEQ ID NO: 94. Preferably, a sequence variant shares, in particular over the whole length of the sequence, at least 70%, at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity with a reference sequence, whereby sequence identity is calculated as described below. In particular, a sequence variant preserves the specific function of the reference sequence. Sequence identity is calculated as described below. In particular, an amino acid sequence variant has an altered sequence in which one or more of the amino acids in the reference sequence is deleted or substituted, or one or more amino acids are

inserted into the sequence of the reference amino acid sequence. As a result of the alterations, the amino acid sequence variant has an amino acid sequence which is at least 70%, at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% identical to the reference sequence. For example, variant sequences which are at least 90% identical have no more than 10 alterations, i.e. any combination of deletions, insertions or substitutions, per 100 amino acids of the reference sequence.

**[0057]** In the context of the present invention, an amino acid sequence "sharing a sequence identity" of at least, for example, 95% to a query amino acid sequence of the present invention, is intended to mean that the sequence of the subject amino acid sequence is identical to the query sequence except that the subject amino acid sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain an amino acid sequence having a sequence of at least 95% identity to a query amino acid sequence, up to 5% (5 of 100) of the amino acid residues in the subject sequence may be inserted or substituted with another amino acid or deleted, preferably within the above definitions of variants or fragments. The same, of course, also applies similarly to nucleic acid sequences.

**[0058]** For (amino acid or nucleic acid) sequences without exact correspondence, a "% identity" of a first sequence may be determined with respect to a second sequence. In general, these two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may then be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.

**[0059]** Methods for comparing the identity and homology of two or more sequences are well known in the art. The percentage to which two sequences are identical can e.g. be determined using a mathematical algorithm. A preferred, but not limiting, example of a mathematical algorithm which can be used is the algorithm of Karlin et al. (1993), PNAS USA, 90:5873-5877. Such an algorithm is integrated in the BLAST family of programs, e.g. BLAST or NBLAST program (see also Altschul et al., 1990, J. Mol. Biol. 215, 403-410 or Altschul et al. (1997), Nucleic Acids Res, 25:3389-3402), accessible through the home page of the NCBI at world wide web site [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov)) and FASTA (Pearson (1990), Methods Enzymol. 183, 63-98; Pearson and Lipman (1988), Proc. Natl. Acad. Sci. U. S. A 85, 2444-2448.). Sequences which are identical to other sequences to a certain extent can be identified by these programmes. Furthermore, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux et al., 1984, Nucleic Acids Res., 387-395), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % homology or identity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of (Smith and Waterman (1981), J. Mol. Biol. 147, 195-197.) and finds the best single region of similarity between two sequences.

**[0060]** More preferably, the fragments of the cell penetrating peptide according to the invention or the variants thereof as described above further retain said peptide's ability to present a cargo molecule such as antigens or antigenic epitopes at the surface of a cell, such as an antigen-presenting cell, in the context of MHC class I and/or MHC class II molecules. The ability of a cell penetrating peptide or complex comprising said cell penetrating peptide to present a cargo molecule such as antigens or antigenic epitopes at the surface of a cell in the context of MHC class I and/or MHC class II molecules can be checked by standard methods known to one skilled in the art, including capacity to stimulate proliferation and/or function of MHC-restricted CD4<sup>+</sup> or CD8<sup>+</sup> T cells with specificity for these epitopes.

**[0061]** The preferred cell penetrating peptide, which

1. i) has a length of the amino acid sequence of said peptide of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total; and/or
2. ii) has an amino acid sequence comprising a fragment of the minimal domain of ZEBRA, said minimal domain extending from residue 170 to residue 220 of the ZEBRA amino acid sequence according to SEQ ID NO: 3, wherein, optionally, 1, 2, 3, 4, or 5 amino acids have been substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, or a variant of such a fragment

preferably comprises an amino acid sequence having at least one conservatively substituted amino acid compared to the referenced sequence, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics.

**[0062]** Generally, substitutions for one or more amino acids present in the referenced amino acid sequence should be made conservatively. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity properties, are well known (Kyte and Doolittle, 1982, J. Mol. Biol. 157(1):105- 132). Substitutions of one or more L-amino acids with one or more D-amino acids are to be considered as conservative substitutions in the context of the present invention. Exemplary amino acid substitutions are presented in Table 1 below:

(Table 1)

| Original residues | Examples of substitutions |
|-------------------|---------------------------|
| Ala (A)           | Val, Leu, Ile, Gly        |
| Arg (R)           | His, Lys                  |
| Asn (N)           | Gln                       |
| Asp (D)           | Glu                       |
| Cys (C)           | Ser                       |
| Gln (Q)           | Asn                       |

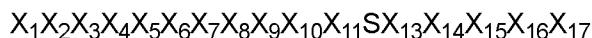
| Original residues | Examples of substitutions    |
|-------------------|------------------------------|
| Glu (E)           | Asp                          |
| Gly (G)           | Pro, Ala                     |
| His (H)           | Lys, Arg                     |
| Ile (I)           | Leu, Val, Met, Ala, Phe      |
| Leu (L)           | Ile, Val, Met, Ala, Phe      |
| Lys (K)           | Arg, His                     |
| Met (M)           | Leu, Ile, Phe                |
| Phe (F)           | Leu, Val, Ile, Tyr, Trp, Met |
| Pro (P)           | Ala, Gly                     |
| Ser (S)           | Thr                          |
| Thr (T)           | Ser                          |
| Trp (W)           | Tyr, Phe                     |
| Tyr (Y)           | Trp, Phe                     |
| Original residues | Examples of substitutions    |
| Val (V)           | Ile, Met, Leu, Phe, Ala      |

**[0063]** Particularly preferably, the preferred cell penetrating peptide, which

1. i) has a length of the amino acid sequence of said peptide of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total; and/or
2. ii) has an amino acid sequence comprising a fragment of the minimal domain of ZEBRA, said minimal domain extending from residue 170 to residue 220 of the ZEBRA amino acid sequence according to SEQ ID NO: 3, wherein, optionally, 1, 2, 3, 4, or 5 amino acids have been substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, or a variant of such a fragment

comprises a Cys (C) substituted into a Ser (S), at the equivalent of position 189 relative to ZEBRA amino acid sequence of SEQ ID NO: 3.

**[0064]** Thereby, it is preferred that such a preferred cell penetrating peptide has an amino acid sequence comprising a sequence according to the following general formula (I):



with 0, 1, 2, 3, 4, or 5 amino acids which are substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, wherein

$X_1$ , is K, R, or H, preferably  $X_1$ , is K or R;

$X_2$  is R, K, or H, preferably  $X_2$  is R or K;

$X_3$  is Y, W, or F, preferably  $X_3$  is Y, W, or F;

$X_4$  is K, R, or H, preferably  $X_4$  is K or R;

$X_5$  is N or Q;

$X_6$  is R, K, or H, preferably  $X_6$  is R or K;

$X_7$  is V, I, M, L, F, or A, preferably  $X_7$  is V, I, M or L;

$X_8$  is A, V, L, I, or G, preferably  $X_8$  is A or G;

$X_9$  is S or T;

$X_{10}$  is R, K, or H, preferably  $X_{10}$  is R or K;

$X_{11}$  is K, R, or H, preferably  $X_{11}$  is K or R;

$X_{13}$  is R, K, or H, preferably  $X_{13}$  is R or K;

$X_{14}$  is A, V, L, I, or G, preferably  $X_{14}$  is A or G;

$X_{15}$  is K, R, or H, preferably  $X_{15}$  is K or R;

$X_{16}$  is F, L, V, I, Y, W, or M, preferably  $X_{16}$  is F, Y or W; and

$X_{17}$  is K, R, or H, preferably  $X_{17}$  is K or R.

**[0065]** Preferably, such a peptide, polypeptide or protein is either (entirely) composed of L-amino acids or (entirely) of D-amino acids, thereby forming "retro-inverso peptide sequences". The term "retro-inverso (peptide) sequences" refers to an isomer of a linear peptide sequence in which the direction of the sequence is reversed and the chirality of each amino acid residue is inverted (see e.g. Jameson et al., *Nature*, 368,744-746 (1994); Brady et al., *Nature*, 368,692-693 (1994)).

**[0066]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein  $X_1$  is K.

**[0067]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein  $X_2$  is R.

**[0068]** In a particular embodiment, the cell penetrating peptide according to the invention is as

generically defined above by general formula (I), wherein X<sub>3</sub> is Y.

**[0069]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>4</sub> is K.

**[0070]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>5</sub> is N.

**[0071]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>6</sub> is R.

**[0072]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>7</sub> is V.

**[0073]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>8</sub> is A.

**[0074]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>9</sub> is S.

**[0075]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>10</sub> is R.

**[0076]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>11</sub> is K.

**[0077]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>13</sub> is R.

**[0078]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>14</sub> is A.

**[0079]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>15</sub> is K.

**[0080]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>16</sub> is F.

**[0081]** In a particular embodiment, the cell penetrating peptide according to the invention is as generically defined above by general formula (I), wherein X<sub>17</sub> is K.

**[0082]** In a particular embodiment, the cell penetrating peptide according to the invention is as

generically defined above by general formula (I), wherein the amino acid at position equivalent to position 12 relative to general formula (I) is a Ser (S).

**[0083]** It is also particularly preferred, that the preferred cell penetrating peptide, which

1. i) has a length of the amino acid sequence of said peptide of 5 to 50 amino acids in total, preferably of 10 to 45 amino acids in total, more preferably of 15 to 45 amino acids in total; and/or
2. ii) has an amino acid sequence comprising a fragment of the minimal domain of ZEBRA, said minimal domain extending from residue 170 to residue 220 of the ZEBRA amino acid sequence according to SEQ ID NO: 3, wherein, optionally, 1, 2, 3, 4, or 5 amino acids have been substituted, deleted, and/or added without abrogating said peptide's cell penetrating ability, or a variant of such a fragment

comprises or consists of an amino acid sequence selected from the group consisting of amino acid sequences according to SEQ ID NO: 4 - 13, or sequence variants thereof without abrogating said peptide's cell penetrating ability, preferably sequence variants having 0, 1, 2, 3, 4, or 5 amino acids substituted, deleted and/or added without abrogating said peptide's cell penetrating ability.

CPP1 (Z11):

KRYKNRVASRKCRAFKQLLQHYREVAAKSSENDRLRLLLKQMC (SEQ ID NO: 4)

CPP2 (Z12):

KRYKNRVASRKCRAFKQLLQHYREVAAKSSENDRLRLLLK

(SEQ ID NO: 5)

CPP3 (Z13):

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLK

(SEQ ID NO: 6)

CPP4 (Z14):

KRYKNRVASRKSRAFKQLLQHYREVAAAK

(SEQ ID NO: 7)

CPP5 (Z15):

KRYKNRVASRKSRAFK

(SEQ ID NO: 8)

CPP6 (Z16):

QHYREVAAKSSEND

(SEQ ID NO: 9)

CPP7 (Z17):

QLLQHYREVAAAK

(SEQ ID NO: 10)

CPP8 (Z18):

REVAAAKSS END RLRLLLK

(SEQ ID NO: 11)

CPP9 (Z19):

KRYKNRVA

(SEQ ID NO: 12)

CPP10 (Z20):

VASRKSRACK

(SEQ ID NO: 13)

**[0084]** Thereby, a cell penetrating peptide is particularly preferred, which has an amino acid sequence comprising or consisting of an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13), SEQ ID NO: 7 (CPP4/Z14), SEQ ID NO: 8 (CPP5/Z15), or SEQ ID NO: 11 (CPP8/Z18), or sequence variants thereof without abrogating said peptide's cell penetrating ability, preferably sequence variants having 0, 1, 2, 3, 4, or 5 amino acids substituted, deleted and/or added without abrogating said peptide's cell penetrating ability. Moreover, a cell penetrating peptide is more preferred, which has an amino acid sequence comprising or consisting of an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13) or SEQ ID NO: 7 (CPP4/Z14) or sequence variants thereof without abrogating said peptide's cell penetrating ability, preferably sequence variants having 0, 1, 2, 3, 4, or 5 amino acids substituted, deleted and/or added without abrogating said peptide's cell penetrating ability.

**[0085]** Moreover, a cell penetrating peptide is most preferred, which has an amino acid sequence comprising or consisting of an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13) or sequence variants thereof without abrogating said peptide's cell penetrating ability, preferably sequence variants having 0, 1, 2, 3, 4, or 5 amino acids substituted, deleted and/or added without abrogating said peptide's cell penetrating ability. Accordingly, it is most preferred that the cell penetrating peptide comprises or consists of a peptide having an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13) or a functional sequence variant thereof having at least 70% sequence identity, preferably at least 75% sequence identity, more preferably at least 80% sequence identity, even more preferably at least 85% sequence identity, still more preferably at least 90% sequence identity, particularly preferably at least 95% sequence identity, most preferably at least 99% sequence identity. In this context, a "functional sequence variant" is a sequence variant wherein said peptide's cell penetrating ability is maintained.

**[0086]** In one preferred embodiment, the cell penetrating peptide according to the invention has an amino acid sequence comprising or consisting of SEQ ID NO: 6 (CPP3/Z13).

**[0087]** In another preferred embodiment, the cell penetrating peptide according to the invention has an amino acid sequence comprising or consisting of SEQ ID NO: 7 (CPP4/Z14).

**[0088]** In another preferred embodiment, the cell penetrating peptide according to the invention has an amino acid sequence comprising or consisting of SEQ ID NO: 8 (CPP5/Z15).

**[0089]** In another preferred embodiment, the cell penetrating peptide according to the invention has an amino acid sequence comprising or consisting of SEQ ID NO: 11 (CPP8/Z18).

**[0090]** It will be understood by one skilled in the art that the primary amino acid sequence of the cell penetrating peptide of the invention may further be post-translationally modified, such as by glycosylation or phosphorylation, without departing from the invention.

**[0091]** In a further embodiment, the cell penetrating peptide according to the invention optionally further comprises, in addition to its amino acid sequence as described above, any one of, or any combination of:

1. (i) a nuclear localization signal (NLS). Such signals are well known to the skilled person and are described in Nair et al. (2003, Nucleic Acids Res. 31(1): 397-399)
2. (ii) a targeting peptide, including tumor homing peptides such as those described in Kapoor et al. (2012, PLoS ONE 7(4): e35787) and listed in <http://crdd.osdd.net/raghava/tumorhope/general.php>?

**[0092]** Preferably, the cell penetrating peptide according to the invention is linked to an antigen or antigenic epitope and facilitates the cellular internalization of said antigen or antigenic epitope.

**[0093]** The complex for use according to the present invention may comprise one single cell penetrating peptide or more than one cell penetrating peptides. Preferably, the complex for use according to the present invention comprises no more than five cell penetrating peptides, more preferably the complex for use according to the present invention comprises no more than four cell penetrating peptides, even more preferably the complex for use according to the present invention comprises no more than three cell penetrating peptides, particularly preferably the complex for use according to the present invention comprises no more than two cell penetrating peptides and most preferably the complex for use according to the present invention comprises one single cell penetrating peptide.

**Component b) - Antigen /antigenic epitope**

**[0094]** The complex for use according to the present invention comprises as component b) at least one antigen or antigenic epitope, wherein the complex comprises an epitope of ASCL2.

**[0095]** As used herein, an "antigen" is any structural substance which serves as a target for the receptors of an adaptive immune response, in particular as a target for antibodies, T cell

receptors, and/or B cell receptors. An "epitope", also known as "antigenic determinant", is the part (or fragment) of an antigen that is recognized by the immune system, in particular by antibodies, T cell receptors, and/or B cell receptors. Thus, one antigen has at least one epitope, i.e. a single antigen has one or more epitopes. In the context of the present invention, the term "epitope" is mainly used to designate T cell epitopes, which are presented on the surface of an antigen-presenting cell, where they are bound to Major Histocompatibility Complex (MHC). T cell epitopes presented by MHC class I molecules are typically, but not exclusively, peptides between 8 and 11 amino acids in length, whereas MHC class II molecules present longer peptides, generally, but not exclusively, between 12 and 25 amino acids in length.

**[0096]** In the complex for use according to the present invention, the at least one antigen or antigenic epitope is an ASCL2 epitope. In addition, the at least one antigen may be selected from the group consisting of: (i) a peptide, a polypeptide, or a protein, (ii) a polysaccharide, (iii) a lipid, (iv) a lipoprotein or a lipopeptide, (v) a glycolipid, (vi) a nucleic acid, and (vii) a small molecule drug or a toxin. Thus, the at least one antigen or antigenic epitope may be a peptide, a protein, a polysaccharide, a lipid, a combination thereof including lipoproteins and glycolipids, a nucleic acid (e.g. DNA, siRNA, shRNA, antisense oligonucleotides, decoy DNA, plasmid), or a small molecule drug (e.g. cyclosporine A, paclitaxel, doxorubicin, methotrexate, 5-aminolevulinic acid), or any combination thereof in particular if more than one antigen or antigenic epitope is comprised by the inventive complex.

**[0097]** It is understood that the complex comprising an epitope of ASCL2 may comprise at least one further antigen or antigenic epitope, for example at least one, i.e. one or more, peptides, polypeptides or proteins linked together and/or at least one, i.e. one or more, nucleic acids, e.g. where each one encodes one peptide or polypeptide. Also the at least one antigen or antigenic epitope can be a combination of a protein, a lipid, and/or a polysaccharide including lipoproteins and glycolipids. Thus, in particular if the complex for use according to the present invention comprises more than one antigen or antigenic epitope, i.e. an additional antigen or antigenic epitope other than an epitope of ASCL2, it can comprise more than one peptide, polypeptide, or protein, more than one polysaccharide, more than one lipid, more than one lipoprotein, more than one glycolipid, more than one nucleic acid, more than one small molecule drug or toxin, or a combination thereof.

**[0098]** The complex for use according to the invention comprises at least one antigen or antigenic epitope comprising one or more epitope(s) from a cancer/tumor-associated antigen, a cancer/tumor-specific antigen, wherein the complex comprises an epitope of ASCL2.,

**[0099]** More preferably, the at least one antigen or antigenic epitope comprises or consists of at least one cancer/tumor epitope, in particular at least one tumor epitope, wherein the complex comprises an epitope of ASCL2. Most preferably, the at least one antigen or antigenic epitope comprises or consists of at least one cancer/tumor epitope, in particular at least one tumor epitope.

**[0100]** It is particularly preferred that the complex for use according to the present invention comprises only such antigen(s) or antigenic epitope(s), which are cancer/tumor-associated antigen(s), cancer/tumor-specific antigen(s) and/or cancer/tumor epitope(s); in particular, which are tumor-associated antigen(s), tumor-specific antigen(s), and/or tumor epitope(s).

**[0101]** As used herein, "cancer epitope" means an epitope from a cancer-associated antigen or from a cancer-specific antigen. Accordingly, "tumor epitope" means an epitope from a tumor-associated antigen or from a tumor-specific antigen. Such epitopes are typically specific (or associated) for a certain kind of cancer/tumor. In particular, cancer/tumor-associated (also cancer/tumor-related) antigens are antigens, which are expressed by both, cancer/tumor cells and normal cells. Accordingly, those antigens are normally present since birth (or even before). Accordingly, there is a chance that the immune system developed self-tolerance to those antigens. Cancer/tumor-specific antigens, in contrast, are antigens, which are expressed specifically by cancer/tumor cells, but not by normal cells. Cancer/tumor-specific antigens include in particular neoantigens. In general neoantigens are antigens, which were not present before and are, thus, "new" to the immune system. Neoantigens are typically due to somatic mutations. In the context of cancer/tumors, cancer/tumor-specific neoantigens were typically not present before the cancer/tumor developed and cancer/tumor-specific neoantigens are usually encoded by somatic gene mutations in the cancerous cells/tumor cells. Since neoantigens are new to the immune system, the risk of self-tolerance of those antigens is considerably lower as compared to cancer/tumor-associated antigens. However, every cancer's set of tumor-specific mutations appears to be unique. Accordingly, in the context of the present invention it is preferred that such cancer/tumor-specific antigens, in particular neoantigens, are identified in a subject diagnosed with colorectal cancer by methods known to the skilled person, e.g., cancer genome sequencing. After identification, the respective cancer/tumor-specific neoantigens and/or cancer/tumor-specific neoantigenic epitopes are used in a complex for use according to the present invention.

**[0102]** Preferably, a complex for use according to the present invention comprises one or more cancer/tumor-associated epitopes and/or one or more cancer/tumor-associated antigens (but preferably no cancer/tumor-specific epitopes), wherein the complex according to the invention comprises an epitope of ASCL2. It is also preferred that a complex for use according to the present invention comprises one or more cancer/tumor-specific epitopes and/or one or more cancer/tumor-specific antigens (but preferably no cancer/tumor-associated epitopes), wherein the complex according to the invention comprises an epitope of ASCL2. A complex for use according to the present invention may also preferably comprise both, (i) one or more cancer/tumor-associated epitopes and/or one or more cancer/tumor-associated antigens and (ii) one or more cancer/tumor-specific epitopes and/or one or more cancer/tumor-specific antigens, wherein the complex comprises an epitope of ASCL2.

**[0103]** In particular, the cancer/tumor with which the antigens or antigenic epitopes are associated or for which the antigens or antigenic epitopes are specific is colorectal cancer as described herein. Thus, the antigens are preferably CRC-associated or CRC-specific antigens and the epitopes are preferably CRC-associated or CRC-specific epitopes.

**[0104]** Suitable cancer/tumor epitopes can be retrieved for example from cancer/tumor epitope databases, e.g. from van der Bruggen P, Stroobant V, Vigneron N, Van den Eynde B. Peptide database: T cell-defined tumor antigens. *Cancer Immun* 2013; URL: <http://www.cancerimmunity.org/peptide/>, wherein human tumor antigens recognized by CD4+ or CD8+ T cells are classified into four major groups on the basis of their expression pattern, or from the database "T antigen" (TANTIGEN version 1.0, Dec 1, 2009; developed by Bioinformatics Core at Cancer Vaccine Center, Dana-Farber Cancer Institute; URL: <http://cvc.dfci.harvard.edu/tadb/>). Examples of cancer/tumor epitopes include e.g. TRP2-derived epitopes, glycoprotein 100 (gp100) melanoma antigen-derived epitopes, glycoprotein 70 (gp70) antigen-derived epitopes, survivin epitopes, IEa epitopes, IL13ra2, EphA2 (ephrin type-A receptor 2), immunogenic fragments thereof, and fusions of such antigens and/or fragments. Furthermore, examples of cancer/tumor epitopes include epitopes of neoantigens, such as, for example, a neoantigen from MC-38 tumor cell line as described by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6. As described above, neoantigens are antigens, which are entirely absent from the normal human genome. As compared with nonmutated self-antigens, neoantigens are of relevance to tumor control, as the quality of the T cell pool that is available for these antigens is not affected by central T cell tolerance. In particular, neoantigens may be based on individual tumor genomes. Potential neoantigens may be predicted by methods known to the skilled person, such as cancer genome sequencing or deep-sequencing technologies identifying mutations within the protein-coding part of the (cancer) genome.

**[0105]** Specific examples of cancer/tumor-associated, in particular tumor-related, or tissue-specific antigens useful in a complex for use according to the present invention other than ASCL2 include, but are not limited to, the following antigens: Her-2/neu, SPAS-1, TRP-2, tyrosinase, Melan A/Mart-1, gp100, BAGE, GAGE, GM2 ganglioside, kinesin 2, TATA element modulatory factor 1, tumor protein D52, MAGE D, ING2, HIP-55, TGF-1 anti-apoptotic factor, HOM-Mel-40/SSX2, epithelial antigen (LEA 135), DF31MUC1 antigen (Apostolopoulos et al., 1996 *Immunol. Cell. Biol.* 74: 457-464; Pandey et al., 1995, *Cancer Res.* 55: 4000-4003), MAGE-1, HOM-Mel-40/SSX2, NY-ESO-1, EGFR, CEA, EphA2, EphA4, PCDGF, HAAH, Mesothelin; EPCAM; NY-ESO-1, glycoprotein MUC1 and NIUC10 mucins p5 (especially mutated versions), EGFR, cancer-associated serum antigen (CASA) and cancer antigen 125 (CA 125) (Kierkegaard et al., 1995, *Gynecol. Oncol.* 59: 251-254), the epithelial glycoprotein 40 (EGP40) (Kievit et al., 1997, *Int. J. Cancer* 71: 237-245), squamous cell carcinoma antigen (SCC) (Lozza et al., 1997 *Anticancer Res.* 17: 525-529), cathepsin E (Mota et al., 1997, *Am. J Pathol.* 150: 1223-1229), tyrosinase in melanoma (Fishman et al., 1997 *Cancer* 79: 1461-1464), cell nuclear antigen (PCNA) of cerebral cavernomas (Notelet et al., 1997 *Surg. Neurol.* 47: 364-370), a 35 kD tumor-associated autoantigen in papillary thyroid carcinoma (Lucas et al., 1996 *Anticancer Res.* 16: 2493-2496), CDC27 (including the mutated form of the protein), antigens triosephosphate isomerase, 707-AP, A60 mycobacterial antigen (Macs et al., 1996, *J. Cancer Res. Clin. Oncol.* 122: 296-300), Annexin II, AFP, ART-4, BAGE,  $\beta$ -catenin/m, BCL-2, bcr-abl, bcr-abl p190, bcr-abl p210, BRCA-1, BRCA-2, CA 19-9 (Tolliver and O'Brien, 1997, *South Med. J.* 90: 89-90; Tsuruta et al., 1997 *Urol. Int.* 58: 20-24), CAMEL, CAP-1, CASP-8,

CDC27/m, CDK-4/m, CEA (Huang et al., Exper Rev. Vaccines (2002)1:49-63), CT9, CT10, Cyp-B, Dek-cain, DAM-6 (MAGE-B2), DAM-10 (MAGE-B1), EphA2 (Zantek et al., Cell Growth Differ. (1999) 10:629-38; Carles-Kinch et al., Cancer Res. (2002) 62:2840-7), EphA4 (Cheng et al., 2002, Cytokine Growth Factor Rev. 13:75-85), tumor associated Thomsen-Friedenreich antigen (Dahlenborg et al., 1997, Int. J Cancer 70: 63-71), ELF2M, ETV6-AML1, G250, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7B, GAGE-8, GnT-V, gp100 (Zajac et al., 1997, Int. J Cancer 71: 491-496), HAGE, HER2/neu, HLA-A\*0201-R170I, HPV-E7, HSP70-2M, HST-2, hTERT, hTRT, iCE, inhibitors of apoptosis (e.g., survivin), KH-1 adenocarcinoma antigen (Deshpande and Danishefsky, 1997, Nature 387: 164-166), KIAA0205, K-ras, LAGE, LAGE-1, LDLR/FUT, MAGE-1, MAGE-2, MAGE-3, MAGE-6, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MAGE-B5, MAGE-B6, MAGE-C2, MAGE-C3, MAGE D, MART-1, MART-1/Melan-A(Kawakami and Rosenberg, 1997, Int. Rev. Immunol. 14: 173-192), MC1R, MDM-2, Myosin/m, MUC1, MUC2, MUM-1, MUM-2, MUM-3; neo-polyA polymerase, NA88-A, NY-ESO-1, NY-ESO-1a (CAG-3), PAGE-4, PAP, Proteinase 3 (Molldrem et al., Blood (1996) 88:2450-7; Molldrem et al., Blood (1997) 90:2529-34), P15, p190, Pm1/RAR $\alpha$ , PRAME, PSA, PSM, PSMA, RAGE, RAS, RCAS1, RU1, RU2, SAGE, SART-1, SART-2, SART-3, SP17, SPAS-1, TEL/AML1, TPI/m, Tyrosinase, TARP, TRP-1 (gp75), TRP-2, TRP-2/INT2, WT-1, and alternatively translated NY-ESO-ORF2 and CAMEL proteins, derived from the NY-ESO-1 and LAGE-1 genes. In a complex for use according to the present invention an epitope of the antigen ASCL2 (Achaete-scute homolog 2) is comprised. Numerous other cancer antigens are well known in the art.

**[0106]** Preferably, the cancer/tumor antigen or the cancer/tumor epitope is a recombinant cancer/tumor antigen or a recombinant cancer/tumor epitope. Such a recombinant cancer/tumor antigen or a recombinant cancer/tumor epitope may be designed by introducing mutations that change (add, delete or substitute) particular amino acids in the overall amino acid sequence of the native cancer/tumor antigen or the native cancer/tumor epitope. The introduction of mutations does not alter the cancer/tumor antigen or the cancer/tumor epitope so much that it cannot be universally applied across a mammalian subject, and preferably a human or dog subject, but changes it enough that the resulting amino acid sequence breaks tolerance or is considered a foreign antigen in order to generate an immune response. Another manner may be creating a consensus recombinant cancer/tumor antigen or cancer/tumor epitope that has at least 85% and up to 99% amino acid sequence identity to its' corresponding native cancer/tumor antigen or native cancer/tumor epitope; preferably at least 90% and up to 98% sequence identity; more preferably at least 93% and up to 98% sequence identity; or even more preferably at least 95% and up to 98% sequence identity. In some instances the recombinant cancer/tumor antigen or the recombinant cancer/tumor epitope has 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to its' corresponding native cancer/tumor antigen or cancer/tumor epitope. The native cancer/tumor antigen is the antigen normally associated with the particular cancer or cancer tumor. Depending upon the cancer/tumor antigen, the consensus sequence of the cancer/tumor antigen can be across mammalian species or within subtypes of a species or across viral strains or serotypes. Some cancer/tumor antigen do not vary greatly from the wild type amino acid sequence of the cancer/tumor antigen. The aforementioned approaches can be combined so that the final recombinant

cancer/tumor antigen or cancer/tumor epitope has a percent similarity to native cancer antigen amino acid sequence as discussed above. Preferably, however, the amino acid sequence of an epitope of a cancer/tumor antigen as described herein is not mutated and, thus, identical to the reference epitope sequence.

**[0107]** As used herein "pathogen epitope" (not claimed; for illustrative purposes only) means an epitope from an antigenic protein, an antigenic polysaccharide, an antigenic lipid, an antigenic lipoprotein or an antigenic glycolipid from a pathogen including viruses, bacteria, fungi, protozoa and multicellular parasites. Antigenic proteins, polysaccharides, lipids, lipoproteins or glycolipids from pathogens include, herewith, proteins, polysaccharides, lipids, lipoproteins and glycolipids, respectively, from pathogens responsible of diseases which can be a target for vaccination including, for instance, Amoebiasis, Anthrax, Buruli Ulcer (*Mycobacterium ulcerans*), Caliciviruses associated diarrhoea, *Campylobacter* diarrhoea, Cervical Cancer (Human papillomavirus), *Chlamydia trachomatis* associated genital diseases, Cholera, Crimean-Congo haemorrhagic fever, Dengue Fever, Diphtheria, Ebola haemorrhagic fever, Enterotoxigenic *Escherichia coli* (ETEC) diarrhoea, Gastric Cancer (*Helicobacter pylori*), Gonorrhoea, Group A *Streptococcus* associated diseases, Group B *Streptococcus* associated diseases, *Haemophilus influenzae* B pneumonia and invasive disease, Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis E diarrhoea, Herpes simplex type 2 genital ulcers, HIV/AIDS, Hookworm Disease, Influenza, Japanese encephalitis, Lassa Fever, Leishmaniasis, Leptospirosis, Liver cancer (Hepatitis B), Liver Cancer (Hepatitis C), Lyme Disease, Malaria, Marburg haemorrhagic fever, Measles, Mumps, Nasopharyngeal cancer (Epstein-Barr virus), *Neisseria meningitidis* Meningitis, Parainfluenza associated pneumonia, Pertussis, Plague, Poliomyelitis, Rabies, Respiratory syncytial virus (RSV) pneumonia, Rift Valley fever, Rotavirus diarrhoea, Rubella, Schistosomiasis, Severe Acute Respiratory Syndrome (SARS), Shigellosis, Smallpox, *Staphylococcus aureus* associated diseases, Stomach Cancer (*Helicobacter pylori*), *Streptococcus pneumoniae* and invasive disease, Tetanus, Tick-borne encephalitis, Trachoma, Tuberculosis, Tularaemia, Typhoid fever, West-Nile virus associated disease, Yellow fever.

**[0108]** Preferably, the at least one antigen or antigenic epitope will be presented at the cell surface in an MHC class I and/or MHC class II context and/or in a CD1 context, whereby presentation at the cell surface in an MHC class I and/or MHC class II context is preferred. The phrase "epitope presentation in the MHC class I context" refers in particular to a CD8<sup>+</sup> epitope lying in the groove of a MHC class I molecule at the surface of a cell. The phrase "epitope presentation in the MHC class II context" refers in particular to a CD4<sup>+</sup> epitope lying in the groove of a MHC class II molecule at the surface of a cell. The phrase "epitope presentation in the CD1 context" refers in particular to a lipidic epitope lying in the groove of a cluster of differentiation 1 molecule at the surface of a cell.

**[0109]** Advantageously, the complex for use according to the invention comprises a cell penetrating peptide and at least one antigen or antigenic epitope, wherein the complex comprises an epitope of ASCL2, and allows the transport and presentation of said epitopes at the cell surface of antigen presenting cells in an MHC class I and MHC class II context, and is, thus, useful in vaccination and immunotherapy.

**[0110]** Preferably, the complex for use according to the present invention comprises at least one antigen or antigenic epitope, which is at least one CD4<sup>+</sup> epitope and/or at least one CD8<sup>+</sup> epitope.

**[0111]** The terms "CD4<sup>+</sup> epitope" or "CD4<sup>+</sup>-restricted epitope", as used herein, designate an epitope recognized by a CD4<sup>+</sup> T cell, said epitope in particular consisting of an antigen fragment lying in the groove of a MHC class II molecule. A single CD4<sup>+</sup> epitope comprised in the complex for use according to the present invention preferably consists of about 12-25 amino acids. It can also consist of, for example, about 8-25 amino acids or about 6-100 amino acids.

**[0112]** The terms "CD8<sup>+</sup> epitope" or "CD8<sup>+</sup>-restricted epitope", as used herein, designate an epitope recognized by a CD8<sup>+</sup> T cell, said epitope in particular consisting of an antigen fragment lying in the groove of a MHC class I molecule. A single CD8<sup>+</sup> epitope comprised in the complex for use according to the present invention preferably consists of about 8-11 amino acids. It can also consist of, for example, about 8-15 amino acids or about 6-100 amino acids.

**[0113]** Preferably, the at least one antigen can comprise or the at least one antigenic epitope can consist of a CD4<sup>+</sup> epitope and/or a CD8<sup>+</sup> epitope corresponding to antigenic determinants(s) of a cancer/tumor-associated antigen, a cancer/tumor-specific antigen, or an antigenic protein from a pathogen. More preferably, the at least one antigen can comprise or the at least one antigenic epitope can consist of a CD4<sup>+</sup> epitope and/or a CD8<sup>+</sup> epitope corresponding to antigenic determinant(s) of a cancer/tumor-associated antigen or a cancer/tumor-specific antigen. Most preferably, the at least one antigen can comprise or the at least one antigenic epitope can consist of a CD4<sup>+</sup> epitope and/or a CD8<sup>+</sup> epitope corresponding to antigenic determinant(s) of a tumor-associated antigen or a tumor-specific antigen.

**[0114]** It is also preferred that the complex for use according to the present invention comprises at least two antigens or antigenic epitopes, wherein at least one antigen or antigenic epitope comprises or consists a CD4<sup>+</sup> epitope and at least one antigen or antigenic epitope comprises or consists a CD8<sup>+</sup> epitope. It is now established that T<sub>h</sub> cells (CD4<sup>+</sup>) play a central role in the anti-tumor immune response both in DC licensing and in the recruitment and maintenance of CTLs (CD8<sup>+</sup>) at the tumor site. Therefore, a complex for use according to the present invention comprising at least two antigens or antigenic epitopes, wherein at least one antigen or antigenic epitope comprises or consists of a CD4<sup>+</sup> epitope and at least one antigen or antigenic epitope comprises or consists a CD8<sup>+</sup> epitope, provides an integrated immune response allowing simultaneous priming of CTLs and T<sub>h</sub> cells and is thus preferable to immunity against only one CD8<sup>+</sup> epitope or only one CD4<sup>+</sup> epitope. For example, the complex

for use according to the present invention may preferably comprise an Ealpha-CD4<sup>+</sup> epitope and a gp100-CD8<sup>+</sup> epitope.

**[0115]** Preferably, the complex for use according to the present invention comprises at least two antigens or antigenic epitopes, wherein the complex comprises an epitope of ASCL2, wherein the at least two antigens or antigenic epitopes comprise or consist of at least two, e.g. 2, 3, 4, 5, 6, 7, 8, 9, or more, CD4<sup>+</sup> epitopes and/or at least two, e.g. 2, 3, 4, 5, 6, 7, 8, 9, or more, CD8<sup>+</sup> epitopes. Thereby, the at least two antigens or antigenic epitopes are preferably different antigens or antigenic epitopes, more preferably the at least two antigens or antigenic epitopes are different from each other but relating to the same kind of tumor. A multi-antigenic vaccine will (i) avoid outgrowth of antigen-loss variants, (ii) target different tumor cells within a heterogeneous tumor mass and (iii) circumvent patient-to-patient tumor variability. Thus, the complex for use according to the present invention particularly preferably comprises at least four antigens or antigenic epitopes, in particular with at least two CD8<sup>+</sup> epitopes and at least two CD4<sup>+</sup> epitopes. Such a complex for use according to the present invention induces multi-epitopic CD8 CTLs and CD4 T<sub>h</sub> cells to function synergistically to counter tumor cells and promote efficient anti-tumor immunity. T<sub>h</sub> cells are also involved in the maintenance of long-lasting cellular immunity that was monitored after vaccination. Such a complex for use according to the present invention induces polyclonal, multi-epitopic immune responses and poly-functional CD8<sup>+</sup> and CD4<sup>+</sup> T cells, and thus efficacious anti-tumor activity.

**[0116]** Preferably, the complex for use according to the present invention comprises at least two antigens or antigenic epitopes, wherein the complex comprises an epitope of ASCL2, more preferably the complex for use according to the present invention comprises at least three antigens or antigenic epitopes, even more preferably the complex for use according to the present invention comprises at least four antigens or antigenic epitopes, particularly preferably the complex for use according to the present invention comprises at least five antigens or antigenic epitopes and most preferably the complex for use according to the present invention comprises at least six antigens or antigenic epitopes. The antigens or antigenic epitopes comprised by the complex for use according to the present invention may be the same or different, preferably the antigens or antigenic epitopes comprised by the complex for use according to the present invention are different from each other. Preferably, the complex for use according to the present invention comprises at least one CD4<sup>+</sup> epitope and at least one CD8<sup>+</sup> epitope.

**[0117]** Preferably, the complex for use according to the present invention comprises more than one CD4<sup>+</sup> epitope, wherein the complex comprises an epitope of ASCL2, e.g. two or more CD4<sup>+</sup> epitopes from the same antigen or from different antigens, and preferably no CD8<sup>+</sup> epitope. It is also preferred that the complex for use according to the present invention comprises more than one CD8<sup>+</sup> epitope, e.g. two or more CD8<sup>+</sup> epitopes from the same antigen or from different antigens, and preferably no CD4<sup>+</sup> epitope. Most preferably, however,

the complex for use according to the present invention comprises (i) at least one CD4<sup>+</sup> epitope, e.g. two or more CD4<sup>+</sup> epitopes from the same antigen or from different antigens, and (ii) at least one CD8<sup>+</sup> epitope, e.g. two or more CD8<sup>+</sup> epitopes from the same antigen or from different antigens.

**[0118]** For example, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may preferably comprise a gp100-CD8<sup>+</sup> epitope, an Ealpha-CD4<sup>+</sup> epitope, and a further CD4<sup>+</sup> epitope and a further CD8<sup>+</sup> epitope. Even more preferably, the complex for use according to the present invention may comprise a polypeptide or protein comprising a gp100-CD8<sup>+</sup> epitope and an Ealpha-CD4<sup>+</sup> epitope. For example, such a polypeptide or protein comprised by the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 14 or sequence variants thereof as defined above:

ESLKIS QAVHAAHAEI NEAGREVVGV GALKVPRNQD WLGVPRFAKF ASFEAQGALA

NIAVDKANLD VEQLESIINF EKLTEWTGS

SEQ ID NO: 14 (MAD5-cargo comprising OVA-CD4<sup>+</sup>, gp100-CD8<sup>+</sup>, Ealpha-CD4<sup>+</sup>, and OVA-CD8<sup>+</sup> epitopes)

**[0119]** For example, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may also comprise a gp70-CD8<sup>+</sup> epitope and/or a gp70-CD4<sup>+</sup> epitope. In particular, the complex for use according to the present invention may comprise a polypeptide or protein comprising a gp70-CD8<sup>+</sup> epitope and/or a gp70-CD4<sup>+</sup> epitope. For example, such a polypeptide or protein comprised by the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 43 or sequence variants thereof as defined above:

VTYHSPSYAYHQFERRAILNRLVQFIKDR

SEQ ID NO: 43 (Mad8-cargo comprising a gp70-CD8<sup>+</sup> and a gp70-CD4<sup>+</sup> epitope)

**[0120]** For example, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may preferably comprise at least one survivin epitope, such as a survivin CD8<sup>+</sup> epitope and/or a survivin CD4<sup>+</sup> epitope. More preferably, the complex for use according to the present invention may comprise a polypeptide or protein comprising a survivin CD8<sup>+</sup> epitope and/or a survivin CD4<sup>+</sup> epitope. More preferably, the complex for use according to the present invention may comprise a polypeptide or protein comprising more than one survivin CD8<sup>+</sup> epitope and/or more than one survivin CD4<sup>+</sup> epitope, such as two different survivin CD8<sup>+</sup> epitopes. For example, such a polypeptide or protein comprised by the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 44 or sequence variants thereof as defined above:

NYRIATFKNWPFLEDCAMEELTVSEFLKLDQR

SEQ ID NO: 44 (Mad11-cargo comprising survivin CD8<sup>+</sup> epitope 1 and survivin CD8<sup>+</sup> epitope 2)

**[0121]** For example, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may preferably comprise an epitope from a neoantigen. Even more preferably, the complex for use according to the present invention may comprise a polypeptide or protein comprising an epitope from a neoantigen, such as the neoantigen from MC-38 tumor cell line identified by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6. For example, such a polypeptide or protein comprised by the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 42 or sequence variants thereof as defined above:

HLELASMTNTELMSIV

SEQ ID NO: 42 (Mad9-cargo comprising the epitope from a neoantigen as described by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6).

**[0122]** For example, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may preferably comprise more than one, e.g. two or three, epitopes from neoantigens. Even more preferably, the complex for use according to the present invention may comprise a polypeptide or protein comprising more than one, e.g. two or three, epitopes from neoantigens, such as the neoantigens from MC-38 tumor cell line identified by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6. For example, such a polypeptide or protein comprised by the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 63 or sequence variants thereof as defined above:

LFRAAQLANDVVLQIMEHLELASMTNTELMSIVVISASIIVFNLLELEG

SEQ ID NO: 63 (Mad12-cargo comprising the epitope from a neoantigen as described by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6).

**[0123]** Preferably, the at least one antigen or antigenic epitope comprised by the complex for use according to the present invention is a peptide, polypeptide, or a protein. Examples of antigen or antigenic epitope of peptidic, polypeptidic, or proteic nature useful in the invention, include cancer/tumor antigens or antigenic epitopes thereof. Also disclosed are allergy antigens or antigenic epitopes thereof, auto-immune self-antigens or antigenic epitopes thereof, pathogenic antigens or antigenic epitopes thereof, and antigens or antigenic epitopes thereof from viruses, preferably from cytomegalovirus (CMV), orthopox variola virus, orthopox alastrim virus, parapox ovis virus, molluscum contagiosum virus, herpes simplex virus 1, herpes simplex virus 2, herpes B virus, varicella zoster virus, pseudorabies virus, human cytomegaly virus, human herpes virus 6, human herpes virus 7, Epstein-Barr virus, human herpes virus 8, hepatitis B virus, chikungunya virus, O'nyong'nyong virus, rubivirus, hepatitis C virus, GB virus C, West Nile virus, dengue virus, yellow fever virus, louping ill virus, St. Louis encephalitis virus, Japan B encephalitis virus, Powassan virus, FSME virus, SARS, SARS-associated corona virus, human corona virus 229E, human corona virus Oc43, Torovirus, human T cell lymphotropic virus type I, human T cell lymphotropic virus type II, HIV (AIDS), i.e. human immunodeficiency virus type 1 or human immunodeficiency virus type 2, influenza

virus, Lassa virus, lymphocytic choriomeningitis virus, Tacaribe virus, Junin virus, Machupo virus, Borna disease virus, Bunyamwera virus, California encephalitis virus, Rift Valley fever virus, sand fly fever virus, Toscana virus, Crimean-Congo haemorrhagic fever virus, Hazara virus, Khasan virus, Hantaan virus, Seoul virus, Prospect Hill virus, Puumala virus, Dobrava Belgrade virus, Tula virus, sin nombre virus, Lake Victoria Marburg virus, Zaire Ebola virus, Sudan Ebola virus, Ivory Coast Ebola virus, influenza virus A, influenza virus B, influenza viruses C, parainfluenza virus, malaria parasite (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi*), Marburg virus, measles virus, mumps virus, respiratory syncytial virus, human metapneumovirus, vesicular stomatitis Indiana virus, rabies virus, Mokola virus, Duvenhage virus, European bat lyssavirus 1 + 2, Australian bat lyssavirus, adenoviruses A-F, human papilloma viruses, condyloma virus 6, coriclyoma virus 11, polyoma viruses, adenoassociated virus 2, rotaviruses, orbiviruses, varicella including varizella zoster, etc., or antigens or antigenic epitopes from leishmania, trypanosomes, amibes, bacteria, etc., or may be selected from epitopes or from variants of the above antigens or antigenic epitopes. Preferably, epitopes as well as variants of antigens as defined above exhibit a sequence homology or identity of about 10 %, in particular at least 10 %, about 20 %, in particular at least 20 %, about 30 %, in particular at least 30 %, about 40 %, in particular at least 40 %, about 50 %, in particular at least 50 %, about 60 %, in particular at least 60 %, about 70 %, in particular at least 70 %, about 80 %, in particular at least 80 %, about 90 % in particular at least 90 %, at least 95 % or at least 98 % with one of the antigens or antigen sequences as shown or described above. In this context, the definition of epitopes and variants similarly applies as defined.

**[0124]** Examples of antigens or antigenic epitopes (not claimed) in the category of peptide, polypeptide or protein include a combination of multiple glioma epitopes such as those described in Novellino et al. (2005, *Cancer Immunol Immunother*, 54(3):187-207), Vigneron et al. (2013, *Cancer Immun*.13:15). However, a single complex may also comprise only a subset, i.e. one or more of all of said glioma epitopes. In such a case preferably different complexes comprise different subsets of all of said glioma epitopes, so that for example a vaccine comprising such different complexes comprises all of said glioma epitopes but distributed in the different complexes.

**[0125]** Moreover, a complex for use according to the invention, wherein the complex comprises an epitope of ASCL2, may also comprise at least one antigen or antigenic epitope, wherein said antigen or antigenic epitope is a polysaccharide, a lipid, a lipoprotein, and/or a glycolipid, in particular a polysaccharidic, lipidic, lipoproteic, and/or glycolipidic epitope.

**[0126]** In particular, the complex for use according to the invention, wherein the complex comprises an epitope of ASCL2, may comprise at least one antigen or antigenic epitope, wherein said antigen or antigenic epitope is polysaccharidic, lipidic, lipoproteic, and/or glycolipidic.

**[0127]** Preferably, said epitopes will be presented at the cell surface in an MHC class I and/or MHC class II context.

**[0128]** Preferably, said lipidic epitopes will be presented at the cell surface in a CD1 (cluster of differentiation 1) context.

**[0129]** The complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may also comprise at least one antigen or antigenic epitope, wherein said antigen or antigenic epitope is a small molecule drug or toxin.

**[0130]** Examples of cargo molecules within the category of small molecule drugs or toxins useful in the invention include cyclosporine A, paclitaxel, doxorubicin, methotrexate, 5-aminolevulinic acid, diphtheria toxin, sunitinib and those molecules reviewed in De wit Amer (2010, Neuro Oncol, 12(3):304-16).

**[0131]** The complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one antigen or antigenic epitope, preferably the complex for use according to the present invention comprises more than one antigen or antigenic epitope, in particular 2, 3, 4, 5, 6, 7, 8, 9, 10 or more antigens or antigenic epitopes, more preferably the complex for use according to the present invention comprises (at least) two or three antigens or antigenic epitopes, even more preferably the complex for use according to the present invention comprises (at least) four or five antigens or antigenic epitopes.

**[0132]** If more than one antigen or antigenic epitope is comprised by the complex for use according to the present invention it is understood that said antigen or antigenic epitope is in particular also covalently linked in the complex for use according to the present invention, e.g. to another antigen or antigenic epitope and/or to a component a), i.e. a cell penetrating peptide, and/or to a component c), i.e. a TLR peptide agonist.

**[0133]** The various antigens or antigenic epitopes comprised by the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, may be the same or different. Preferably, the various antigens or antigenic epitopes comprised by the complex for use according to the present invention are different from each other, thus providing a multi-antigenic and/or multi-epitopic complex.

**[0134]** Moreover, it is preferred that the more than one antigen or antigenic epitope, in particular 2, 3, 4, 5, 6, 7, 8, 9, 10 or more antigens or antigenic epitopes, are positioned consecutively in the complex for use according to the present invention. This means in particular that all antigens and/or antigenic epitopes comprised by the complex are positioned in a stretch, which is neither interrupted by component a), i.e. a cell penetrating peptide, nor by component c), i.e. a TLR peptide agonist. Rather, component a) and component c) are positioned in the complex for example before or after such a stretch of all antigens and/or antigenic epitopes. However, the antigens and/or antigenic epitopes positioned consecutively in such a way may be linked to each other for example by a spacer or linker as described below, which is neither component a), i.e. a cell penetrating peptide, nor component c), i.e. a

TLR peptide agonist.

**[0135]** Moreover, it is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises one or more epitopes of an antigen. Accordingly, the complex preferably comprises one or more epitopes of one or more antigens. For example the complex may comprise one or more epitopes of a first antigen and one or more epitopes of a second antigen; or the complex may comprise one or more epitopes of a first antigen, one or more epitopes of a second antigen, and one or more epitopes of a third antigen; or the complex may comprise one or more epitopes of a first antigen, one or more epitopes of a second antigen, one or more epitopes of a third antigen, and one or more epitopes of a fourth antigen; and so on. In a preferred embodiment, the complex comprises more than one epitope of one or more antigens; more preferably the antigenic epitopes of the same antigen are positioned consecutively (or overlapping) in the complex for use according to the present invention. This means in particular that all antigenic epitopes of the same antigen comprised by the complex are positioned in a stretch, which is neither interrupted by component a), i.e. a cell penetrating peptide, nor by component c), i.e. a TLR peptide agonist, nor by any other antigenic epitopes (i.e. epitopes from another antigen).

**[0136]** Alternatively, however, the various antigens and/or antigenic epitopes may also be positioned in any other way in the complex for use according to the present invention, for example with component a) and/or component c) positioned in between two or more antigens and/or antigenic epitopes, i.e. with one or more antigens and/or antigenic epitopes positioned between component a) and component c) (or vice versa) and, optionally, one or more antigens and/or antigenic epitopes positioned at the respective other end of component a) and/or component c).

**[0137]** It is understood that a number of different antigens or antigenic epitopes relating to colorectal cancer may be advantageously comprised by a single complex for use according to the present invention. Alternatively, a number of different antigens or antigenic epitopes relating to colorectal cancer may be distributed to subsets of different antigens or antigenic epitopes, in particular subsets complementing each other in the context of colorectal cancer which are comprised by different complexes according to the present invention, whereby such different complexes comprising different subsets may advantageously be administered simultaneously, e.g. in a single vaccine, to a subject in need thereof.

**[0138]** Preferably, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor epitope, which is an epitope of an antigen selected from the group consisting of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART and IL13Ralpha2. More preferably, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor epitope, which is an epitope of an antigen selected from the group consisting of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART and IL13Ralpha2. Those antigens are particularly useful

in the context of colorectal cancer. It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor antigen selected from the group consisting of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART and IL1 3Ralpha2, or a fragment thereof, or a sequence variant of a tumor antigen or a sequence variant of a fragment thereof. It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor antigen selected from the group consisting of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART and IL13Ralpha2, or a fragment thereof, or a sequence variant of a tumor antigen or a sequence variant of a fragment thereof. As used herein, a "fragment" of an antigen comprises at least 10 consecutive amino acids of the antigen, preferably at least 15 consecutive amino acids of the antigen, more preferably at least 20 consecutive amino acids of the antigen, even more preferably at least 25 consecutive amino acids of the antigen and most preferably at least 30 consecutive amino acids of the antigen. A "sequence variant" is as defined above, namely a sequence variant has an (amino acid) sequence which is at least 70%, preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% identical to the reference sequence. A "functional" sequence variant means in the context of an antigen/antigen fragment/epitope, that the function of the epitope(s), e.g. comprised by the antigen (fragment), is not impaired or abolished. Preferably, however, the amino acid sequence of the epitope(s), e.g. comprised by the cancer/tumor antigen (fragment) as described herein, is not mutated and, thus, identical to the reference epitope sequence.

**[0139]** As described above, suitable cancer/tumor epitopes of those antigens are known from the literature or can be identified by using cancer/tumor epitope databases, e.g. from van der Bruggen P, Stroobant V, Vigneron N, Van den Eynde B. Peptide database: T cell-defined tumor antigens. *Cancer Immun* 2013; URL: <http://www.cancerimmunity.org/peptide/>, wherein human tumor antigens recognized by CD4+ or CD8+ T cells are classified into four major groups on the basis of their expression pattern, or from the database "Tantigen" (TANTIGEN version 1.0, Dec 1, 2009; developed by Bioinformatics Core at Cancer Vaccine Center, Dana-Farber Cancer Institute; URL: <http://cvc.dfci.harvard.edu/tadb/>).

### EpCAM

**[0140]** Ep-Cam is a glycoprotein mediating cellular adhesion. The amino acid sequence of EpCAM is shown in the following:

·MAPPQVLAFGILLLAAATATFAAAQEECVGENYKLAVNGFVNNNRQCQGTSVGAQNTVICSKL ·  
AAKCLVMKAEMNGSKLGRRAKPEGALQNNDGLYDPDCDESGLFKAKQCNGTSMCWCVNT  
AGVRRRTDKDTEITCSERVRTYWIIIELKHKAREKPYDSKSLRTALQKEITTRYQLDPKFITSILYENN  
VITIDLVQNNSQKTQNDVDIADVAYYFEKDVKGESLFHSKKMDLTVNGEQLLDPCQTLIYVV  
DEKAPEFSMQGLKAGVIAVIVVVIAVVAGIVVLVISRKKRMAKYEKAEIKEMGEMHRELNA

[SEQ ID NO: 47]

**[0141]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 47 or a fragment or a variant thereof as described herein.

**[0142]** Several epitopes of EpCAM are known to the skilled person. A preferred EpCAM epitope, which is preferably comprised by the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, includes the following epitope (the epitope sequence shown in the following is a fragment of the above EpCAM sequence and is, thus, shown in the above EpCAM sequence underlined; the following epitope sequence may refer to one epitope or more than one (overlapping) epitopes):

**GLKAGVIAV**

[SEQ ID NO: 48]

**[0143]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 48 or a fragment or a variant thereof as described herein.

**HER-2/neu**

**[0144]** Her-2 belongs to the EGFR (epidermal growth factor receptor) family. Many HLA-A epitopes are known to the skilled person. The amino acid sequence of HER2 is shown in the following:

MELAALCRWGLLLALLPPGAASTQVCTGTDMLRHLQGCQVVQGNLE  
LTYLPTNASLFLQDIQEVTQGYVIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLN  
NTTPVTGASPQGLRELQLRSLTEIKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTIDTNR  
SRACHPCSPMCKGSRCWGESSEDQSLRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKH  
SDCLACLFHNHSIGICEHCPALVTYNTDTFESMPNPEGRYTFGASCYTACPVNYLSTDVGSCTL  
VCPLHNQEVTAEDEGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFGCKKIFGSLAF  
LPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLQDLSVFQNLQVIRGRILHNGAY  
SLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVWPWDQLFRNPHQALLHTANRPEDEC  
VGEGLACHQLCARGHCWGPCTQCVNCQFLRGQECVEECRVLQQLPREYVNRHCLPCH  
PECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSCVKPDLSYMPIWKFPDEEGACQP  
CPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRRLL  
QETELVEPLTPSGAMPNQAQMRLKTELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLR

ENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLTQLMPYGCLLDHVRENRGRLGS  
 QDLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHWKITDFGLARLLDIDETEYHAD  
 GGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQ  
 PPICTIDVYIMVKCWMIDSECPRFRELVSEFSRMARDPQRFVVIQNEGLGPASPLDSTFYRSL  
 LEDDDMGDLVDAEYLVPPQQGFFCPDPAPGAGGMVHHRHRSSTRSGGGDLTLGEPSEE  
 APRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPQLQRYSEDPTVPLPSETDGYVAPLT  
 CSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAGGAVENPE  
 YLTPQGGAAPQPHPPAFSPAFDNLYYWDQDPERGAPPSTFKGTPTAENPEYLGLDVPV

[SEQ ID NO: 70]

**[0145]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 70 or a fragment or a variant thereof as described herein. As described above, suitable cancer/tumor epitopes of Her-2 are known from the literature or can be identified by using cancer/tumor epitope databases, e.g. from van der Bruggen P, Stroobant V, Vigneron N, Van den Eynde B. Peptide database: T cell-defined tumor antigens. *Cancer Immun* 2013; URL: <http://www.cancerimmunity.org/peptide/>, wherein human tumor antigens recognized by CD4+ or CD8+ T cells are classified into four major groups on the basis of their expression pattern, or from the database "Tantigen" (TANTIGEN version 1.0, Dec 1, 2009; developed by Bioinformatics Core at Cancer Vaccine Center, Dana-Farber Cancer Institute; URL: <http://cvc.dfci.harvard.edu/tadb/>).

### Mucin-1 (MUC-1)

**[0146]** MUC-1 is a human epithelial mucin, acting on cell adhesion. The amino acid sequence of MUC-1 is shown in the following:

MTPGTQSPFFLLLLTVLTVVTGSGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSVLSHSP  
 GGSSTTQGQDVT LAPATEPASGSAATWGQDVTSPVTRPALGSTTPPAHDVTSAPDNKPAP  
GSTAPPAHGVTSAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGST  
 APPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPP  
 AHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAH  
 GVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT  
 SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAP  
 DTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTR  
 PAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAP  
 GSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGST  
 APPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPP  
 AHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAH  
 GVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT  
 SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAP  
 DTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDTRPAPGSTAPPAHGVT SAPDNR

PALGSTAPPVHNVTSACGSASGSASTLVHNGTSARATTPASKSTPFSIPSHHSDTPTLASHSTK  
 TDASSTHHSVPPLTSSNHSTSPQLSTGVSFFFLFHISNLQFNSSLEDPSTDYYQELQRDIEMFL  
 QIYKQCGFLGLSNIKFRPGSVVQLTLAFRECTINHDVETQFNQYKTEAASRYNLTISDVSVS  
 DVPFPFSAQSGAGVPGWGIALLVLVCVLVALAIVYLIALAVCQCRRKNYGQLDIFPARDTYHP  
 MSEYPTYHTHGRYVPPSSTDSPYEVSAGNGGSSLSYTNPAVAATSANL

[SEQ ID NO: 49]

**[0147]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 49 or a fragment or a variant thereof as described herein.

**[0148]** Several epitopes of MUC-1 are known to the skilled person. Preferred MUC-1 epitopes, which are preferably comprised by the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, include the following epitopes (the epitope sequences shown in the following are fragments of the above MUC-1 sequence and are, thus, shown in the above MUC-1 sequence underlined; each of the following epitope sequences may refer to one epitope or more than one (overlapping) epitopes):

GSTAPPVHN

[SEQ ID NO: 50]

TAPPAHGVTS

[SEQ ID NO: 51]

**[0149]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 50 and/or an amino acid sequence according to SEQ ID NO: 51.

#### TOMM34

**[0150]** TOMM34 is involved in the import of precursor proteins into mitochondria. Many epitopes thereof are known to the skilled person.

#### RNF 43

**[0151]** RNF43 is a RING-type E3 ubiquitin ligase and is predicted to contain a transmembrane domain, a protease-associated domain, an ectodomain, and a cytoplasmic RING domain. RNF43 is thought to negatively regulate Wnt signaling, and expression of RNF43 results in an increase in ubiquitination of frizzled receptors, an alteration in their subcellular distribution, resulting in reduced surface levels of these receptors. Many epitopes thereof are known to the skilled person.

**KOC1**

**[0152]** KOC1, also known as insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3) is an mRNA binding protein. No expression data are however available.

**Vascular endothelial growth factor (VEGF)/ Vascular endothelial growth factor receptor (VEGFR)**

**[0153]** Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. VEGF's normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels (collateral circulation) to bypass blocked vessels. There are three main subtypes of the receptors for VEGF (VEGFR), namely VEGFR1, VEGFR2 and VEGFR3.

**Beta subunit of human chorionic gonadotropin (βHCG)**

**[0154]** Human chorionic gonadotropin (hCG) is a hormone produced by the embryo following implantation. Some cancerous tumors produce this hormone; therefore, elevated levels measured when the patient is not pregnant can lead to a cancer diagnosis. hCG is heterodimeric with an  $\alpha$  (alpha) subunit identical to that of luteinizing hormone (LH), folliclestimulating hormone (FSH), thyroid-stimulating hormone (TSH), and  $\beta$  (beta) subunit that is unique to hCG. The  $\beta$ -subunit of hCG gonadotropin (beta-hCG) contains 145 amino acids and is encoded by six highly homologous genes.

**Survivin**

**[0155]** Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5, is a member of the inhibitor of apoptosis (IAP) family. The survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. The amino acid sequence of survivin is shown in the following:

MGAPTLPPAWQPFLKDHRISTFKNWPFLLEGCACTPERMAEGFIHCPTENEPDLAQCFCCFKEL  
EGWEPDDDPIEEHKKHSSGCAFLSVKKQFEELTLGEFLKLDRERAKNKIAKETNNKKFEETAKK  
VRRAIEQLAAMD

[SEQ ID NO: 52]

**[0156]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 52 or a fragment or a variant thereof as described herein.

**[0157]** Several epitopes of survivin are known to the skilled person. A preferred survivin epitope, which is preferably comprised by the complex for use according to the present invention, includes the following epitope (the epitope sequence shown in the following is a fragment of the above survivin sequence and is, thus, shown in the above survivin sequence underlined; the following epitope sequence may refer to one epitope or more than one (overlapping) epitopes):

RISTFKNWPF

[SEQ ID NO: 53]

**[0158]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 53.

**[0159]** Accordingly, it is preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises-an epitope of survivin. More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 52, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity). Even more preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 53. Most preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 95 or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

#### Carcino-embryonic antigen (CEA)

**[0160]** CEA is an intracellular adhesion glycoprotein. CEA is normally produced in gastrointestinal tissue during fetal development, but the production stops before birth. Therefore, CEA is usually present only at very low levels in the blood of healthy adults. The amino acid sequence of CEA is shown in the following:

MESPSAPPHRCIPWQRLLL TASLLTFWNPP TAKLTIE STPFNVAEGKEVLLL VHNLPQHLFGY  
SWYKGERVDG NRQIIGYVIGTQQATPGPAYS G REIIYPNASL LIQNI IQNDTGFYTLHVI KSDLV  
NEEATGQFRVYPELPKPSI SSNNSKPVEDKDAV AFTCEPETQD ATYLWV VNNQSLPVSPRLQLS  
NGNRTLTFNVTRNDT ASYKCETQNP VSARRSDS VILNVLYGPDAPTISPLNTSYRSGENNLSC

HAASNPPAQYSWFVNQTFQQSTQELFIPNITVNNSGSYTCQAHNSDTGLNRTTVTTITVYAEP  
 PKPFITSNNSNPVEDEDAVALTCEPEIQNTTYLWWVNNQSLPVSPRLQLSNDNRTLTLGVTRN  
 DVGPYECGIQNKLSDHSDPVILNVLYCPDDPTISPSYTYRPGVNLSCHAASNPPAQYSW  
 LIDGNIQQHTQELFISNITEKNSGLYTCQANNASGHSRTTVKTITVSAELPKPSISSNNSKPVED  
 KDAVAFTCEPEAQNTTYLWWVNGQSLPVSPRLQLSNGNRTLTLNVTRNDARAYVCGIQNS  
 VSANRSDPVTLDVLYCPDTPISPPDSSYLSGANLNLSCHASNPSPQYSWRINGIPQQHTQVL  
 FIAKITPNNNGTYACFVSNLATGRNNNSIVKSITVSASGTSPGLSAGATVGIMIGVLVGVAL

[SEQ ID NO: 54]

**[0161]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 54 or a fragment or a variant thereof as described herein.

**[0162]** Several epitopes of CEA are known to the skilled person. Preferred CEA epitopes, which are preferably comprised by the complex for use according to the present invention, include the following epitopes (the epitope sequences shown in the following are fragments of the above CEA sequence and are, thus, shown in the above CEA sequence underlined; each of the following epitope sequences may refer to one epitope or more than one (overlapping) epitopes):

YLSGANLNLS

[SEQ ID NO: 55]

SWRINGIPQQ

[SEQ ID NO: 56]

**[0163]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 55 and/or an amino acid sequence according to SEQ ID NO: 56.

**[0164]** Accordingly, it is preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an epitope of CEA. More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 54, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity). Even more preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 55 and/or a peptide having an amino acid sequence according to SEQ ID NO: 56. Most

preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 96 or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

**Transforming growth factor beta receptor 2 (TGF $\beta$ R2)**

**[0165]** TGF $\beta$  receptors are single pass serine/threonine kinase receptors. They exist in several different isoforms. TGF $\beta$ R2 is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with another receptor protein, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation.

**P53**

**[0166]** P53 is a tumor suppressor protein having a role in preventing genome mutation. P53 has many mechanisms of anticancer function and plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. In its anti-cancer role, p53 works through several mechanisms: it activates DNA repair proteins when DNA has sustained damage; it can arrest growth by holding the cell cycle at the G1/S regulation point on DNA damage recognition; and it can initiate apoptosis.

**Kirsten Ras (KRas)**

**[0167]** GTPase KRas also known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and KRAS, performs an essential function in normal tissue signaling, and the mutation of a KRAS gene is an essential step in the development of many cancers. Like other members of the ras subfamily, the KRAS protein is a GTPase and is an early player in many signal transduction pathways. KRAS is usually tethered to cell membranes because of the presence of an isoprene group on its C-terminus. The amino acid sequence of KRas is shown in the following:

MTEYKLVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEY  
SAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVDSEDPVMVLVGNKCDLPSRTVD  
TKQAQDLARSYGIPFIETSAKTRQRVEDAFYTLVREIRQYRLKKISKEEKTPGCVKIKKCIIM

[SEQ ID NO: 57]

**[0168]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to

SEQ ID NO: 57 or a fragment or a variant thereof as described herein.

**[0169]** Several epitopes of Kirsten Ras are known to the skilled person. A preferred Kirsten Ras epitope, which is preferably comprised by the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, includes the following epitope (the epitope sequence shown in the following is a fragment of the above Kirsten Ras sequence and is, thus, shown in the above Kirsten Ras sequence underlined; the following epitope sequence may refer to one epitope or more than one (overlapping) epitopes):

VVVGAGGVG

[SEQ ID NO: 58]

**[0170]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 58.

#### **O-Linked N-Acetylglucosamine (GlcNAc) Transferase (OGT)**

**[0171]** OGT (O-Linked N-Acetylglucosamine (GlcNAc) Transferase, O-GlcNAc transferase, OGTase, O-linked N-acetylglucosaminyltransferase, uridine diphospho-N-acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase, protein O-linked beta-N-acetylglucosamine transferase) is an enzyme with system name UDP-N-acetyl-D-glucosamine:protein-O-beta-N-acetyl-D-glucosaminyl transferase) is an enzyme with system name "UDP-N-acetyl-D-glucosamine:protein-O-beta-N-acetyl-D-glucosaminyl transferase". OGT catalyzes the addition of a single N-acetylglucosamine in O-glycosidic linkage to serine or threonine residues of intracellular proteins. OGT is a part of a host of biological functions within the human body. OGT is involved in the resistance of insulin in muscle cells and adipocytes by inhibiting the Threonine 308 phosphorylation of AKT, increasing the rate of IRS1 phosphorylation (at Serine 307 and Serine 632/635), reducing insulin signaling, and glycosylating components of insulin signals. Additionally, OGT catalyzes intracellular glycosylation of serine and threonine residues with the addition of N-acetylglucosamine. Studies show that OGT alleles are vital for embryogenesis, and that OGT is necessary for intracellular glycosylation and embryonic stem cell vitality. OGT also catalyzes the posttranslational modification that modifies transcription factors and RNA polymerase II, however the specific function of this modification is mostly unknown.

#### **Caspase 5 (CASPS)**

**[0172]** Caspase 5 is an enzyme that proteolytically cleaves other proteins at an aspartic acid residue, and belongs to a family of cysteine proteases called caspases. It is an inflammatory caspase, along with caspase 1, caspase 4 and the murine caspase 4 homolog caspase 11, and has a role in the immune system.

**Colorectal tumor-associated antigen-1 (COA-1)**

**[0173]** COA-1 was identified in 2003 by Maccalli et al. (Maccalli, C., et al., Identification of a colorectal tumor-associated antigen (COA-1) recognized by CD4(+) T lymphocytes. *Cancer Res*, 2003. 63(20): p. 6735-43) as strongly expressed by colorectal and melanoma cells (no data available). Its mutation may interfere with the differential recognition of tumor and normal cells.

**Melanoma-associated antigen (MAGE)**

**[0174]** The mammalian members of the MAGE (melanoma-associated antigen) gene family were originally described as completely silent in normal adult tissues, with the exception of male germ cells and, for some of them, placenta. By contrast, these genes were expressed in various kinds of tumors. Therefore, the complex for use according to the present invention preferably comprises an antigen of the MAGE-family (a "MAGE" antigen) or an epitope thereof. Of the MAGE family, in particular MAGE-A3 and MAGE-D4 are preferred, and MAGE-A3 is particularly preferred. The normal function of MAGE-A3 in healthy cells is unknown. MAGE-A3 is a tumor-specific protein, and has been identified on many tumors. The amino acid sequence of MAGE-A3 is shown in the following:

MPLEQRSQHCKPEEGLEARGEALGLVGAQAPATEEQEAASSSTLVEVTLGEVPAAESPDPPQS  
 PQGASSLPTTMNYPLWSQSYEDSSNQEEGPSTFPDLESEFQAALSRKVAELVHFLLKYRAREP  
 VTKAEMLGSVVGWNWQYFFPVIFSKAFSSLQLVFGIELMEVDPIGHLYIFATCLGLSYDGLLGDN  
 QIMPKAGLLIIVLAIIAREGDCAPEEKIWEELSVELVFEGRREDISILGDPKKLLTQHFVQENYLEYRQ  
 VPGSDPACYEFLWGPRALVETSYVKVLHHMVKISGGPHIS YPPLHEWVLREGEE

[SEQ ID NO: 59]

**[0175]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 59.

**[0176]** Several epitopes of MAGE-A3 are known to the skilled person. A preferred MAGE-A3 epitope, which is preferably comprised by the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, includes the following epitope (the epitope sequence shown in the following is a fragment of the above MAGE-A3 sequence and is, thus, shown in the above MAGE-A3 sequence underlined; the following epitope sequence may refer to one epitope or more than one (overlapping) epitopes):

KVAfLVHFL

[SEQ ID NO: 60]

**[0177]** Accordingly, a preferred complex for use according to the present invention, wherein

the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 60.

**Squamous cell carcinoma antigen recognized by T-cells (SART)**

**[0178]** Within the SART family, SART-3 is most preferred. Thus, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, preferably comprises an antigen of the SART-family (a "SART" antigen) or an epitope thereof; the complex for use according to the present invention more preferably comprises SART-3 or an epitope thereof. Squamous cell carcinoma antigen recognized by T-cells 3 possesses tumor epitopes capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes in cancer patients. SART-3 is thought to be involved in the regulation of mRNA splicing.

**IL13Ralpha2**

**[0179]** IL13Ralpha2 binds interleukin 13 (IL-13) with very high affinity (and can therefore sequester it) but does not allow IL-4 binding. It acts as a negative regulator of both IL-13 and IL-4, however the mechanism of this is still undetermined. The amino acid sequence of IL13Ralpha2 is shown in the following:

MAFVCLAIGCLYTFLISTFGCTSSSDTEIKVNPPQDFEIVDPGYLGYLYLQWQPPLSLDHFKECT  
VEYELKYRNIGSETWKTIIKNLHYKDGFDLNKGIEAKIHTLLPWQCTNGSEVQSSWAETTYWIS  
PQGIPETKVQDMDCVYYNWQYLLCSWKGIGVLLDTNYNLFYWYEGLDHALQCVDYIKAD  
GQNIGCRFPYLEASDYKDFYICVNGSSENKPIRSSYFTFQLQNIVKPLPPVYLFTRESSCEIKLKW  
SIPLGPIPACRFDYIEIREDDTTLVTATVENETYTLKTTNETRQLCFVVRSKVNIYCSDDGIWSEW  
SDKQCWEGEDLSKKTLLRFWLPFGFILLIVFVTG LLLRKPNTPKMIPEFFCDT

[SEQ ID NO: 61]

**[0180]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 61 or a fragment or a variant thereof as described herein.

**[0181]** Several epitopes of IL13Ralpha2 are known to the skilled person. A preferred IL13Ralpha2 epitope, which is preferably comprised by the complex for use according to the present invention, includes the following epitope (the epitope sequence shown in the following is a fragment of the above IL13Ralpha2 sequence and is, thus, shown in the above IL13Ralpha2 sequence underlined; the following epitope sequence may refer to one epitope or more than one (overlapping) epitopes):

**LPFGFIL**

[SEQ ID NO: 62]

**[0182]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 62.

**ASCL2 (Achaete-scute homolog 2)**

**[0183]** ASCL2 is a basic helix-loop-helix transcription factor essential for the maintenance of proliferating trophoblasts during placental development. ASCL2 was found to be a putative regulator of proliferation that is overexpressed in intestinal neoplasia. The amino acid sequence of ASCL2 is shown in the following:

MDGGTLPRSAPPAPPVPGCAARRPASPELLRCSRRRPATAETGGAAVARRNERERNRV  
KLVNLGFQALRQHVPHGGASKKLSKVETLRSAVEYIRALQRLLAEHDAVRNALAGGLRPQAVR  
PSAPRGPPGTTPVAASPSRASSSPGRGGSSEPGSPRSAYSSDDSGCEGALSPAERELLDFSSWLG  
GY

[SEQ ID NO: 92]

**[0184]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 92 or a fragment or a variant thereof as described herein.

**[0185]** Several epitopes of ASCL2 are known to the skilled person. Preferred ASCL2 epitopes, which are preferably comprised by the complex for use according to the present invention, include the following epitopes (the epitope sequences shown in the following are fragments of the above ASCL2 sequence and are, thus, shown in the above ASCL2 sequence underlined; each of the following epitope sequences may refer to one epitope or more than one (overlapping) epitopes):

SAVEYIRALQ

[SEQ ID NO: 93]

ERELLDFSSW

[SEQ ID NO: 94]

**[0186]** Accordingly, a preferred complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises an amino acid sequence according to SEQ ID NO: 93 and/or an amino acid sequence according to SEQ ID NO: 94.

**[0187]** Accordingly, the complex for use according to the present invention comprises an epitope of ASCL2. Preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 92, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity). More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 93 and/or a peptide having an amino acid sequence according to SEQ ID NO: 94. Most preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 97 or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

**[0188]** Preferably, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor epitope, which is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin, CEA, KRas, MAGE-A3 and IL13Ralpha2, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55, 56, 58, 60 and 62; preferably, the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises at least one tumor epitope, which is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin, CEA, KRas, MAGE-A3, and IL13Ralpha2, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55, 56, 58, 60, 62, 93 and 94; more preferably the at least one tumor epitope is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin, CEA, KRas and MAGE-A3, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55, 56, 58 and 60; more preferably the at least one tumor epitope is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin, CEA, KRas, and MAGE-A3, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55, 56, 58, 60, 93 and 94; even more preferably the at least one tumor epitope is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin and CEA, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55 and 56; even preferably the at least one tumor epitope is an epitope of an antigen selected from the group consisting of EpCAM, MUC-1, survivin and CEA, such as an epitope according to any of SEQ ID NOs 48, 50, 51, 53, 55, 56, 93 and 94; and most preferably the at least one tumor epitope is an epitope of an antigen selected from the group consisting of EpCAM, survivin, and CEA, such as an epitope according to any of SEQ ID NOs 48, 53, 55, 56, 93 and 94.

**[0189]** It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises

1. i) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

2. ii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;
3. iii) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof;
4. iv) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof;
5. v) one or more epitopes of KRas (such as the epitope according to SEQ ID NO: 58) or functional sequence variants thereof; and/or
6. vi) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0190]** It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises

- vii) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;
- viii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;
- ix) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof;
- x) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof;
- xi) one or more epitopes of KRas (such as the epitope according to SEQ ID NO: 58) or functional sequence variants thereof; and/or
- xii) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0191]** The complex according to the invention comprises one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0192]** As described above, further epitopes of those antigens (in addition to the exemplified epitopes) can easily be retrieved from cancer/tumor epitope databases, e.g. from van der Bruggen P, Stroobant V, Vigneron N, Van den Eynde B. Peptide database: T cell-defined tumor antigens. *Cancer Immun* 2013; URL: <http://www.cancerimmunity.org/peptide/>, or from the database "Tantigen" (TANTIGEN version 1.0, Dec 1, 2009; developed by Bioinformatics Core at Cancer Vaccine Center, Dana-Farber Cancer Institute; URL: <http://cvc.dfcf.harvard.edu/tadb/>).

**[0193]** A "sequence variant" is as defined above, namely a sequence variant has an (amino acid) sequence which is at least 70%, at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% identical to the reference sequence. A "functional" sequence variant means in the context of an epitope, that the function as an epitope is not impaired or abolished. Preferably, however, the amino acid sequence of an epitope of a cancer/tumor antigen as described herein is not mutated and, thus, identical to the reference epitope sequence.

**[0194]** It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises

- a fragment of EpCAM comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of MUC-1 comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of survivin comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of CEA comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of KRas comprising one or more epitopes or a functional sequence variant thereof; and/or
- a fragment of MAGE-A3 comprising one or more epitopes or a functional sequence variant thereof.

**[0195]** It is also preferred that the complex for use according to the present invention, wherein the complex comprises an epitope of ASCL2, comprises

- a fragment of EpCAM comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of MUC-1 comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of survivin comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of CEA comprising one or more epitopes or a functional sequence variant thereof;
- a fragment of KRas comprising one or more epitopes or a functional sequence variant thereof; and/or
- a fragment of MAGE-A3 comprising one or more epitopes or a functional sequence variant thereof.

**[0196]** The complex according to the invention comprises a fragment of ASCL2 comprising one or more epitopes or a functional sequence variant thereof.

**[0197]** As used herein, a "fragment" of an antigen comprises at least 10 consecutive amino acids of the antigen, preferably at least 15 consecutive amino acids of the antigen, more preferably at least 20 consecutive amino acids of the antigen, even more preferably at least 25 consecutive amino acids of the antigen and most preferably at least 30 consecutive amino acids of the antigen. Accordingly, a fragment of EpCAM comprises at least 10 consecutive amino acids of EpCAM (SEQ ID NO: 47), preferably at least 15 consecutive amino acids of EpCAM (SEQ ID NO: 47), more preferably at least 20 consecutive amino acids of EpCAM (SEQ ID NO: 47), even more preferably at least 25 consecutive amino acids of EpCAM (SEQ ID NO: 47) and most preferably at least 30 consecutive amino acids of EpCAM (SEQ ID NO: 47); a fragment of MUC-1 comprises at least 10 consecutive amino acids of MUC-1 (SEQ ID NO: 49), preferably at least 15 consecutive amino acids of MUC-1 (SEQ ID NO: 49), more preferably at least 20 consecutive amino acids of MUC-1 (SEQ ID NO: 49), even more preferably at least 25 consecutive amino acids of MUC-1 (SEQ ID NO: 49) and most preferably at least 30 consecutive amino acids of MUC-1 (SEQ ID NO: 49); a fragment of survivin comprises at least 10 consecutive amino acids of survivin (SEQ ID NO: 52), preferably at least 15 consecutive amino acids of survivin (SEQ ID NO: 52), more preferably at least 20 consecutive amino acids of survivin (SEQ ID NO: 52), even more preferably at least 25 consecutive amino acids of survivin (SEQ ID NO: 52) and most preferably at least 30 consecutive amino acids of survivin (SEQ ID NO: 52); a fragment of CEA comprises at least 10 consecutive amino acids of CEA (SEQ ID NO: 54), preferably at least 15 consecutive amino acids of CEA (SEQ ID NO: 54), more preferably at least 20 consecutive amino acids of CEA (SEQ ID NO: 54), even more preferably at least 25 consecutive amino acids of CEA (SEQ ID NO: 54) and most preferably at least 30 consecutive amino acids of CEA (SEQ ID NO: 54); a fragment of KRas comprises at least 10 consecutive amino acids of KRas (SEQ ID NO: 57), preferably at least 15 consecutive amino acids of KRas (SEQ ID NO: 57), more preferably at least 20 consecutive amino acids of KRas (SEQ ID NO: 57), even more preferably at least 25 consecutive amino acids of KRas (SEQ ID NO: 57) and most preferably at least 30 consecutive amino acids of KRas (SEQ ID NO: 57); and a fragment of MAGE-A3 comprises at least 10 consecutive amino acids of MAGE-A3 (SEQ ID NO: 59), preferably at least 15 consecutive amino acids of MAGE-A3 (SEQ ID NO: 59), more preferably at least 20 consecutive amino acids of MAGE-A3 (SEQ ID NO: 59), even more preferably at least 25 consecutive amino acids of MAGE-A3 (SEQ ID NO: 59) and most preferably at least 30 consecutive amino acids of MAGE-A3 (SEQ ID NO: 59). Moreover, a fragment of ASCL2 comprises at least 10 consecutive amino acids of ASCL2 (SEQ ID NO: 92), preferably at least 15 consecutive amino acids of ASCL2 (SEQ ID NO: 92), more preferably at least 20 consecutive amino acids of ASCL2 (SEQ ID NO: 92), even more preferably at least 25 consecutive amino acids of ASCL2 (SEQ ID NO: 92) and most preferably at least 30 consecutive amino acids of ASCL2 (SEQ ID NO: 92).

**[0198]** A functional sequence variant of such a fragment has an (amino acid) sequence, which is at least 70%, at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99%

identical to the reference sequence, and the epitope function of at least one, preferably all, epitope(s) comprised by the fragment is maintained.

**[0199]** Preferably, such a complex as defined by claim 1 may also comprise

xiii) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xiv) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof; and

xv) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0200]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2.

**[0201]** It is also preferred that such a complex as defined by claim 1 may also comprise

xvi) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xvii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xviii) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof; and

xix) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0202]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2.

**[0203]** It is also preferred that such a complex as defined by claim 1 may also comprise

xx) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or

functional sequence variants thereof;

xxi) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xxii) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof; and

xxiii) one or more epitopes of KRas (such as the epitope according to SEQ ID NO: 58) or functional sequence variants thereof.

**[0204]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR, (3hCG, survivin, TGF $\beta$ R2, p53, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0205]** It is also preferred that such a complex as defined by claim 1 may also comprise

xxiv) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xxv) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof;

xxvi) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof; and

xxvii) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0206]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL 13Ralpha2.

**[0207]** It is also preferred that such a complex as defined by claim 1 may also comprise

xxviii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xxix) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and

xxx) one or more epitopes of MAGE-A3 (such as the epitope according to SEQ ID NO: 60) or functional sequence variants thereof.

**[0208]** Such a complex does preferably not comprise any epitope of EpCAM, HER-2, TOMM34, RNF 43, KOC1, VEGFR, PHCG, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of EpCAM, HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, SART or IL 13Ralpha2.

**[0209]** More preferably, such a complex as defined by claim 1 may also comprise

xxxi) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xxxii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xxxiii) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and/or

xxxiv) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0210]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR, PHCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0211]** More preferably, such a complex as defined by claim 1 may also comprise one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof and, in addition,

xxv) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xxvi) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xxvii) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof;

; and/or

xxxviii) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0212]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0213]** More preferably, such a complex as defined in claim 1 comprises one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof; and, in addition,

xxxix) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xl) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and/or

xli) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0214]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0215]** Particularly preferably, such a complex as defined by claim 1 may also comprise

xlii) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

xliii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof;

xliv) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and

xlv) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0216]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2,

TOMM34, RNF 43, KOC1, VEGFR, (3hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0217]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

xlvi) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof;

xlvii) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof;

xlviii) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof; and

xlix) one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0218]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, (3hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0219]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

i) one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof;

ii) one or more epitopes of MUC-1 (such as the epitope according to SEQ ID NO: 50 and/or the epitope according to SEQ ID NO: 51) or functional sequence variants thereof; and

iii) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0220]** Such a complex does preferably not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR, (3hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of HER-2, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0221]** It is also particularly preferred that such a complex as defined by claim 1 comprises

iii) one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the

epitope according to SEQ ID NO: 56) or functional sequence variants thereof;

liv) one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and

lv) one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0222]** Such a complex does preferably not comprise any epitope of HER-2, EpCAM, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0223]** Even more preferably, the complex as defined by claim 1 comprises in N- to C-terminal direction:

- one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof;
- one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and
- one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0224]** Such a complex does preferably not comprise any epitope of HER-2, EpCAM, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL 13Ralpha2

**[0225]** Even more preferably, the complex as defined by claim 1 comprises in N- to C-terminal direction:

1. i) a peptide having an amino acid sequence according to SEQ ID NO: 54, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
2. ii) a peptide having an amino acid sequence according to SEQ ID NO: 52, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly

preferably at least 95%, most preferably at least 99% sequence identity); and

3. iii) a peptide having an amino acid sequence according to SEQ ID NO: 92, or a fragment thereof having a length of at least 10 amino acids (preferably at least 15 amino acids, more preferably at least 20 amino acids, even more preferably at least 25 amino acids and most preferably at least 30 amino acids), or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

**[0226]** Such a complex does preferably not comprise any further antigen or further epitopes of antigens other than CEA, survivin and ASCL2, more preferably such a complex does not comprise any other (tumor) epitope.

**[0227]** Preferably, in such a complex, the C-terminus of (i) the peptide having an amino acid sequence according to SEQ ID NO: 54 or the fragment or variant thereof is directly linked to the N-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 52 or the fragment or variant thereof; and the C-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 52 or the fragment or variant thereof is directly linked to the N-terminus of (iii) the peptide having an amino acid sequence according to SEQ ID NO: 92 or the fragment or variant thereof.

**[0228]** Still more preferably, the complex as defined by claim 1 comprises in N- to C-terminal direction:

1. i) a peptide having an amino acid sequence according to SEQ ID NO: 96, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
2. ii) a peptide having an amino acid sequence according to SEQ ID NO: 95, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity); and
3. iii) a peptide having an amino acid sequence according to SEQ ID NO: 97, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

**[0229]** Such a complex does preferably not comprise any further antigen or further epitopes of

antigens other than CEA, survivin and ASCL2, more preferably such a complex does not comprise any other (tumor) epitope.

**[0230]** Preferably, in such a complex, the C-terminus of (i) the peptide having an amino acid sequence according to SEQ ID NO: 96 or the variant thereof is directly linked to the N-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 95 or the variant thereof; and the C-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 95 or the variant thereof is directly linked to the N-terminus of (iii) the peptide having an amino acid sequence according to SEQ ID NO: 97 or the variant thereof.

**[0231]** Most preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 98 or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity). Such a complex does preferably not comprise any further antigen or further epitopes of antigens other than CEA, survivin and ASCL2, more preferably such a complex does not comprise any other (tumor) epitope.

**[0232]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

- one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof; and
- one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0233]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0234]** It is also particularly preferred that such a complex as defined by claim 1 comprises

- one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or functional sequence variants thereof; and
- one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0235]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, CEA, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-

1, MAGE, SART or IL 13Ralpha2.

**[0236]** It is also particularly preferred that such a complex as defined by claim 1 comprises

- one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof; and
- one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0237]** Such a complex does preferably not comprise any epitope of HER-2, EpCAM, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL 13Ralpha2.

**[0238]** It is also particularly preferred that such a complex as defined by claim 1 comprises

- one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and
- one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0239]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, EpCAM, CEA, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0240]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

- one or more epitopes of survivin (such as the epitope according to SEQ ID NO: 53) or functional sequence variants thereof; and
- one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0241]** Such a complex does preferably not comprise any epitope of HER-2, EpCAM, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0242]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

- one or more epitopes of EpCAM (such as the epitope according to SEQ ID NO: 48) or

functional sequence variants thereof.

**[0243]** Such a complex does preferably not comprise any epitope of HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, CEA, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0244]** It is also particularly preferred that such a complex as defined by claim 1 may also comprise

- one or more epitopes of CEA (such as the epitope according to SEQ ID NO: 55 and/or the epitope according to SEQ ID NO: 56) or functional sequence variants thereof.

**[0245]** Such a complex does preferably not comprise any epitope of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2. More preferably, such a complex does not comprise any epitope of EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF $\beta$ R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**[0246]** It is also particularly preferred that such a complex as defined by claim 1 comprises

- one or more epitopes of ASCL2 (such as the epitope according to SEQ ID NO: 93 and/or the epitope according to SEQ ID NO: 94) or functional sequence variants thereof.

**[0247]** Such a complex does preferably not comprise any epitope of EpCAM, HER-2, MUC-1, CEA, TOMM34, RNF 43, KOC1, VEGFR,  $\beta$ hCG, survivin, TGF(3R2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART or IL13Ralpha2.

**Component c) - TLR peptide agonist**

**[0248]** In the complex for use according to the present invention, the TLR peptide agonist allows an increased targeting of the vaccine towards dendritic cells along with self-adjuvancy. Physical linkage of a TLR peptide agonist to the CPP and the at least one antigen or antigenic epitope according to the present invention in the complex for use according to the present invention provides an enhanced immune response by simultaneous stimulation of antigen presenting cells, in particular dendritic cells, that internalize, metabolize and display antigen(s).

**[0249]** As used in the context of the present invention, a "TLR peptide agonist" is an agonist of a Toll-like receptor (TLR), i.e. it binds to a TLR and activates the TLR, in particular to produce a biological response. Moreover, the TLR peptide agonist is a peptide, a polypeptide or a protein as defined above. Preferably, the TLR peptide agonist comprises from 10 to 150 amino acids, more preferably from 15 to 130 amino acids, even more preferably from 20 to 120 amino acids, particularly preferably from 25 to 110 amino acids, and most preferably from 30 to 100 amino acids.

**[0250]** Toll like receptors (TLRs) are transmembrane proteins that are characterized by extracellular, transmembrane, and cytosolic domains. The extracellular domains containing leucine-rich repeats (LRRs) with horseshoe-like shapes are involved in recognition of common molecular patterns derived from diverse microbes. Toll like receptors include TLRs1 - 10. Compounds capable of activating TLR receptors and modifications and derivatives thereof are well documented in the art. TLR1 may be activated by bacterial lipoproteins and acetylated forms thereof, TLR2 may in addition be activated by Gram positive bacterial glycolipids, LPS, LP A, LTA, fimbriae, outer membrane proteins, heat shock proteins from bacteria or from the host, and Mycobacterial lipoarabinomannans. TLR3 may be activated by dsRNA, in particular of viral origin, or by the chemical compound poly(LC). TLR4 may be activated by Gram negative LPS, LTA, Heat shock proteins from the host or from bacterial origin, viral coat or envelope proteins, taxol or derivatives thereof, hyaluronan containing oligosaccharides and fibronectins. TLR5 may be activated with bacterial flagellae or flagellin. TLR6 may be activated by mycobacterial lipoproteins and group B streptococcus heat labile soluble factor (GBS- F) or staphylococcus modulins. TLR7 may be activated by imidazoquinolines. TLR9 may be activated by unmethylated CpG DNA or chromatin - IgG complexes.

**[0251]** According to the invention, the TLR peptide agonist comprised by the complex for use according to the present invention is an agonist of TLR, 2 and/or 4, and, optionally, TLR1, 5, 6 and/or 10. TLRs are expressed either on the cell surface (TLR1, 2, 4, 5, 6, and 10) or on membranes of intracellular organelles, such as endosomes (TLR3, 4, 7, 8, and 9). The natural ligands for the endosomal receptors turned out to be nucleic acid-based molecules (except for TLR4). The cell surface-expressed TLR1, 2, 4, 5, 6, and 10 recognize molecular patterns of extracellular microbes (Monie, T. P., Bryant, C. E., et al. 2009: Activating immunity: Lessons from the TLRs and NLRs. Trends Biochem. Sci. 34(11), 553-561). TLRs are expressed on several cell types but virtually all TLRs are expressed on DCs allowing these specialized cells to sense all possible pathogens and danger signals.

**[0252]** However, TLR2, 4, and 5 are constitutively expressed at the surface of DCs. The TLR peptide agonist comprised by the complex for use according to the present invention is a peptide agonist of TLR2, and/or TLR4.. Particularly preferably, the TLR peptide agonist is a TLR4 peptide agonist. Most preferably, the TLR peptide agonist is one TLR peptide agonist, which is both, a TLR2 and a TLR4 agonist. TLR2 can detect a wide variety of ligands derived from bacteria, viruses, parasites, and fungi. The ligand specificity is often determined by the interaction of TLR2 with other TLRs, such as TLR1, 6, or 10, or non-TLR molecules, such as dectin-1, CD14, or CD36. The formation of a heterodimer with TLR1 enables TLR2 to identify

triacyl lipoproteins or lipopeptides from (myco)bacterial origin, such as Pam3CSK4 and peptidoglycan (PGA; Gay, N. J., and Gangloff, M. (2007): Structure and function of Toll receptors and their ligands. *Annu. Rev. Biochem.* 76, 141-165; Spohn, R., Buwitt-Beckmann, U., et al. (2004): Synthetic lipopeptide adjuvants and Toll-like receptor 2-Structure-activity relationships. *Vaccine* 22(19), 2494-2499). Heterodimerization of TLR2 and 6 enables the detection of diacyl lipopeptides and zymosan. Lipopolysaccharide (LPS) and its derivatives are ligands for TLR4 and flagellin for TLR5 (Bryant, C. E., Spring, D. R., et al. (2010). The molecular basis of the host response to lipopolysaccharide. *Nat. Rev. Microbiol.* 8(1), 8-14).

**[0253]** TLR2 interacts with a broad and structurally diverse range of ligands, including molecules expressed by microbes and fungi. Multiple TLR2 agonists have been identified, including natural and synthetic lipopeptides (e.g. *Mycoplasma fermentas* macrophage-activating lipopeptide (MALP-2)), peptidoglycans (PG such as those from *S. aureus*), lipopolysaccharides from various bacterial strains (LPS), polysaccharides (e.g. zymosan), glycosylphosphatidylinositol-anchored structures from gram positive bacteria (e.g. lipoteichoic acid (LTA) and lipoarabinomannan from mycobacteria and lipomannas from *M. tuberculosis*). Certain viral determinants may also trigger via TLR2 (Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nat Immunol.* 2009; 10(11):1200-7). Bacterial lipopeptides are structural components of cell walls. They consist of an acylated s-glycerylcysteine moiety to which a peptide can be conjugated via the cysteine residue. Examples of TLR2 agonists, which are bacterial lipopeptides, include MALP-2 and its synthetic analogue di-palmitoyl-S-glyceryl cysteine (Pam<sub>2</sub>Cys) or tri-palmitoyl-S-glyceryl cysteine (Pam<sub>3</sub>Cys).

**[0254]** A diversity of ligands interact with TLR4, including Monophosphoryl Lipid A from *Salmonella minnesota* R595 (MPLA), lipopolysaccharides (LPS), mannans (*Candida albicans*), glycoinositolphospholipids (*Trypanosoma*), viral envelope proteins (RSV and MMTV) and endogenous antigens including fibrinogen and heat-shock proteins. Such agonists of TLR4 are for example described in Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell.* Feb 24; 2006: 124(4):783-801 or in Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun.* Oct 30; 2009 388(4):621-5. LPS, which is found in the outer membrane of gram negative bacteria, is the most widely studied of the TLR4 ligands. Suitable LPS-derived TLR4 agonist peptides are described for example in WO 2013/120073 (A1).

**[0255]** TLR5 is triggered by a region of the flagellin molecule expressed by nearly all motile bacteria. Thus, flagellin, or peptides or proteins derived from flagellin and/or variants or fragments of flagellin are also suitable as TLR peptide agonists which may additionally be comprised by the complex for use according to the present invention.

**[0256]** Examples of TLR peptide agonists thus include the TLR2 lipopeptide agonists MALP-2, Pam<sub>2</sub>Cys and Pam<sub>3</sub>Cys or modifications thereof, different forms of the TLR4 agonist LPS, e.g. *N. meningitidis* wild-type L3-LPS and mutant penta-acylated LpxL1-LPS, and the TLR5 agonist flagellin.

**[0257]** However, it is preferred that the TLR peptide agonist comprised by the complex for use according to the present invention is neither a lipopeptide nor a lipoprotein, neither a glycopeptide nor a glycoprotein, more preferably, the TLR peptide agonist comprised by the complex for use according to the present invention is a classical peptide, polypeptide or protein as defined herein.

**[0258]** A preferred TLR2 peptide agonist is annexin II or an immunomodulatory fragment thereof, which is described in detail in WO 2012/048190 A1 and US patent application 13/0331546, in particular a TLR2 peptide agonist comprising an amino acid sequence according to SEQ ID NO: 4 or SEQ ID NO: 7 of WO 2012/048190 A1 or fragments or variants thereof are preferred.

**[0259]** Thereby, a TLR2 peptide agonist comprising or consisting of an amino acid sequence according to SEQ ID NO: 15 or a sequence variant thereof as described above is particularly preferred as component c), i.e. as the at least one TLR peptide agonist, comprised by the complex for use according to the present invention.

STVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 15 (TLR2 peptide agonist Anaxa)

**[0260]** A particularly preferred functional sequence variant of the TLR peptide agonist according to SEQ ID NO: 15 is the TLR peptide agonist according to SEQ ID NO: 71:

STVHEILSKLSLEGDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 71

**[0261]** Accordingly, a TLR2 peptide agonist comprising or consisting of an amino acid sequence according to SEQ ID NO: 71 or a sequence variant thereof as described above is particularly preferred as component c), i.e. as the at least one TLR peptide agonist, comprised by the complex. In other words, the TLR peptide agonist in the complex most preferably comprises or consists of a peptide having an amino acid sequence according to SEQ ID NO: 71, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity).

**[0262]** Regarding TLR4, TLR peptides agonists are particularly preferred, which in particular correspond to motifs that bind to TLR4, in particular (i) peptides mimicking the natural LPS ligand (RS01: Gln-Glu-Ile-Asn-Ser-Ser- Tyr and RS09: Ala-Pro-Pro-His-Ala-Leu-Ser) and (ii) Fibronectin derived peptides. The cellular glycoprotein Fibronectin (FN) has multiple isoforms generated from a single gene by alternative splicing of three exons. One of these isoforms is the extra domain A (EDA), which interacts with TLR4.

**[0263]** Further suitable TLR peptide agonists comprise a fibronectin EDA domain or a fragment or variant thereof. Such suitable fibronectin EDA domains or a fragments or variants

thereof are disclosed in EP 1 913 954 B1, EP 2 476 440 A1, US 2009/0220532 A1, and WO 2011/101332 A1. Thereby, a TLR4 peptide agonist comprising or consisting of an amino acid sequence according to SEQ ID NO: 45 or a sequence variant thereof as described above is particularly preferred as component c), i.e. as the at least one TLR peptide agonist, comprised by the complex for use according to the present invention.

NIIDRPKGGLAFTDVDSIKIAWESPQGQVSRVTYSSPEDGIRELFPAPDGEDDTAELQGLRP

GSEYTVSVVALHDDMESQPLIGIQRST

SEQ ID NO: 45 (TLR4 peptide agonist EDA)

**[0264]** In addition, high-mobility group box 1 protein (HMGB1) and peptide fragments thereof are assumed to be TLR4 agonists. Such HMGB1-derived peptides are for example disclosed in US 201110236406 A1.

**[0265]** Moreover, also the TLR agonist according to SEQ ID NO: 15 and the TLR agonist according to SEQ ID NO: 71 may act as TLR4 agonist. Accordingly, a TLR4 peptide agonist comprising or consisting of an amino acid sequence according to SEQ ID NO: 15 or 71 or a functional sequence variant thereof is particularly preferred as component c), i.e. as the at least one TLR peptide agonist, comprised by the complex.

**[0266]** The complex for use according to the present invention comprises at least one TLR2 and/or TLR4 peptide agonist, preferably the complex for use according to the present invention may also comprise more than one TLR peptide agonist, in particular 2, 3, 4, 5, 6, 7, 8, 9, 10 or more TLR peptide agonists, more preferably the complex for use according to the present invention comprise (at least) two or three TLR peptide agonists, even more preferably the complex for use according to the present invention comprises (at least) four or five TLR peptide agonists. If more than one TLR peptide agonist is comprised by the complex for use according to the present invention it is understood that said TLR peptide agonist is in particular also covalently linked in the complex for use according to the present invention, e.g. to another TLR peptide agonist and/or to a component a), i.e. a cell penetrating peptide, and/or to a component b), i.e. an antigen or antigenic epitope.

**[0267]** In a particularly preferred embodiment, the complex for use according to the present invention comprises one single TLR2 or 4 peptide agonist. In particularly, in this particularly preferred embodiment, the complex for use according to the present invention comprises one single TLR 2 or 4 peptide agonist and no further component having TLR agonist properties except the one single TLR peptide agonist as described.

**[0268]** The various TLR peptide agonists comprised by the complex for use according to the present invention may be the same or different. Preferably, the various TLR peptide agonists comprised by the complex for use according to the present invention are different from each other.

**[0269]** Moreover, it is preferred that the more than one antigen or antigenic epitope, in

particular 2, 3, 4, 5, 6, 7, 8, 9, 10 antigens or antigenic epitopes, or more TLR peptide agonists, in particular 2, 3, 4, 5, 6, 7, 8, 9, 10 TLR agonists, are positioned consecutively in the complex for use according to the present invention. This means in particular that all TLR peptide agonists comprised by the complex are positioned in a stretch, which is neither interrupted by component a), i.e. a cell penetrating peptide, nor by component b), i.e. at least one antigen or antigenic epitope. Rather, component a) and component b) are positioned in the complex for example before or after such a stretch of all TLR peptide agonists. However, the TLR peptide agonists positioned consecutively in such a way may be linked to each other for example by a spacer or linker as described below, which is neither component a), i.e. a cell penetrating peptide, nor component b), i.e. at least one antigen or antigenic epitope.

**[0270]** Alternatively, however, the various TLR peptide agonists may also be positioned in any other way in the complex for use according to the present invention, for example with component a) and/or component b) positioned in between two or more TLR peptide agonists, i.e. with one or more TLR peptide agonist positioned between component a) and component b) (or vice versa) and, optionally, one or more TLR peptide agonists positioned at the respective other end of component a) and/or component b).

**[0271]** It is understood that a number of different TLR peptide agonists activating the same or different TLR receptors may be advantageously comprised by a single complex for use according to the present invention. Alternatively, a number of different TLR peptide agonists activating the same or different TLR receptors may be distributed to subsets of different TLR peptide agonists activating the same or different TLR receptors, which are comprised by different complexes according to the present invention, whereby such different complexes comprising different subsets may advantageously be administered simultaneously, e.g. in a single vaccine, to a subject in need thereof.

**Linkage of components a), b), and c) in the complex for use according to the present invention**

**[0272]** In the complex for use according to the present invention, components a), b) and c) are covalently linked, i.e. the linkage between two out of the three components a), b), and c) of the complex for use according to the present invention is a covalent linkage. Preferably, two out of the three components a), b), and c) of the complex for use according to the present invention are covalently linked to each other (i.e. the "first" and the "second" component), and the third component out of the three components a), b), and c) is covalently linked either to the first component out of the three components a), b), and c) or to the second component out of the three components a), b), and c). Thereby, preferably a linear molecule is formed. However, it is also conceivable that each of the three components a), b), and c) is covalently linked to both of the other components out of the three components a), b), and c).

**[0273]** A "covalent linkage" (also covalent bond), as used in the context of the present invention, refers to a chemical bond that involves the sharing of electron pairs between atoms.

A "covalent linkage" (also covalent bond) in particular involves a stable balance of attractive and repulsive forces between atoms when they share electrons. For many molecules, the sharing of electrons allows each atom to attain the equivalent of a full outer shell, corresponding to a stable electronic configuration. Covalent bonding includes many kinds of interactions, including for example  $\sigma$ -bonding,  $\pi$ -bonding, metal-to-metal bonding, agostic interactions, and three-center two-electron bonds. Accordingly, the complex for use according to the present invention may also be referred to as "compound", in particular it may be referred to as "molecule".

**[0274]** Preferably, in the complex for use according to the present invention, components a), b), and c) are covalently linked by chemical coupling in any suitable manner known in the art, such as cross-linking methods. However, attention is drawn to the fact that many known chemical cross-linking methods are non-specific, i.e., they do not direct the point of coupling to any particular site on the components a), b), and c). Thus, the use of non-specific cross-linking agents may attack functional sites or sterically block active sites, rendering the fused components of the complex for use according to the present invention biologically inactive. It is referred to the knowledge of the skilled artisan to block potentially reactive groups by using appropriate protecting groups. Alternatively, the use of the powerful and versatile oxime and hydrazone ligation techniques, which are chemo-selective entities that can be applied for the cross-linking of components a), b), and c) may be employed. This linking technology is described e.g. by Rose et al. (1994), JACS 116, 30.

**[0275]** Coupling specificity can be increased by direct chemical coupling to a functional group found only once or a few times in components a), b), and/or c), which functional group is to be cross-linked to the another of the components a), b), and c). As an example, the cystein thiol group may be used, if just one cystein residue is present in a certain component a), b), or c) of complex for use according to the present invention. Also, for example, if a certain component a), b), or c) contains no lysine residues, a cross-linking reagent specific for primary amines will be selective for the amino terminus of the respective component. Alternatively, cross-linking may also be carried out via the side chain of a glutamic acid residue placed at the N-terminus of the peptide such that a amide bond can be generated through its side-chain. Therefore, it may be advantageous to link a glutamic acid residue to the N-terminus of a certain component a), b), or c). However, if a cysteine residue is to be introduced into a certain component a), b), or c), introduction at or near its N- or C-terminus is preferred. Conventional methods are available for such amino acid sequence alterations based on modifications of certain component a), b), or c) by either adding one or more additional amino acids, e.g. *inter alia* an cystein residue, to the translocation sequence or by substituting at least one residue of the translocation sequence(s) being comprised in the respective component. In case a cystein side chain is used for coupling purposes, a certain component a), b), or c) has preferably one cystein residue. Any second cystein residue should preferably be avoided and can, optionally, be replaced when they occur in the respective component comprised by the complex for use according to the present invention. When a cysteine residue is replaced in the original sequence of a certain component a), b), or c), it is typically desirable to minimize resulting changes in the peptide folding of the respective component. Changes in folding are minimized

when the replacement is chemically and sterically similar to cysteine. Therefore, serine is preferred as a replacement for cysteine.

**[0276]** Coupling of two out of the three components a), b), and c) can be accomplished via a coupling or conjugating agent including standard peptide synthesis coupling reagents such as HOBt, HBTU, DICI, TBTU. There are several intermolecular cross-linking agents which can be utilized, see for example, Means and Feeney, Chemical Modification of Proteins, Holden-Day, 1974, pp. 39-43. Among these reagents are, for example, N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or N,N'-(1,3-phenylene)bismaleimide; N,N'-ethylenbis(iodoacetamide) or other such reagent having 6 to 11 carbon methylene bridges; and 1,5-difluoro-2,4-dinitrobenzene. Other cross-linking agents useful for this purpose include: p,p'-difluoro-m,m'-dinitrodiphenylsulfone; dimethyl adipimidate; phenol-1,4-disulfonylchloride; hexamethylenediisocyanate or diisothiocyanate, or azophenyl-p-diisocyanate; glutaraldehyde and disdiazobenzidine. Cross-linking agents may be homobifunctional, i.e., having two functional groups that undergo the same reaction. A preferred homobifunctional cross-linking agent is bismaleimidohexane (BMH). BMH contains two maleimide functional groups, which react specifically with sulfhydryl-containing compounds under mild conditions (pH 6.5-7.7). The two maleimide groups are connected by a hydrocarbon chain. Therefore, BMH is useful for irreversible cross-linking of proteins (or polypeptides) that contain cysteine residues. Cross-linking agents may also be heterobifunctional. Heterobifunctional crosslinking agents have two different functional groups, for example an amine-reactive group and a thiol-reactive group, that will cross-link two proteins having free amines and thiols, respectively. Examples of heterobifunctional cross-linking agents are Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and succinimide 4-(p-maleimidophenyl)butyrate (SMPB), an extended chain analog of MBS. The succinimidyl group of these cross-linkers reacts with a primary amine, and the thiol-reactive maleimide forms a covalent bond with the thiol of a cysteine residue. Because cross-linking agents often have low solubility in water, a hydrophilic moiety, such as a sulfonate group, may be added to the cross-linking agent to improve its water solubility. Sulfo-MBS and sulfo-SMCC are examples of cross-linking agents modified for water solubility. Many cross-linking agents yield a conjugate that is essentially non-cleavable under cellular conditions. Therefore, some cross-linking agents contain a covalent bond, such as a disulfide, that is cleavable under cellular conditions. For example, Traut's reagent, dithiobis (succinimidylpropionate) (DSP), and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) are well-known cleavable cross-linkers. The use of a cleavable cross-linking agent permits the cell penetrating peptide, the at least one antigen or antigenic epitope and the at least one TLR peptide agonist comprised by the complex for use according to the present invention to separate from each other after delivery into the target cell. For this purpose, direct disulfide linkage may also be useful. Chemical cross-linking may also include the use of spacer arms. Spacer arms provide intramolecular flexibility or adjust intramolecular distances between conjugated moieties and thereby may help preserve biological activity. A spacer arm may be in the form of a protein (or polypeptide) moiety that includes spacer amino acids, e.g. proline. Alternatively, a spacer arm may be part of the crosslinking agent, such as in "long-chain SPDP" (Pierce Chem. Co., Rockford, Ill., cat. No. 21651 H). Numerous cross-linking agents, including the ones discussed above, are

commercially available. Detailed instructions for their use are readily available from the commercial suppliers. More detailed information on protein cross-linking and conjugate preparation, which is useful in the context of linkage of components a), b), and c) comprised by the complex for use according to the present invention can be retrieved from: Wong, Chemistry of Protein Conjugation and Cross-Linking, CRC Press (1991).

**[0277]** Cross-linking agents for peptide or protein crosslinking include for example (i) amine-to-amine crosslinkers, e.g. homobifunctional amine-specific protein crosslinking reagents based on NHS-ester and imidoester reactive groups for selective conjugation of primary amines; available in short, long, cleavable, irreversible, membrane permeable, and cell surface varieties; (ii) sulfhydryl-to-carbohydrate crosslinkers, e.g. crosslinking reagents based on maleimide and hydrazide reactive groups for conjugation and formation of covalent crosslinks; (iii) sulfhydryl-to-sulfhydryl crosslinkers, e.g. homobifunctional sulfhydryl-specific crosslinking reagents based on maleimide or pyridylthiol reactive groups for selective covalent conjugation of protein and peptide thiols (reduced cysteines) to form stable thioether bonds; (iv) photoreactive crosslinkers, e.g. aryl azide, diazirine, and other photo-reactive (light-activated) chemical heterobifunctional crosslinking reagents to conjugate proteins, nucleic acids and other molecular structures involved in receptor-ligand interaction complexes via two-step activation; (v) amine-to-sulfhydryl crosslinkers, e.g. heterobifunctional protein crosslinking reagents for conjugation between primary amine (lysine) and sulfhydryl (cysteine) groups of proteins and other molecules; available with different lengths and types of spacer arms; and (vi) amine-to-amine crosslinkers, e.g. carboxylto-amine crosslinkers, e.g. Carbodiimide crosslinking reagents, DCC and EDC (EDAC), for conjugating carboxyl groups (glutamate, aspartate, C-termini) to primary amines (lysine, N-termini) and also N-hydroxysuccinimide (NHS) for stable activation of carboxylates for amine-conjugation.

**[0278]** Examples of crosslinkers in general, which can be used in the complex for use according to the present invention, include *N*-( $\alpha$ -Maleimidoacetoxy)-succinimide ester, *N*-5-Azido-2-nitrobenzyloxy-succinimide, 1,4-*Bis*-Maleimidobutane, 1,4-*Bis*-Maleimimidyl-2,3-dihydroxy-butane, *Bis*-Maleimidohexane, *Bis*-Maleimidoethane, *N*-( $\beta$ -Maleimidopropionic acid)hydrazide\*TFA, *N*-( $\beta$ -Maleimidopropoxy)succinimide ester, 1,8-*Bis*-Maleimidodiethylene-glycol, 1,11-*Bis*-Maleimidotriethyleneglycol, *Bis* (sulfosuccinimidyl)suberate, *Bis* (sulfosuccinimidyl)glutarate-d0, *Bis* (sulfosuccinimidyl)2,2,4,4-glutarate-d4, *Bis* (sulfosuccinimidyl)suberate-d0, *Bis* (sulfosuccinimidyl)2,2,7,7-suberate-d4, *Bis* (NHS)PEG5, *Bis* (NHS)PEG9, *Bis* (2-[succinimidoxycarbonyloxy]ethyl)sulfone, *N,N*-Dicyclohexylcarbodiimide, 1-5-Difluoro-2,4-dinitrobenzene, Dimethyl adipimidate\*2HCl, Dimethyl pimelimidate\*2HCl, Dimethyl suberimidate\*2HCl, Disuccinimidyl glutarate, Dithiobis(succimidylpropionate) (Lomant's Reagent), Disuccinimidyl suberate, Disuccinimidyl tartarate, Dimethyl 3,3'-dithiobispropionimidate\*2HCl, Dithiobis-maleimidoethane, 3,3'-Dithiobis (sulfosuccinimidylpropionate), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Ethylene glycol *bis* (succinimidylsuccinate), *N*- $\varepsilon$ -Maleimidocaproic acid, *N*-( $\varepsilon$ -Maleimidocaproic acid)hydrazide, *N*- $\varepsilon$ -Maleimidocaproxyloxy)succinimide ester, *N*-( $\gamma$ -Maleimidobutyryloxy)succinimide ester, *N*-( $\kappa$ -Maleimidoundecanoic acid)hydrazide, NHS-LC-Diazirine, Succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxy-(6-amidocaproate,

Succinimidyl 6-(3'-[2-pyridyldithio] propionamido)hexanoate, L-Photo-Leucine, L-Photo-Methionine, *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester, 4-(4-*N*-Maleimidophenyl)-butyric acid hydrazide\*HCl, 2-[*N*2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysyl]ethylmethanethiosulfate, 2-{*N*2-[*N*6-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysyl]}ethylmethanethiosulfate, *N*-Hydroxysuccinimide, *N*-hydroxysuccinimide ester ethane azide, *N*-hydroxysuccinimide ester tetraoxapentadecane azide, *N*-hydroxysuccinimide ester dodecaoxanonatriacontane azide, NHS-Phosphine, 3-(2-Pyridyldithio)propionylhydrazide, 2-pyridyldithioltetraoxatetradecane-*N*-hydroxysuccinimide, 2-pyridyldithiol-tetraoxaoctatriacontane-*N*-hydroxysuccinimide, *N*-(*p*-Maleimidophenyl)isocyanate, Succinimidyl 3-(bromoacetamido)propionate, NHS-Diazirine, NHS-SS-Diazirine, *N*-succinimidyl iodoacetate, *N*-Succinimidyl(4-iodoacetyl)aminobenzoate, Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, NHS-PEG2-Maliemide, NHS-PEG4-Maliemide, NHS-PEG6-Maleimide, NHs-PEG8-Maliemide, NHS-PEG12-Malimide, NES-PEG24-Maleimide, Succinimidyl 4-(*p*-maleimido-phenyl)butyrate, Succinimidyl-6-( $\beta$ -maleimidopropionamido)hexanoate, 4-Succinimidylloxycarbonyl-methyl- $\alpha$ -(2-pyridyldithio)toluene, Succinimidyl-(4-psoralen-8-yloxy)butyrate, *N*-succinimidyl 3-(2-pyridyldithio)propionate, Ethylene glycol bis (sulfo-succinimidyl succinate), *N*-( $\epsilon$ -Maleimidocaproyloxy)sulfosuccinimide ester, *N*-( $\gamma$ -Maleimidobutryloxy)sulfosuccinimide ester, *N*-( $\kappa$ -Maleimidoundecanoyloxy)sulfosuccinimide ester, Sulfo-NHS-LC-Diazirine, Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]propionamido)hexanoate, *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester, *N*-Hydroxysuccinimide, Sulfo-NHS-Phosphine, Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate, Sulfo-NHS-(2-6-[Biotinamido]-2-(*p*-azidobezamido), Sulfo-NHS-Diazirine, Sulfo-NHS-SS-Diazirine, Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate, Sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, Sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate, *Tris*-(2-Maleimidoethyl)amine (Trifunctional), and *Tris*-(succimidyl aminotriacetate) (Trifunctional).

**[0279]** The linkage between two out of the three components a), b), and c) of the complex for use according to the present invention may be directly or indirectly, i.e. two components directly adjoin or they may be linked by an additional component of the complex, e.g. a spacer or a linker.

**[0280]** A direct linkage may be realized preferably by an amide bridge, if the components to be linked have reactive amino or carboxy groups. More specifically, if the components to be linked are peptides, polypeptides or proteins, a peptide bond is preferred. Such a peptide bond may be formed using a chemical synthesis involving both components (an N-terminal end of one component and the C-terminal end of the other component) to be linked, or may be formed directly via a protein synthesis of the entire peptide sequence of both components, wherein both (protein or peptide) components are preferably synthesized in one step. Such protein synthesis methods include e.g., without being limited thereto, liquid phase peptide synthesis methods or solid peptide synthesis methods, e.g. solid peptide synthesis methods according to Merrifield, *t*-Boc solid-phase peptide synthesis, Fmoc solid-phase peptide synthesis, BOP (Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate) based solid-phase peptide synthesis, etc. Alternatively, ester or ether linkages are preferred.

**[0281]** As the components to be linked are peptides, polypeptides or proteins, a linkage may occur via the side chains, e.g. by a disulfide bridge. Further components of other chemical nature, e.g. the at least one antigen or antigenic epitope if it is not of peptidic nature, may be likewise attached to the components of peptidic nature, e.g. the cell penetrating peptide, the at least one TLR peptide agonist, and the at least one antigen or antigenic epitope if it is of peptidic nature. The linkage via a side chain will preferably be based on side chain amino, thiol or hydroxyl groups, e.g. via an amide or ester or ether linkage. A linkage of a peptidic main chain with a peptidic side chain of another component may also be via an isopeptide bond. An isopeptide bond is an amide bond that is not present on the main chain of a protein. The bond forms between the carboxyl terminus of one peptide or protein and the amino group of a lysine residue on another (target) peptide or protein.

**[0282]** The complex for use according to the present invention may optionally comprise a spacer or linker, which are non-immunologic moieties, which are preferably cleavable, and which link component a) and b) and/or component a) and c), and/or component b) and c), and/or link consecutive antigens or antigenic epitopes, and/or link consecutive TLR peptide agonists, and/or link consecutive cell penetrating peptides, and/or which can be placed at the C-terminal part of components b) and/or c). A linker or spacer may preferably provide further functionalities in addition to linking of the components, and preferably being cleavable, more preferably naturally cleavable inside the target cell, e.g. by enzymatic cleavage. However, such further functionalities do in particular not include any immunological functionalities. Examples of further functionalities, in particular regarding linkers in fusion proteins, can be found in Chen X. et al., 2013: Fusion Protein Linkers: Property, Design and Functionality. *Adv Drug Deliv Rev.* 65(10): 1357 - 1369, wherein for example also in vivo cleavable linkers are disclosed. Moreover, Chen X. et al., 2013: Fusion Protein Linkers: Property, Design and Functionality. *Adv Drug Deliv Rev.* 65(10): 1357 - 1369 also discloses various linkers, e.g. flexible linkers and rigid linkers, and linker designing tools and databases, which can be useful in the complex for use according to the present invention or to design a linker to be used in the complex for use according to the present invention.

**[0283]** Said spacer is peptidic. Preferably, a peptidic spacer consists of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids, more preferably of about 1, 2, 3, 4, or 5 amino acids. The amino acid sequence of the peptidic spacer may be identical to that of the N-terminal or C-terminal flanking region of any of the components a), b), or c). Alternatively a peptidic spacer can consist of non-natural amino acid sequences such as an amino acid sequence resulting from conservative amino acid substitutions of said natural flanking regions or sequences of known cleavage sites for proteases such as an enterokinase target site (amino acid sequence: DDDK, SEQ ID NO: 16), factor Xa target site (amino acid sequence: IEDGR, SEQ ID NO: 17), thrombin target site (amino acid sequence: LVPRGS, SEQ ID NO: 18), protease TEV target site (amino acid sequence: ENLYFQG, SEQ ID NO: 19), PreScission protease target site (amino acid sequence LEVLFQGP, SEQ ID NO: 20), polycationic amino acids, e.g. poly K, furin target site (amino acid sequence RX(R/K)R, SEQ ID NO: 21). In a particular embodiment, the peptidic spacer does not contain any Cys (C) residues. In a preferred embodiment the linker sequence

contains at least 20%, more preferably at least 40% and even more preferably at least 50% Gly or  $\beta$ -alanine residues, e.g. GlyGlyGlyGly (SEQ ID NO: 22), GlyGlyGlyGly (SEQ ID NO: 23), GlyGlyGly, CysGlyGly or GlyGlyCys, etc. Appropriate linker sequences can be easily selected and prepared by a person skilled in the art. They may be composed of D and/or L amino acids. Further examples of a peptidic spacer include the amino acid sequences EQLE (SEQ ID NO: 24) or TEWT (SEQ ID NO: 25) or any conservative substitutions thereof.

**[0284]** A non-peptidic spacer can include or may be an ester, a thioester, and a di-sulfide.

**[0285]** In particular, the complex for use according to the invention may comprise a spacer or linker, in particular a peptidic spacer, placed between component a) and b) and/or between component a) and c), and/or between component b) and c). This peptidic spacer can be chosen by one skilled in the art so that it may be cut by the cell machinery once the complex comprising the cell penetrating peptide and the cargo molecule has been internalized.

**[0286]** When the complex comprises several antigens or antigenic epitopes or when the complex comprises several TLR peptide agonists, it will be clear for one skilled in the art that each of the antigens or antigenic epitopes and/or each of the TLR peptide agonists comprised in the complex of the invention can be either directly linked to each other or linked via spacers or linkers such as, e.g., a peptidic spacer consisting of a few amino acids. Alternatively, when the complex for use according to the present invention comprises several antigens or antigenic epitopes or when the complex comprises several TLR peptide agonists, it is also possible that some antigens or antigenic epitopes and/or some TLR peptide agonists comprised by the complex of the invention are directly linked to each other and some other antigens or antigenic epitopes and/or some other TLR peptide agonists are linked via spacers or linkers such as a peptidic spacer consisting of a few amino acids.

**[0287]** For example, two successive antigens or antigenic epitopes or two successive TLR peptide agonists comprised in the complex of the invention are linked to each other by spacers consisting of the natural flanking regions of said antigens or antigenic epitopes or of said TLR peptide agonists, respectively. For example, the spacer used to link a first antigen/antigenic epitope or a first TLR peptide agonist to a second antigen/antigenic epitope or to a second TLR peptide agonist, respectively, may consist of up to about 8 amino acids corresponding to up to about 4 amino acids of the N-terminal or C-terminal flanking region of the first antigen/antigenic epitope or the first TLR peptide agonist, followed by up to about 4 amino acids of the N-terminal or C-terminal flanking region of the second antigen/antigenic epitope or the second TLR peptide agonist. In an illustration of the present invention, the spacer used to link a first antigen/antigenic epitope or a first TLR peptide agonist ("antigen/epitope/TLR peptide agonist 1") to a second epitope ("antigen/epitope/TLR peptide agonist 2") consists of about 8 amino acids corresponding to any possible combination ranging from: 0 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 8 flanking amino acids of antigen/epitope/TLR peptide agonist 2, to 8 flanking amino acids of antigen/epitope/TLR peptide agonist 1 and 0 flanking amino acid of antigen/epitope/TLR peptide agonist 2, i.e. including 1 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 7 flanking amino

acids of antigen/epitope/TLR peptide agonist 2, 2 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 6 flanking amino acids of antigen/epitope/TLR peptide agonist 2, 3 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 5 flanking amino acids of antigen/epitope/TLR peptide agonist 2, 4 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 4 flanking amino acids of antigen/epitope/TLR peptide agonist 2, 5 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 3 flanking amino acids of antigen/epitope/TLR peptide agonist 2, 6 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 2 flanking amino acids of antigen/epitope/TLR peptide agonist 2, 7 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 1 flanking amino acid of antigen/epitope/TLR peptide agonist 2, 8 flanking amino acid of antigen/epitope/TLR peptide agonist 1 and 0 flanking amino acids of antigen/epitope/TLR peptide agonist 2. It will be understood that the total of 8 amino acids constituting a spacer linking two consecutive antigen/epitope/TLR peptide agonist is not an absolute value and the spacer could also be composed of a total of, for instance, 3 amino acids, 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 9 amino acids or 10 amino acids. Similarly, equivalent combinations as mentioned above are also an illustration of the invention in the situation where a spacer has less or more than 8 amino acids.

**[0288]** In another particular illustration of the present invention, the spacer used to link a first antigen/antigenic epitope or a first TLR peptide agonist ("antigen/epitope/TLR peptide agonist 1") to a second antigen/antigenic epitope or to a second TLR peptide agonist, respectively, ("antigen/epitope/TLR peptide agonist 2") consists of e.g. 1, 2, 3, 4, or 5 amino acids. More particularly, said spacer's amino acid sequence can correspond to the 4 amino acids of the N-terminal or C-terminal flanking region of antigen/epitope/TLR peptide agonist 1 or antigen/epitope/TLR peptide agonist 2. A spacer as described above may also be placed at the C-terminal part of the last antigen/epitope/TLR peptide agonist comprised in the complex for use according to the present invention.

**[0289]** The technics for linking two of the three components a), b), and c) are well documented in the literature and can depend on the nature of the at least one antigen or antigenic epitope. For instance, linkages between two of the three components a), b), and c) can be achieved via cleavable disulphide linkages through total stepwise solid-phase synthesis or solutionphase or solid-phase fragment coupling, stable amide, thiazolidine, oxime and hydrazine linkage, disulphide linkage, stable thiomaleimide linkage, peptide bond (including peptide bonds between amino acids of a fusion protein), or electrostatic or hydrophobic interactions.

**[0290]** The at least one antigen or antigenic epitope comprised by the complex for use according to the present invention as well as any optional spacer or linker comprised by the complex for use according to the present invention are of peptidic nature. All components of the complex for use according to the present invention, e.g. the cell penetrating peptide, the at least one antigen or antigenic epitope, which is a peptide, polypeptide or protein, the at least one TLR peptide agonist and any optional peptidic linker or spacer are linked in the complex for use according to the present invention by a peptide bond. The complex for use according to the present invention is thus a polypeptide or protein, such as a fusion protein, e.g. a

recombinant fusion protein.

**[0291]** In this context, a complex comprising an amino acid sequence according to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, SEQ ID NO: 69, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, or SEQ ID NO: 87; or a complex comprising an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, SEQ ID NO: 69, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, or SEQ ID NO: 87 is preferred. Moreover, a complex comprising an amino acid sequence according to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, SEQ ID NO: 69, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90 or SEQ ID NO: 91; or a complex comprising an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, SEQ ID NO: 69, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90 or SEQ ID NO: 91 is preferred. Furthermore, a complex comprising an amino acid sequence according to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, or SEQ ID NO: 69; or a complex comprising an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 46, or SEQ ID NO: 69 is preferred. A complex comprising an amino acid sequence according to SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41 or SEQ ID NO: 69 or a complex comprising an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41 or SEQ ID NO: 69 is more preferred. A complex comprising an amino acid sequence according to SEQ ID NO: 27, SEQ





sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 83. Most preferably, the complex for use according to the present invention comprises an amino acid sequence according to SEQ ID NO: 84 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 84. Most preferably, the complex for use according to the present invention comprises an amino acid sequence according to SEQ ID NO: 85 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 85. Most preferably, the complex for use according to the present invention comprises an amino acid sequence according to SEQ ID NO: 86 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 86. Most preferably, the complex for use according to the present invention comprises an amino acid sequence according to SEQ ID NO: 87 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 87. Most preferably, the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 88 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 88. Most preferably, the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 89 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 89. Most preferably, the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 90 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 90. Most preferably, the complex for use according to the present invention comprises or consists of an amino acid sequence according to SEQ ID NO: 91 or of an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with SEQ ID NO: 91.

**[0292]** It is particularly preferred that the complex for use according to the present invention comprises or consists of a polypeptide having an amino acid sequence according to SEQ ID NO: 89, or a functional sequence variant thereof having at least 50% sequence identity (preferably at least 60%, more preferably at least 70%, more preferably at least 75%, even more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95% and most preferably at least 98% or 99% sequence identity).

SEQ ID NO: 26:

MHHHHHHHNID RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI  
 RELFPAPDGEDDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTKRY KNRVASRKSR  
 AKFKOI LOHY REVAAKSSF NDRIRLLIKE SIKISOAVHA AHAFINFAGR FVVGVGAI KV

PRNQDWLGV P RFAKFASFEA QGALANIAVD KANLDVEQLE SIINFEKLTE WTGS

SEQ ID NO: 27:

MHHHHHHHSTV HEILCKLSLE GDHSTPPSAY GSVKPYTNFD AEKRYKNRVA SRKSRAFKQ

LLQHYREVAA AKSENDRLR LLLKESLKIS QAVHAAHAEI NEAGREVVG V GALKVPRNQD  
WLCVPRFAKF ASFEAQGALA NIAVDKANLD VEQLESIINF EKLTEWTGS

SEQ ID NO: 28:

MHHHHHHHKRYKNRVA SRKSRAFKQ LLQHYREVAA AKSENDRLR LLLKESLKIS  
QAVHAAHAEI NEAGREVVG V GALKVPRNQD WLCVPRFAKF ASFEAQGALA  
NIAVDKANLD VEQLESIINF EKLTEWTGSS TVHEILCKLS LEGDHSTPPS AYGSVKPYTN FDAE

SEQ ID NO: 33:

MHHHHHHHKRY KNRVASRKS RAKFQQLQHY REVAAAKESL KISQAVHAAH AEINEAGREV  
VGVGALKVPR NQDWLGVPRF AKFASFEAQG ALANIAVDKA NLDVEQLESI INFEKLTEWT  
GSSTVHEILC KLSLEGDHST PPSAYGSVKP YTNFdae

SEQ ID NO: 34:

MHHHHHHHREV AAAKSSENDR LRLLKESLK ISQAVHAAHA EINEAGREVVG VVGALKVPRN  
QDWLGVPRFA KFASFEAQGA LANIAVDKAN LDVEQLESII NFEKLTEWTG SSTVHEILCK  
LSLEGDHSTP PSAYGSVKP YTNFdae

SEQ ID NO: 37:

MHHHHHHHNID RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI RELFPAPDGE  
DDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTKRY KNRVASRKS RAKFQQLQHY  
REVAAAKESL KISQAVHAAH AEINEAGREV VGVGALKVPR NQDWLGVPRF AKFASFEAQG  
ALANIAVDKA NLDVEQLESI INFEKLTEWT GS

SEQ ID NO: 38:

MHHHHHHHNID RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI RELFPAPDGE  
DDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTREV AAAKSSENDR LRLLKESLK  
ISQAVHAAHA EINEAGREVVG VVGALKVPRN QDWLGVPRFA KFASFEAQGA LANIAVDKAN  
LDVEQLESII NFEKLTEWTG S

SEQ ID NO: 39:

KRYKNRVA SRKSRAFKQQLQHYREVAAAKSENDRLRLLLKVTYHSPSYAYHQFERRAILNRLV

QFIKDRISVVQALVLTSTVHEILCKLSLEGDHSTPPSAYGSVKP YTN Fdae

SEQ ID NO: 40:

KRYKNRVA SRKSRAFKQQLQHYREVAAAKSENDRLRLLLKNYRIATFKNWPFL EDCA MEELT  
VSEFLKLDQRQ RSTVHEILCKLSLEGDHSTPPSAYGSVKP YTN Fdae

SEQ ID NO: 41:

KRYKNRVA SRKSRAFKQQLQHYREVAAAKSENDRLRLLLKHELASMTNMELMSSIVSTVHEI  
LCKLSLEGDHSTPPSAYGSVKP YTN Fdae

SEQ ID NO: 46:

RKKRRQRRRVKRISQAVHAAHAEINEAGRRVKRKVRNQDWLRVKRASFEAQGALANIAVD  
KARVKRSIINFEKLRVKRSTVHEILCKSLEGDHSTPPSAYGSVKPYTNFdae

SEQ ID NO: 69:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKFLRAAQLANDVVLQIMEHLELA  
SMTNMELMSSIVVISASIIVFNLELEGSTVHEILCKSLEGDHSTPPSAYGSVKPYTNFdae

SEQ ID NO: 72:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKAPPQVLAFGLLAAATAYVDEK  
APEFSMQGLKAGVIAVIVVSTVHEILCKSLEGDHSTPPSAYGSVKPYTNFdae

SEQ ID NO: 73:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNGTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSAGATVGIMIGVLVGVALLGDPKKLLTQHFVQENYLE  
YRQVPGSDPASYEFLWGPRALVETSYVKVALSRKVAELVHFLLKYRAREPVTKAEMLGSVVA  
QVLAFGLLAAATAYVDEKAPEFSMQGLKAGVIAVIVVSTVHEILCKSLEGDHSTPPSAYGSVK  
PYTNFdae

SEQ ID NO: 74:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNGTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSLGDPKKLLTQHFVQENYLEYRQVPGSDPASYEFLWG  
PRALVETSYVKVALSRKVAELVHFLLKYRAREPVTKAEMLGSVVA  
QVLAFGLLAAATAYVDEKAPEFSMQGLKAGVIAVIVVSTVHEILCKSLEGDHSTPPSAYGSVK  
PYTNFdae

SEQ ID NO: 75:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNGTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSLGDPKKLLTQHFVQENYLEYRQVPGSDPASYEFLWG  
PRALVETSYVKVALSRKVAELVHFLLKYRAREPVTKAEMLGSVVA  
QVLAFGLLAAATAYVDEKAPEFSMQGLKAGVIAVIVVAPGSTAPPAHGVT  
TAPPVHNNTSSTVHEILCKSLEGDHSTPPSAYGSVKPYTNFdae

SEQ ID NO: 76:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNGTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSAPLPPAWQPFLKDHRISTFKNWPFL  
EELTLGEFLKLDRERAPPQVLAFGLLAAATAYVDEKAPEFSMQGLKAGVIAVIVVAPGSTAPPAHG  
VTSAPDTRPAPGSTAPPAHGVT  
TAPPVHNNTSSTVHEILCKSLEGDHSTPPSAY  
GSVKPYTNFdae

SEQ ID NO: 77:

KRYKNRVASRKSRAFKQLLQHYREVAAAKSENDRLRLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNGTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSEYKLVVVGAVGVGKSALTAPPQVLAFGLLAAATAYV  
DEKAPEFSMQGLKAGVIAVIVVAPGSTAPPAHGVT  
TAPPVHNNTSSTVHEILCKSLEGDHSTPPSAY  
GSVKPYTNFdae

GSTAPPVHNNTSSTVHEILCKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 78:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNNTYACF

VSNLATGRNNSIVKSITVSASGTSPGLSAPTLPPAWQPFLKDHRISTFKNWPFLGSAVKKQFEEL  
TLGEFLKLDRERAPPQVLAFGLLLAAATAYVDEKAPEFSMQGLKAGVIAVIVVLGDPKKLLTQH  
FVQENYLEYRQPGSDPASYEFLWGPRALVETSYVKVALSRKVAELVHFLLKYRAREPVTKAEM  
LGSVVSTVHEILCKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 79:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNNTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSAPPQVLAFGLLLAAATAYVDEKAPEFSMQGLKAGVIA  
VIVVAPGSTAPPAHGVTSAPDTRPAGSTAPPAHGVTSAPDRPALGSTAPPVHNNTSSTVHEIL  
CKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 80:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNNTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSAPPQVLAFGLLLAAATAYVDEKAPEFSMQGLKAGVIA  
VIVVSTVHEILCKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 81:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKLGDPKKLLTQHFVQENYLEYRQ  
VPGSDPASYEFLWGPRALVETSYVKVALSRKVAELVHFLLKYRAREPVTKAEMGSVVAPTLPP  
AWQPFLKDHRISTFKNWPFLGSAVKKQFEELTLGEFLKLDRERAPGSTAPPAHGVTSAPDTRP  
APGSTAPPAHGVTSAPDRPALGSTAPPVHNNTSSTVHEILCKLSLECDHSTPPSAYGSVKPYTNF  
DAE

SEQ ID NO: 82:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKRTLTLFNVTRNDARAYVSGIQN  
SVSANRSDPVTDPSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNNNTYACF  
VSNLATGRNNSIVKSITVSASGTSPGLSSTVHEILCKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 83:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKNRTLTLFNVTRNDARAYVSGIQ  
NSVSANRSDPVTLDVLPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNN  
CTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAGATVGIMIGVLVGVALIAPGSTAPPAHGV  
SAPDTRPAGSTAPPAHGVTSAPDRPALGSTAPPVHNNTSAPPQVLAFGLLLAAATALIYYVDE  
KAPEFSMQGLKAGVIAVIVVSTVHEILCKLSLECDHSTPPSAYGSVKPYTNFDAE

SEQ ID NO: 84:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKNRTLTLFNVTRNDARAYVSGIQ  
NSVSANRSDPVTLDVLPDSSYLSGANLNLSHSASPQYSWRINGIPQQHTQVLFIAKITPNNN  
CTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPGSTAPPAHGVTSAPDTRPAGSTAPPA

HGVTsapDRPAlgSTAPPVHNVTsAPPQVLAfGLLAAATAliYYVDEKAPEfSMQGLKAGVIA  
VIVVSTVHEILCKLsLEGDHSTPPSAYGSVKPYTNFdaE

SEQ ID NO: 85:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLfIAkITpnnn  
GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPGStappaHGVtsapDTRPAPGStappa  
HGVTsapDRPAlgSTAPPVHNVTsAPPQVLAfGLLAAATAliYYVDEKAPEfSMQGLKAGVIA  
VIVVSTVHEILCKLsLEGDHSTPPSAYGSVKPYTNFdaE

SEQ ID NO: 86:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLfIAkITpnnn  
GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFkDHRISTfKNWPflegsa  
VKKQFEELTLGEFLKLDRERAPGStappaHGVtsapDTRPAPGStappaHGVtsapDRPAlgSt  
APPVHNVTsAPPQVLAfGLLAAATAliYYVDEKAPEfSMQGLKAGVIAVIVVSTVHEILCKLsLE  
GDHSTPPSAYGSVKPYTNFdaE

SEQ ID NO: 87:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLfIAkITpnnn  
GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFkDHRISTfKNWPflegsa  
VKKQFEELTLGEFLKLDRERAPGStappaHGVtsapDTRPAPGStappaHGVtsapDRPAlgSt  
APPVHNVTsAPPQVLAfGLLAAATAliYYVDEKAPEfSMQGLKAGVIAVIVVSTVHEILCKLsLE  
GDHSTPPSAYGSVKPYTNFdaE

SEQ ID NO: 88:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLfIAkITpnnn  
GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFkDHRISTfKNWPflegsa  
VKKQFEELTLGEFLKLDRERAPPQVLAfGLLAAATAliYYVDEKAPEfSMQGLKAGVIAVIVVAA  
VARRNERERNRVKLVLNLGFQALRQHVPHGGASKKLSKVETLRSAVEYIRALQRLLAEHDAVRN  
ALAGGLRPQAVRPSAPRGPSEGALSPAERELLDfSSWLGGYSTVHEILCKLsLEGDHSTPPSAYG  
SVKPYTNFdaE

SEQ ID NO: 89:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLfIAkITpnnn  
GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFkDHRISTfKNWPflegsa  
VKKQFEELTLGEFLKLDRERAAVARRNERERNRVKLVLNLGFQALRQHVPHGGASKKLSKVETLR  
SAVEYIRALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGPSEGALSPAERELLDfSSWLGGYST  
VHEILCKLsLEGDHSTPPSAYGSVKPYTNFdaE

SEQ ID NO: 90:

KRYKNRVArksRAkfkQllQHyRevAAkssendrlrlLKNRtLfnvtrndarayvsgiq  
NSVSArSDPVTLDVLPDSSYLSGANLNLSCHSASPOYSWRINGIPOOHTOVLfIAkITpnnn

GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFLKDHRISTFKNWPFLECSA  
 VKKQFEELTLGEFLKLDRERAKNIAAVARRNERERNRKLVNLGFQALRQHVPHGGASKKLSK  
 VETLRSAYEYIRALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGSEGALSPAERELLDFSSWLG  
 GYSTVHEILSKLSLEGDHSTPPSAYGSVKPYTNFADAE

SEQ ID NO: 91:

KRYKNRVASRKSRACKQLLQHYREVAAKSSENDRLRLLLKNRTLTFNVTRNDARAYVSGIQ  
 NSVSANRSDPVTLVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLFIAKITPNNN

GTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAAPTLPPAWQPFLKDHRISTFKNWPFLECSA  
 VKKQFEELTLGEFLKLDRERAKNIAAVARRNERERNRKLVNLGFQALRQHVPHGGASKKLSK  
 VETLRSAYEYIRALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGPPGTTVAASPSRASSSPGR  
 GGSSEPGSPRSAYSSDDSGSEGALSPAERELLDFSSWLGYSTVHEILSKLSLEGDHSTPPSAYGS  
 VKPYTNFADAE

**[0293]** Most preferably, the complex for use according to the present invention comprises (or consists of) the following components:

1. (i) a peptide having an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13) or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
2. (ii) a peptide having an amino acid sequence according to SEQ ID NO: 96, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
3. (iii) a peptide having an amino acid sequence according to SEQ ID NO: 95, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
4. (iv) a peptide having an amino acid sequence according to SEQ ID NO: 97, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity); and
5. (v) a peptide having an amino acid sequence according to SEQ ID NO: 71; or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least

99% sequence identity).

**[0294]** Preferably, the complex comprises components (i) - (v) (as described above) in N- to C-terminal direction. However, the components may also be arranged differently, as will be described below.

**[0295]** In a further aspect, the present invention also provides a complex comprising:

a cell penetrating peptide;

at least three antigenic epitopes; and

at least one TLR2 or 4 peptide agonist,

wherein the components a) - c) are covalently linked, and

wherein the at least three antigenic epitopes comprise (i) one or more epitopes of survivin or (a) functional sequence variant(s) thereof, (ii) one or more epitopes of CEA or (a) functional sequence variant(s) thereof, and (iii) one or more epitopes of ASCL2 or (a) functional sequence variant(s) thereof, wherein the complex is a polypeptide or protein.

**[0296]** Preferred embodiments of such a complex according to the present invention correspond to preferred embodiments of the complex for use according to the present invention as described above.

**[0297]** For example, the complex according to the present invention preferably comprises a peptide having an amino acid sequence according to SEQ ID NO: 52, or a fragment thereof having a length of at least 10 amino acids, or a functional sequence variant thereof having at least 70% sequence identity, as described above. More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 53. Even more preferably, the complex the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 95 or a functional sequence variant thereof having at least 70% sequence identity, as described above.

**[0298]** For example, the complex according to the present invention preferably comprises a peptide having an amino acid sequence according to SEQ ID NO: 54, or a fragment thereof having a length of at least 10 amino acids, or a functional sequence variant thereof having at least 70% sequence identity, as described above. More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 55 and/or a peptide having an amino acid sequence according to SEQ ID NO: 56. Even more preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 96 or a functional sequence variant thereof having at least 70% sequence identity, as described above.

**[0299]** For example, the complex according to the present invention preferably comprises a peptide having an amino acid sequence according to SEQ ID NO: 92, or a fragment thereof having a length of at least 10 amino acids, or a functional sequence variant thereof having at least 70% sequence identity, as described above. More preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 93 and/or a peptide having an amino acid sequence according to SEQ ID NO: 94. Even more preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 97 or a functional sequence variant thereof having at least 70% sequence identity, as described above.

**[0300]** It is also preferred that the complex comprises in N- to C-terminal direction:

one or more epitopes of CEA or functional sequence variants thereof as described above;  
one or more epitopes of survivin or functional sequence variants thereof as described above;  
and  
one or more epitopes of ASCL2 or functional sequence variants thereof as described above.

**[0301]** More preferably, the complex comprises in N- to C-terminal direction:

- a peptide having an amino acid sequence according to SEQ ID NO: 54, or a fragment thereof having a length of at least 10 amino acids as described above, or a functional sequence variant thereof having at least 70% sequence identity as described above;
- a peptide having an amino acid sequence according to SEQ ID NO: 52, or a fragment thereof having a length of at least 10 amino acids as described above, or a functional sequence variant thereof having at least 70% sequence identity as described above; and
- a peptide having an amino acid sequence according to SEQ ID NO: 92, or a fragment thereof having a length of at least 10 amino acids as described above, or a functional sequence variant thereof having at least 70% sequence identity as described above.

**[0302]** Even more preferably, the C-terminus of (i) the peptide having an amino acid sequence according to SEQ ID NO: 54 or the fragment or variant thereof as described above is directly linked to the N-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 52 or the fragment or variant thereof as described above; and the C-terminus of (ii) the peptide having an amino acid sequence according to SEQ ID NO: 52 or the fragment or variant thereof as described above is directly linked to the N-terminus of (iii) the peptide having an amino acid sequence according to SEQ ID NO: 92 or the fragment or variant thereof as described above.

**[0303]** It is also preferred that the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 96 or a functional sequence variant thereof having at least 70% sequence identity as described above; a peptide having an amino acid sequence

according to SEQ ID NO: 95 or a functional sequence variant thereof having at least 70% sequence identity as described above; and a peptide having an amino acid sequence according to SEQ ID NO: 97 or a functional sequence variant thereof having at least 70% sequence identity as described above.

**[0304]** Most preferably, the complex comprises a peptide having an amino acid sequence according to SEQ ID NO: 98 or a functional sequence variant thereof having at least 70% sequence identity as described above.

**[0305]** Preferably, the complex is a recombinant polypeptide or a recombinant protein as described above.

**[0306]** Moreover, the cell penetrating peptide of the complex according to the present invention preferably corresponds to the cell penetrating peptide of the complex for use according to the present invention as described above. This applies in particular to preferred embodiments of the cell penetrating peptide.

**[0307]** In addition, the TLR peptide agonist of the complex according to the present invention preferably corresponds to the TLR peptide agonist of the complex for use according to the present invention as described above. This applies in particular to preferred embodiments of the TLR peptide agonist.

**[0308]** For example, the complex according to the present invention comprises preferably in N-to C-terminal direction, the following components, as described above:

1. (i) a peptide having an amino acid sequence according to SEQ ID NO: 6 (CPP3/Z13) or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
2. (ii) a peptide having an amino acid sequence according to SEQ ID NO: 96, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
3. (iii) a peptide having an amino acid sequence according to SEQ ID NO: 95, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity);
4. (iv) a peptide having an amino acid sequence according to SEQ ID NO: 97, or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least

99% sequence identity); and

5. (v) a peptide having an amino acid sequence according to SEQ ID NO: 71; or a functional sequence variant thereof having at least 70% sequence identity (preferably at least 75%, more preferably at least 80%, even preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95%, most preferably at least 99% sequence identity),

wherein components (i) - (v) are optionally linked by a linker or spacer.

**[0309]** Most preferably, the complex comprises or consists of a polypeptide having an amino acid sequence according to SEQ ID NO: 89, or a functional sequence variant thereof having at least 50% sequence (preferably at least 60%, more preferably at least 70%, more preferably at least 75%, even more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95% and most preferably at least 98% or 99% sequence identity).

**[0310]** The complex according to the present invention is used in the prevention and/or treatment of colorectal cancer as described herein.

**Arrangement of components a), b), and c) in the complex for use according to the present invention**

**[0311]** The components a), b), and c) may be arranged in the complex for use according to the present invention in any way.

**[0312]** In particular if more than one cell penetrating peptide and/or more than one antigen or antigenic epitope and/or more than one TLR peptide agonist are comprised by the complex for use according to the present invention, the more than one cell penetrating peptide may be positioned in a non-consecutive manner, i.e. at least one antigen or antigenic epitope (component b)) and/or at least one TLR peptide agonist (component c)) may interrupt a stretch of consecutively positioned cell penetrating peptides and/or the cell penetrating peptides may be positioned with component b) and/or with component c) in an alternating manner. Similarly, the more than one antigen or antigenic epitope may be positioned in a non-consecutive manner, i.e. at least one cell penetrating peptide (component a)) and/or at least one TLR peptide agonist (component c)) may interrupt a stretch of consecutively positioned antigens or antigenic epitopes and/or the antigens or antigenic epitopes may be positioned with component a) and/or with component c) in an alternating manner. Similarly, the more than one TLR peptide agonist may be positioned in a non-consecutive manner, i.e. at least one cell penetrating peptide (component a)) and/or at least one antigen or antigenic epitope (component b)) may interrupt a stretch of consecutively positioned TLR peptide agonists and/or the TLR peptide agonists may be positioned with component a) and/or with component b) in an alternating manner.

**[0313]** However, it is preferred that the more than one cell penetrating peptide is positioned in the complex for use according to the present invention in a consecutive manner and/or the more than one antigen or antigenic epitope is positioned in the complex for use according to the present invention in a consecutive manner and/or the more than one TLR peptide agonist is positioned in the complex for use according to the present invention in a consecutive manner. This means in particular that all single units of a certain component, i.e. all cell penetrating peptides, all antigens or antigenic epitopes or all TLR peptide agonists, which are comprised by the complex are positioned in a stretch, which is not interrupted by any of the other two components. Rather, the other two components are positioned in the complex for example before or after such a stretch of all single units of said certain component. However, the single units of said certain component positioned consecutively in such a way may be linked to each other for example by a spacer or linker as described herein, which is not of the other two components.

**[0314]** It is particularly preferred that each of the components a), b), and c) is positioned in a consecutive manner.

**[0315]** Structurally each component a), b), and c) typically comprises a single main chain and at least one side chain. The term "main chain" (also "backbone chain"), as used in the context of the present invention, refers to the main continuous chain of covalently bond atoms in a molecule. For example, in peptides, polypeptides and proteins, the main chain (backbone) typically comprises alpha-carbon atoms and nitrogen atoms of the constituent amino acids linked by the peptide bond. The backbone does not include the side chains. The term "side chain" (also "pendant chain"), as used in the context of the present invention, refers to a chemical group that is attached to a core part of the molecule called "main chain" or backbone. For example, in peptides, polypeptides and proteins, the side chains typically represent the (main) parts of the constituent amino acids, which are attached to the alpha-carbon atoms of the backbone.

**[0316]** In the complex for use according to the present invention, the components a), b), and c) may be covalently linked via a linker or spacer as described herein or they may be directly covalently linked. Independently of whether a spacer or linker is used for covalent linkage or not, there are in principle four options of how two of the three components are linked to each other in the complex for use according to the present invention, namely:

1. (i) via main-chain/main-chain linkage,
2. (ii) via main-chain/side-chain linkage,
3. (iii) via side-chain/main-chain linkage or
4. (iv) via side-chain/side chain linkage.

**[0317]** Preferably, all three components a), b), and c) are linked via main-chain/main-chain linkage, thus resulting in particular in a main chain of the complex for use according to the present invention, which comprises the main chain of one or more cell penetrating peptide(s),

the main chain of one or more antigen(s) or antigenic epitope(s), and the main chain of one or more TLR peptide agonist(s). In other words, the main chain of one or more cell penetrating peptide(s), the main chain of one or more antigen(s) or antigenic epitope(s), and the main chain of one or more TLR peptide agonist(s) constitute the main chain of the complex for use according to the present invention, optionally together with further components, for example linker(s), spacer(s), etc.. Accordingly, the following arrangements of the components a), b), and c) are preferred, in particular as the at least one antigen or antigenic epitope is a polypeptide or protein, whereby said preferred arrangements are shown below in N-terminus -> C-terminus direction of the main chain of the complex and wherein all three components a), b), and c) are linked via main-chain/main-chain linkage and may be optionally linked by a linker, a spacer or another additional component:

- (α) component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - component c) (at least one TLR peptide agonist);
- (β) component c) (at least one TLR peptide agonist) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope);
- (γ) component a) (cell penetrating peptide) - component c) (at least one TLR peptide agonist) - component b) (at least one antigen or antigenic epitope);
- (δ) component c) (at least one TLR peptide agonist) - component b) (at least one antigen or antigenic epitope) - component a) (cell penetrating peptide);
- (ε) component b) (at least one antigen or antigenic epitope) - component a) (cell penetrating peptide) - component c) (at least one TLR peptide agonist); or
- (ζ) component b) (at least one antigen or antigenic epitope) - component c) (at least one TLR peptide agonist) - component a) (cell penetrating peptide).

**[0318]** In particular if all three components a), b), and c) are linked via main-chain/main-chain linkage, it is preferred that the at least one antigen or antigenic epitope is positioned C-terminally of the cell penetrating peptide, whereby the cell penetrating peptide and the at least one antigen or antigenic epitope are optionally linked by a further component, e.g. a linker, a spacer, or by the at least one TLR peptide agonist. Accordingly, this corresponds to the arrangements (α), (β), and (γ) from the arrangements shown above, i.e. from the above arrangements arrangements (α), (β), and (γ) are more preferred.

**[0319]** Even more preferably, the at least one antigen or antigenic epitope is positioned C-terminally of the cell penetrating peptide, whereby the cell penetrating peptide and the at least one antigen or antigenic epitope are optionally linked by a further component, e.g. a linker, a spacer, but not by the at least one TLR peptide agonist. Accordingly, this corresponds to the arrangements (α) and (β) from the arrangements shown above, i.e. from the above arrangements arrangements (α) and (β) are even more preferred. Particularly preferably, the

complex for use according to the present invention is a recombinant polypeptide or a recombinant protein and the components a) to c) are positioned in N-terminus -> C-terminus direction of the main chain of said complex in the order:

(a) component a) - component b) - component c); or

(β) component c) - component a) - component b),

wherein the components may be linked by a further component, in particular by a linker or a spacer.

**[0320]** Particularly preferred is arrangement (a), wherein the at least one TLR agonist comprises or consists of at least one TLR2 agonist, for example:

(a1) component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s);

(a2) component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s), one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s);

(a3) component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s) and one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s); or

(a4) component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s).

**[0321]** Alternatively, in such an arrangement comprising a TLR2 peptide agonist, additional TLR peptide agonists may also be arranged at other positions in the complex, for example:

(a5) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s);

(a6) one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s); or

(a7) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s).

**[0322]** Particularly preferred is arrangement (β), wherein the at least one TLR agonist comprises or consists of at least one TLR4 agonist, for example:

(β1) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope);

(β2) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s), one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope);

(β3) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) and one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope); or

(β4) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope).

**[0323]** Alternatively, in such an arrangement comprising a TLR4 peptide agonist, additional TLR peptide agonists may also be arranged at other positions in the complex, for example:

(β5) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s);

(β6) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s); or

(β7) one or more TLR4 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR4 peptide agonist(s) - component a) (cell penetrating peptide) - component b) (at least one antigen or antigenic epitope) - one or more TLR2 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR2 peptide agonist(s) and one or more TLR5 peptide agonist, e.g. 1, 2, 3, 4, or 5 TLR5 peptide agonist(s).

**[0324]** Alternatively, only two of the three components a), b), and c) are linked via main-chain/main-chain linkage in the complex for use according to the present invention.

**[0325]** For example components a) and b) are linked via main-chain/main-chain linkage, resulting thus in the following arrangements of the components a) and b) in the complex,

shown in N-terminus -> C-terminus direction of the main chain of the complex, whereby the components

1. a) and b) may be optionally linked by a further component, e.g. a linker, a spacer etc.:
  1. (1) cell penetrating peptide (a) - antigen/antigenic epitope (b); or
  2. (2) antigen/antigenic epitope (b) - cell penetrating peptide (a).

**[0326]** In such a case, component c), i.e. the at least one TLR peptide agonist, may then be arranged via main-chain/side-chain linkage, via side-chain/main-chain linkage or via side-chain/side chain linkage to either the cell penetrating peptide (a) or to the antigen/antigenic epitope (b) or, if present, to an additional component like a spacer or linker, which may be, for example, positioned between the cell penetrating peptide (a) and the antigen/antigenic epitope (b). This includes the following arrangements:

1. (i) component c) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component a), i.e. the main chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of the cell penetrating peptide;
2. (ii) component c) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component a), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the main chain of the cell penetrating peptide;
3. (iii) component c) may be linked - optionally via a spacer or a linker-via side-chain/side-chain linkage to component a), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of the cell penetrating peptide;
4. (iv) component c) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component b), i.e. the main chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one antigen or antigenic epitope;
5. (v) component c) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component b), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the main chain of the at least one antigen or antigenic epitope;
6. (vi) component c) may be linked - optionally via a spacer or a linker - via side-chain/side-chain linkage to component b), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one antigen or antigenic epitope;
7. (vii) component c) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to a linker or a spacer positioned between component a) and component b), i.e. the main chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component a) and component b);

8. (viii) component c) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to a linker or a spacer positioned between component a) and component b), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the main chain of a linker or a spacer positioned between component a) and component b); or
9. (ix) component c) may be linked - optionally via a spacer or a linker- via side-chain/side-chain linkage to a linker or a spacer positioned between component a) and component b), i.e. the side chain of the at least one TLR peptide agonist is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component a) and component b).

**[0327]** For example components b) and c) are linked via main-chain/main-chain linkage, resulting thus in the following arrangements of the components b) and c) in the complex, shown in N'- terminus → C-terminus direction of the main chain of the complex, whereby the components b) and c) may be optionally linked by a further component, e.g. a linker, a spacer etc.:

- (3) antigen/antigenic epitope (b) - TLR peptide agonist (c); or
- (4) TLR peptide agonist (c) - antigen/antigenic epitope (b).

**[0328]** In such a case, component a), i.e. the cell penetrating peptide, may then be arranged via main-chain/side-chain linkage, via side-chain/main-chain linkage or via side-chain/side chain linkage to either the antigen/antigenic epitope (b) or to the TLR peptide agonist (c) or, if present, to an additional component like a spacer or linker, which may be, for example, positioned between the antigen/antigenic epitope (b) and the TLR peptide agonist (c). This includes the following arrangements:

(x) component a) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component b), i.e. the main chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one antigen or antigenic epitope;

(xi) component a) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component b), i.e. the side chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the main chain of the at least one antigen or antigenic epitope;

(xii) component a) may be linked - optionally via a spacer or a linker- via side-chain/side-chain linkage to component b), i.e. the side chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one antigen or antigenic epitope;

(xiii) component a) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component c), i.e. the main chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one TLR peptide agonist;

(xiv) component a) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component c), i.e. the side chain the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the main chain of the at least one TLR peptide agonist;

(xv) component a) may be linked - optionally via a spacer or a linker - via side-chain/side-chain linkage to component c), i.e. the side chain of the cell penetrating peptide is covalently linked optionally via a spacer or a linker to the side chain of the at least one TLR peptide agonist;

(xvi) component a) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to a linker or a spacer positioned between component b) and component c), i.e. the main chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component b) and component c);

(xvii) component a) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to a linker or a spacer positioned between component b) and component c), i.e. the side chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the main chain of a linker or a spacer positioned between component b) and component c); or

(xviii) component a) may be linked - optionally via a spacer or a linker - via side-chain/side-chain linkage to a linker or a spacer positioned between component b) and component c), i.e. the side chain of the cell penetrating peptide is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component b) and component c).

**[0329]** For example components a) and c) are linked via main-chain/main-chain linkage, resulting thus in the following arrangements of the components a) and b) in the complex, shown in N-terminus → C-terminus direction of the main chain of the complex, whereby the components a) and c) may be optionally linked by a further component, e.g. a linker, a spacer etc.:

(5) cell penetrating peptide (a) - TLR peptide agonist (c); or

(6) TLR peptide agonist (c) - cell penetrating peptide (a).

**[0330]** In such a case, component b), i.e. the at least one antigen or antigenic epitope, may

then be arranged via main-chain/side-chain linkage, via side-chain/main-chain linkage or via side-chain/side chain linkage to either the cell penetrating peptide (a) or to the TLR peptide agonist (c) or, if present, to an additional component like a spacer or linker, which may be, for example, positioned between the cell penetrating peptide (a) and the TLR peptide agonist (c). This includes the following arrangements:

(xix) component b) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component a), i.e. the main chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of the cell penetrating peptide;

(xx) component b) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component a), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the main chain of the cell penetrating peptide;

(xxi) component b) may be linked - optionally via a spacer or a linker - via side-chain/side-chain linkage to component a), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of the cell penetrating peptide;

(xxii) component b) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to component c), i.e. the main chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one TLR peptide agonist;

(xxiii) component b) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to component c), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the main chain of the at least one TLR peptide agonist;

(xxiv) component b) may be linked - optionally via a spacer or a linker - via side-chain/side-chain linkage to component c), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of the at least one TLR peptide agonist;

(xxv) component b) may be linked - optionally via a spacer or a linker - via main-chain/side-chain linkage to a linker or a spacer positioned between component a) and component c), i.e. the main chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component a) and component c);

(xxvi) component b) may be linked - optionally via a spacer or a linker - via side-chain/main-chain linkage to a linker or a spacer positioned between component a) and component c), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the main chain of a linker or a spacer positioned between component a) and component c); or

(xxvii) component b) may be linked - optionally via a spacer or a linker via side-chain/side-chain linkage to a linker or a spacer positioned between component a) and component c), i.e. the side chain of the at least one antigen or antigenic epitope is covalently linked - optionally via a spacer or a linker - to the side chain of a linker or a spacer positioned between component a) and component c).

**[0331]** Alternatively, it is also conceivable that in the complex for use according to the present invention all three of the components a), b), and c) are arranged via main-chain/side-chain linkage, via side-chain/main-chain linkage or via side-chain/side chain linkage, optionally linked by an additional component, e.g. a spacer or a linker.

#### **Colorectal cancer**

**[0332]** The present invention provides the complex as described above for use in the prevention and/or treatment of colorectal cancer.

**[0333]** Colorectal cancer (CRC, also known as "bowel cancer") is a cancer that comprises colon cancers and rectal cancers (CC). Both individual cancers have many features in common, but the cancer starting point. According to Siegel, R., C. Desantis, and A. Jemal, Colorectal cancer statistics, 2014. CA Cancer J Clin, 2014. 64(2): p. 104-17, in the United States between 2006 and 2010, the incidence by tumor site is slightly more important in the proximal colon (first and middle parts of the colon). With about 19 cases on 100,000 people, it represents 42% of the cases. It is followed by the rectal cancer, with 28% of the cases and the distal colon (bottom part of the colon) with an incidence of 10 cases on 100,000 people.

**[0334]** Anatomically, the term "colorectal cancer" includes (i) cancers of colon, such as cancers of cecum (including cancers the ileocecal valve), appendix, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon (including cancers of sigmoid (flexure)) as well as cancers of overlapping sites of colon; (ii) cancers of rectosigmoid junction, such as cancers of colon and rectum and cancers of rectosigmoid; and (iii) cancers of rectum, such as cancers of rectal ampulla.

**[0335]** Preferably, the colorectal cancer is a cancer of colon, such as a cancer of cecum (including cancer the ileocecal valve), cancer of appendix, cancer of ascending colon, cancer of hepatic flexure, cancer of transverse colon, cancer of splenic flexure, cancer of descending colon, cancer of sigmoid colon (including cancers of sigmoid (flexure)) or a combination thereof.

**[0336]** It is also preferred that the colorectal cancer is a cancer of rectosigmoid junction, such as (i) a cancer of colon and rectum or (ii) a cancer of rectosigmoid.

**[0337]** Furthermore, it is also preferred that the colorectal cancer is a cancer of rectum, such as a cancer of rectal ampulla.

**[0338]** Regarding the cell type, colorectal cancers include colorectal adenocarcinoma, colorectal stromal tumors, primary colorectal lymphoma, colorectal leiomyosarcoma, colorectal melanoma, colorectal squamous cell carcinoma and colorectal carcinoid tumors, such as, for example, carcinoid tumors of cecum, appendix, ascending colon, transverse colon, descending colon, sigmoid colon and/or rectum. Thus, preferred types of colorectal cancers include colorectal adenocarcinoma, colorectal stromal tumors, primary colorectal lymphoma, colorectal leiomyosarcoma, colorectal melanoma, colorectal squamous cell carcinoma and colorectal carcinoid tumors, such as, for example, carcinoid tumors of cecum, appendix, ascending colon, transverse colon, descending colon, sigmoid colon and/or rectum. More preferably, the colorectal cancer is a colorectal adenocarcinoma or a colorectal carcinoid carcinoma. Even more preferably, the colorectal cancer is a colorectal adenocarcinoma.

**[0339]** More than 95% of CRCs are adenocarcinomas. Colorectal adenocarcinomas typically start from glandular cells that make mucus to lubricate the colon or rectum. CRC typically starts in the innermost layer and can grow through some or all of the other layers. In rare cases, CRC could form in a polyp, which facilitates its growth into the wall of starting region. In advanced stage (stage III and IV), the cancer travels to nearby lymph nodes or to distant parts of the body through blood vessels.

**[0340]** For example, in colorectal cancer, the TNM staging system includes the following stages for primary tumors ("T" stages): TX - Primary tumour cannot be assessed, T0 - No evidence of primary tumour, Ta - Non-invasive papillary carcinoma, Tis - Carcinoma in situ: intraepithelial or invasion of lamina propria, T1 - Tumour invades submucosa, T2 - Tumour invades muscularis propria, T3 - Tumour invades through the muscularis propria into the pericolorectal tissues, T4a - Tumour penetrates to the surface of the visceral peritoneum and T4b - Tumour directly invades or is adherent to other organs or structures; following stages for lymph nodes ("N" stages): NX - Regional lymph nodes cannot be assessed, N0 - No regional lymph node metastasis, N1 - Metastasis in 1-3 regional lymph nodes with N1a - Metastasis in 1 regional lymph node, N1b - Metastasis in 2-3 regional lymph nodes and N1c - Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis, N2 - Metastasis in 4 or more lymph nodes with N2a - Metastasis in 4-6 regional lymph nodes and N2b - Metastasis in 7 or more regional lymph nodes; and the following stages for distant metastasis ("M" stages): M0 - No distant metastasis and M1 - Distant metastasis with M1a - Metastasis confined to 1 organ or site (eg, liver, lung, ovary, nonregional node) and M1b - Metastases in more than 1 organ/site or the peritoneum. This stages can be integrated into the following numerical staging of colorectal cancer: Stage 0: Tis, N0, M0; Stage I: T1, N0, M0 or T2, N0, M0; Stage IIA: T3, N0, M0; Stage IIB: T4a, N0, M0; Stage IIC: T4b, N0, M0; Stage IIIA: T1-T2, N1/N1c, M0 or T1, N2a, M0; Stage IIIB: T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0; Stage IIIC: T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0; Stage IVA: any T, any N, M1a and Stage IVB: any T, any N, M1b. Briefly, in Stage 0, the cancer has not grown beyond the inner layer of the colon or rectum; in

Stage I the cancer has spread from the mucosa to the muscle layer; in Stage II the cancer has spread through the muscle layer to the serosa nearby organs; in Stage III the cancer has spread to nearby lymph node(s) or cancer cells have spread to tissues near the lymph nodes; and in Stage IV the cancer has spread through the blood and lymph nodes to other parts of the body.

**[0341]** Despite the term "cancer", colorectal cancer includes all numerical stages as described above, and, thus, a preferred stage of colorectal cancer may be selected from the group consisting of Stage 0 (Tis, N0, M0), Stage I (T1, N0, M0 or T2, N0, M0), Stage IIA (T3; N0, M0), Stage IIB (T4a, N0, M0), Stage IIC (T4b, N0, M0), Stage IIIA (T1-T2, N1/N1c, M0 or T1, N2a, M0), Stage IIIB (T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0), Stage IIIC (T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0), Stage IVA (any T, any N, M1a) and Stage IVB (any T, any N, M1b). More preferably, the colorectal cancer is selected from the group consisting of Stage I (T1, N0, M0 or T2, N0, M0), Stage IIA (T3, N0, M0), Stage IIB (T4a, N0, M0), Stage IIC (T4b, N0, M0), Stage IIIA (T1-T2, N1/N1c, M0 or T1, N2a, M0), Stage IIIB (T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0), Stage IIIC (T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0), Stage IVA (any T, any N, M1a) and Stage IVB (any T, any N, M1b). Even more preferably, the colorectal cancer is selected from the group consisting of Stage IIA (T3, N0, M0), Stage IIB (T4a, N0, M0), Stage IIC (T4b, N0, M0), Stage IIIA (T1-T2, N1/N1c, M0 or T1, N2a, M0), Stage IIIB (T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0), Stage IIIC (T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0), Stage IVA (any T, any N, M1a) and Stage IVB (any T, any N, M1b). Most preferably, the colorectal cancer is (i) Stage III colorectal cancer, such as Stage IIIA (T1-T2, N1/N1c, M0 or T1, N2a, M0), Stage IIIB (T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0), or Stage IIIC (T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0), or (ii) Stage IV colorectal cancer, such as Stage IVA (any T, any N, M1a) and Stage IVB (any T, any N, M1b).

***Nucleic acid encoding the peptides and protein complexes***

**[0342]** In another aspect the present invention provides a nucleic acid, in particular a nucleic acid molecule, encoding the complex as described herein, wherein the complex is a polypeptide or a protein, for use in the prevention and/or treatment of colorectal cancer. In particular, the present invention provides polynucleotides for use in the prevention and/or treatment of colorectal cancer, said polynucleotides encoding the complex as defined above. In other words, the present invention provides a nucleic acid, in particular a nucleic acid molecule, comprising a polynucleotide, wherein the polynucleotide encodes the complex as described herein and wherein the complex is a polypeptide or a protein, for use in the prevention and/or treatment of colorectal cancer.

**[0343]** The present invention also provides a nucleic acid, in particular a nucleic acid molecule, encoding the complex according to the present invention as described above, wherein the complex is a polypeptide or a protein. In particular, the present invention provides polynucleotides encoding the complex as defined above. In other words, the present invention

provides a nucleic acid, in particular a nucleic acid molecule, comprising a polynucleotide, wherein the polynucleotide encodes the complex according to the present invention as described herein and wherein the complex is a polypeptide or a protein. Such a nucleic acid may be used in the prevention and/or treatment of colorectal cancer.

**[0344]** Nucleic acids preferably comprise single stranded, double stranded or partially double stranded nucleic acids, preferably selected from genomic DNA, cDNA, RNA, siRNA, antisense DNA, antisense RNA, ribozyme, complimentary RNA/DNA sequences with or without expression elements, a mini-gene, gene fragments, regulatory elements, promoters, and combinations thereof. Further preferred examples of nucleic acid (molecules) and/or polynucleotides include, e.g., a recombinant polynucleotide, a vector, an oligonucleotide, an RNA molecule such as an rRNA, an mRNA, an miRNA, an siRNA, or a tRNA, or a DNA molecule as described above. It is thus preferred that the nucleic acid (molecule) is a DNA molecule or an RNA molecule; preferably selected from genomic DNA; cDNA; siRNA; rRNA; mRNA; antisense DNA; antisense RNA; ribozyme; complimentary RNA and/or DNA sequences; RNA and/or DNA sequences with or without expression elements, regulatory elements, and/or promoters; a vector; and combinations thereof.

**[0345]** The invention relates to a nucleic acid (for use) according to the present invention, as defined by claim 24 ... If more than one antigen or antigenic epitope, which is a polypeptide or protein, is comprised by said complex, the more than one antigens or antigenic epitopes are also covalently linked, optionally with peptidic spacer(s) or linker(s) as described herein. Similarly, if more than one TLR peptide agonist is comprised by said complex, the more than one TLR peptide agonists are also covalently linked, optionally with peptidic spacer(s) or linker(s) as described herein.

**[0346]** The nucleic acid (for use) according to the present invention and as defined by claim 24 encodes a complex which is a (recombinant) fusion protein comprising (a) a cell penetrating peptide as described above, (b) at least one, preferably at least two, more preferably at least three, even more preferably at least four, particularly preferably at least five, most preferably at least six antigens or antigenic epitopes as described above, preferably arranged in a consecutive manner as described above and (c) at least one TLR agonist as described above.

**[0347]** Most preferably, the nucleic acid (molecule) as claimed encodes an exemplified complex as described herein or a functional sequence variant thereof. Accordingly, the nucleic acid (molecule) most preferably comprises a polynucleotide encoding an amino acid sequence according to any of SEQ ID NOs: 26 - 28, 33 - 34, 37 - 41, 46, 69, or 72 - 87 or an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NOs: 26 - 28, 33 - 34, 37 - 41, 46, 69, or 72 - 87. In particular, the nucleic acid (molecule) most preferably comprises a polynucleotide encoding an amino acid sequence according to any of SEQ ID NOs: 26 - 28, 33 - 34, 37 - 41, 46, 69, or 72 - 91 or an amino acid sequence sharing at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% sequence identity with any of SEQ ID NOs: 26 - 28, 33 - 34, 37-41,

46, 69, or 72-91.

**[0348]** It is particularly preferred that the nucleic acid (molecule) according to the present invention comprises a polynucleotide encoding an amino acid sequence according to SEQ ID NO: 89, or a functional sequence variant thereof having at least 50% sequence identity (preferably at least 60%, more preferably at least 70%, more preferably at least 75%, even more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, particularly preferably at least 95% and most preferably at least 98% or 99% sequence identity).

#### ***Production and purification of the complexes***

**[0349]** According to a further aspect the present invention provides a vector, preferably for use in the prevention and/or treatment of colorectal cancer, in particular a recombinant vector, comprising a nucleic acid as claimed.

**[0350]** The term "vector", as used in the context of the present invention, refers to a nucleic acid molecule, preferably to an artificial nucleic acid molecule, i.e. a nucleic acid molecule which does not occur in nature. A vector in the context of the present invention is suitable for incorporating or harboring a desired nucleic acid sequence. Such vectors may be storage vectors, expression vectors, cloning vectors, transfer vectors etc. A storage vector is a vector which allows the convenient storage of a nucleic acid molecule. Thus, the vector may comprise a sequence corresponding, e.g., to a desired complex according to the present invention. An expression vector may be used for production of expression products such as RNA, e.g. mRNA, or peptides, polypeptides or proteins. For example, an expression vector may comprise sequences needed for transcription of a sequence stretch of the vector, such as a promoter sequence. A cloning vector is typically a vector that contains a cloning site, which may be used to incorporate nucleic acid sequences into the vector. A cloning vector may be, e.g., a plasmid vector or a bacteriophage vector. A transfer vector may be a vector which is suitable for transferring nucleic acid molecules into cells or organisms, for example, viral vectors. A vector in the context of the present invention may be, e.g., an RNA vector or a DNA vector. Preferably, a vector is a DNA molecule. For example, a vector in the sense of the present application comprises a cloning site, a selection marker, such as an antibiotic resistance factor, and a sequence suitable for multiplication of the vector, such as an origin of replication. Preferably, a vector in the context of the present application is a plasmid vector. Preferably, a vector in the context of the present application is an expression vector.

**[0351]** Cells transformed with a vector as described above, preferably for use in the prevention and/or treatment of colorectal cancer, are also included within the scope of the invention. Examples of such cells include, but are not limited to, bacterial cells, e.g. *E. coli*, and eukaryotic cells, e.g., yeast cells, animal cells or plant cells. In one embodiment the cells are mammalian, e.g., human, CHO, HEK293T, PER.C6, NS0, myeloma or hybridoma cells. Accordingly, the present invention also relates to a cell expressing the complex (for use) according to the

present invention; or comprising the vector (for use) according to the present invention.

**[0352]** In particular, a cell may be transfected with a vector as described above, preferably with an expression vector. The term "transfection" refers to the introduction of nucleic acid molecules, such as DNA or RNA (e.g. mRNA) molecules, into cells, preferably into eukaryotic cells. In the context of the present invention, the term "transfection" encompasses any method known to the skilled person for introducing nucleic acid molecules into cells, preferably into eukaryotic cells, such as into mammalian cells. Such methods encompass, for example, electroporation, lipofection, e.g. based on cationic lipids and/or liposomes, calcium phosphate precipitation, nanoparticle based transfection, virus based transfection, or transfection based on cationic polymers, such as DEAE-dextran or polyethylenimine etc. Preferably, the introduction is non-viral.

**[0353]** Numerous expression systems can be used, including without limitation chromosomes, episomes, and derived viruses. More particularly, the vector as described above, in particular the recombinant vector used, can be derived from bacterial plasmids, transposons, yeast episomes, insertion elements, yeast chromosome elements, viruses such as baculovirus, papilloma viruses such as SV40, vaccinia viruses, adenoviruses, fox pox viruses, pseudorabies viruses, retroviruses.

**[0354]** For example, such vectors, in particular recombinant vectors, can equally be cosmid or phagemid derivatives. The nucleotide sequence, in particular the nucleic acid according to the present invention, may be inserted in the recombinant expression vector by methods well known to a person skilled in the art such as, for example, those described in MOLECULAR CLONING: A LABORATORY MANUAL, Sambrook et al., 4th Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001.

**[0355]** The vector, in particular the recombinant vector, may also include nucleotide sequences that control the regulation of the expression, in particular of the nucleic acid (for use) according to the present invention, as well as nucleotide sequences permitting the expression, the transcription, and the translation, in particular of the nucleic acid (for use) according to the present invention. Typically, these sequences are selected according to the host cells used.

**[0356]** Thus, for example, an appropriate secretion signal can be integrated in the vector (for use) according to the present invention, in particular in a recombinant vector, so that the polypeptide or protein encoded by the nucleic acid (for use) according to the present invention, will be directed, for example towards the lumen of the endoplasmic reticulum, towards the periplasmic space, on the membrane or towards the extracellular environment. The choice of an appropriate secretion signal may facilitate subsequent protein purification.

**[0357]** In yet another aspect the present invention provides a host cell, preferably for use in the prevention and/or treatment of colorectal cancer, the host cell comprising a vector, in particular a recombinant vector, as claimed.

**[0358]** The introduction of the vector, in particular the recombinant vector, into a host cell can be carried out according to methods that are well known to a person skilled in the art, such as those described in BASIC METHODS IN MOLECULAR BIOLOGY, Davis et al., 2nd ed., McGraw-Hill Professional Publishing, 1995, and MOLECULAR CLONING: A LABORATORY MANUAL, supra, including for example transfection as described above, e.g. by calcium phosphate, by DEAE dextran, or by cationic lipids; microinjection, electroporation, transduction or infection.

**[0359]** The host cell can be, for example, bacterial cells such as *E. coli*, cells of fungi such as yeast cells and cells of *Aspergillus*, *Streptomyces*, insect cells, and/or any cell line, e.g. Chinese Hamster Ovary cells (CHO), C127 mouse cell line, BHK cell line of Syrian hamster cells, Human Embryonic Kidney 293 (HEK 293) cells. Preferably, the host cell (for use) according to the present invention is mammalian, e.g., human, CHO, HEK293T, PER.C6, NS0, myeloma or hybridoma cells. Dendritic cells and dendritic cell lines are particularly preferred as a host cell. Typically, the choice of a culture medium depends in particular on the choice of the cell type and/or the cell line, whereby the skilled person is aware of suitable culture media, which are appropriate for a selected cell type and/or cell line.

**[0360]** The host cells can be used, for example, to express a polypeptide or protein, in particular the complex (for use) according to the present invention, on the basis of the vector and/or the nucleic acid as described herein. After purification by standard methods, the expressed polypeptide or protein, in particular the complex (for use) according to the present invention, can be used as described herein.

**[0361]** A method for preparing the complex as defined herein is disclosed (not claimed), in particular wherein the complex is a polypeptide or protein. Said method comprises the steps of:

1. (i) cultivating a host cell as described above in a culture medium; and
2. (ii) separating the complex as defined herein from the culture medium or separating the complex as defined herein from the host cell lysate after host cell lysis.

**[0362]** Thus, the complex obtained by such a method is in particular a complex (for use) according to the present invention as described herein.

**[0363]** For protein extraction commercially available kits and/or reagents may be used, for example BugBuster™ from Novagen.

**[0364]** The method for preparing the complex as defined herein may further comprise the following step:

(iii) solubilization of the complex as defined herein, e.g. by resuspension in solutions containing urea or guanidine hydrochloride (GuHCl), wherein step (iii) follows step (ii) as described above.

**[0365]** Moreover, the method for preparing the complex as defined herein may further comprise the following step:

(iv) purification of the complex as defined herein, preferably by one-step affinity chromatography, wherein step (iv) follows step (ii), or, if present, step (iii) as described above.

**[0366]** In addition, the complex as defined herein may also be prepared by synthetic chemistry methods, for example by solid-phase peptide synthesis.

**[0367]** Purification of those peptides or proteins may be carried out by means of any technique known in the art for protein/peptide purification. Exemplary techniques include ion-exchange chromatography, hydrophobic interaction chromatography, and immunoaffinity methods.

**[0368]** Thus, a method for preparing the complex as defined herein is disclosed (not claimed) comprising the steps of:

1. (i) chemically synthesizing said complex; and
2. (ii) purifying said complex.

**[0369]** In particular, in the method for preparing a complex as defined herein, the complex chemically synthesized in step (i) and purified in step (ii) comprises an amino acid sequences as described herein for a cell penetrating peptide, an amino acid sequence as described herein for a TLR peptide agonist, and, optionally if the at least one antigen and/or antigenic epitope is a peptide or a protein, an amino acid sequence as described herein for an antigen or antigenic epitope.

**[0370]** Alternatively, a method for preparing the complex as defined herein is disclosed (not claimed), wherein

1. (i) the cell penetrating peptide, the at least one antigen or antigenic fragment and/or the at least one TLR peptide agonist are synthesized separately;
2. (ii) optionally, the cell penetrating peptide, the at least one antigen or antigenic fragment and/or the at least one TLR peptide agonist are purified; and
3. (iii) the cell penetrating peptide, the at least one antigen or antigenic fragment and/or the at least one TLR peptide agonist are covalently linked as described above, optionally by a spacer or linker or by a cross-linking agent as described above.

***Cells loaded with the complexes according to the invention***

**[0371]** In yet another aspect the present invention relates to a cell loaded with the complex as claimed, preferably for use in the prevention and/or treatment of colorectal cancer. For

example, the cells loaded with the complex as defined herein are cells from a subject to be treated, in particular isolated cells from a subject to be treated, i.e. cells isolated from a subject to be treated.

**[0372]** As used in the context of the present invention, the term "subject" refers in particular to mammals. For example, mammals contemplated by the present invention include human, primates, domesticated animals such as cattle, sheep, pigs, horses, laboratory rodents and the like. More preferably, the term "subject" refers to a human subject.

**[0373]** As used in the context of the present invention, "treatment" and "treating" and the like generally mean obtaining a desired pharmacological and physiological effect. The effect may be prophylactic in terms of preventing or partially preventing a disease, a symptom or a condition thereof and/or may be therapeutic in terms of a partial or complete cure of a disease, a condition, a symptom or an adverse effect attributed to the disease. The term "treatment" as used herein covers any treatment of a disease in a mammal, in particular in a human, and includes: (a) preventing the disease from occurring in a subject who may be predisposed to the disease but the outbreak of the disease has not yet occurred and/or the disease has not yet been diagnosed in this subject, for example a preventive early asymptomatic intervention; (b) inhibiting the disease, i.e., arresting or slowing down its development; or (c) relieving the disease, i.e., causing an at least partial regression of the disease and/or of at least one of its symptoms or conditions such as improvement or remediation of damage. In particular, the methods, uses, formulations and compositions according to the invention are useful in the treatment of cancers or infectious diseases and/or in the prevention of evolution of cancers into an advanced or metastatic stage in subjects with early stage cancer, thereby improving the staging of the cancer. When applied to cancers, prevention of a disease or disorder includes the prevention of the appearance or development of a cancer in an individual identified as at risk of developing said cancer, for instance due to past occurrence of said cancer in the circle of the individual's relatives, and prevention of infection with tumor promoting pathogens such as, for example, Epstein-Barr virus (EBV), Human papillomavirus (HPV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Herpes virus 8 (HHV8), human T-cell leukemia virus type 1 (HTLV-1), Merkel cell polyomavirus (MCV) and Helicobacter pylori. Also covered by the terms "prevention/treatment" of a cancer is the stabilization or delay of an already diagnosed cancer in an individual. By "stabilization", it is meant the prevention of evolution of cancer into advanced or metastatic stage in subjects with early stage cancer.

**[0374]** Preferably, the cell loaded with the complex as defined herein is an antigen-presenting cell (APC). Preferably, the antigen presenting cell is selected from the group consisting of a dendritic cell (DC), a macrophage and a B-cell. Dendritic cells, in particular dendritic cells (conventional and/or plasmacytoid) isolated from a subject to be treated, are more preferred.

**[0375]** Methods to isolate antigen-presenting cells, in particular dendritic cells, from a subject are known to the skilled person. They include harvesting monocytes or hematopoietic stem cells from bone marrow, cord blood, or peripheral blood. They also include the use of embryonic stem (ES) cells and induced pluripotent stem cells (iPS). Antigen presenting cells, in

particular dendritic cells or their precursors, can be enriched by methods including elutriation and magnetic bead based separation, which may involve enrichment for CD14<sup>+</sup> precursor cells.

**[0376]** Methods to load the complex as defined herein into the cells, preferably into the above-mentioned antigen presenting cells, more preferably into dendritic cells, and further to prepare such cells before administration to a subject are known to one skilled in the art. For example, preparation of dendritic cells can include their culture or differentiation using cytokines that may include for example GM-CSF and IL-4. Dendritic cell lines may also be employed. Loading of the complex of the invention into the cells, preferably into APC, more preferably into the dendritic cells, can involve co-incubation of the complex of the invention with the cells in culture, making use of the intrinsic properties of the cell penetrating peptide comprised by the complex as defined herein (i.e. its internalization ability). Further culture of the cells, e.g. the dendritic cells, thus loaded to induce efficient maturation can include addition of cytokines including IL-1 $\beta$ , IL-6, TNF $\alpha$ , PGE2, IFN $\alpha$ , and adjuvants which may include poly-IC, poly-ICLC (i.e. a synthetic complex of carboxymethylcellulose, polyinosinic-polycytidylic acid, and poly-L-lysine double-stranded RNA), and further TLR agonists and NLR (nucleotide-binding oligomerization domain-like receptors) agonists.

**[0377]** A method for preparing cells, in particular antigen presenting cells, loaded with the complex as defined herein may comprise the steps of:

1. (i) transducing or transfecting said cells with the complex of the invention;
2. (ii) cultivating said cells in a culture medium; and
3. (iii) separating said cells from the culture medium.

**[0378]** Preferably, the cells are loaded with a complex as claimed, wherein the complex is a polypeptide or a protein and are used in the prevention and/or treatment of colorectal cancer.

**[0379]** Preferably, the cells loaded with a complex(es) according to the invention and used in the prevention and/or treatment of colorectal cancer present the at least one antigen or antigenic epitope comprised by said complex at the cell surface in an MHC class I context and/or in an MHC class II context.

***Compositions and kits according to the present invention***

**[0380]** According to another aspect, the invention provides a composition, preferably for use in the prevention and/or treatment of colorectal cancer, the composition comprising at least one component selected from:

1. (i) a complex as claimed,

2. (ii) a nucleic acid as claimed,
3. (iii) a vector as claimed,
4. (iv) a host cell as claimed, and
5. (v) a cell loaded with a complex as claimed.

**[0381]** Preferably, the composition according to the present invention comprises the complex as claimed.

**[0382]** The composition (for use) according to the present invention may also comprises more than one of the above components (i) to (v). For example, the composition (for use) according to the present invention may comprise at least two different complexes under (i), at least two different nucleic acids under (ii), at least two different vectors under (iii), at least two different host cells under (iv), and/or at least two different cells under (v); e.g., the composition (for use) according to the invention may comprise at least two different complexes (i) and/or at least two different nucleic acids (ii).

**[0383]** For example, the different complexes (i) comprised by the composition as described above may differ in either component a), i.e. in the cell penetrating peptides, in component b), i.e. in the antigens or antigenic epitopes or in the subsets of more than one antigen or antigenic epitope, or in component c), i.e. in the TLR peptide agonist or in the subset of more than one TLR peptide agonist; or the different complexes (i) comprised by the composition as described above may differ in two out of the three components a), b), and c); or the different complexes (i) comprised by the composition as described above may differ in all three components a), b), and c) of the complex. Accordingly, the different nucleic acids (ii) comprised by the composition as described above may differ in that they encode such different complexes; the different vectors (iii) comprised by the composition as described above may differ in that they comprise such different nucleic acids; the different host cells (iv) comprised by the composition as described above may differ in that they comprise such different vectors; and the different cells loaded with a complex (v) comprised by the composition as described above may differ in that they are loaded with such different complexes.

**[0384]** The present invention also provides a vaccine, preferably for use in the prevention and/or treatment of colorectal cancer, the vaccine comprising at least one component selected from:

1. (i) a complex as claimed,
2. (ii) a nucleic acid as claimed,
3. (iii) a vector as claimed,
4. (iv) a host cell as claimed, and
5. (v) a cell loaded with a complex as claimed.

**[0385]** Preferably, the vaccine (for use) according to the present invention comprises the complex as claimed.

**[0386]** Thereby, the above details described for the composition (for use) according to the present invention regarding more than one of the components (i) to (v), also apply for the vaccine (for use) according to the present invention.

**[0387]** As used in the context of the present invention, the term "vaccine" refers to a biological preparation that provides innate and/or adaptive immunity, typically to a particular disease, preferably cancer. Thus, a vaccine supports in particular an innate and/or an adaptive immune response of the immune system of a subject to be treated. For example, the antigen or antigenic epitope of the complex as defined herein typically leads to or supports an adaptive immune response in the patient to be treated, and the TLR peptide agonist of the complex as defined herein may lead to or support an innate immune response.

**[0388]** The inventive composition, in particular the inventive vaccine, may also comprise a pharmaceutically acceptable carrier, adjuvant, and/or vehicle as defined below for the inventive pharmaceutical composition. In the specific context of the inventive composition, in particular of the inventive vaccine, the choice of a pharmaceutically acceptable carrier is determined in principle by the manner in which the inventive composition, in particular the inventive vaccine, is administered. The inventive composition, in particular the inventive vaccine, can be administered, for example, systemically or locally. Routes for systemic administration in general include, for example, transdermal, oral, parenteral routes, including subcutaneous, intravenous, intramuscular, intraarterial, intradermal and intraperitoneal injections and/or intranasal administration routes. Routes for local administration in general include, for example, topical administration routes but also intradermal, transdermal, subcutaneous, or intramuscular injections or intralesional, intracranial, intrapulmonary, intracardial, intranodal and sublingual injections. More preferably, inventive composition, in particular the vaccines, may be administered by an intradermal, subcutaneous, intranodal or intramuscular route. Even more preferably, the inventive composition, in particular the vaccine, may be administered by subcutaneous, intranodal or intramuscular route. Particularly preferably, the inventive composition, in particular the vaccines, may be administered by subcutaneous or intranodal route. Most preferably, the inventive composition, in particular the vaccines may be administered by subcutaneous route. Inventive composition, in particular the inventive vaccines, are therefore preferably formulated in liquid (or sometimes in solid) form.

**[0389]** The suitable amount of the inventive composition, in particular the inventive vaccine, to be administered can be determined by routine experiments with animal models. Such models include, without implying any limitation, rabbit, sheep, mouse, rat, dog and non-human primate models. Preferred unit dose forms for injection include sterile solutions of water, physiological saline or mixtures thereof. The pH of such solutions should be adjusted to about 7.4. Suitable carriers for injection include hydrogels, devices for controlled or delayed release, polylactic acid and collagen matrices. Suitable pharmaceutically acceptable carriers for topical application include those which are suitable for use in lotions, creams, gels and the like. If the inventive

composition, in particular the inventive vaccine, is to be administered orally, tablets, capsules and the like are the preferred unit dose form. The pharmaceutically acceptable carriers for the preparation of unit dose forms which can be used for oral administration are well known in the prior art. The choice thereof will depend on secondary considerations such as taste, costs and storability, which are not critical for the purposes of the present invention, and can be made without difficulty by a person skilled in the art.

**[0390]** The inventive composition, in particular the inventive vaccine, can additionally contain one or more auxiliary substances in order to further increase its immunogenicity. A synergistic action of the inventive complex as defined above and of an auxiliary substance, which may be optionally contained in the inventive vaccine as described above, is preferably achieved thereby. Depending on the various types of auxiliary substances, various mechanisms can come into consideration in this respect. For example, compounds that permit the maturation of dendritic cells (DCs), for example lipopolysaccharides, TNF-alpha or CD40 ligand, form a first class of suitable auxiliary substances. In general, it is possible to use as auxiliary substance any agent that influences the immune system in the manner of a "danger signal" (LPS, GP96, etc.) or cytokines, such as GM-CSF, which allow an immune response produced by the immune-stimulating adjuvant according to the invention to be enhanced and/or influenced in a targeted manner. Particularly preferred auxiliary substances are cytokines, such as monokines, lymphokines, interleukins or chemokines, that further promote the innate immune response, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IFN-alpha, IFN-beta, IFN-gamma, GM-CSF, G-CSF, M-CSF, LT-beta or TNF-alpha, growth factors, such as hGH.

**[0391]** Further additives which may be included in the inventive vaccine are emulsifiers, such as, for example, Tween<sup>®</sup>; wetting agents, such as, for example, sodium lauryl sulfate; colouring agents; taste-imparting agents, pharmaceutical carriers; tablet-forming agents; stabilizers; antioxidants; preservatives.

**[0392]** The inventive composition, in particular the inventive vaccine, can also additionally contain any further compound, which is known to be immune-stimulating due to its binding affinity (as ligands) to human Toll-like receptors TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, or due to its binding affinity (as ligands) to murine Toll-like receptors TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 or TLR13.

**[0393]** Another class of compounds, which may be added to an inventive composition, in particular to an inventive vaccine, in this context, may be CpG nucleic acids, in particular CpG-RNA or CpG-DNA. A CpG-RNA or CpG-DNA can be a single-stranded CpG-DNA (ss CpG-DNA), a double-stranded CpG-DNA (dsDNA), a single-stranded CpG-RNA (ss CpG-RNA) or a double-stranded CpG-RNA (ds CpG-RNA). The CpG nucleic acid is preferably in the form of CpG-RNA, more preferably in the form of single-stranded CpG-RNA (ss CpG-RNA). The CpG nucleic acid preferably contains at least one or more (mitogenic) cytosine/guanine dinucleotide sequence(s) (CpG motif(s)). According to a first preferred alternative, at least one CpG motif

contained in these sequences, in particular the C (cytosine) and the G (guanine) of the CpG motif, is unmethylated. All further cytosines or guanines optionally contained in these sequences can be either methylated or unmethylated. According to a further preferred alternative, however, the C (cytosine) and the G (guanine) of the CpG motif can also be present in methylated form.

**[0394]** The present invention also provides a pharmaceutical composition, preferably for use in the prevention and/or treatment of colorectal cancer, in particular a vaccine composition as claimed. Use in a method for treating a subject, preferably a mammalian subject, and most preferably a human subject, who is suffering from colorectal cancer, is described herein.

**[0395]** In particular, the present invention provides a pharmaceutical composition (for use) in the prevention and/or treatment of colorectal cancer comprising at least one complex as claimed or at least one cell loaded with a complex as claimed, and optionally a pharmaceutically acceptable carrier and/or vehicle, or any excipient, buffer, stabilizer or other materials well known to those skilled in the art, in particular the pharmaceutical composition comprising at least one complex as claimed or at least one cell loaded with a complex as claimed and a pharmaceutically acceptable carrier.

**[0396]** As a further ingredient, the inventive pharmaceutical composition may in particular comprise a pharmaceutically acceptable carrier and/or vehicle. In the context of the present invention, a pharmaceutically acceptable carrier typically includes the liquid or non-liquid basis of the inventive pharmaceutical composition. If the inventive pharmaceutical composition is provided in liquid form, the carrier will typically be pyrogen-free water; isotonic saline or buffered (aqueous) solutions, e.g. phosphate, citrate etc. buffered solutions. Particularly for injection of the inventive pharmaceutical composition, water or preferably a buffer, more preferably an aqueous buffer, may be used, containing a sodium salt, preferably at least 30 mM of a sodium salt, a calcium salt, preferably at least 0.05 mM of a calcium salt, and optionally a potassium salt, preferably at least 1 mM of a potassium salt. According to a preferred embodiment, the sodium, calcium and, optionally, potassium salts may occur in the form of their halogenides, e.g. chlorides, iodides, or bromides, in the form of their hydroxides, carbonates, hydrogen carbonates, or sulfates, etc. Without being limited thereto, examples of sodium salts include e.g. NaCl, NaI, NaBr, Na<sub>2</sub>CO<sub>3</sub>, NaHCO<sub>3</sub>, Na<sub>2</sub>SO<sub>4</sub>, examples of the optional potassium salts include e.g. KCl, KI, KBr, K<sub>2</sub>CO<sub>3</sub>, KHCO<sub>3</sub>, K<sub>2</sub>SO<sub>4</sub>, and examples of calcium salts include e.g. CaCl<sub>2</sub>, CaI<sub>2</sub>, CaBr<sub>2</sub>, CaCO<sub>3</sub>, CaSO<sub>4</sub>, Ca(OH)<sub>2</sub>. Furthermore, organic anions of the aforementioned cations may be contained in the buffer. According to a more preferred embodiment, the buffer suitable for injection purposes as defined above, may contain salts selected from sodium chloride (NaCl), calcium chloride (CaCl<sub>2</sub>) and optionally potassium chloride (KCl), wherein further anions may be present additional to the chlorides. CaCl<sub>2</sub> can also be replaced by another salt like KCl. Typically, the salts in the injection buffer are present in a concentration of at least 30 mM sodium chloride (NaCl), at least 1 mM potassium chloride (KCl) and at least 0.05 mM calcium chloride (CaCl<sub>2</sub>). The injection buffer may be hypertonic, isotonic or hypotonic with reference to the specific reference medium, i.e.

the buffer may have a higher, identical or lower salt content with reference to the specific reference medium, wherein preferably such concentrations of the afore mentioned salts may be used, which do not lead to damage of cells due to osmosis or other concentration effects. Reference media are e.g. liquids occurring in "*in vivo*" methods, such as blood, lymph, cytosolic liquids, or other body liquids, or e.g. liquids, which may be used as reference media in "*in vitro*" methods, such as common buffers or liquids. Such common buffers or liquids are known to a skilled person. Saline (0.9% NaCl) and Ringer-Lactate solution are particularly preferred as a liquid basis.

**[0397]** Preferably, the (pharmaceutical) composition, which comprises the complex as described herein, further comprises arginine, such as L-arginine.

**[0398]** However, one or more compatible solid or liquid fillers or diluents or encapsulating compounds may be used as well for the inventive pharmaceutical composition, which are suitable for administration to a subject to be treated. The term "compatible" as used herein means that these constituents of the inventive pharmaceutical composition are capable of being mixed with the complex as defined herein as defined above in such a manner that no interaction occurs which would substantially reduce the pharmaceutical effectiveness of the inventive pharmaceutical composition under typical use conditions. Pharmaceutically acceptable carriers, fillers and diluents must, of course, have sufficiently high purity and sufficiently low toxicity to make them suitable for administration to a subject to be treated. Some examples of compounds which can be used as pharmaceutically acceptable carriers, fillers or constituents thereof are sugars, such as, for example, lactose, glucose and sucrose; starches, such as, for example, corn starch or potato starch; cellulose and its derivatives, such as, for example, sodium carboxymethylcellulose, ethylcellulose, cellulose acetate; powdered tragacanth; malt; gelatin; tallow; solid glidants, such as, for example, stearic acid, magnesium stearate; calcium sulfate; vegetable oils, such as, for example, groundnut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil from theobroma; polyols, such as, for example, polypropylene glycol, glycerol, sorbitol, mannitol and polyethylene glycol; alginic acid.

**[0399]** The inventive pharmaceutical composition may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional, intracranial, transdermal, intradermal, intrapulmonary, intraperitoneal, intracardial, intraarterial, intranodal and sublingual injection or infusion techniques. Preferably, the inventive pharmaceutical composition may be administered intradermally, intramuscularly, intranodally or subcutaneously. More preferably the inventive pharmaceutical composition may be administered intramuscularly, intranodally or subcutaneously. Even more preferably the inventive pharmaceutical composition may be administered intranodally or subcutaneously. Most preferably, the inventive pharmaceutical composition may be administered subcutaneously. It is also particularly preferred that the inventive pharmaceutical composition may be administered subcutaneously or intradermally.

**[0400]** Preferably, the inventive pharmaceutical composition may be administered by parenteral injection, more preferably by subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional, intracranial, transdermal, intradermal, intrapulmonary, intraperitoneal, intracardial, intraarterial, intranodal and sublingual injection or via infusion techniques. Sterile injectable forms of the inventive pharmaceutical compositions may be aqueous or oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents that are commonly used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation of the inventive pharmaceutical composition.

**[0401]** For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will preferably be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included, as required. Whether it is a polypeptide, peptide, or nucleic acid molecule, other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated.

**[0402]** The inventive pharmaceutical composition as defined above may also be administered orally in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions are required for oral use, the active ingredient, i.e. the inventive transporter cargo conjugate molecule as defined above, is combined with

emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

**[0403]** The inventive pharmaceutical composition may also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, e.g. including diseases of the skin or of any other accessible epithelial tissue. Suitable topical formulations are readily prepared for each of these areas or organs. For topical applications, the inventive pharmaceutical composition may be formulated in a suitable ointment, containing the inventive immunostimulatory composition, particularly its components as defined above, suspended or dissolved in one or more carriers. Carriers for topical administration include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively, the inventive pharmaceutical composition can be formulated in a suitable lotion or cream. In the context of the present invention, suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

**[0404]** In this context, prescription of treatment, e.g. decisions on dosage etc. when using the above pharmaceutical composition is typically within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in REMINGTON'S PHARMACEUTICAL SCIENCES, 16th edition, Osol, A. (ed), 1980.

**[0405]** Accordingly, the inventive pharmaceutical composition typically comprises a "safe and effective amount" of the components of the inventive pharmaceutical composition, in particular of the complex as defined herein as defined above and/or cells loaded with said complex. As used herein, a "safe and effective amount" means an amount of the complex as defined herein that is sufficient to significantly induce a positive modification of a disease or disorder, i.e. an amount of the complex as defined herein or cells loaded with said complex, that elicits the biological or medicinal response in a tissue, system, animal or human that is being sought. An effective amount may be a "therapeutically effective amount" for the alleviation of the symptoms of the disease or condition being treated and/or a "prophylactically effective amount" for prophylaxis of the symptoms of the disease or condition being prevented. The term also includes the amount of active complex sufficient to reduce the progression of the disease, notably to reduce or inhibit the tumor growth or infection and thereby elicit the response being sought, in particular such response could be an immune response directed against the antigens or antigenic epitopes comprised in by the complex (i.e. an "inhibition effective amount"). At the same time, however, a "safe and effective amount" is small enough to avoid serious side-effects, that is to say to permit a sensible relationship between advantage and risk. The determination of these limits typically lies within the scope of sensible medical judgment. A "safe and effective amount" of the components of the inventive pharmaceutical composition, particularly of the complex as defined herein as defined above, will furthermore vary in connection with the particular condition to be treated and also with the age and physical

condition of the patient to be treated, the body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, the activity of the specific components a), b), and c) of the complex as defined herein as defined above, the severity of the condition, the duration of the treatment, the nature of the accompanying therapy, of the particular pharmaceutically acceptable carrier used, and similar factors, within the knowledge and experience of the accompanying doctor. The inventive pharmaceutical composition may be used for human and also for veterinary medical purposes, preferably for human medical purposes, as a pharmaceutical composition in general or as a vaccine.

**[0406]** Pharmaceutical compositions, in particular vaccine compositions, or formulations according to the invention may be administered as a pharmaceutical formulation which can contain a complex as claimed in any form described herein.

**[0407]** The terms "pharmaceutical formulation" and "pharmaceutical composition" as used in the context of the present invention refer in particular to preparations which are in such a form as to permit biological activity of the active ingredient(s) to be unequivocally effective and which contain no additional component which would be toxic to subjects to which the said formulation would be administered.

**[0408]** In the context of the present invention, an "efficacy" of a treatment can be measured based on changes in the course of a disease in response to a use or a method according to the present invention. For example, the efficacy of a treatment of cancer can be measured by a reduction of tumor volume, and/or an increase of progression free survival time, and/or a decreased risk of relapse post-resection for primary cancer. More specifically for cancer treated by immunotherapy, assessment of efficacy can be by the spectrum of clinical patterns of antitumor response for immunotherapeutic agents through novel immune-related response criteria (irRC), which are adapted from Response Evaluation Criteria in Solid Tumors (RECIST) and World Health Organization (WHO) criteria (J. Natl. Cancer Inst. 2010, 102(18): 1388-1397). The efficacy of prevention of infectious disease is ultimately assessed by epidemiological studies in human populations, which often correlates, with titres of neutralizing antibodies in sera, and induction of multifunctional pathogen specific T cell responses. Preclinical assessment can include resistance to infection after challenge with infectious pathogen. Treatment of an infectious disease can be measured by inhibition of the pathogen's growth or elimination of the pathogen (and, thus, absence of detection of the pathogen), correlating with pathogen specific antibodies and/or T cell immune responses.

**[0409]** Pharmaceutical compositions, in particular vaccine compositions, or formulations according to the invention may also be administered as a pharmaceutical formulation which can contain antigen presenting cells loaded with a complex according to the invention in any form described herein.

**[0410]** The vaccine and/or the composition (for use) according to the present invention may also be formulated as pharmaceutical compositions and unit dosages thereof, in particular together with a conventionally employed adjuvant, immunomodulatory material, carrier, diluent

or excipient as described above and below, and in such form may be employed as solids, such as tablets or filled capsules, or liquids such as solutions, suspensions, emulsions, elixirs, or capsules filled with the same, all for oral use, or in the form of sterile injectable solutions for parenteral (including subcutaneous and intradermal) use by injection or continuous infusion.

**[0411]** In the context of the present invention, in particular in the context of a pharmaceutical composition and vaccines according to the present invention, injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art. Such pharmaceutical compositions and unit dosage forms thereof may comprise ingredients in conventional proportions, with or without additional active compounds or principles, and such unit dosage forms may contain any suitable effective amount of the active ingredient commensurate with the intended daily dosage range to be employed.

**[0412]** Examples of suitable adjuvants and/or immunomodulatory materials in the context of the present invention include MPL<sup>®</sup> (Corixa), aluminum-based minerals including aluminum compounds (generically called Alum), ASO1-4, MF59, CalciumPhosphate, Liposomes, Iscom, polyinosinic:polycytidylic acid (polyIC), including its stabilized form poly-ICLC (Hiltonol), CpG oligodeoxynucleotides, Granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS), Montanide, polylactide co-glycolide (PLG), Flagellin, Soap Bark tree saponins (QS21), amino alkyl glucosamide compounds (e.g. RC529), two component antibacterial peptides with synthetic oligodeoxynucleotides (e.g. IC31), Imiquimod, Resiquimod, Immunostimulatory sequences (ISS), monophosphoryl lipid A (MPLA), Fibroblast-stimulating lipopeptide (FSL1), and anti-CD40 antibodies.

**[0413]** Compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention may be liquid formulations including, but not limited to, aqueous or oily suspensions, solutions, emulsions, syrups, and elixirs. The compositions may also be formulated as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain additives including, but not limited to, suspending agents, emulsifying agents, non-aqueous vehicles and preservatives. Suspending agents include, but are not limited to, sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminum stearate gel, and hydrogenated edible fats. Emulsifying agents include, but are not limited to, lecithin, sorbitan monooleate, and acacia. Preservatives include, but are not limited to, methyl or propyl p-hydroxybenzoate and sorbic acid. Dispersing or wetting agents include but are not limited to poly(ethylene glycol), glycerol, bovine serum albumin, Tween<sup>®</sup>, Span<sup>®</sup>.

**[0414]** Compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention may also be formulated as a depot preparation, which may be administered by implantation or by intramuscular injection.

**[0415]** Compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention may also be solid compositions, which may be in the form of

tablets or lozenges formulated in a conventional manner. For example, tablets and capsules for oral administration may contain conventional excipients including, but not limited to, binding agents, fillers, lubricants, disintegrants and wetting agents. Binding agents include, but are not limited to, syrup, accacia, gelatin, sorbitol, tragacanth, mucilage of starch and polyvinylpyrrolidone. Fillers include, but are not limited to, lactose, sugar, microcrystalline cellulose, maize starch, calcium phosphate, and sorbitol. Lubricants include, but are not limited to, magnesium stearate, stearic acid, talc, polyethylene glycol, and silica.

**[0416]** Disintegrants include, but are not limited to, potato starch and sodium starch glycollate. Wetting agents include, but are not limited to, sodium lauryl sulfate. Tablets may be coated according to methods well known in the art.

**[0417]** Compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention may also be administered in sustained release forms or from sustained release drug delivery systems.

**[0418]** Moreover, the compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention may be adapted for delivery by repeated administration.

**[0419]** Further materials as well as formulation processing techniques and the like, which are useful in the context of compositions, in particular pharmaceutical compositions and vaccines, (for use) according to the present invention or in the context of their preparation are set out in "Part 5 of Remington's "The Science and Practice of Pharmacy", 22nd Edition, 2012, University of the Sciences in Philadelphia, Lippincott Williams & Wilkins".

**[0420]** Also disclosed (not claimed) is a kit-of-parts, preferably for use in the prevention and/or treatment of colorectal cancer, the kit of parts comprising at least one of:

1. (i) a complex as described above,
2. (ii) a nucleic acid as described above,
3. (iii) a vector as described above,
4. (iv) a host cell as described above, and
5. (v) a cell loaded with a complex as described above.

**[0421]** In particular, the kit-of-parts of the disclosure may comprise more than one component (i) to (v). For example, the kit-of-parts according to the present disclosure may comprise at least two different complexes under (i), at least two different nucleic acids under (ii), at least two different vectors under (iii), at least two different host cells under (iv), and/or at least two different cells under (v); e.g., the kit-of-parts of the invention may comprise at least two different complexes (i) and/or at least two different nucleic acids (ii).

**[0422]** For example, the different complexes (i) comprised by the kit-of-parts as described

above may differ in either component a), i.e. in the cell penetrating peptides, in component b), i.e. in the antigens or antigenic epitopes or in the subsets of more than one antigen or antigenic epitope, or in component c), i.e. in the TLR peptide agonist or in the subset of more than one TLR peptide agonist; or the different complexes (i) comprised by the kit-of-parts as described above may differ in two out of the three components a), b), and c); or the different complexes (i) comprised by the kit-of-parts as described above may differ in all three components a), b), and c) of the complex. Accordingly, the different nucleic acids (ii) comprised by the kit-of-parts as described above may differ in that they encode such different complexes; the different vectors (iii) comprised by the kit-of-parts as described above may differ in that they comprise such different nucleic acids; the different host cells (iv) comprised by the kit-of-parts as described above may differ in that they comprise such different vectors; and the different cells loaded with a complex (v) comprised by the kit-of-parts as described above may differ in that they are loaded with such different complexes.

**[0423]** The various components of the kit-of-parts may be packaged in one or more containers. The above components may be provided in a lyophilized or dry form or dissolved in a suitable buffer. The kit may also comprise additional reagents including, for instance, preservatives, growth media, and/or buffers for storage and/or reconstitution of the above-referenced components, washing solutions, and the like. In addition, the kit-of-parts according to the present disclosure may optionally contain instructions of use.

**[0424]** Moreover, the present disclosure also provides a vaccination kit (not claimed) for treating, preventing and/or stabilizing colorectal cancer, comprising the pharmaceutical composition as described herein or a vaccine as described herein and instructions for use of said pharmaceutical composition or of said vaccine in the prevention and/or treatment of colorectal cancer.

**[0425]** Thus, the present disclosure also provides a kit (not claimed) comprising the complex as described herein, the cell as described herein, the composition as described herein, the vaccine as described herein, and/or the pharmaceutical composition as described herein.

**[0426]** In particular, such a kit further comprises a package insert or instruction leaflet with directions to treat colorectal cancer by using the complex (for use) as described herein, the cell as described herein, the composition as described herein, the vaccine as described herein, and/or the pharmaceutical composition as described herein.

**[0427]** In addition to any of components (i) - (v) as described above, the kit may comprise a chemotherapeutic agent, a targeted drug and/or an immunotherapeutic agent, such as an immune checkpoint modulator, as described herein in the context of the combination for use as described herein.

***Use and use in methods according to the invention***

**[0428]** In another aspect, the present invention provides the use of any one of: (i) a complex as claimed, and/or (ii) cells, such as antigen-presenting cells, loaded with a complex as claimed, for the prevention, treatment or stabilization of colorectal cancer. Accordingly, the present invention provides any one of: (i) a complex as claimed, and/or (ii) cells, such as antigen-presenting cells, loaded with a complex as claimed, for use in the prevention, treatment or stabilization of colorectal cancer.

**[0429]** The present invention also provides a complex for use according to the present invention, which allows the transport and presentation of the at least one antigen or antigenic epitope comprised by the complex at the cell surface of antigen presenting cells in an MHC class I and/or MHC class II context, for use in vaccination and/or immunotherapy.

**[0430]** According to another aspect, the present invention provides a use in method of preventing, treating or repressing colorectal cancer, wherein said method comprises administering any one of: (i) a complex of the invention, (ii) cells, such as antigen-presenting cells, loaded with a complex of the invention, or (iii) a pharmaceutical formulation of (i) to (ii), to said subject.

**[0431]** Moreover, the present disclosure (not claimed) provides a method for eliciting or improving, in a subject, an immune response against one or multiple epitopes that is dependent on CD4<sup>+</sup> helper T cells and/or CD8<sup>+</sup> cytotoxic T cells, wherein said method comprises administering any one of: (i) a complex for use as described herein, and/or (ii) cells, such as antigen-presenting cells, loaded with said complex, or (iii) a pharmaceutical formulation of (i) to (ii), to said subject.

**[0432]** An immune response that is dependent on CD4<sup>+</sup> and/or CD8<sup>+</sup> response can be determined by evaluating an inflammatory response, a pro-inflammatory cytokine response, including an increase in the expression of one or more of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 mRNA or protein relative to the level before administration of the compounds of the invention. It can also be measured by an increase in the frequency or absolute number of antigen-specific T cells after administration of the compounds of the invention, measured by HLA-peptide multimer staining, ELISPOT assays, and delayed type hypersensitivity tests. It can also be indirectly measured by an increase in antigen-specific serum antibodies that are dependent on antigen-specific T helper cells.

**[0433]** The present disclosure (not claimed) also provides a method for eliciting or improving, in a subject, an immune response against one or multiple antigens or antigenic epitopes that is restricted by multiple MHC class I molecules and/or multiple MHC class II molecules, wherein said method comprises administering any one of: (i) a complex for use as described herein, and/or (ii) cells, such as antigen-presenting cells, loaded with said complex, or (iii) a pharmaceutical formulation of (i) to (ii), to said subject.

**[0434]** A method for eliciting or improving, in a subject, an immune response against multiple

epitopes (not claimed) as described herein, that is restricted by multiple MHC class I molecules and/or multiple MHC class II molecules can be determined by evaluating a cytokine response, including an increase in the expression of one or more of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 mRNA or protein relative to the level before administration of the compounds of the invention, after in vitro stimulation of T cells with individual peptides binding to discrete MHC class I and class II molecules on antigen presenting cells. Restriction to different MHC molecules can also be validated by using antigen presenting cells expressing different MHC molecules, or by using MHC blocking antibodies. It can also be measured by an increase in the frequency or absolute number of antigen-specific T cells after administration of the compounds of the invention, measured by HLA-peptide multimer staining, which uses multimers assembled with discrete MHC molecules.

**[0435]** Specifically, in the methods for eliciting or improving an immune response against one or multiple antigens or antigenic epitopes according to the present invention, the immune response is directed against one or multiple epitopes of a tumor-associated antigen or a tumorspecific antigen as, for instance, a combination of epitopes as described herein.

**[0436]** Alternatively or additionally, the immune response may be directed against multiple epitopes of an antigenic protein from a pathogen.

**[0437]** The methods as described herein, may be for eliciting or improving, in a subject, an immune response against one or multiple epitopes that is restricted by MHC class I molecules and/or MHC class II molecules.

**[0438]** In particular, the present invention thus provides use in a method for preventing and/or treating colorectal cancer or initiating in a subject in need thereof comprising administering to the subject a complex comprising:

a cell penetrating peptide;

at least one antigen or antigenic epitope; and

at least one TLR peptide agonist,

wherein the components a) - c) are covalently linked,

wherein the TLR peptide agonist is a TLR2 peptide agonist and/or a TLR4 peptide agonist, and

wherein the complex is a polypeptide or a protein, in particular a recombinant polypeptide or a recombinant protein.

**[0439]** Use in such a method it is preferred that the complex (for use) according to the present invention as described herein, the nucleic acid as described herein, the cell as described herein, the composition as described herein, the vaccine as described herein, and/or the

pharmaceutical composition as described herein is administered to the subject.

**[0440]** Preferably, the subject has colorectal cancer and/or was diagnosed with colorectal cancer.

**[0441]** The present disclosure provides the use of any one of: (i) a complex as described herein, and/or (ii) cells, such as antigen-presenting cells, loaded with the complex as described herein, for the preparation of an imaging composition for imaging techniques in the context of (diagnosis of) colorectal cancer or for the preparation of a diagnosis composition ("diagnostic compositions") for diagnosing colorectal cancer (not claimed). A diagnostic composition for diagnosing colorectal cancer according to the present disclosure (not claimed) comprises at least one component selected from:

1. (i) a complex as described above,
2. (ii) a nucleic acid as described above,
3. (iii) a vector as described above,
4. (iv) a host cell as described above, and
5. (v) a cell loaded with a complex as described above.

**[0442]** Specifically, the diagnostic composition according to the present invention comprises the complex as described above.

**[0443]** In particular, the complex (for use) according to the present invention, the nucleic acid as described herein, the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention, the inventive composition, the inventive pharmaceutical composition or the inventive vaccine or the diagnostic composition may be utilized in diagnosis as a diagnostic tool (not claimed), e.g. in (*in vivo* or *in vitro*) assays, e.g. in immunoassays, to detect, prognose, diagnose, or monitor colorectal cancer.

**[0444]** As an example, (*in vitro*) assays may be performed (not claimed) by delivering the complex (for use) according to the present invention, the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention, the inventive composition, the inventive pharmaceutical composition or the inventive vaccine or the diagnostic composition to target cells typically selected from e.g. cultured animal cells, human cells or microorganisms, and to monitor the cell response by biophysical methods typically known to a skilled person. The target cells typically used therein may be cultured cells (*in vitro*), e.g. cells isolated from human or animal body, such as blood cells isolated from human or animal body, or *in vivo* cells, i.e. cells composing the organs or tissues of living animals or humans, or microorganisms found in living animals or humans. Particularly preferable in this context are so called markers or labels, which may be contained in the complex (for use) according to the present invention and, in particular, in the diagnostic composition according to the present disclosure (not claimed).

**[0445]** The disclosure provides a method of diagnosing colorectal cancer in a subject (not claimed), wherein said method comprises administering any one of: (i) a complex of the invention, (ii) cells, such as antigen-presenting cells, loaded with the complex of the invention, or (iii) a pharmaceutical formulation of (i) to (ii), to said subject or to said subject's sample *ex vivo*.

**[0446]** Preferably, uses and use methods according to the present invention comprise administration of a complex (for use) according to the invention.

**[0447]** Moreover, uses and use in methods according to the present invention comprise administration of more than one complex, cells, or pharmaceutical formulation according to the invention. For example, in the uses and use in methods according to the present invention, at least two different complexes are used or administered, wherein each complex comprises at least one antigen or antigenic epitope and said antigen or antigenic epitope or (if more than one antigen or antigenic epitope is comprised by said complex) said subset of antigens or antigenic epitopes are different between the two complexes.

**[0448]** For example, the different complexes (i) comprised by the composition as described above may differ in either component a), i.e. in the cell penetrating peptides, in component b), i.e. in the antigens or antigenic epitopes or in the subsets of more than one antigen or antigenic epitope, or in component c), i.e. in the TLR peptide agonist or in the subset of more than one TLR peptide agonist; or the different complexes (i) comprised by the composition as described above may differ in two out of the three components a), b), and c); or the different complexes (i) comprised by the composition as described above may differ in all three components a), b), and c) of the complex. Accordingly, the different nucleic acids (ii) comprised by the composition as described above may differ in that they encode such different complexes; the different vectors (iii) comprised by the composition as described above may differ in that they comprise such different nucleic acids; the different host cells (iv) comprised by the composition as described above may differ in that they comprise such different vectors; and the different cells loaded with a complex (v) comprised by the composition as described above may differ in that they are loaded with such different complexes.

**[0449]** Moreover, in the uses and use in methods according to the present invention, the cells according to the present invention may be antigen presenting cells, in particular dendritic cells, more preferably dendritic cells from the subject to be treated.

#### ***Mode of administration***

**[0450]** The complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered in any manner as described above, including enterally, such as orally or rectally, and parenterally, such as

intravenously or combinations thereof. Parenteral administration includes, but is not limited to, intravenous, intra-arterial, intra-peritoneal, subcutaneous, intradermal and intramuscular. Preferably, the complex for use according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex for use according to the present invention; the inventive composition; the inventive pharmaceutical composition and/or the inventive vaccine are administered via an enteral route of administration, such as oral, sublingual and rectal. The complex (for use) according to the present invention; the nucleic acid ((for use)) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may also be preferably administered via topical, intratumoral, intradermal, subcutaneous, intramuscular, intranasal, or intranodal route. The complex (for use) according to the present invention; the nucleic acid ((for use)) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may also be administered in the form of an implant, which allows slow release of the compositions as well as a slow controlled i.v. infusion. For example, the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered subcutaneously or intradermally.

**[0451]** The administration of complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may require multiple successive injections/administrations. Thus, the administration may be repeated at least two times, for example once as primary immunization injections/administration and, later, as booster injections/administration.

**[0452]** In particular, the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered repeatedly or continuously. The complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered repeatedly or continuously for a period of at least 1, 2, 3, or 4 weeks; 2, 3, 4, 5, 6, 8, 10, or 12 months; or 2, 3, 4, or 5 years. For example, the complex (for use) according to the present invention may be administered twice per day, once per day, every two days, every three days, once per week, every two weeks, every three weeks, once per month or every two months. Preferably, the complex (for use) according to the present invention may be administered repeatedly, for example once per week or (once) every two weeks.

**[0453]** Moreover, the cell penetrating peptide, components a), b), and c), i.e. the at least one antigen or antigenic epitope and the at least one TLR peptide agonist, composing the complex (for use) according to the present invention may be contained in separate compositions which are mixed just before administration or which are administered simultaneously to the subject in need thereof.

**[0454]** According to one approach, the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered directly to a patient using the administration routes as described above, in particular for pharmaceutical compositions. Alternatively, the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine may be administered to a patient using an *ex vivo* approach, e.g. by introducing the pharmaceutical composition, the vaccine or the inventive transporter cargo conjugate molecule as defined above into cells, preferably autologous cells, i.e. cells derived from the patient to be treated, and transplanting these cells into the site of the patient to be treated, optionally subsequent to storing and/or culturing these cells prior to treatment.

**[0455]** The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factors, including pharmacokinetic properties, subject conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired.

**[0456]** Typically, for cancer treatment, the therapeutically effective dose of a complex (for use) according to the present invention is from about 0.01 mg to 5 mg per injection, in particular from about 0.1mg to 2 mg per injection, or from about 0.01 nmol to 1 mmol per injection, in particular from 1 nmol to 1 mmol per injection, preferably from 1  $\mu$ mol to 1 mmol per injection.

**[0457]** Typically, for cancer treatment, the therapeutically effective dose of an antigen presenting cell loaded with a complex (for use) according to the present invention is from about 0.2 million cells to 2 million cells per injection.

### ***Combination therapy***

**[0458]** The administration of the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine in the use in methods and for uses according to the invention can be carried out alone or in combination

with a co-agent useful for treating and/or stabilizing colorectal cancer.

**[0459]** For instance, in the case of treatment, prevention, or stabilization of a colorectal cancer, the administration of the pharmaceutical compositions in the methods and uses according to the invention can be carried out in combination with substances used in conventional chemotherapy directed against solid colorectal tumors and for control of establishment of metastases or any other molecule that act by triggering programmed cell death e.g. for example a co-agent selected from Tumor Necrosis Family Members including, but not limited, to Fas Ligand and tumor necrosis factor (TNF)-related apoptosis inducing (TRAIL) ligand. According to a further embodiment, the administration of the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine for use in the methods and for uses according to the present invention can be carried out in parallel of radiotherapy.

**[0460]** The invention encompasses the administration of the complex (for use) according to the present invention; the nucleic acid (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine, wherein it is administered to a subject prior to, simultaneously or sequentially with other therapeutic regimens or co-agents useful for treating, and/or stabilizing a colorectal cancer and/or preventing colorectal cancer relapsing (e.g. multiple drug regimens), in a therapeutically effective amount. Said complex, cell, composition, vaccine or pharmaceutical composition, that is administered simultaneously with said co-agents can be administered in the same or different composition(s) and by the same or different route(s) of administration.

**[0461]** Said other therapeutic regimens or co-agents may be selected from the group consisting of radiation therapy, chemotherapy, surgery, targeted therapy (including small molecules, peptides and monoclonal antibodies), and anti-angiogenic therapy. Anti-angiogenic therapy is defined herein as the administration of an agent that directly or indirectly targets tumor-associated vasculature.

**[0462]** Accordingly, the present invention also provides a combination of

1. (i) a complex as claimed; and
2. (ii) a chemotherapeutic agent, a targeted drug and/or an immunotherapeutic agent, such as an immune checkpoint modulator.

**[0463]** It may be used in the prevention and/or treatment of colorectal cancer.

**[0464]** Traditional chemotherapeutic agents are cytotoxic, i.e. they act by killing cells that divide rapidly, one of the main properties of most cancer cells. Preferred chemotherapeutic

agents for combination with the complex as defined herein are such chemotherapeutic agents known to the skilled person for treatment of colorectal cancer. Preferred chemotherapeutic agents for combination include 5-Fluorouracil (5-FU), Capecitabine (Xeloda<sup>®</sup>), Irinotecan (Camptosar<sup>®</sup>) and Oxaliplatin (Eloxatin<sup>®</sup>). It is also preferred that the complex as defined herein is combined with a combined chemotherapy, preferably selected from (i) FOLFOX (5-FU, leucovorin, and oxaliplatin); (ii) CapeOx (Capecitabine and oxaliplatin); (iii) 5-FU and leucovorin; (iv) FOLFOXIRI (leucovorin, 5-FU, oxaliplatin, and irinotecan); and (v) FOLFIRI (5-FU, leucovorin, and irinotecan). In non-spread cancer, a combination with (i) FOLFOX (5-FU; leucovorin, and oxaliplatin); (ii) CapeOx (Capecitabine and oxaliplatin); or (iii) 5-FU and leucovorin is preferred. For cancer that has spread, a combination with (iv) FOLFOXIRI (leucovorin, 5-FU, oxaliplatin, and irinotecan); (i) FOLFOX (5-FU, leucovorin, and oxaliplatin); or (v) FOLFIRI (5-FU, leucovorin, and irinotecan) is preferred.

**[0465]** Targeted drugs for combination with the complex as defined herein for treatment of colorectal cancer include VEGF-targeted drugs and EGFR-targeted drugs. Preferred examples of VEGF-targeted drugs include Bevacizumab (Avastin<sup>®</sup>), ramucirumab (Cyramza<sup>®</sup>) or ziv-aflibercept (Zaltrap<sup>®</sup>). Preferred examples of EGFR-targeted drugs include Cetuximab (Erbitux<sup>®</sup>), panitumumab (Vectibix<sup>®</sup>) or Regorafenib (Stivarga<sup>®</sup>).

**[0466]** Immunotherapeutic agents for combination with the complex as defined herein for treatment of colorectal cancer include vaccines, chimeric antigen receptors (CARs), checkpoint modulators and oncolytic virus therapies.

**[0467]** Preferred vaccines for combination with the complex as defined herein for treatment of colorectal cancer include TroVax, OncoVax, IMA910, ETBX-011, MicOryx, EP-2101, MKC1106-PP, CDX-1307, V934/V935, MelCancerVac, Imprime PGG, FANG, Tecemotide, AlloStim, DCVax, GI-6301, AVX701, OCV-C02.

**[0468]** Artificial T cell receptors (also known as chimeric T cell receptors, chimeric immunoreceptors, chimeric antigen receptors (CARs)) are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. Artificial T cell receptors (CARs) are preferred in the context of adoptive cell transfer. To this end, T cells are removed from a patient and modified so that they express receptors specific to colorectal cancer. The T cells, which can then recognize and kill the cancer cells, are reintroduced into the patient.

**[0469]** As used herein, the term "immune checkpoint modulator" (also referred to as "checkpoint modulator") refers to a molecule or to a compound that modulates (e.g., totally or partially reduces, inhibits, interferes with, activates, stimulates, increases, reinforces or supports) the function of one or more checkpoint molecules. Thus, an immune checkpoint modulator may be an "immune checkpoint inhibitor" (also referred to as "checkpoint inhibitor" or "inhibitor") or an "immune checkpoint activator" (also referred to as "checkpoint activator" or "activator"). An "immune checkpoint inhibitor" (also referred to as "checkpoint inhibitor" or "inhibitor") totally or partially reduces, inhibits, interferes with, or negatively modulates the

function of one or more checkpoint molecules. An "immune checkpoint activator" (also referred to as "checkpoint activator" or "activator") totally or partially activates, stimulates, increases, reinforces, supports or positively modulates the function of one or more checkpoint molecules. Immune checkpoint modulators are typically able to modulate (i) self-tolerance and/or (ii) the amplitude and/or the duration of the immune response. Preferably, the immune checkpoint modulator used according to the present invention modulates the function of one or more human checkpoint molecules and is, thus, a "human checkpoint inhibitor".

**[0470]** Checkpoint molecules are molecules, such as proteins, are typically involved in immune pathways and, for example, regulate T-cell activation, T-cell proliferation and/or T-cell function. Accordingly, the function of checkpoint molecules, which is modulated (e.g., totally or partially reduced, inhibited, interfered with, activated, stimulated, increased, reinforced or supported) by checkpoint modulators, is typically the (regulation of) T-cell activation, T-cell proliferation and/or T cell function. Immune checkpoint molecules thus regulate and maintain self-tolerance and the duration and amplitude of physiological immune responses. Many of the immune checkpoint molecules belong to the B7:CD28 family or to the tumor necrosis factor receptor (TNFR) super family and, by the binding of specific ligands, activate signaling molecules that are recruited to the cytoplasmic domain (cf. Susumu Suzuki et al., 2016: Current status of immunotherapy. Japanese Journal of Clinical Oncology, 2016: doi: 10.1093/jjco/hyv201 [Epub ahead of print]; in particular Table 1).

**[0471]** Preferably, the immune checkpoint modulator for combination with the complex as claimed for treatment of colorectal cancer is an activator or an inhibitor of one or more immune checkpoint point molecule(s) selected from CD27, CD28, CD40, CD122, CD137, OX40, GITR, ICOS, A2AR, B7-H3, B7-H4, BTLA, CD40, CTLA-4, IDO, KIR, LAG3, PD-1, TIM-3, VISTA, CEACAM1, GARP, PS, CSF1R, CD94/NKG2A, TDO, GITR, TNFR and/or FasR/DcR3; or an activator or an inhibitor of one or more ligands thereof.

**[0472]** More preferably, the immune checkpoint modulator is an activator of a (co-)stimulatory checkpoint molecule or an inhibitor of an inhibitory checkpoint molecule or a combination thereof. Accordingly, the immune checkpoint modulator is more preferably (i) an activator of CD27, CD28, CD40, CD122, CD137, OX40, GITR and/or ICOS or (ii) an inhibitor of A2AR, B7-H3, B7-H4, BTLA, CD40, CTLA-4, IDO, KIR, LAG3, PD-1, PDL-1, PD-L2, TIM-3, VISTA, CEACAM1, GARP, PS, CSF1R, CD94/NKG2A, TDO, TNFR and/or FasR/DcR3.

**[0473]** Even more preferably, the immune checkpoint modulator is an inhibitor of an inhibitory checkpoint molecule (but preferably no inhibitor of a stimulatory checkpoint molecule). Accordingly, the immune checkpoint modulator is even more preferably an inhibitor of A2AR, B7-H3, B7-H4, BTLA, CTLA-4, IDO, KIR, LAG3, PD-1, PDL-1, PD-L2, TIM-3, VISTA, CEACAM1, GARP, PS, CSF1R, CD94/NKG2A, TDO, TNFR and/or DcR3 or of a ligand thereof.

**[0474]** It is also preferred that the immune checkpoint modulator is an activator of a stimulatory or costimulatory checkpoint molecule (but preferably no activator of an inhibitory checkpoint molecule). Accordingly, the immune checkpoint modulator is more preferably an activator of

CD27, CD28, CD40, CD122, CD137, OX40, GITR and/or ICOS or of a ligand thereof.

**[0475]** It is even more preferred that the immune checkpoint modulator is a modulator of the CD40 pathway, of the IDO pathway, of the CTLA-4 pathway and/or of the PD-1 pathway. In particular, the immune checkpoint modulator is preferably a modulator of CD40, CTLA-4, PD-L1, PD-L2, PD-1 and/or IDO, more preferably the immune checkpoint modulator is an inhibitor of CTLA-4, PD-L1, PD-L2, PD-1 and/or IDO or an activator of CD40, even more preferably the immune checkpoint modulator is an inhibitor of CTLA-4, PD-L1, PD-1 and/or IDO and most preferably the immune checkpoint modulator is an inhibitor of CTLA-4 and/or PD-1.

**[0476]** It is even more preferred that the immune checkpoint modulator is a modulator of the CD40 pathway, of the IDO pathway, of the LAG3 pathway, of the CTLA-4 pathway and/or of the PD-1 pathway. In particular, the immune checkpoint modulator is preferably a modulator of CD40, LAG3, CTLA-4, PD-L1, PD-L2, PD-1 and/or IDO, more preferably the immune checkpoint modulator is an inhibitor of CTLA-4, PD-L1, PD-L2, PD-1, LAG3, and/or IDO or an activator of CD40, even more preferably the immune checkpoint modulator is an inhibitor of CTLA-4, PD-L1, PD-1, LAG3 and/or IDO, even more preferably the immune checkpoint modulator is an inhibitor of LAG3, CTLA-4 and/or PD-1, and most preferably the immune checkpoint modulator is an inhibitor of CTLA-4 and/or PD-1.

**[0477]** Accordingly, the checkpoint modulator for combination with the complex as claimed for treatment of colorectal cancer may be selected from known modulators of the CD40 pathway, the CTLA-4 pathway or the PD-1 pathway. Preferably, the checkpoint modulator for combination with the complex as claimed for treatment of colorectal cancer may be selected from known modulators of the CD40 pathway, the LAG3 pathway, the CTLA-4 pathway or the PD-1 pathway. Particularly preferably, the immune checkpoint modulator is a PD-1 inhibitor. Preferred inhibitors of the CTLA-4 pathway and of the PD-1 pathway include the monoclonal antibodies Yervoy® (Ipilimumab; Bristol Myers Squibb) and Tremelimumab (Pfizer/MedImmune) as well as Opdivo® (Nivolumab; Bristol Myers Squibb), Keytruda® (Pembrolizumab; Merck), Durvalumab (MedImmune/AstraZeneca), MEDI4736 (AstraZeneca; cf. WO 2011/066389 A1), MPDL3280A (Roche/Genentech; cf. US 8,217,149 B2), Pidilizumab (CT-011; CureTech), MEDI0680 (AMP-514; AstraZeneca), MSB-0010718C (Merck), MIH1 (Affymetrix) and Lambrolizumab (e.g. disclosed as hPD109A and its humanized derivatives h409AII, h409A16 and h409A17 in WO2008/156712; Hamid et al., 2013; N. Engl. J. Med. 369: 134-144). More preferred checkpoint inhibitors include the CTLA-4 inhibitors Yervoy® (Ipilimumab; Bristol Myers Squibb) and Tremelimumab (Pfizer/MedImmune) as well as the PD-1 inhibitors Opdivo® (Nivolumab; Bristol Myers Squibb), Keytruda® (Pembrolizumab; Merck), Pidilizumab (CT-011; CureTech), MEDI0680 (AMP-514; AstraZeneca), AMP-224 and Lambrolizumab (e.g. disclosed as hPD109A and its humanized derivatives h409AII, h409A16 and h409A17 in WO2008/156712; Hamid O. et al., 2013; N. Engl. J. Med. 369: 134-144). As described above, a preferred example of a LAG3 inhibitor is the anti-LAG3 monoclonal antibody BMS-986016 (Bristol-Myers Squibb). Other preferred examples of a LAG3 inhibitor include LAG525 (Novartis), IMP321 (ImmuneTep) and LAG3-Ig as disclosed in WO 2009/044273

A2 and in Brignon et al., 2009, Clin. Cancer Res. 15: 6225-6231 as well as mouse or humanized antibodies blocking human LAG3 (e.g., IMP701 as described in WO 2008/132601 A1), or fully human antibodies blocking human LAG3 (such as disclosed in EP 2320940 A2).

**[0478]** Preferably, the immune checkpoint modulator is not a modulator of CD40. In particular, it is preferred that the immune checkpoint modulator is not a CD40 ligand. It is also preferred that the immune checkpoint modulator is not an anti-CD40 antibody.

**[0479]** It is also preferred that the immune checkpoint modulator for combination with the complex as claimed for treatment of colorectal cancer is selected from the group consisting of Pembrolizumab, Ipilimumab, Nivolumab, MPDL3280A, MEDI4736, Tremelimumab, Avelumab, PDR001, LAG525, INCB24360, Varlilumab, Urelumab, AMP-224 and CM-24.

**[0480]** Oncolytic viruses are engineered to cause cell lysis by replicating in tumors, thus activating an antitumor immune response. An oncolytic virus therapy for combination with the complex as defined herein for treatment of colorectal cancer is preferably selected from the group consisting of JX594 (Thymidine Kinase-Deactivated Vaccinia Virus), ColoAd1 (adenovirus), NV1020 (HSV-derived), ADXS11-001 (attenuated Listeria vaccine), Reolysin® (special formulation of the human reovirus), PANVAC (recombinant vaccinia-virus CEA-MUC-1-TRICOM), Ad5-hGCC-PADRE (recombinant adenovirus vaccine) and vvDD-CDSR (vaccinia virus).

**[0481]** Preferably, (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are administered at about the same time.

**[0482]** "At about the same time", as used herein, means in particular simultaneous administration or that directly after administration of (i) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, (ii) the complex is administered or directly after administration of (i) the complex (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, is administered. The skilled person understands that "directly after" includes the time necessary to prepare the second administration - in particular the time necessary for exposing and disinfecting the location for the second administration as well as appropriate preparation of the "administration device" (e.g., syringe, pump, etc.). Simultaneous administration also includes if the periods of administration of (i) the complex and of (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, overlap or if, for example, one component is administered over a longer period of time, such as 30 min, 1 h, 2 h or even more, e.g. by infusion, and the other component is administered at some time during such a long period. Administration of (i) the complex and of (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, at about the same time is in particular preferred if different routes of administration and/or different administration sites are used.

**[0483]** It is also preferred that (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are administered consecutively. This means that (i) the complex is administered before or after (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator. In consecutive administration, the time between administration of the first component and administration of the second component is preferably no more than one week, more preferably no more than 3 days, even more preferably no more than 2 days and most preferably no more than 24 h. It is particularly preferred that (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are administered at the same day with the time between administration of the first component (the checkpoint modulator of the complex) and administration of the second component (the other of the checkpoint modulator and the complex) being preferably no more than 6 hours, more preferably no more than 3 hours, even more preferably no more than 2 hours and most preferably no more than 1 h.

**[0484]** Preferably, (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are administered via the same route of administration. It is also preferred that (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are administered via distinct routes of administration.

**[0485]** Moreover, (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are preferably provided in distinct compositions. Alternatively, (i) the complex and (ii) the chemotherapeutic agent, the targeted drug and/or the immunotherapeutic agent, such as an immune checkpoint modulator, are preferably provided in the same composition.

**[0486]** Accordingly, the present invention provides a pharmaceutical formulation comprising a complex (for use) according to the invention or a cell (for use) according to the invention, in particular an antigen-presenting cell (for use) according to the invention, combined with at least one co-agent useful for treating and/or stabilizing a cancer and/or preventing colorectal cancer relapsing, and at least one pharmaceutically acceptable carrier.

**[0487]** Moreover, the complex (for use) according to the present invention; the cell, such as antigen-presenting cell, loaded with the complex (for use) according to the present invention; the inventive composition; the inventive pharmaceutical composition or the inventive vaccine can be administered after surgery where solid tumors have been removed as a prophylaxis against relapsing and/or metastases.

**[0488]** Moreover, the administration of the imaging or diagnosis composition in the methods and uses according to the invention can be carried out alone or in combination with a co-agent useful for imaging and/or diagnosing colorectal cancer.

## Subjects

**[0489]** The present invention can be used for the prevention and/or treatment of any subject suffering from colorectal cancer or at risk to develop colorectal cancer. In particular, the therapeutic effect of said complex may be to elicit an immune response directed against said antigens or antigenic epitopes, in particular a response that is dependent on CD4<sup>+</sup> helper T cells and/or CD8<sup>+</sup> cytotoxic T cells and/or that is restricted by MHC class I molecules and/or MHC class II molecules.

**[0490]** It is also preferred that subjects according to the invention have been subjected to a surgical removal of a tumor.

**[0491]** The present invention is e.g. described by the specific embodiments. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures.

**[0492]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only.

## BRIEF DESCRIPTION OF THE FIGURES

**[0493]** In the following a brief description of the appended figures will be given. The figures are intended to illustrate the present invention in more detail.

### Figure 1

shows for Example 1 (not falling under the claims) expression of activation marker CD40 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of EDAZ13Mad5, Z13Mad5, Mad5 or 25ng/ml of LPS during 48h. Isotype staining for each condition was also performed (isotype is not shown in the Fig. 1) (one experiment).

### Figure 2

shows for Example 1 (not falling under the claims) expression of activation marker CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of EDAZ13Mad5, Z13Mad5, Mad5 or 25ng/ml of LPS during 48h. Isotype staining for each condition was also performed (isotype is not shown in the Fig. 2) (one experiment).

### Figure 3

shows for Example 1 (not falling under the claims) expression of activation marker

HLADR by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of EDAZ13Mad5, Z13Mad5, Mad5 or 25ng/ml of LPS during 48h. Isotype staining for each condition was also performed (isotype is not shown in the Fig. 3) (one experiment).

Figure 4

shows for Example 1 (not falling under the claims) expression of activation marker CD83 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of EDAZ13Mad5, Z13Mad5, Mad5 or 25ng/ml of LPS during 48h. Isotype staining for each condition was also performed (isotype is not shown in the Fig. 4) (one experiment).

Figure 5

shows for Example 2 (not falling under the claims) functional MHC class I-restricted cross-presentation in a murine *in an vitro* system using bone marrow derived dendritic cells (BMDCs) and splenocytes from different TCR transgenic mice. To this end, BMDCs were loaded overnight with 300nM of EDAZ13Mad5, EDAMad5 or Mad5. Efficient MHC class I-restricted presentation of OVACD8 epitope and gp100 epitope was monitored after 4 days with CFSE-labeled OT1 cells and P-Mel cells respectively. Efficient MHC class II-restricted presentation of OVACD4 epitope was monitored after 4 days with CFSE-labeled OT2 cells. As control, BMDCs were pulsed for 1 h with SuM peptide (one experiment representative of 2 individual experiments).

Figure 6

shows the results for the 2 nmol groups for Example 3 (not falling under the claims). C57BL/6 mice were vaccinated twice (Wk0 and Wk2) with 2 nmol of EDAMad5 or EDAZ13Mad5. Positive control group was vaccinated with Mad5 and MPLA (equimolar to EDA). Mice were bled 7 days after last vaccination and pentamer staining was performed (3-4 mice per group, one experiment).

Figure 7

shows the results for the 10 nmol groups for Example 3 (not falling under the claims). C57BL/6 mice were vaccinated twice (Wk0 and Wk2) with 10 nmol of EDAMad5 or EDAZ13Mad5. Positive control group was vaccinated with Mad5 and MPLA (equimolar to EDA). Mice were bled 7 days after last vaccination and pentamer staining was performed (3-4 mice per group, one experiment).

Figure 8

shows for Example 3 (not falling under the claims) the percentage of pentamer positive CD8+ T cells for all groups tested. C57BL/6 mice were vaccinated twice (Wk0 and Wk2) with 2 nmol or 10 nmol of EDAMad5 or EDAZ13Mad5. Positive control group - was vaccinated with Mad5 and MPLA (equimolar to EDA). Mice were bled 7 days after last vaccination and pentamer staining was performed (one experiment with 3-4 mice per group).

Figure 9

shows for Example 4 (not falling under the claims) the tumor growth of 7 mice per group (mean  $\pm$  SEM); \*, p<0.05 EDAZ13Mad5 versus control group (2-way Anova test). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 10nmol of EDAZ13Mad5,

EDAMad5, Mad5 or Mad5 and MPLA (equimolar to EDA) s.c. in the right flank. Tumor size was measured with a caliper.

Figure 10

shows for Example 4 (not falling under the claims) individual tumor growth curves (7 individual mice per group). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 10nmol of EDAZ13Mad5, EDAMad5, Mad5 or Mad5 and MPLA (equimolar to EDA) s.c. in the right flank. Tumor size was measured with a caliper.

Figure 11

shows for Example 4 (not falling under the claims) (A) the survival curve of 7 mice per group; \*, p<0.05 EDAZ13Mad5 versus control group (Log-rank test) and (B) the tumor-free progression curve of 7 mice per group; \*, p<0.05 EDAZ13Mad5 versus control group (Log-rank test).

Figure 12

shows for Example 5 (not falling under the claims) the number of metastasis for every experimental group. C57BL/6 mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and vaccinated twice (d0 and d9) by subcutaneous injection of 2 nmol of EDAZ13Mad5, EDAMad5 or Z13Mad5 + MPLA (equimolar to EDA) or MPLA alone s.c. in the right flank. Mice were euthanized at day 13 and lung recovered. Number of metastasis foci was counted for each lung. \*\*, p<0.01; \*\*\*, p<0.0001 (Unpaired T test).

Figure 13

shows for Example 6 (not falling under the claims) the number of metastasis for every experimental group. C57BL/6 mice were vaccinated twice (d-21 and d-7) by subcutaneous injection of 2nmoles of EDAZ13Mad5, EDAMad5 or Z13Mad5 + MPLA (equimolar to EDA) s.c. in the right flank. At day 0, mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells. Mice were euthanized at day 14 and lung recovered. Number of metastasis foci was counted for each lung. \*, p<0.05. \*\*, p<0.001 (Unpaired T test).

Figure 14:

shows the results for Example 8 (not falling under the claims). HEK-hTLR2 cell lines were seeded in flat 96-well plate in culture medium, stimulated with 0.3  $\mu$ M, 1  $\mu$ M or 3  $\mu$ M of Anaxaz13Mad5 or Z13Mad5Anaxaz and incubated at 37°C for 24h. Positive control was performed with 500ng/ml of Pam3CSK4. (A) Twenty microliters of supernatant were added to QuantiBlue® detection medium and incubated at 37°C for 1h before OD reading (620nm). (B) Quantification of IL-8 secretion (by ELISA) in the supernatant.

Figure 15:

shows the results for Example 9 (not falling under the claims). C57BL/6 mice were vaccinated twice (Wk0 and Wk2) with 2nmoles of Z13Mad5Anaxaz or Anaxaz13Mad5. Mice were bled 7 days after last vaccination and pentamer staining was performed (one experiment).

Figure 16:

shows the results for Example 9 (not falling under the claims). C57BL/6 mice were

vaccinated twice (Wk0 and Wk2) with 2nmoles Z13Mad5Anaxa or AnaxaZ13Mad5. Mice were bled 7 days after last vaccination and pentamer staining was performed (one experiment with 4 mice per group). \*, p<0.05.

Figure 17:

shows for Example 10 (not falling under the claims) the tumor growth of 7 mice per group (mean  $\pm$  SEM). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 10 nmol of either AnaxZ13Mad5, Z13Mad5Anaxa or co-injection of Z13Mad5 + Pam3CSK4 (equimolar to Anaxa) in the right flank. Tumor size was measured with a caliper. \*, p<0.05; \*\*, p<0.01, \*\*\*, p<0.001, \*\*\*\*, p<0.0001.

Figure 18:

shows for Example 10 (not falling under the claims) the individual tumor growth curves (7 individual mice per group). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 10 nmol of either AnaxZ13Mad5, Z13Mad5Anaxa or co-injection of Z13Mad5 + Pam3CSK4 (equimolar to Anaxa) s.c. in the right flank. Tumor size was measured with a caliper.

Figure 19:

shows for Example 10 (not falling under the claims) the survival curve of 7 mice per group. C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 10 nmol of either AnaxZ13Mad5, Z13Mad5Anaxa or co-injection of Z13Mad5 + Pam3CSK4 (equimolar to Anaxa) in the right flank. Tumor size was measured with a caliper. \*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001 (Log-rank test).

Figure 20:

shows for Example 11 (not falling under the claims) the tumor growth of 7 mice per group (mean  $\pm$  SEM). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 2nmoles of Hp91Z13Mad5, EDAZ13Mad5, Z13Mad5Anaxa, Z13Mad5EDA or Z13Mad5 and MPLA (equimolar to EDA) in the right flank. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001 (2-way Anova test at day 23).

Figure 21:

shows for Example 11 (not falling under the claims) the individual tumor growth curves (7 individual mice per group). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of 2nmoles of Hp91Z13Mad5, EDAZ13Mad5, Z13Mad5Anaxa, Z13Mad5EDA or Z13Mad5 and MPLA (equimolar to EDA) s.c. in the right flank.

Figure 22:

shows for Example 11 (not falling under the claims) the survival curves of all 7 mice per group. Median survival is indicated on the graph (m.s.). \*, p<0.05; \*\*, p<0.01 (Log-rank test).

Figure 23:

shows for Example 12 (not falling under the claims) the tumor growth of 7 mice per

group (mean $\pm$ SEM); \*\*\*\*, p<0.0001 (Log-rank test). C57BL/6 mice were implanted s.c. with  $3\times10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (once at d5 and once at d13) by subcutaneous injection of either 0.5 nmol, 2 nmol or 10 nmol of Z13Mad5Anaxa in the right flank. Tumor size was measured with a caliper.

Figure 24:

shows for Example 13 (not falling under the claims) the SIINFEKL-specific CD8 T cell responses detected in the blood of C57BL/6 mice vaccinated three times (once at Wk0, once at Wk2 and once at Wk4) s.c., i.d. or i.m. with 0.5 nmol (A) or 2nmol (B) of Z13Mad5Anaxa. Blood was obtained from mice 7 days after the 2nd and the 3rd vaccination and multimer staining was performed (one experiment with 4 mice per group). \*, p<0.05.

Figure 25:

shows for Example 13 (not falling under the claims) KLRG1 expression (A) and PD-1 expression (B), which were analyzed on multimer-positive CD8 T cells (one experiment with 4 mice per group). Briefly, C57BL/6 mice were vaccinated three times (once at Wk0, once at Wk2 and once at Wk4) s.c., i.d. or i.m. with 2nmol of Z13Mad5Anaxa. Blood was obtained from mice 7 days after the 2nd and the 3rd vaccination and FACS staining was performed.

Figure 26:

shows for Example 14 (not falling under the claims) SIINFEKL-specific CD8 T cell responses in C57BL/6 mice vaccinated two times (once at Wk0 and once at Wk2) intranodally with 0.5 nmol of Z13Mad5Anaxa. Blood was obtained from mice 7 days after the 2nd vaccination and multimer staining was performed (3 mice per group).

Figure 27:

shows for Example 15 (not falling under the claims) the percentage of pentamer-positive cells among CD8 T cells (A and B; \*, p<0.05) and KLRG1 geomean of pentamer-positive CD8 T cells (C and D). Briefly, C57BL/6 mice were vaccinated 3 times (A and C: Wk0, Wk2 and Wk4; B and D: Wk0, Wk2 and Wk8) s.c. with 2 nmol of Z13Mad5Anaxa. Mice were bled 7 days after last vaccination and pentamer staining was performed (one experiment with 4 mice per group).

Figure 28:

shows for Example 15 (not falling under the claims) the percentage of multimer-positive cells among CD8 T cells (A and D); KLRG1 geomean of multimer-positive CD8 T cells (B and E) and PD1 geomean of multimer-positive CD8 T cells (C and F). A-C, C57BL/6 mice were vaccinated 3 times at Day0, Day3 and Day7 and bled at Day7 and Day14. D-F, C57BL/6 mice were vaccinated 3 times at Day0, Day7 and Day14 and bled at Day14 and Day21. Vaccination was performed s.c. with 0.5 nmol of Z13Mad5Anaxa. Multimer staining was performed on blood samples (one experiment with 4 mice per group).

Figure 29:

shows for Example 16 (not falling under the claims) the IL-6 secretion indicating the APC activation after incubation of BMDCs with various constructs as indicated in the Figure. Briefly, BMDCs were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of Z13Mad5Anaxa, Mad5Anaxa, Z13Mad5, EDAZ13Mad5 or EDAMad5 and incubated for 24h at 37°C. IL-6 secretion was quantified by ELISA in the supernatant. Mean  $\pm$  SEM

of 2 to 3 individual experiments.

Figure 30:

shows for Example 16 (not falling under the claims) the TNF- $\alpha$  secretion indicating the APC activation after incubation of Raw 264.7 cells with various constructs as indicated in the Figure. Briefly, Raw 264.7 cells were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of Z13Mad5Anax, Mad5Anax or Z13Mad5 and incubated for 24h at 37°C. TNF- $\alpha$  secretion was quantified by ELISA in the supernatant. Mean  $\pm$  SEM of 2 to 3 individual experiments.

Figure 31:

shows for Example 17 (not falling under the claims) the IL-8 secretion indicating TLR4 binding after incubation of HEK-hTLR4 cells with various constructs as indicated in the Figure. Briefly, HEK-hTLR4 were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of Z13Mad5Anax, Mad5Anax, Z13Mad5, EDAZ13Mad5 or EDAMad5 and incubated 24h at 37°C. IL-8 secretion was quantified by ELISA in the supernatant. Mean  $\pm$  SEM of 2 individual experiments.

Figure 32:

shows for Example 18 (not falling under the claims) the number of metastasis in a lung metastasis model with semitherapeutic settings. Briefly, C57BL/6 mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and vaccinated twice (d0 and d9) by subcutaneous injection of 2 nmol of EDAZ13Mad5, Z13Mad5 + MPLA (equimolar to EDA) or MPLA alone s.c. in the right flank. Mice were euthanized at day 13 and lung recovered. Number of metastasis foci was counted for each lung. \*\*, p<0.01 (One-way Anova with Tukey's multiple comparisons test).

Figure 33:

shows for Example 19 (not falling under the claims) the number of metastasis in a lung metastasis model with semitherapeutic settings. Briefly, C57BL/6 mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and vaccinated twice (d0 and d9) by subcutaneous injection of 0.5 nmol of Z13Mad5Anax, Mad5Anax or Z13Mad5 + Pam3CSK4 (equimolar to Anax) s.c. in the right flank. Mice were euthanized at day 21 and lung recovered. Number of metastasis foci was counted for each lung. \*, p<0.05; \*\*, p<0.01 (Unpaired t-test).

Figure 34:

shows for Example 20 (not falling under the claims) the quantification of SIINFEKL-specific CD8 T cells in a Quad-GI261 glioblastoma model. Briefly, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  GI261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anax or 2 nmol of Z13Mad5 and 2 nmol of Anax. SIINFEKL-specific CDS T cells were quantified in blood and in BILs at d28 by multimer staining (5-8 mice per group).

Figure 35:

shows for Example 20 (not falling under the claims) the cytokine secretion. Briefly, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  GI261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anax or 2 nmol of Z13Mad5 and 2 nmol of Anax. BILs were isolated and cultured during 6h with matured BMDCs

loaded or not with SIINFEKL peptide in presence of BrefeldinA before intracellular staining for cytokines. % of CD8 T cells secreting cytokine (5-8 mice per group).

Figure 36:

shows for Example 21 (not falling under the claims) the effect of Z13Mad5Anaxa on survival in the Quad-GI261 glioblastoma model. Briefly, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  GI261-Quad tumor cells and vaccinated three times (d7, d21 and d35) by s.c. injection of 2 nmol of Z13Mad5Anaxa. Mice were weight daily and euthanized when weight loss reached more than 15%.

Figure 37:

shows for Example 22 (not falling under the claims) the effect of Z13Mad5Anaxa on tumor growth and survival in subcutaneous EG7-OVA tumor model in a prophylactic setting. Briefly, C57BL/6 mice were vaccinated twice (d-21 and d-7) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa in the right flank and then implanted at day0 s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank. Tumor size was measured with a caliper. (A) Tumor growth of 7 mice per group (mean  $\pm$  SEM); \*\*\*\*, p<0.0001 (2-way Anova test at day 30). (B) Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.). \*\*, p<0.001 (Log-rank test).

Figure 38:

shows for Example 23 (not falling under the claims) the effect of Z13Mad5Anaxa on tumor growth and survival in subcutaneous B16-OVA tumor model in a therapeutic setting on an established tumor. Briefly, C57BL/6 mice were implanted s.c. with  $1 \times 10^5$  B16-OVA tumor cells in the left flank and vaccinated twice (d14 and d21) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa in the right flank. (A) Tumor growth of 7 mice per group (mean  $\pm$  SEM); \*, p<0.05 (2-way Anova test at day 32). (B) Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.).

Figure 39:

shows for Example 24 (not falling under the claims) the effect of the CPP in Z13Mad5Anaxa on tumor growth and survival in subcutaneous EG7-OVA tumor model. Briefly, C57BL/6 mice were implanted at day0 s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and then vaccinated twice (d5 and d13) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa or Mad5Anaxa in the right flank. Tumor size was measured with a caliper. (A) Tumor growth of 7 mice per group (mean  $\pm$  SEM); \*\*\*\*, p<0.0001. (B) Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.). \*\*, p<0.01; \*\*\*, p<0.001.

Figure 40:

shows for Example 25 (not falling under the claims) the effect of complexes having different CPPs on the immune response. C57BL/6 mice were vaccinated five times (Wk0, Wk2, Wk4, Wk6 and Wk8) s.c. with either 2nmol (A) or 0.5 nmol (B) of Z13Mad5Anaxa, Z14Mad5Anaxa or Z18Mad5Anaxa. Mice were bled 7 days after the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> vaccination and multimer staining was performed (one experiment with 4 mice per group). \*, p<0.05 between vaccinated versus naïve mice at each time point except after Vac2 for Z18Mad5Anaxa-vaccinated mice.

Figure 41:

shows for Example 26 (not falling under the claims) the effect of complexes having different CPPs on CD8 T cells in spleen (A), draining lymph nodes (B) and bone marrow (C). C57BL/6 mice were vaccinated five times (Wk0, Wk2, Wk4, Wk6 and Wk8) s.c. with 2 nmol of Z13Mad5Anaxa or Z14Mad5Anaxa. Nine days after the 5<sup>th</sup> vaccination, mice were euthanized, organs recovered and multimer staining was performed.

Figure 42:

shows for Example 26 (not falling under the claims) the effect of complexes having different CPPs on T cells in spleen (CD8 T cell response (A) and CD4 T cell response (B)). C57BL/6 mice were vaccinated five times (Wk0, Wk2, Wk4, Wk6 and Wk8) s.c. with 2 nmol of Z13Mad5Anaxa or Z14Mad5Anaxa. (A) nine days after the 5<sup>th</sup> vaccination, Elispot assay was performed on spleen cells stimulated with SIINFEKL OVACD8 peptide. (B) nine days after the 5<sup>th</sup> vaccination, Elispot assay was performed on spleen cells stimulated with OVACD4 peptide.

Figure 43:

shows for Example 26 (not falling under the claims) the effect of complexes having different CPPs on CD8 T cell effector function. C57BL/6 mice were vaccinated five times (Wk0, Wk2, Wk4, Wk6 and Wk8) s.c. with 2 nmol of Z13Mad5Anaxa or Z14Mad5Anaxa. Nine days after the 5<sup>th</sup> vaccination, intracellular staining was performed on spleen cells stimulated with SIINFEKL OVACD8 peptide.

Figure 44:

shows for Example 27 (not falling under the claims) the effect of complexes having different CPPs on tumor growth (A) and survival rates (B). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa or Z14Mad5Anaxa in the right flank. (A) Tumor growth of 7 mice per group (mean  $\pm$  SEM); \*, p<0.05; \*\*\*\*, p<0.0001 (2-way Anova test at day 28). (B) Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.). \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001 (Log-rank test).

Figure 45:

shows for Example 28 (not falling under the claims) the effect of complexes having different CPPs on the immune response. C57BL/6 mice were vaccinated three times (Wk0, Wk2 and Wk4) s.c. with 2 nmol (A) or 0.5 nmol (B) of EDAZ13Mad5, EDAZ14Mad5 or EDAZ18Mad5. Mice were bled 7 days after the 3<sup>rd</sup> vaccination and multimer staining was performed (one experiment with 4 mice per group). \*, p<0.05

Figure 46:

shows for Example 29 (not falling under the claims) the effect of EDAZ14Mad5 on tumor growth (A) and survival rates (B). C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by s.c. injection of 2nmoles of EDAZ14Mad5 in the right flank. Left panel: Tumor growth of 7 mice per group (mean  $\pm$  SEM); \*\*, p<0.01 (2-way Anova test at day 27). Right panel: Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.).

Figure 47:

shows for Example 30 (not falling under the claims) the quantification of SIINFEKL-

specific CD8 T cells in a Quad-GI261 glioblastoma model. Briefly, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  GI261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anaxa or 2 nmol of Z13Mad5 and 2 nmol of Anaxa. SIINFEKL-specific CD8 T cells were quantified in blood and in BILs at d28 by multimer staining (7-16 mice per group).

Figure 48:

shows for Example 30 (not falling under the claims) the cytokine secretion. Briefly, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  GI261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anaxa or 2 nmol of Z13Mad5 and 2 nmol of Anaxa. BILs were isolated and cultured during 6h with matured BMDCs loaded or not with SIINFEKL peptide in presence of BrefeldinA before intracellular staining for cytokines. % of CD8 T cells secreting cytokine (7-16 mice per group).

Figure 49:

shows for Example 31 (not falling under the claims) the effect of Z13Mad8Anaxa on T cells in spleen (CD8 T cell response (A) and CD4 T cell response (B)). C57BL/6 mice were vaccinated four times (Wk0, Wk2, Wk4 and Wk6) s.c. with 2 nmol of Z13Mad8Anaxa. (A) one week after the 4<sup>th</sup> vaccination, Elispot assay was performed on spleen cells stimulated gp70CD8 peptide. (B) one week after the 4<sup>th</sup> vaccination, Elispot assay was performed on spleen cells stimulated with gp70CD4 peptide.

Figure 50:

shows for Example 32 (not falling under the claims) the effect of Z13Mad11Anaxa on the number of metastasis in the B16 lung metastasis model (A) and on the T cell response in spleen (B). C57BL/6 mice were vaccinated two times (day0, day10) s.c. with 1 nmol of Z13Mad11Anaxa.

Figure 51:

shows for Example 33 (not falling under the claims) the effect of Z13Mad9Anaxa on T cells in spleen (CD8 T cell response. C57BL/6 mice were vaccinated four times (Wk0, Wk2, Wk4 and Wk6) s.c. with 2 nmol of Z13Mad9Anaxa. One week after the 4<sup>th</sup> vaccination, Elispot assay was performed on spleen cells stimulated with adpgk peptide.

Figure 52:

shows for Example 34 (not falling under the claims) the effect of complexes having different CPPs on the immune response. C57BL/6 mice were vaccinated two times (Wk0 and Wk2) s.c. with 2 nmol of either Z13Mad5Anaxa or TatFMad5Anaxa. Mice were bled 7 days after the 2<sup>nd</sup> vaccination and multimer staining was performed (one experiment with 8 mice per group).

Figure 53:

shows for Example 35 (not falling under the claims) the quantification of SIINFEKL-specific CD8 T cells in naive mice. Briefly, C57BL/6 mice were vaccinated once (day0) by s.c. injection of 2 nmol of Z13Mad5Anaxa (group "Z13Mad5Anaxa") or 2 nmol of Z13Mad5 and 2 nmol of Anaxa (group "Z13Mad5+Anaxa"). SIINFEKL-specific CD8 T cells were quantified in blood at d7 by multimer staining (4-8 mice per group).

Figure 54:

shows for Example 36 (not falling under the claims) the effect of Z13Mad12Anaxa on T

cells in blood (CD8 T cell response). C57BL/6 mice were vaccinated twice (Wk0 and Wk2) s.c. with 2 nmol of Z13Mad12Anaxa. One week after the 2nd vaccination, multimer staining for the neoantigen reps1 was performed on blood cells.

Figure 55:

shows for Example 37 (not falling under the claims) expression of activation marker HLA-DR, CD83, CD80 and CD86 (from left to right) by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of Z13Mad5Anaxa (lower panels) or Z13Mad5 (upper panels) during 48h. Isotype staining for each condition was also performed as shown.

Figure 56:

shows for Example 38 (not falling under the claims) the percentage of multimer-positive cells (% of CD8 T cells) over the course of repeated vaccination. Briefly, C57BL/6 mice were vaccinated subcutaneously 6 times (weeks 0, 2, 4, 8, 12, 16) with 2 nmol of Z13Mad5Anaxa. Mice were bled 7 days after each vaccination or before vaccination and pentamer staining was performed (two experiment with 4 mice per group). The arrows under the time axis indicate the vaccination time points.

Figure 57:

shows for Example 39 (not falling under the claims) the tumor volume (A) and the survival rate (B) of mice vaccinated with Z13Mad11Anaxa and control mice in the MC-38 tumor model. Briefly, C57BL/6 mice were implanted s.c. with  $2 \times 10^5$  MC38 tumor cells in the left flank and vaccinated twice (-d21 & -d7 before tumor implantation) by s.c. injection of 2 nmol of Z13Mad11Anaxa in the right flank. (A) Tumor growth and (B) Survival curve of 7 mice per group. Median survival is indicated on the graph (m.s.). \*, p<0.05; \*\*, p<0.01 (Log-rank test).

Figure 58:

shows for Example 40 (not falling under the claims) the release of selected cytokines after administration of the complex according to the present invention. Briefly, C57BL/6 mice were injected i.v. with 10 nmol of Z13Mad5Anaxa. 0.5, 1 and 3h post-administration, blood samples were taken to monitor IL-6, TNF $\alpha$ , IFNY, IL1- $\beta$  and IL12 in the serum using a multiplex from Luminex (n=4).

Figure 59:

shows for Example 41 (not falling under the claims) neoantigen adpgk-specific immune response at the tumor site (Tumor-infiltrating cells, TILs) for control (column (A)) and Z13Mad12Anaxa-vaccinated mice (column (B)). FACS dot plots of TILs are shown. Percentage of multimer-positive cells (in % of CD8 T cells) is indicated for each dot plot.

Figure 60:

shows for Example 41 (not falling under the claims) neoantigen reps1-specific immune response at the tumor site (Tumor-infiltrating cells, TILs) for control (column (A)) and Z13Mad12Anaxa-vaccinated mice (column (B)). FACS dot plots of blood cells are shown. Percentage of multimer-positive cells (in % of CD8 T cells) is indicated for each dot plot.

Figure 61:

shows for Example 41 (not falling under the claims) neoantigen-specific immune response at the tumor site (Tumor-infiltrating cells, TILs) for control and

Z13Mad12Anaxa-vaccinated mice. Percentage of multimer-positive cells (in % of CD8 T cells) is shown for each epitope (adpgk (A) and reps1 (B)).

Figure 62:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP110 during over night. Isotype staining for each condition was also performed as shown.

Figure 63:

shows for Example 42(not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP112 during over night. Isotype staining for each condition was also performed as shown.

Figure 64:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP115 during over night. Isotype staining for each condition was also performed as shown.

Figure 65:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP117 during over night. Isotype staining for each condition was also performed as shown.

Figure 66:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP118 during over night. Isotype staining for each condition was also performed as shown.

Figure 67:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP119 during over night. Isotype staining for each condition was also performed as shown.

Figure 68:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP120 during over night. Isotype staining for each condition was also performed as shown.

Figure 69:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP122 during over night. Isotype staining for each condition was also performed as shown.

Figure 70:

shows for Example 42 (not falling under the claims) expression of activation markers

HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 300nM of ATP123 during over night. Isotype staining for each condition was also performed as shown.

Figure 71:

shows for Example 42 (not falling under the claims) expression of activation markers HLA-DR, CD83, CD80 and CD86 by human blood monocyte-derived dendritic cells (DCs) from one single buffy. The DCs were stimulated with 600nM of ATP125 during over night. Isotype staining for each condition was also performed as shown.

Figure 72

shows for Example 43 (not falling under the claims) the tumor growth (A) and the survival rate (B) of 13 to 14 mice per group (mean  $\pm$  SEM). C57BL/6 mice were implanted s.c. with  $2 \times 10^5$  MC-38 tumor cells on the back. Mice of the groups "Z13Mad12Anaxa" and "Z13Mad12Anaxa + anti-PD1" were vaccinated 3 times (d3, d10 and d17) by subcutaneous injection of 2 nmol of Z13Mad12Anaxa at the tail base. 200  $\mu$ g of anti-PD1 antibody were administered i.p. on each of days 6, 10, 13, 17, 20, 24 and 27 to mice of groups "anti-PD1" and "Z13Mad12Anaxa + anti-PD1". Tumor size was measured with a caliper. The number of tumor-free mice of each group is indicated for each tumor growth curve. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

Figure 73

shows for Example43 (not falling under the claims) individual tumor growth curves of 13 to 14 mice per group. C57BL/6 mice were implanted s.c. with  $2 \times 10^5$  MC-38 tumor cells on the back. Mice of the groups "Z13Mad12Anaxa" and "Z13Mad12Anaxa + anti-PD1" were vaccinated 3 times (d3, d10 and d17) by subcutaneous injection of 2 nmol of Z13Mad12Anaxa at the tail base. 200  $\mu$ g of anti-PD1 antibody were administered i.p. on each of days 6, 10, 13, 17, 20, 24 and 27 to mice of groups "anti-PD1" and "Z13Mad12Anaxa + anti-PD1". Tumor size was measured with a caliper.

Figure 74

shows for the results for Example 44. C57BL/6 mice were vaccinated once with 4nmol of ATP128. Mice were bled 7 days later and Elispot assay was performed on blood cells stimulated with dendritic cells loaded with ATP128 (one experiment).

Figure 75

shows for Example 45 the Activation Index of human blood monocyte-derived dendritic cells (DCs) from ten different buffys. The DCs were stimulated with 300nM of ATP128 overnight. Negative and positive controls were performed by incubating cells with buffer and MPLA, respectively.

Figure 76

shows for Example 46 the regions of ATP128 containing peptides that were found presented on MHC class I (A) and class II (B) at the surface of human dendritic cells from two different donors, donor 9 (solid line) and donor 10 (dotted line), after overnight loading with ATP128 and the respective number of peptides presented on MHC class I and class II (C). Underlined numbers correspond to peptides previously described in the literature as immunogenic peptides.

## EXAMPLES

**[0494]** In the following, particular examples illustrating various embodiments and aspects of the invention are presented. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They are intended as illustrations of single aspects of the invention only. Indeed, various modifications of the invention in addition to those described herein will become readily apparent to those skilled in the art from the foregoing description, accompanying figures and the examples below.

**Example 1: In vitro human dendritic cell maturation (not falling within the claims)**

**[0495]** The goal of this study was to investigate the capacity of a complex for use to induce maturation of dendritic cells. In the present study, the complex for use is a fusion protein, comprising the cell-penetrating peptide "Z13", a protein "MADS", which consists of different CD8<sup>+</sup> and CD4<sup>+</sup> epitopes from various antigens, and the TLR4 peptide agonist "EDA". Accordingly, a fused protein with the EDA peptide at the N-terminal position and different control conjugated proteins without Z13 or EDA or both were designed.

**[0496]** Namely, the following constructs were designed, whereby in the amino acid sequence the cell-penetrating peptide "Z13" is shown underlined and the TLR peptide agonist "EDA" is shown in italics:

**EDAZ13Mad5**

**[0497] Sequence:**

MHHHHHHH*NID* RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI  
*RELFPA*PAPDGEDDTAELQGLR PGSEYTVSVV ALHDDMESQP *LIGIQSTKRY* KNRVASRKS*R*  
*AKFKQ*LLQHY REVAAKSSE NDRLRLLL*KE* SLKISQAVHA AHAEINEAGR EVVGVGALKV  
 PRNQDWLGVP RFAKFASFEA QGALANIAAVD KANLDVEQL*E* SIINFEKLT*E* WTGS \*\*\*  
 [SEQ ID NO: 26]

**Molecular weight: 25'057 Da**

**[0498] Characteristics:**

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes

- Contains EDA TLR agonist (Lasarte, J.J., et al., The extra domain A from fibronectin targets antigens to TLR4-expressing cells and induces cytotoxic T cell responses in vivo. J Immunol, 2007. 178(2): p. 748-56)
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 1 M L-Arginine, pH 8
- Endotoxin level: < 0.01EU/ug

### Z13Mad5

#### [0499] Sequence:

MHHHHHHHKRY KNRVASRKS~~R~~ AKFKQLLQHY REVAAKSSE NDRLRLLLKE SLKISQAVHA  
AHAEINEAGR EVVGVGALKV PRNQDWLGVP RFAKFASFEA QGALANIAVD KANLDVEQLE  
SIINFEKLTE WTGS

[SEQ ID NO: 29]

**Molecular weight: 15'196 Da**

#### [0500] Characteristics:

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 1 M L-Arginine, pH 9
- Endotoxin level:
  - Batch 1: 0.32EU/mg
  - Batch 2: 0.44EU/mg

### Mad5

#### [0501] Sequence:

MHHHHHHHE SLKISQAVHA AHAEINEAGR EVVGVGALKV PRNQDWLGVP RFAKFASFEA  
QGALANIAVD KANLDVEQLE SIINFEKLTE WTGS

[SEQ ID NO: 30]

**Molecular weight: 10'154.6 Da**

**[0502] Characteristics:**

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 0.5 M L-Arginine, pH 8
- Endotoxin level: 0.069EU/mg

**[0503]** The EDAZ13Mad5, Z13Mad5 and Mad5 proteins were investigated for their capacity to induce human dendritic cell (DC) maturation. After incubation during 48h with 300nM of protein, activation markers expression (CD86, CD40, CD83 and HLA-DR) was assessed on the human DCs by FACS (Figures 1 - 4). Specific buffers of each protein were used as negative controls.

**[0504]** Results are shown for CD40 in Fig. 1, for CD86 in Fig. 2, for HLA-DR in Fig. 3, and for CD83 in Fig. 4. Whereas EDAZ13Mad5 induced maturation of human DCs, shown by the up-regulation of CD86, HLA-DR and CD83, Z13Mad5 and Mad5 proteins were not able to activate human DCs. These results indicate that the EDA portion of the protein is responsible for the up-regulation of the activation markers on the human DCs.

**Example 2: In vitro epitope presentation (MHC I) (not falling within the claims)**

**[0505]** The goal of this study was to assess functional MHC class I-restricted cross-presentation in a murine *in an vitro* system using bone marrow derived dendritic cells (BMDCs) and splenocytes from different TCR transgenic mice. To this end, the constructs EDAZ13Mad5 and Mad5 (described above in Example 1) and the construct EDAMad5 were used:

**EDAMad5**

**[0506] Sequence**

MHHHHHHH*NID* RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI  
 RELFPAPDGEDDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTE SLKISQAVHA  
 AHAEINEAGR EVVGVGALKV PRNQDWLGVP RFAKFASFEA QGALANIAVD KANLDVEQLE  
 SIINFEKLTE WTGS

[SEQ ID NO: 31]

**Molecular weight: 20'017 Da**

[0507] Characteristics:

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Contains EDA TLR agonist
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 0.5 M L-Arginine, pH 8
- Endotoxin level: 1.8EU/mg

[0508] BMDCs were loaded overnight with 300nM of with the EDAMad5, EDAZ13Mad5 and MadS proteins containing OVACD8, OVACD4 and gp100 epitopes. Processing and presentation of these MHC I-restricted OVACD8 and gp100 epitopes were monitored by measuring the *in vitro* proliferation of naïve OVA<sub>257-264</sub>-specific CD8<sup>+</sup>T cells from OT-1 T cell receptor (TCR) transgenic mice and gp100-specific CD8<sup>+</sup> T cells from P-mel T cell TCR transgenic mice respectively. Accordingly, efficient MHC class I-restricted presentation of OVACD8 epitope and gp100 epitope was monitored after 4 days with CFSE-labeled OT1 cells and P-Mel cells respectively. Processing and presentation of MHC II-restricted OVACD4 epitope was monitored by measuring the *in vitro* proliferation of naïve OVA<sub>323-339</sub>-specific CD4<sup>+</sup>T cells from OT-2 T cell receptor (TCR) transgenic mice. Accordingly, efficient MHC class II-restricted presentation of OVACD4 epitope was monitored after 4 days with CFSE-labeled OT2 cells. As control, BMDCs were pulsed for 1h with 5uM peptide (one experiment representative of 2 individual experiments).

[0509] Results are shown in Fig. 5. Similar cross-presentation and processing capacity of all assessed MadS-based proteins were observed.

**Example 3: CD8 T cell immune response (not falling within the claims)**

[0510] To investigate the efficacy of EDA-conjugated proteins in inducing polyclonal CD8<sup>+</sup> T cell response, C57BL/6 mice were vaccinated twice (Wk0 and Wk2), by subcutaneous injection of either 2 nmol or 10 nmol of the constructs EDAZ13Mad5 or EDAMad5 (described in Examples 1 and 2). Positive control group was vaccinated with Mad5 and the TLR4 agonist MPLA (equimolar to EDA). Two doses were assessed 2 nmol of the construct (Fig. 6) and 10 nmol of the construct (Fig. 7). 3 - 4 mice were used per group.

[0511] Seven days after the last vaccination, mice were bled and pentamer staining was performed to monitor the OVA-specific immune response in the blood. In Fig. 8, the percentage of pentamer positive CD8+ T cells is shown for all groups and both doses tested.

[0512] These data show that interestingly the immune response is lower at 10nmol compared

to 2nmol. At both doses, 2nmol and 10nmol, the vaccine mediated immune response was observed more consistently in the EDAZ13Mad5 group in contrast to the EDAMad5 group. Moreover, there is an increased immune response when the TLR4 agonist is conjugated with the vaccine.

**Example 4: Vaccine efficacy on tumor growth in a benchmark EG.7-OVA tumor model (not falling within the claims)**

**[0513]** To evaluate the effect of EDA construct proteins on tumor growth control, the s.c. model of EG.7-OVA thymoma cells was chosen. C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank. After tumor implantation, mice were vaccinated at day 5 and 13 with 10 nmol of one of the following constructs (cf. Examples 1 and 2 for construct description): EDAZ13Mad5, EDAMad5, Mad5, or Mad5 and MPLA (equimolar to EDA) s.c. in the right flank. Tumor size was measured with a caliper.

**[0514]** Fig. 9 shows the tumor growth of 7 mice per group (mean  $\pm$  SEM); \*, p<0.05 EDAZ13Mad5 versus control group (2-way Anova test). Fig. 10 shows individual tumor growth curves (7 individual mice per group). Fig. 11 A shows the survival curve of 7 mice per group; \*, p<0.05 EDAZ13Mad5 versus control group (Log-rank test). Fig. 11 B shows the tumor-free progression curve of 7 mice per group; \*, p<0.05 EDAZ13Mad5 versus control group (Log-rank test).

**[0515]** The results show that in a therapeutic setting, EDAZ13Mad5 was the only protein vaccine to significantly control the tumor growth compared to the control group with a significant better tumor free progression curve and survival curve.

**[0516]** The results therefore suggest that the construct protein EDAZ13Mad5 is a highly potent vaccine for controlling the tumor growth in a therapeutic setting.

**Example 5: Vaccine efficacy on tumor growth in a melanoma metastasis model (not falling within the claims)**

**[0517]** To assess the efficacy in a lung metastasis model using B16-OVA tumor cells in a semitherapeutic setting, different construct proteins were used: EDAMad5, EDAZ13Mad5, Z13Mad5 + MPLA (cf. Examples 1 and 2 for design of the constructs), and MPLA alone. C57BL/6 mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and at the same time (d0) 2 nmol of the vaccine (EDAMad5, EDAZ13Mad5, Z13Mad5 + MPLA, MPLA alone) was administered by subcutaneous injection in the right flank. Nine days later, mice were vaccinated a second time with the same dose. Further control groups were vaccinated with 2 nmol of Z13Mad5 and the TLR4 agonist MPLA (equimolar to EDA) or MPLA alone.

**[0518]** Mice were euthanized at day 13 and lung recovered. Number of metastasis foci was counted for each lung. The results are shown in Fig. 12.

**[0519]** The results show that the conjugate EDAZ13Mad5 is as potent as Z13Mad5 + MPLA to inhibit tumor metastasis in the lung. Furthermore, EDA-Mad5 is less potent than EDAZ13Mad5, indicating a crucial role of Z13 in vaccine efficacy.

**Example 6: Vaccine efficacy on tumor growth in a melanoma metastasis model - prophylactic setting (not falling within the claims)**

**[0520]** Furthermore, the efficacy of the different construct proteins EDAMad5, EDAZ13Mad5, and Z13Mad5 + MPLA (cf. Examples 1 and 2 for design of the constructs) was assessed in a lung metastasis model in a prophylactic setting. C57BL/6 mice were vaccinated 21 and 7 days before implantation of tumor cells (d-21 and d-7) by subcutaneous injection of 2 nmol of EDAZ13Mad5, EDAMad5 or Z13Mad5 + MPLA (equimolar to EDA) s.c. in the right flank. At day 0, mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells. Mice were euthanized at day 14 and lung recovered. Results are shown in Fig. 13.

**Example 7: Design of further constructs comprising a TLR2 peptide agonist (not falling within the claims)**

**[0521]** Herein, the complex for use is again a fusion protein, comprising the cell-penetrating peptide "Z13", the protein "MADS", which consists of different CD8<sup>+</sup> and CD4<sup>+</sup> epitopes from various antigens, and the TLR2 peptide agonist "Anaxxa". Accordingly, fused proteins with the Anaxxa peptide at the C-terminal or N-terminal position were designed.

**[0522]** Namely, the following constructs were designed, whereby in the amino acid sequence the cell-penetrating peptide "Z13" is shown underlined and the TLR peptide agonist "Anaxxa" is shown in italics:

**AnaxxaZ13Mad5**

**[0523] Sequence:**

MHHHHHHHSTV HEILCKLSLE GDHSTPPSAY GSVKPYTNFD AEKRYKNRVA SRKSRAKFQ  
LLQHYREVAA AKSSENDRLR LLLKESLKIS QAVHAAHAEI NEAGREVVGV GALKVPRNQD  
WLGVPRFAKF ASFEAQGALA NIAVDKANLD VEQLESIINF EKLTEWTGS  
[SEQ ID NO: 27]

**Molecular weight: 18973 Da**

**[0524] Characteristics:**

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Contains the 35-mer peptide of Annexin
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 0.5 M L-Arginine, pH 8
- Endotoxin level: 5.17 EU/mg

**Z13Mad5Anaxa****[0525] Sequence:**

MHHHHHHHKRYKNRVA SRKSRAKFQO LLOHYREVAA AKSSENDRLR LLLKESLKIS  
 QAVHAAHAEI NEAGREVVGV GALKVPRNQD WLGVPRFAKF ASFEAQGALA  
 NIAVDKANLD VEQLESIINF EKLTEWTGSS TVHEILCKLS LEGDHSTPPS AYGCSVKPYTN FDAE  
 [SEQ ID NO: 28]

**Molecular weight: 18973 Da****[0526] Characteristics:**

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Contains the 35-mer peptide of Annexin
- Storage buffer: 50 mM Tris-HCl, , 150 mM NaCl, 10% Glycerol, 2 mM DTT, 0.5 M L-Arginine, pH 8

**Endotoxin level: 3.1 EU/mg****Example 8: TLR2 binding (HEK-hTLR2 cell lines) (not falling within the claims)**

**[0527]** The goal of this study was to assess whether the Z13Mad5Anaxa and AnaxaZ13Mad5 construct proteins (cf. Example 7 for design of these construct proteins) were able to bind

TLR2 as an agonist. HEK-Blue™ hTLR2 were seeded in flat 96-well plate in culture medium, stimulated with 0.3  $\mu$ M, 1  $\mu$ M or 3  $\mu$ M of AnaxaZ13Mad5 or Z13Mad5Anaxa and incubated at 37°C for 24h. Positive control was performed with 500ng/ml of Pam3CSK4, a TLR2 agonist.

**[0528]** To monitor the activation of NF- $\kappa$ B/AP1, twenty microliters of the supernatant were added to QuantiBlue® detection medium and incubated at 37°C for 1h before OD reading (620nm). Results are shown in Figure 14 A.

**[0529]** The secretion of IL-8 in the supernatant was quantified by ELISA. Results are shown in Figure 14 B.

**[0530]** Results (Fig. 14 A, B) showed that Z13Mad5Anaxa and AnaxaZ13Mad5 are similarly able to bind to TLR2 in a dose dependent manner.

**Example 9: *In vivo* induction of specific CD8 $^{\pm}$  T cells (not falling within the claims)**

**[0531]** To investigate the efficacy of the Anaxa-conjugated proteins of Example 7 in the induction of CD8 $^+$  T cell responses, C57BL/6 mice were vaccinated twice (Wk0 and Wk2), by subcutaneous injection of 2 nmol of AnaxaZ13Mad5 or 2 nmol of Z13Mad5Anaxa. Seven days after the last vaccination, mice were bled and to monitor the OVA-specific immune response in the blood, pentamer staining was performed (one experiment with 4 mice per group). Results are shown in Figures 15 and 16.

**[0532]** These data indicate that both, the Z1 3Mad5Anaxa vaccine and the AnaxaZ13Mad5 construct, elicit a strong immune response.

**Example 10: Therapeutic effect on tumor growth (not falling within the claims)**

**[0533]** To evaluate the effect of the Anaxa-conjugated construct proteins designed in Example 7 on tumor growth control, a benchmark tumor model was used, namely the s.c. implantation of EG.7-OVA thymoma cells.

**[0534]** C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank. After tumor implantation, the three groups of 7 mice each were vaccinated s.c. in the right flank at day 5 and 13 by subcutaneous injection of 10 nmol of either AnaxaZ13Mad5 (group 1), Z13Mad5Anaxa (group 2) or Z13Mad5 and Pam3CSK4 (equimolar to Anaxa; group 3). In order to compare the effect to a protein mixed with an external adjuvant, a control group was vaccinated with Z13Mad5 and Pam3CSK4 (equimolar to Anaxa). Tumor size was measured with a caliper. Results are shown in Fig. 17 - 19.

[0535] In a therapeutic schedule, Z13Mad5Anaxa and AnaxaZ13Mad5 are better protein vaccines for controlling tumor growth compared to the control group, i.e. co-injection of Z13Mad5 and Pam3CSK showing a significant better survival curve. In particular, Z13Mad5Anaxa and AnaxaZ13Mad5 demonstrate significantly higher efficacy than Z13Mad5 administrated separately with Pam3CSK4. The results therefore suggest that the construct proteins Z13Mad5Anaxa and AnaxaZ13Mad5 are promising conjugate-vaccines for controlling the tumor growth in a therapeutic setting.

**Example 11: Therapeutic effect on tumor growth - comparison of constructs with different TLR agonists (not falling within the claims)**

[0536] The goal of this study was to compare the efficacy of the different construct protein vaccines conjugated to different TLR agonist, namely EDAZ13Mad5 and Z13Mad5Anaxa of Example 1 and 7, on tumor growth control. To this end, C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG.7-OVA thymoma cells in the left flank as described previously in Example 10. Mice (7 individual mice per group) were vaccinated s.c. in the right flank at day 5 and 13 with 2 nmol of either EDAZ13Mad5, Z13Mad5Anaxa or co-injection of Z13Mad5+MPLA (equimolar to EDA).

[0537] Results are shown in Figures 20, 21 and 22. In this experimental setting, Z13Mad5Anaxa, EDAZ13Mad5, and Z13Mad5+MPLA were similarly able to significantly control tumor growth. Moreover, these data indicate that Z13Mad5Anaxa is the best construct to significantly control tumor growth and EDAZ13Mad5 was slightly better than Z13Mad5+MPLA in this experimental setting.

**Example 12: Dose effect of Z13Mad5Anaxa on tumor growth control (not falling within the claims)**

[0538] To identify the optimal dose of the conjugate vaccine, three different doses (0.5 nmol, 2 nmol and 10 nmol) of Z13Mad5Anaxa (cf. Example 7) were assessed for their ability to control tumor growth. The dose effect of Z13Mad5Anaxa construct was evaluated in the s.c. model of EG.7 -OVA thymoma cells as described previously in Example 10. After tumor implantation, mice were vaccinated twice (at day 5 and at day 13 after tumor implantation) in a therapeutic setting at 0.5, 2 or 10 nmol of Z13Mad5Anaxa.

[0539] C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and vaccinated twice (d5 and d13) by subcutaneous injection of either 0.5 nmol, 2 nmol or 10 nmol of Z13Mad5Anaxa in the right flank. Tumor size was measured with a caliper.

[0540] The tumor growth of 7 mice per group is depicted in Figure 23. Those data show that the doses of 0.5 and 2 nmol are at least as efficacious as 10 nmol for controlling tumor growth.

**Example 13: Effect of different routes of administration of Z13Mad5Anaxa (not falling within the claims)**

**[0541]** This study was based on the previous Examples demonstrating the efficacy of Z13Mad5Anaxa conjugate vaccine (cf. Example 7), which is able to elicit specific immune responses and is efficacious for controlling tumor growth in the subcutaneous tumor model EG7 as shown above.

**[0542]** To investigate the effect of subcutaneous, intramuscular and intradermal routes of administration, immune responses elicited by subcutaneous, intramuscular and intradermal injection were compared. Intradermal injections were performed using the PLEASE® device from Pantec Biosolutions.

**[0543]** Mice were vaccinated three times every two weeks (Wk0, Wk2 and Wk4) with 0.5 or 2 nmol of Z13Mad5Anaxa (cf. Example 7). In order to target several lymph nodes, the 1st and the 3rd vaccinations were performed in the right flank whereas the 2nd was done in the left flank. SIINFEKL-specific CD8+ T cell response was analyzed 1 week after the 2nd and the 3rd vaccination in the blood. Figure 24 shows the SIINFEKL-specific CD8 T cell responses after each vaccination detected in the blood of C57BL/6 mice vaccinated three times (Wk0, Wk2 and Wk4) s.c., i.d. or i.m. with 0.5 nmol (Figure 24 A) or 2nmol (Figure 24 B) of Z13Mad5Anaxa. Blood was obtained from mice 7 days after the 2nd and the 3rd vaccination and multimer staining was performed (one experiment with 4 mice per group).

**[0544]** The results indicate that at the two doses assessed (0.5 and 2nmol), (i) all routes of administration tested elicited a SIINFEKL-specific CD8 immune response and (ii) the subcutaneous vaccination elicited the strongest SIINFEKL-specific CD8 immune response. For subcutaneous administration, the maximum response was reached after the 3rd vaccination and still maintained after the 3rd vaccination. The SIINFEKL-specific CD8 immune response after the 2nd vaccination elicited by intradermal and intramuscular vaccinations is lower compared to subcutaneous vaccination and is not enhanced after the 3rd vaccination.

**[0545]** Next, the effector function and the exhaustion status of SIINFEKL-specific CD8 T cells was evaluated by analyzing KLRG 1 (Killer cell lectin-like receptor subfamily G member 1) and PD-1 respectively.

**[0546]** To this end, C57BL/6 mice were vaccinated three times (Wk0, Wk2 and Wk4) s.c., i.d. or i.m. with 2nmol of Z13Mad5Anaxa (cf. Example 7). Blood was obtained from mice 7 days after the 2nd and the 3rd vaccination and FACS staining was performed. KLRG1 and PD-1 expression were analyzed on multimer-positive CD8 T cells (one experiment with 4 mice per group). Results are shown in Figure 25.

**[0547]** These data indicate that the expression of KLRG 1 is strongly increasing on SIINFEKL-

specific CD8 T cells after subcutaneous vaccination. After i.d. or i.m. vaccination, the observed effects were lower. The percentage of KLRG 1-positive cells among SIINFEKL-specific CD8 T cells is also enhanced after s.c. vaccination (data not shown).

**[0548]** In contrast to KLRG 1, PD-1 expression is decreasing with the time and the vaccinations, for subcutaneous and intramuscular vaccination routes. This suggests that SIINFEKL-specific CD8 T cells are not exhausted. The percentage of PD1-positive cells among SIINFEKL-specific CD8 T cells is also reduced after s.c. and i.m. vaccination (data not shown). It is important to note that PD-1 expression is higher after the 2nd vaccination when mice were vaccinated subcutaneously, reflecting the early activation status of specific T cells (Keir, M.E., et al., PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*, 2008. 26: p. 677-704).

**[0549]** The expression of the late exhaustion marker Tim-3 was also analyzed. A very low expression as observed for all groups.

**[0550]** Taken together, results indicate that subcutaneous vaccination elicits the best specific CD8 immune response compared to intramuscular or intradermal injections.

**Example 14: Intranodal route of administration (not falling within the claims)**

**[0551]** Based on the previous experiments (Example 13), the intranodal route of administration was additionally investigated. To this end, the immune response elicited by intranodal injection of Z13Mad5Anaxa (cf. Example 7) was investigated.

**[0552]** For this purpose, mice were first injected with Evans Blue subcutaneously in order to allow easily visualizing the lymph nodes for injection and inject intranodally without invasive surgery, for example as described in Jewell, C.M., S.C. Lopez, and D.J. Irvine, *In situ engineering of the lymph node microenvironment via intranodal injection of adjuvant-releasing polymer particles*. *Proc Natl Acad Sci US A*, 2011. 108(38): p. 15745-50.

**[0553]** C57BL/6 mice were vaccinated two times every two weeks (Wk0 and Wk2) intranodally with 0.5 nmol of Z13Mad5Anaxa (cf. Example 7). The 1st vaccination was performed in the right inguinal lymph node, whereas the second vaccination was done in the left inguinal lymph node. Blood was obtained from mice 7 days after the 2nd vaccination and multimer staining was performed (3 mice per group). In other words, SIINFEKL-specific CD8+ T cell response was analyzed one week after the 2nd vaccination in the blood. Figure 26 shows the SIINFEKL-specific CD8 T cell responses. Those data indicate that also intranodal injection was able to elicit SIINFEKL-specific CD8 T cells.

**Example 15: Vaccination schedule (not falling within the claims)**

**[0554]** The vaccination schedule evaluation work was initiated with the objective to identify the impact of the third vaccination using the same Z13Mad5Anaxa construct as described above (cf. Example 7). The subcutaneous route was chosen given the previous results.

**[0555]** In the experiment first two vaccinations were performed at wk0 and wk2 with a 3rd vaccination either at wk4 (Figure 27 A) or at wk8 (Figure 27B). Thus, C57BL/6 mice were vaccinated three times (Figure 27 A and C: Wk0, Wk2 and Wk4 and Figure 27 B and D: Wk0, Wk2 and Wk8) s.c. with 2 nmol of Z13Mad5Anaxa. Blood was obtained from mice 7 days after last vaccination and pentamer staining was performed (one experiment with 4 mice per group). Accordingly, SIINFEKL-specific CD8+ T cell response was analyzed 1 week after the 2nd and the 3rd vaccination (Figure 27 A and B). Additionally, the effector function of SIINFEKL-specific T cells was evaluated by analyzing the expression of KLRG 1 on specific CD8 T cells (Figure 27C and D).

**[0556]** The data indicate that compared to control the percentage of SIINFEKL-specific CD8 T cells was significantly increased at all time points tested (Vac2 and Vac3) as well as in both vaccination schedules (Figure 27 A and B).

**[0557]** Interestingly, the third vaccination at Wk4 allowed to most prominently increasing the percentage of SIINFEKL-specific CD8 T cells (Figure 27 A). The same cells also demonstrate an improved effector function through higher KLRG 1 expression (Figure 27 C). In contrast, with a third vaccination performed at Wk8 no improvement from the second to the third vaccination could be observed in the SIINFEKL-specific immune response and in the KLRG 1 expression.

**[0558]** Taken together, these results indicate that the CD8 immune response could be increased by shorten the delay between the second and the third vaccination.

**[0559]** Given that an earlier third vaccination seems to increase immune response, in the next study two short schedules of vaccination were investigated:

1. i) three vaccinations at day 0, day 3 and day 7 and
2. ii) three vaccinations at day 0, day 7 and day 14.

**[0560]** Again, C57BL/6 mice were used and vaccination was performed s.c. with 0.5 nmol of Z13Mad5Anaxa (cf. Example 7). Multimer staining was performed on blood samples obtained one week after the 2nd and the 3rd vaccination (one experiment with 4 mice per group).

**[0561]** Thus, SIINFEKL-specific CD8+ T cell response was analyzed one week after the 2nd and the 3rd vaccination (Figure 28A and D): Additionally, the effector function of SHNFEKL-specific T cells was evaluated by analyzing the expression of KLRG 1 on specific CD8 T cells (Figure 28B and 28E) and the exhaustion status by analyzing the PD-1 expression of specific T

cells (Figure 28C and 28F).

**[0562]** The data indicate that - similarly to the first study regarding the vaccination schedule described above - compared to control the percentage of SIINFEKL-specific CD8 T cells was increased at all time points tested (Vac2 and Vac3) as well as in both vaccination schedules (Figure 28 A and B).

**[0563]** However, compared to the schedule wk0-wk2-wk4, a schedule with vaccinations at Day0, Day3 and Day7 did not elicit such a high SIINFEKL-specific CD8 T cell immune response. Concerning the schedule with vaccinations at Day0, Day7 and Day14, the SIINFEKL-specific CD8 T cell immune response elicited is better compared to the previous schedule (d0-d3-d7) but is not maintained after the 3rd vaccination.

**[0564]** Taken together, vaccination schedule data set indicates that the Wk0-Wk2-Wk4 vaccination schedule is the best vaccination schedule for inducing potent OVA-specific CD8 immune response with high effector function.

**Example 16: Capacity of TLR agonist-CPP conjugate constructs to activate murine antigen-presenting cells (APCs) (not falling within the claims)**

**[0565]** To investigate the effect of both, the CPP component and the TLR agonist component in a complex for use, again the fusion proteins as described above (cf. Examples 1, 2 and 7) were used.

**[0566]** In addition, a further "control peptide" was designed, which is also a fusion protein and which comprises the protein "MAD5", which consists of different CD8<sup>+</sup> and CD4<sup>+</sup> epitopes from various antigens, and the TLR2 peptide agonist "Anaxxa" (i.e. without cell penetrating peptide). Accordingly, the following control construct was additionally designed:

**Mad5Anaxxa**

**[0567]** Sequence:

MHHHHHHESL KISQAVHAAH AEINEAGREV VGVGALKVPR NQDWLGVPWF  
AKFASFEAQG ALANIAVDKA NLDVEQLESI INFEKLTEWT GSSTVHEILC KLSLEGDHST  
PPSAYGSVKP YTNFDAE

[SEQ ID NO: 32]

**Molecular weight: 13933 Da**

**[0568]** Characteristics:

- Mad5 cargo contains OVACD4, gp100CD8, EalphaCD4 and OVACD8 epitopes
- Contains the 35-mer peptide of Annexin in C-terminal position
- Storage buffer: 50 mM Tris-HCl, 150 mM NaCl, 10% Glycerol, 2 mM DTT, 0.5 M L-Arginine, pH 8
- Endotoxin level: Batch 1 - 12.15 EU/mg

**[0569]** The aim of this study was to evaluate the capacity of two exemplary complexes, namely EDAZ13Mad5 (cf. Example 1) and Z13Mad5Anax (cf. Example 7), to promote antigen-presenting cells activation in comparison to reference complexes lacking either the cell penetrating peptide component Z13 (Mad5Anax, cf. above; EDAMad5, cf. Example 2) or the TLR agonist (Z13Mad5, cf. Example 1).

**[0570]** To this end, the capacity of the above mentioned constructs to promote antigen-presenting cells (APC) activation was assessed in bone marrow-derived dendritic cells (BMDCs), which express all TLRs except TLR7.

**[0571]** BMDCs were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of either Z13Mad5Anax (cf. Example 7), Mad5Anax (cf. above), Z13Mad5 (cf. Example 1), EDAZ13Mad5 (cf. Example 1) or EDAMad5 (cf. Example 2) and incubated for 24h at 37°C.

**[0572]** The APC activation was investigated by monitoring the secretion of IL-6 in the culture supernatant of BMDCs. IL-6 secretion was quantified by ELISA in the supernatant.

**[0573]** The results are shown in Figure 29. These data clearly show that Z13Mad5Anax was able to activate BMDCs, whereas no such activation was observed when the cells were cultured in presence of Z13Mad5 or Mad5Anax. This suggests that not only the TLR agonist (Anax or EDA) is critical for the activation of macrophages and dendritic cells, but that the CPP is also needed. Also the presence of the CPP without the TLR agonist is not sufficient, but indeed both, CPP and TLR agonist are critical for the activation of macrophages and dendritic cells.

**[0574]** Those results were confirmed by using another cell line, namely in the Raw 264.7 mouse macrophage cell line, which expresses all TLRs except TLR5 (Applequist, S.E., R.P. Wallin, and H.G. Ljunggren, Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol*, 2002. 14(9): p. 1065-74).

**[0575]** Raw 264.7 cells were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of either Z13Mad5Anax (cf. Example 7), Mad5Anax (cf. above) or Z13Mad5 (cf. Example 1) and incubated for 24h at 37°C.

**[0576]** In Raw 264.7 cells the APC activation was investigated by monitoring the secretion of

TNF- $\alpha$  in the culture supernatant of Raw 264.7. TNF- $\alpha$  secretion was quantified by ELISA in the supernatant. The results are shown in Figure 30.

**[0577]** It is thought that the CPP may facilitate the entry of the molecule into the cells, allowing a better targeting of intracellular TLR.

**[0578]** Taken together, the data reveal the critical role of both, CPP and TLR agonist, within the conjugate constructs to activate APC. This effect may be due to helping the entry of the construct into the cells, therefore resulting in an optimal targeting of the intracellular TLR.

**Example 17: Ability of the conjugate constructs to bind to human TLR4 (not falling within the claims)**

**[0579]** It was recently shown that the Anaxa peptide owns an adjuvant activity by signaling through TLR2 (WO 2012/048190 A1), whereas the EDA peptide is a natural ligand for TLR4 (Okamura, Y., et al., The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem*, 2001. 276(13): p. 10229-33).

**[0580]** Moreover, as shown above in Example 8 and Figure 14, a complex for use comprising the Anaxa peptide as TLR agonist, for example Z13Mad5Anaxa, is able to bind to human TLR2 and to promote the secretion of IL-8 by HEK-hTLR2 cells (cf. Example 8, Fig. 14).

**[0581]** In the present study, the ability of complexes comprising either the Anaxa peptide as TLR agonist or the EDA peptide as TLR agonist to bind to human TLR4 was evaluated. To this end, HEK cells transfected with human TLR4 (HEK-hTLR4) were seeded in flat 96-well plate in culture medium, stimulated with 1  $\mu$ M of either Z13Mad5Anaxa (cf. Example 7), Mad5Anaxa (cf. above), Z13Mad5 (cf. Example 1), EDAZ13Mad5 (cf. Example 1) or EDAMad5 (cf. Example 2) and incubated for 24h at 37°C. IL-8 secretion was quantified by ELISA in the supernatant.

**[0582]** Results are shown in Figure 31. As expected, incubation of HEK-hTLR4 with EDAZ13Mad5 resulted in remarkable IL-8 secretion, indicating binding of EDAZ13Mad5 to TLR4. In line with the results obtained in Example 16, the IL-8 secretion of EDAMad5 (without the CPP) was remarkably lower as compared to EDAZ13Mad5, showing the effect of the presence of a CPP. The Z13Mad5 construct, which does not comprise a TLR agonist, showed no IL-8 secretion, indicating - as expected - a lack of binding to TLR4.

**[0583]** Interestingly, incubation of HEK-hTLR4 with the construct Z13Mad5Anaxa resulted in the most pronounced IL-8 secretion, indicating binding of Z13Mad5Anaxa to TLR4. This is astonishing, since Anaxa was previously hypothesized to be a TLR2 agonist. Again, the same construct but without the CPP (Mad5Anaxa) 'resulted in remarkably lower IL-8 secretion, confirming the results obtained in Example 16.

**[0584]** Taken together, these data (i) confirm the results obtained in Example 16, (ii) confirm that EDA is indeed a TLR4 agonist, and (iii) show surprisingly that the Anaxxa peptide is also a TLR4 agonist (in addition to being a TLR2 agonist, cf. Example 8 and Fig. 14).

**Example 18: Vaccine efficacy on tumor growth in a lung metastasis model - semi-therapeutic setting: TLR agonist EDA (not falling within the claims)**

**[0585]** This study is based on Example 6, showing the efficacy of a complex for use namely EDAZ13Mad5, in a melanoma lung metastasis model in a prophylactic setting (cf. Figure 13).

**[0586]** In the present study the same lung metastasis model was used as well as the construct proteins EDAZ13Mad5 and Z13Mad5 + MPLA (cf. Examples 1 and 2 for design of the constructs). However, in the semi-therapeutic setting, C57BL/6 mice were vaccinated at the same time as tumor cells were implanted (d0) and, for a second time, at nine days after implantation (d9). Vaccination was performed by subcutaneous injection of 2 nmol of EDAZ13Mad5, Z13Mad5 + MPLA (equimolar to EDA) or MPLA s.c. in the right flank. At day 0, mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and vaccinated twice (d0 and d9) by subcutaneous injection of 2 nmol of EDAZ13Mad5, Z13Mad5 + MPLA (equimolar to EDA) or MPLA alone s.c. in the right flank. Mice were euthanized at day 13 and lung recovered. Results are shown in Fig. 32.

**[0587]** The results show that EDAZ13Mad5 is slightly more potent than Z13Mad5 + MPLA to inhibit the growth of melanoma metastasis. In addition, no adjuvant effect was observed in mice injected with MPLA only.

**[0588]** Both, EDAZ13Mad5 and Z13Mad5 + MPLA, significantly inhibit the growth of melanoma metastasis in the lung in prophylactic and semitherapeutic settings.

**Example 19: Vaccine efficacy on tumor growth in a lung metastasis model - semi-therapeutic setting: TLR agonist Anaxxa (not falling within the claims)**

**[0589]** This study is based on Example 18 with the same model (semitherapeutic settings) and experimental schedule. However, the effect of complexes comprising the "Anaxxa" peptide as TLR agonist were investigated - instead of the EDA TLR agonist as in Example 18.

**[0590]** To this end, C57BL/6 mice were implanted i.v. with  $1 \times 10^5$  B16-OVA melanoma tumor cells and vaccinated twice (d0 and d9) by subcutaneous injection of 0.5 nmol of Z13Mad5Anaxxa, Mad5Anaxxa or Z1 3Mad5 + Pam3CSK4 (equimolar to Anaxxa) s.c. in the right flank. Mice were euthanized at day 21 and the lung was recovered. Number of metastasis foci was counted for each lung. The results are shown in Figure 33.

**[0591]** The results show that Z13Mad5Anaxa is sensibly more potent than Z13Mad5 + Pam3CSK4 to inhibit the growth of melanoma metastasis. In contrast, Mad5Anaxa was not able to control metastasis growth in the lung, underlining again the importance of CPP.

**[0592]** Altogether, the B1 6-OVA lung metastasis experiment showed that Z13Mad5Anaxa was highly efficacious in inhibiting the growth of melanoma metastasis in the lung.

**Example 20: Vaccine efficacy in a glioblastoma model (not falling within the claims)**

**[0593]** In this study, another cancer model was used, namely a glioblastoma model. Glioma is the most frequent form of primary brain tumors in adults, with glioblastoma multiforme (GBM) being the most lethal. This tumor is notorious for its highly invasive and aggressive behavior. Currently, the best treatment against GBM is a regimen involving a combination of surgery, chemotherapy and radiotherapy, which has a median survival period of only 14.6 months. There is an urgent, unmet medical need for new treatment modalities that improve the prognosis of glioma patients. T-cell mediated immunotherapy is a conceptually attractive treatment option to use in conjunction with existing modalities for glioma, in particular highly invasive GBM.

**[0594]** The G1261 glioma is a carcinogen-induced mouse glioma model. This model represents one of the very few brain tumor models developed in immunocompetent animals, that has growth characteristics similar to human GBM (Newcomb, E. and D. Zagzag, The murine GL261 glioma experimental model to assess novel brain tumor treatments, in CNS Cancer Models, Markers, Prognostic, Factors, Targets, and Therapeutic Approaches, E.G. Van Meir, Editor. 2009, Humana Press: Atlanta. p. 227-241; Jacobs, V.L., et al., Current review of in vivo GBM rodent models: emphasis on the CNS-1 tumour model. ASN Neuro, 2011. 3(3): p. e00063). Low numbers of intracranially transplanted G1261 cells formed intracranial tumors in C57BL/6 mice (Zhu, X., et al., Poly-/CLC promotes the infiltration of effector T cells into intracranial gliomas via induction of CXCL10 in IFN-alpha and IFN-gamma dependent manners. Cancer Immunol Immunother, 2010. 59(9): p. 1401-9; Zhu, X., et al., Toll like receptor-3 ligand poly-/CLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. J Transl Med, 2007. 5: p. 10). The cells are moderately immunogenic: they are able to elicit tumor-specific immune response at the tumor site. However, the tumor-specific immune cells are not capable of complete tumor clearance.

**[0595]** Recently, M. Ollin generated a new G1261 model (Ohlfest, J.R., et al., Vaccine injection site matters: qualitative and quantitative defects in CDB T cells primed as a function of proximity to the tumor in a murine glioma model. J Immunol, 2013. 190(2): p. 613-20) by transfecting G1261 cell line with the "Quad Cassette" expressing four peptides presented by H-2b class I or II molecules: human gp100<sub>25-33</sub>, chicken OVA<sub>257-264</sub>, chicken OVA<sub>323-339</sub>, and mouse I-Ea<sub>52-68</sub>. The Quad-G1261 cell line also stably expresses luciferase, which allows the

follow-up of tumor growth by bioluminescence.

**[0596]** The goal of this study was to assess the efficacy of a complex for its use in the Quad-G1261 glioblastoma model.

**[0597]** The effect of a complex for its use, namely Z13Mad5Anaxa (cf. Example 7) was evaluated in the above described glioblastoma model T cell homing at the tumor site was therefore analyzed in GI261-Quad tumor-bearing mice vaccinated twice (Wk1 and Wk3) with Z13Mad5Anaxa vaccine. A group vaccinated with Z13Mad5 and Anaxa (equimolar to Z13Mad5Anaxa) administrated separately was used as control. Briefly, C57BL/6 mice were implanted i.c. (intracranially) with  $5 \times 10^5$  G1261-Quad tumor cells and vaccinated twice (at d7 and d21 following implantation) by s.c. injection of 2 nmol of Z13Mad5Anaxa (group 1) or 2 nmol of Z13Mad5 and 2 nmol of Anaxa (group 2). At Wk4, the blood and the brain infiltrating leukocytes (BILs) were analyzed, whereby SIINFEKL-specific CD8 T cells were quantified in blood and in BILs at d28 by multimer staining (5-8 mice per group). Results are shown in Figure 34.

**[0598]** In general, low frequency of SIINFEKL-specific CD8 T cells was quantified in the blood. However, a higher percentage of SIINFEKL-specific CD8 T cells was observed in the blood of Z13Mad5Anaxa-vaccinated mice. In all groups, there was a sensibly stronger accumulation of SIINFEKL-specific CD8 T cells in the BILs.

**[0599]** After two vaccinations with Z13Mad5Anaxa, the frequency of SIINFEKL-specific cells CD8+ T cells in the BILs was 2-fold higher (24%) than with Z13Mad5 + Anaxa (12%).

**[0600]** Next, cytokine secretion was assessed. To this end, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  G1261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anaxa or 2 nmol of Z13Mad5 and 2 nmol of Anaxa. BILs were isolated and cultured during 6h with matured BMDCs loaded or not with SIINFEKL peptide in presence of BrefeldinA before intracellular staining for cytokines. Results are shown in Figure 35.

**[0601]** Despite heterogeneity, a high level of cytokine secretion was observed for brain-infiltrating CD8 T cells from mice vaccinated with Z13Mad5Anaxa. These results demonstrate that Z13Mad5Anaxa vaccine was able to elicit a stronger SIINFEKL specific CD8 T cell immune response in the brain of tumor-bearing mice with potent effector function.

**[0602]** The results obtained are indicating that Z13Mad5Anaxa is efficacious for eliciting high brain infiltrating SIINFEKL-specific CD8 immune response. Z13Mad5Anaxa is able to promote the secretion of cytokine by antigen-specific CD8 T cells in the brain.'

**Example 21: Vaccine efficacy on survival in the GI261-Quad glioblastoma model (not falling within the claims)**

**[0603]** In an independent experiment, the survival of control and Z13Mad5Anaxa-vaccinated mice was monitored. The therapeutic settings were three consecutive vaccinations with 2 nmol of Z13Mad5Anaxa at day 7, 21 and 35, post i.c. tumor implantation.

**[0604]** C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  G1261-Quad tumor cells and vaccinated three times (d7, d21 and d35) by s.c. injection of 2 nmol of Z13Mad5Anaxa. Mice were weight daily and euthanized when weight loss reached more than 15%. Results are shown in Figure 36.

**[0605]** The results show that Z13Mad5Anaxa therapeutic vaccination is more efficacious than the control group with a median survival prolonged by 10 days.

**Example 22: Vaccine efficacy in a subcutaneous tumor model - prophylactic setting (not falling within the claims)**

**[0606]** This study is based on the results obtained in Example 10 as shown in Figures 17 - 19.

**[0607]** To evaluate the effect of the Anaxa-conjugated construct proteins designed in Example 7 on tumor growth control, a benchmark tumor model was used, namely the s.c. implantation of EG.7-OVA thymoma cells. In contrast to Example 10, wherein vaccination was performed on days 5 and 13, in the present study a prophylactic setting was evaluated, wherein mice were vaccinated 21 and 7 days before tumor implantation.

**[0608]** C57BL/6 mice were vaccinated twice (d-21 and d-7) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa in the right flank and then implanted at day0 s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and. Tumor size was measured with a caliper.

**[0609]** The results are shown in Figure 37 with tumor volume (Fig. 37 A) and survival rate (Fig. 37 B). The data is showing that prophylactic vaccination with Z13Mad5Anaxa is highly efficacious for controlling tumor growth and survival rate. The volume of the tumor is highly significantly decreased in mice treated with Z13Mad5Anaxa as compared to control mice. The survival rate is highly significantly increased in mice treated with Z13Mad5Anaxa as compared to control mice.

**Example 23: Vaccine efficacy in a subcutaneous tumor model - therapeutic setting with established tumor (not falling within the claims)**

**[0610]** This study is based on the results obtained in Example 10 as shown in Figures 17 - 19 and on the results obtained in Example 22 shown in Figure 37. It was the goal of this study to evaluate the effect of Z13Mad5Anaxa (cf. Example 7) on an established tumor.

[0611] For this purpose, the s.c. model of B16-OVA melanoma cells was used. In this model tumor cells are spreading slowly, therefore allowing a bigger vaccination time window.

[0612] The first vaccination with the low dose of 0.5 nmol of Z13Mad5Anaxa was performed once the tumor was established and visible i.e. at day 14 after tumor cell implantation. A second vaccination was done at day 21.

[0613] Thus, C57BL/6 mice were implanted s.c. with  $1 \times 10^5$  B16-OVA tumor cells in the left flank and vaccinated twice (d14 and d21) by s.c. injection of 0.5 nmol of Z13Mad5Anaxa in the right flank. Tumor growth and survival curves were monitored. Results are shown in Fig. 38.

[0614] The results indicate that Z13Mad5Anaxa efficaciously controls the growth of an established and visible tumor. Moreover, despite an established and visible tumor survival rates increased in mice treated with Z13Mad5Anaxa as compared to controls.

**Example 24: Vaccine efficacy in a subcutaneous tumor model - therapeutic setting: effect of the CPP (not falling within the claims)**

[0615] The protocol of this study corresponds to the study described in Example 10, with the difference that an additional group "Mad5Anaxa" (cf. Example 16) was evaluated.

[0616] Briefly, a benchmark tumor model was used, namely the s.c. implantation of EG.7-OVA thymoma cells. C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank. After tumor implantation, groups of 7 mice each were vaccinated s.c. in the right flank at day 5 and 13 by subcutaneous injection of 0.5 nmol of either Z13Mad5Anaxa (group 1) or Mad5Anaxa (group 2) and compared to a control group. Tumor size was measured with a caliper. Results are shown in Figure 39.

[0617] The results show that the mice treated with Z13Mad5Anaxa show a significantly decreased tumor volume and a significantly increased survival rate compared to both, control mice and mice treated with Mad5Anaxa, i.e. a construct without CPP. These results indicate that the presence of a CPP results in significantly decreased tumor volume and a significantly increased survival rate, i.e. in increased efficiency of vaccination. Therefore, the results indicate - together with the results obtained in Example 10 - that the presence of a CPP and the TLR agonist exert a synergic effect on tumor growth and survival rate.

**Example 25: Comparison of the kinetic of immune responses with complexes having different cell penetrating peptides (not falling within the claims)**

[0618] To investigate the effect of different CPPs in the complex for its use the fusion protein Z13Mad5Anaxa as described above (cf. Example 7) was used.

**[0619]** In addition, further fusion proteins were designed, which comprise CPPs other than Z13 - namely Z14 (SEQ ID NO: 7) or Z18 (SEQ ID NO: 11). Those fusion proteins also comprise the protein "MAD5", which consists of different CD8 and CD4<sup>+</sup> epitopes from various antigens, and the TLR2 peptide agonist "Anaxxa". Accordingly, the following constructs were additionally designed:

**Z14Mad5Anaxxa**

**[0620]** Sequence:

MHHHHHHHKRY KNRVASRKSR AKFKQLLQHY REVAAAKESL KISQAVHAAH AEINEAGREV  
VVGALKVPR NQDWLGVPWF AKFASFEAQG ALANIAVDKA NLDVEQLESI INFEKLTEWT  
CSSTVHEILC KLSLEGDHST PPSAYGSVKP YTNAFDAE  
(SEQ ID NO: 33)

**Z18Mad5Anaxxa**

**[0621]** Sequence:

MHHHHHHHREV AAAKSSENDR LRLLLKESLK ISQAVHAAHA EINEAGREVV GVGALKVPRN  
QDWLGVPFKA KFASFEAQGA LANIAVDKAN LDVEQLESII NFEKLTEWTG SSTVHEILCK  
LSLEGDHSTP PSAYGSVKPY TNFDAE  
(SEQ ID NO: 34)

**[0622]** C57BL/6 mice were assigned to eight different groups (4 mice per group): three groups receiving 2 nmol of either Z13Mad5Anaxxa, Z14Mad5Anaxxa or Z18Mad5Anaxxa and a respective control and three groups receiving 0.5 nmol of Z13Mad5Anaxxa, Z14Mad5Anaxxa or Z18Mad5Anaxxa and a respective control. The mice were vaccinated five times (Week0, Week2, Week4, Week6 and Week8) s.c.. Mice were bled 7 days after the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> vaccination and multimer staining was performed (one experiment with 4 mice per group).

**[0623]** The results are shown in Figure 40. All groups vaccinated with Z13Mad5Anaxxa, Z14Mad5Anaxxa or Z18Mad5Anaxxa showed an increased percentage of multimer-positive cells compared to the control group (except for the second vaccination of Z18Mad5Anaxxa). These results indicate that complexes having different cell penetrating peptides are able to elicit an immune response at different doses.

**Example 26: Comparison of T cell immune responses with complexes having different cell penetrating peptides (not falling within the claims)**

**[0624]** To investigate the CD8 T cell immune responses in more detail, C57BL/6 mice were assigned to three different groups (3-4 mice per group): naïve, Z13Mad5Anaxa or Z14Mad5Anaxa.

**[0625]** C57BL/6 mice of the Z13Mad5Anaxa group and of the Z14Mad5Anaxa group were vaccinated five times (Week0, Week2, Week4, Week6 and Week8) s.c. with 2 nmol of either Z13Mad5Anaxa (cf. Example 7) or Z14Mad5Anaxa (cf. Example 25). Nine days after the 5<sup>th</sup> vaccination, mice were euthanized, organs recovered and multimer staining was performed to identify the percentage of SIINFEKL-specific CD8 T cells in the spleen, bone marrow and draining lymph nodes (inguinal and axillary).

**[0626]** The results are shown in Figure 41. Mice vaccinated with Z13Mad5Anaxa or with Z14Mad5Anaxa showed a similar increase in multimer-positive cells, in particular in the spleen and bone marrow as well as a slight increase in draining lymph nodes.

**[0627]** To further investigate the CD8 T cell effector function after vaccination with complexes with different CPPs, in the same groups of mice as described above Elispot assay was performed on spleen cells stimulated with SIINFEKL OVACD8 peptide (SEQ ID NO: 35) nine days after the 5<sup>th</sup> vaccination in order to quantify IFN-γ producing cells.

**[0628]** The results are shown in Figure 42A. Mice vaccinated with Z13Mad5Anaxa showed a significant increase in IFN-γ producing cells compared to naïve mice. Mice vaccinated with Z14Mad5Anaxa showed also an increase in IFN-γ producing cells compared to naïve mice, however, the increase was not significant, which may be due to the low number of mice (3 mice in Z14Mad5Anaxa group).

**[0629]** To investigate the CD4 T cell responses after vaccination with complexes with different CPPs, in the same groups of mice as described above Elispot assay was performed on spleen cells stimulated with OVACD4 peptide (SEQ ID NO: 36) nine days after the 5<sup>th</sup> vaccination in order to quantify IFN-γ producing cells.

**[0630]** The results are shown in Figure 42B. Mice vaccinated with Z13Mad5Anaxa showed a highly significant increase in IFN-γ producing cells compared to naïve mice. Mice vaccinated with Z14Mad5Anaxa showed also an increase in IFN-γ producing cells compared to naïve mice, however, the increase was not significant, which may be due to the low number of mice (3 mice in Z14Mad5Anaxa group).

**[0631]** In addition, in the above described groups of mice, intracellular staining was performed on spleen cells stimulated with SIINFEKL OVACD8 peptide (SEQ ID NO: 35) to identify CD107a<sup>+</sup>IFN-γ<sup>+</sup>TNF-α<sup>+</sup> cells. Results are shown in Figure 43. Mice vaccinated with Z13Mad5Anaxa or with Z14Mad5Anaxa showed a similar increase in CD107a<sup>+</sup>IFN-γ<sup>+</sup>TNF-α<sup>+</sup>

cells.

**Example 27: Comparison of the effect of complexes having different cell penetrating peptides on tumor growth and survival in the EG.7-OVA s.c. model (not falling within the claims)**

**[0632]** To investigate the effects of complexes having different cell penetrating peptides on tumor growth and survival the EG.7-OVA s.c. model was used. On d0 C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and assigned to three different groups (naive, Z13Mad5Anaxa and Z14Mad5Anaxa). Mice were vaccinated twice at d5 and d13 after tumor implantation by s.c. injection of either 0.5 nmol of Z13Mad5Anaxa or Z14Mad5Anaxa in the right flank.

**[0633]** Results are shown in Figure 44. Vaccination with Z13Mad5Anaxa or with Z14Mad5Anaxa resulted in significantly decreased tumor volumes compared to control mice (Figure 44 A) as well as to significantly increased survival rates compared to control mice (Figure 44 B). Those results indicate that both complexes, Z13Mad5Anaxa and Z14Mad5Anaxa, are able to significantly decrease tumor growth and to significantly prolong survival.

**Example 28: Comparison of the immune responses after vaccination with complexes having different cell penetrating peptides (not falling within the claims)**

**[0634]** In this experiment the effect of different CPPs in the complex for its use was investigated by using a complex with the TLR agonist "EDA". Therefore, the fusion protein EDAZ13Mad5 as described above (cf. Example 1) was used.

**[0635]** In addition, further fusion proteins were designed, which comprise CPPs other than Z13 - namely Z14 (SEQ ID NO: 7) or Z18 (SEQ ID NO: 11). Those fusion proteins also comprise the protein "MAD5", which consists of different CD8<sup>+</sup> and CD4<sup>+</sup> epitopes from various antigens, and the TLR4 peptide agonist "EDA". Accordingly, the following constructs were additionally designed:

**EDAZ14Mad5**

**[0636] Sequence:**

MHHHHHHHNID RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI RELFPAPDGE  
 DDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTKRY KNRVASRKSR AKFKQLLQHY  
 REVAAAKESL KISQAVHAAH AEINEAGREV VGVGALKVPR NQDWLGVPWF AKFASFEAQG  
 ALANIAVDKA NLDVEQLESI INFEKLTEWT GS

(SEQ ID NO: 37)

**EDAZ18Mad5**

**[0637] Sequence:**

MHHHHHHHNID RPKGLAFTDV DVDSIKIAWE SPQGQVSRYR VTYSSPEDGI RELFPAPDGE  
DDTAELQGLR PGSEYTVSVV ALHDDMESQP LIGIQSTREV AAAKSSENDR LRLLLKESLK  
ISQAVHAAHA EINEAGREVV GVGALKVPRN QDWLGVPRFA KFASFEAQGA LANIAVDKAN  
--- LDVEQLESII NFEKLTEWTG \$

(SEQ ID NO: 38)

**[0638]** C57BL/6 mice were assigned to eight different groups (4 mice per group): three groups receiving 2 nmol of either EDAZ13Mad5, EDAZ14Mad5 or EDAZ18Mad5 and a respective control and three groups receiving 0.5 nmol of either EDAZ13Mad5, EDAZ14Mad5 or EDAZ18Mad5 and a respective control group. The mice were vaccinated three times (Week0, Week2 and Week4) s.c.. Mice were bled 7 days after the 2<sup>nd</sup> and 3<sup>rd</sup> vaccination and multimer staining was performed (one experiment with 4 mice per group).

**[0639]** The results are shown in Figure 45. All groups vaccinated with EDAZ13Mad5, EDAZ14Mad5 or EDAZ18Mad5 showed an increased percentage of multimer-positive cells compared to the control group. These results indicate that complexes having different cell penetrating peptides are able to elicit an immune response at different doses.

**Example 29: Effect of EDAZ14Mad5 on tumor growth and survival in the EG.7-OVA s.c. model (not falling within the claims)**

**[0640]** To investigate the effect of EDAZ14Mad5 on tumor growth and survival the EG.7-OVA s.c. model was used (cf. Example 4 and Figures 9-11 for the effect of EDAZ13Mad5 in the same model).

**[0641]** On d0 C57BL/6 mice were implanted s.c. with  $3 \times 10^5$  EG7-OVA tumor cells in the left flank and assigned to two different groups (naive and EDAZ14Mad5). Mice were vaccinated twice at d5 and d13 after tumor implantation by s.c. injection of 0.5 nmol of EDAZ14Mad5 in the right flank.

**[0642]** Results are shown in Figure 46. Similarly to EDAZ13Mad5 (cf. Example 4, Figures 9 - 11) vaccination with EDAZ14Mad5 resulted in significantly decreased tumor volumes compared to control mice (Figure 46 A) as well as to significantly increased survival rates compared to control mice (Figure 46 B). Those results indicate that EDAZ14Mad5 is able to significantly

decrease tumor growth and to significantly prolong survival -similarly to EDAZ13Mad5 (cf. Example 4, Figures 9 - 11).

**Example 30: Superior efficacy of Z13Mad5Anaxa fusion construct compared to Z13Mad5 and Anaxa in a glioblastoma model (not falling within the claims)**

**[0643]** To investigate the efficacy of a complex the glioblastoma model was chosen (cf. Example 20). Namely, Z13Mad5Anaxa (cf. Example 7; SEQ ID NO: 28) was administered to one group of mice, whereas Z13Mad5 (SEQ ID NO: 29) and Anaxa (SEQ ID NO: 15) were administered (both together) to another group of mice.

**[0644]** T cell homing at the tumor site was analyzed in G1261-Quad tumor-bearing mice (7-16 mice per group) vaccinated twice, namely at day 7 and at day 21 after tumor implantation (day 0), with 2 nmol Z13Mad5Anaxa vaccine. A group vaccinated with both, Z13Mad5 and Anaxa (equimolar to Z13Mad5Anaxa), was used as control. Briefly, C57BL/6 mice were implanted i.c. (intracranially) with  $5 \times 10^5$  G1261-Quad tumor cells and vaccinated twice (at d7 and d21 following implantation) by s.c. injection of 2 nmol of Z13Mad5Anaxa (group 1) or 2 nmol of Z13Mad5 and 2 nmol of Anaxa (group 2). At day 28, the blood and the brain infiltrating leukocytes (BILs) were analyzed, whereby SIINFEKL-specific CD8 T cells were quantified in blood and in BILs at d28 by multimer staining (7 - 16 mice per group).

**[0645]** Results are shown in Figure 47. A significantly higher percentage of SIINFEKL-specific CD8 T cells was observed in the blood of Z13Mad5Anaxa-vaccinated mice as compared to mice vaccinated with both, Z13Mad5 and Anaxa (Fig. 47A). Similarly, a stronger accumulation of SHNFEKL-specific CD8 T cells was observed in the BILs of Z13Mad5Anaxa-vaccinated mice as compared to mice vaccinated with Z13Mad5 and Anaxa separately (Fig. 47B,  $p = 0.0539$ ).

**[0646]** Next, cytokine secretion was assessed. To this end, C57BL/6 mice were implanted i.c. with  $5 \times 10^5$  G1261-Quad tumor cells and vaccinated twice (d7 and 21) by s.c. injection of 2 nmol of Z13Mad5Anaxa or 2 nmol of Z13Mad5 and 2 nmol of Anaxa. BILs were isolated and cultured during 6h with matured BMDCs loaded or not with SIINFEKL peptide (SEQ ID NO: 35) in presence of BrefeldinA before intracellular staining for cytokines.

**[0647]** Results are shown in Figure 48. In general, a high level of cytokine secretion was observed for brain-infiltrating CD8 T cells from mice vaccinated with Z13Mad5Anaxa. In particular, a significantly higher secretion of total IFN- $\gamma$  and of IFN- $\gamma$  and TNF- $\alpha$  together was observed for brain-infiltrating CD8 T cells from mice vaccinated with Z13Mad5Anaxa as compared to mice vaccinated with Z13Mad5 and Anaxa separately.

**[0648]** Taken together, these results demonstrate that Z13Mad5Anaxa vaccine (as compared to Z13Mad5 and Anaxa administered separately) was able to elicit a stronger SIINFEKL specific CD8 T cell immune response in the brain of tumor-bearing mice with potent effector

function.

**[0649]** The results obtained are indicating that Z13Mad5Anaxa is efficacious for eliciting high brain infiltrating SIINFEKL-specific CD8 immune response. Z13Mad5Anaxa is able to promote the secretion of cytokine by antigen-specific CD8 T cells in the brain.

**Example 31: Effect of another antigenic cargo in the complex (not falling within the claims)**

**[0650]** To investigate the effect of a different antigenic cargo ("Mad8"), another complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist was designed ("Z13Mad8Anaxa"). Z13Mad8Anaxa differs from Z13Mad5Anaxa (described in Example 7) in the antigenic cargoes. In particular, "Z13Mad8Anaxa" is a fusion protein comprising the cell-penetrating peptide "Z13", the antigenic cargo "MAD8" comprising CD8 and CD4 epitopes of glycoprotein 70, and the TLR peptide agonist "Anax". In the following, the amino acid sequence of Z13Mad8Anaxa is shown with the cell-penetrating peptide "Z13" shown underlined and the TLR peptide agonist "Anax" shown in italics:

KRYKNRVASR KSRAFKQOLL QHYREVAAK SSENDRLRLKK VTYHSPSYAY HQFERRAILN

RLVQFIKDRI SVVQALVLT*S TVHEILCKLS* LECDHSTPPS AYGSVKPYTN FDAE

(SEQ ID NO: 39)

**[0651]** Naïve Balb/c mice (4 mice per group) were vaccinated four times s.c.(week0, week2, week4 and week6 with 2 nmol of Z13Mad8Anaxa.

**[0652]** To investigate the CD4 T cell responses after vaccination, one week after the 4<sup>th</sup> vaccination, mice were euthanized; organs recovered and *ex vivo* Elispot assay was performed on spleen cells stimulated with gp70CD4 peptide (SEQ ID NO: 64) or gp70CD8 peptide (SEQ ID NO: 65) in order to quantify IFN- $\gamma$ -producing epitope-specific CD4 and CD8 T cells.

**[0653]** The results are shown in Figure 49. Mice vaccinated with Z13Mad8Anaxa showed a significant increase in IFN- $\gamma$ -producing cells compared to naive mice. These data show that Z13Mad8Anaxa vaccine was able to elicit potent epitope-specific CD8 and CD4 T cell immune response and thus that the complex is able to elicit self-antigen immune response.

**Example 32: Effect of another antigenic cargo in the complex (not falling within the claims)**

**[0654]** To investigate the effect of a further different antigenic cargo ("Mad11"), another complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist was designed ("Z13Mad11Anaxa"). Z13Mad11Anaxa differs from Z13Mad5Anaxa (described

in Example 7) in the antigenic cargoes. In particular, "Z13Mad11Anaxa" is a fusion protein comprising the cell-penetrating peptide "Z13", the antigenic cargo "MAD11" comprising two CD8 epitopes of survivin as described in Derouazi M, Wang Y, Marlu R, et al. Optimal epitope composition after antigen screening using a live bacterial delivery vector: Application to TRP-2. *Bioengineered Bugs.* 2010;1(1):51-60. doi:10.4161/bbug.1.1.9482, and the TLR peptide agonist "Anax". In the following, the amino acid sequence of Z13Mad11 Anaxa is shown:  
 KRYKNRVASRKSRAFKQQLQHYREVAAKSSENDRLRLLKNYRIATFKNWPFLEDCAMEELT  
 VSEFLKLDQRQRSTVHEILCKSLECDHSTPPSAYGSVKPYTNFDAE  
 (SEQ ID NO: 40)

**[0655]** Naive C57BL/6 mice (5 mice per group) were implanted i.v. with  $1 \times 10^5$  B16 melanoma tumor cells and vaccinated twice (d0 and d10) ' by subcutaneous injection of 1 nmol of Z13Mad11Anaxa.

**[0656]** On day18 mice were euthanized, organs recovered and *ex vivo* Elispot assay was performed on spleen cells stimulated with survivin peptides survivin20-28 (SEQ ID NO: 67) and survivin97-104: (SEQ ID NO: 68) in order to quantify IFN- $\gamma$  producing survivin-specific T cells.

**[0657]** The results are shown in Figure 50. Mice vaccinated with Z13Mad11Anaxa showed less metastasis compared to naive mice (Fig. 50A). Moreover, in the spleen of mice vaccinated with Z13Mad11 Anaxa significantly higher numbers of IFN- $\gamma$  producing survivin-specific T cells were observed (Fig. 49B).

**[0658]** The results obtained show that Z13Mad11Anaxa is efficacious for reducing the number of metastasis and Z13Mad11Anaxa is able to promote the secretion of cytokines by antigen-specific CD8 T cells in the spleen.

**Example 33: Effect of another antigenic cargo in the complex (not falling within the claims)**

**[0659]** To investigate the effect of a further different antigenic cargo ("Mad9"), another complex comprising a cell penetrating peptide, a different antigen and a TLR peptide agonist was designed ("Z13Mad9Anaxa"). Z13Mad9Anaxa differs from Z13Mad5Anaxa (described in Example 7) in the antigenic cargo. In particular, "Z13Mad9Anaxa" is a fusion protein comprising the cell-penetrating peptide "Z13", the antigenic cargo "Mad9" comprising the neoantigen as identified by Yadav et al. *Nature.* 2014 Nov 27;515(7528):572-6 from MC-38 tumor cell line, and the TLR peptide agonist "Anax". In the following, the amino acid sequence of Z13Mad9Anaxa is shown with the cell-penetrating peptide "Z13" shown underlined and the TLR peptide agonist "Anax" shown in italics:

KRYKNRVASRKSRAFKQQLQHYREVAAKSSENDRLRLLKHELEASMTNMELMSSIVSTVHEI  
 LCKLSLECDHSTPPSAYGSVKPYTNFDAE  
 (SEQ ID NO: 41)

**[0660]** Naïve C57BL/6 mice (4 mice per group) were vaccinated four times s.c. (week0, week2, week4 and week6 with 2 nmol of Z13Mad9Anaxa. To investigate the CD8 T cell responses after vaccination, one week after the 4<sup>th</sup> vaccination, mice were euthanized, organs recovered and Elispot assay was performed on spleen cells after a 7-day in vitro restimulation with stimulated with adpgk peptide (SEQ ID NO: 66) in order to quantify IFN- $\gamma$ -producing epitope-specific CD8 T cells .

**[0661]** The results are shown in Figure 51. Mice vaccinated with Z13Mad9Anaxa showed a significant increase in effector neoantigen-specific CD8 T cells compared to naive mice.

**Example 34: Comparison of the immune responses after vaccination with complexes having different cell penetrating peptides (not falling within the claims)**

**[0662]** In this experiment the effect of a further different CPP in the complex was investigated by using a complex with the TLR agonist "Anax". Therefore, the fusion protein Z13Mad5Anaxa as described above (cf. Example 7, SEQ ID NO: 28) was used.

**[0663]** In addition, a further fusion protein was designed, which comprise the TAT CPP combined to furin linkers as described in Lu et al., Multiepitope trojan antigen peptide vaccines for the induction of antitumor CTL and Th immune responses J. Immunol., 172 (2004), pp. 4575-4582. That fusion protein also comprises the protein "MAD5", which consists of different CD8<sup>+</sup> and CD4<sup>+</sup> epitopes from various antigens, and the TLR4 peptide agonist "Anax". Accordingly, the following construct was additionally designed:

**TatFMad5Anaxa**

**[0664] Sequence:**

RKKRRQRRRVKRISQAVHAAHAEINEAGRRVKRKVPRNQDWLRVKRASFEAQGALANIAVD  
KARVKRSIINFEKLRVKRSTVHEILCKSLEGDHSTPPSAYGSVKPYTNFDAE

(SEQ ID NO: 46)

**[0665]** C57BL/6 mice were assigned to three different groups (8 mice per group): one group receiving 2 nmol of Z13Mad5Anaxa, one group receiving 2 nmol of TatFMad5Anaxa and a respective control. The mice were vaccinated two times (Week0 and Week2) s.c. with either 2 nmol of Z13Mad5Anaxa or 2 nmol of TatFMad5Anaxa. Mice were bled 7 days after the 2<sup>nd</sup> vaccination and multimer staining was performed (8 mice per group).

**[0666]** The results are shown in Figure 52. Mice vaccinated with Z13Mad5Anaxa or TatFMad5Anaxa showed an increased percentage of multimer-positive cells compared to the control group. These results indicate that complexes having different cell penetrating peptides are able to elicit an immune response at different doses. However, the CPP derived from ZEBRA (Z13) was better than the TAT CPP.

**Example 35: Superior efficacy of Z13Mad5Anaxa fusion construct compared to Z13Mad5 and Anaxa in naive mice (not falling within the claims)**

**[0667]** Next, the efficacy of a complex was investigated in naive mice. Namely, Z13Mad5Anaxa (cf. Example 7; SEQ ID NO: 28) was administered to one group of mice, whereas Z13Mad5 (SEQ ID NO: 29) and Anaxa (SEQ ID NO: 15) were administered (both together) to another group of mice.

**[0668]** C57BL/6 mice of the Z13Mad5Anaxa group and of the Z13Mad5 + Anaxa group were vaccinated once (Week0) by s.c. injection of 2 nmol of Z13Mad5Anaxa (group 1) or 2 nmol of Z13Mad5 and 2 nmol of Anaxa (group 2). At day 14, the blood was analyzed, whereby SIINFEKL-specific CD8 T cells were quantified in blood by multimer staining (4 - 8 mice per group).

**[0669]** Results are shown in Figure 53. A significantly higher percentage of SIINFEKL-specific CD8 T cells was observed in the blood of Z13Mad5Anaxa-vaccinated mice as compared to mice vaccinated with Z13Mad5 and Anaxa separately (Fig. 53).

**[0670]** Taken together, these results demonstrate that Z13Mad5Anaxa vaccine (as compared to Z13Mad5 and Anaxa administered separately) was able to elicit a stronger SIINFEKL specific CD8 T cell immune response in the periphery.

**Example 36: Effect of another antigenic cargo in the complex (not falling within the claims)**

**[0671]** To investigate the effect of a further different antigenic cargo ("Mad12"), another complex comprising a cell penetrating peptide, a different antigen and a TLR peptide agonist was designed ("Z13Mad12Anaxa"). Z13Mad12Anaxa differs from Z13Mad5Anaxa (described in Example 7) in the antigenic cargo. In particular, "Z13Mad12Anaxa" is a fusion protein comprising the cell-penetrating peptide "Z13", the antigenic cargo "MAD12" comprising three neoantigens as identified by Yadav et al. *Nature*. 2014 Nov 27;515(7528):572-6 from MC-38 tumor cell line, and the TLR peptide agonist "Anaxa". In the following, the amino acid sequence of Z13Mad12Anaxa is shown:

KRYKNRVASRKSRAFKQLLQHYREVAAKSSENDRLRLLLKLFRRAQLANDVVLQIMEHLELA

SMTNMELMSSIVVISASIIVFNLLLEGSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE

/SEQ ID NO: 60

(SEQ ID NO. 03)

**[0672]** Naive C57BL/6 mice (4 mice per group) were vaccinated twice s.c. (week0, week2) with 2 nmol of Z13Mad12Anaxa. To investigate the CD8 T cell responses after vaccination, one week after the 2<sup>nd</sup> vaccination, the blood was analyzed, whereby neoantigen reps1-specific CD8 T cells were quantified in blood by multimer staining (4 mice per group).

**[0673]** The results are shown in Figure 54. Mice vaccinated with Z13Mad12Anaxa showed a significant increase in effector neoantigen-specific CD8 T cells compared to naive mice.

**Example 37: In vitro human dendritic cell maturation (not falling within the claims)**

**[0674]** The goal of this study was to investigate the capacity of a complex for its use ("Z13Mad5Anaxa", SEQ ID NO: 28, cf. Example 7) to induce maturation of dendritic cells in comparison to a complex lacking a TLR peptide agonist ("Z13Mad5", SEQ ID NO: 29, cf. Example 1).

**[0675]** The Z13Mad5Anaxa polypeptide and the Z13Mad5 polypeptide were investigated for their capacity to induce human dendritic cell (DC) maturation. After incubation over night with 300nM of protein, activation markers expression (CD86, CD80, CD83 and HLA-DR) was assessed on the human DCs by FACS (Figure 55). Same buffer volumes of each protein were used as negative controls.

**[0676]** Results are shown in Fig. 55. Whereas Z13Mad5Anaxa induced maturation of human DCs, shown by the up-regulation of CD86, HLADR and CD83, Z13Mad5 was not able to activate human DCs. These results indicate that the Anaxa portion of the protein is responsible for the up-regulation of the activation markers on the human DCs.

**Example 38: CD8 T cell immune response over the course of repeated vaccination (not falling within the claims)**

**[0677]** To investigate whether a pool of effector T cells could be maintained over several months, repeated vaccination was performed. Namely, C57BL/6 mice were vaccinated subcutaneously six times (weeks 0, 2, 4, 8, 12, 16) with 2 nmol of the construct Z13Mad5Anaxa (SEQ ID NO: 28). Seven days after the each vaccination (and before some vaccinations), mice were bled and pentamer staining was performed to monitor the OVA-specific immune response in the blood (two experiments with 4 mice per group). In Fig. 56, the percentage of pentamer positive CD8+ T cells is shown for Z13Mad5Anaxa vaccinated mice and for control mice.

**[0678]** These data show that a pool of effector T cells could be maintained over four months

(17 weeks) with six vaccinations.

**Example 39: Evaluation of the complex in the murine MC38 colorectal cancer model (not falling within the claims)**

**[0679]** To evaluate the effects of the complex in a murine colorectal cancer model, the murine MC38 colorectal cancer model was chosen. MC38 is a colon carcinoma cell line.

**[0680]** Naive C57BL/6 mice were implanted s.c. with  $2 \times 10^5$  MC38 tumor cells in the left flank and vaccinated twice (-day 21 & -day 7 before tumor implantation) by subcutaneous injection of 2 nmol of the construct Z13Mad11 Anaxxa (cf. Example 32, SEQ ID NO: 40) in the right flank. The "Mad11" antigenic cargo contains two survivin epitopes expressed by the murine MC38 colorectal model.

**[0681]** The results are shown in Figure 57. Mice vaccinated with Z13Mad11 Anaxxa showed significantly less tumor volume compared to naïve mice (Fig. 57A). Moreover, mice vaccinated with Z13Mad11Anaxxa showed a significantly higher survival rate (Fig. 57B).

**[0682]** The results obtained show that Z13Mad11Anaxxa was able to significantly reduce and delay the tumor growth as compared to the control. Moreover, the survival rate of mice vaccinated with Z13Mad11Anaxxa was increased.

**Example 40: Preliminary toxicity study in mice (not falling within the claims)**

**[0683]** To assess the toxicity of the complex in mice, Z13Mad5Anaxxa (SEQ ID NO: 28) was injected at 2 nmol s.c. and i.v. in naïve C57BL/6 mice. Blood sampling was performed at 0.5, 1.5 and 3 h after administration. A commercial multiplex kit (Luminex) was used for the detection and quantification of cytokines in blood and four mice were sampled at each time point. However, no expression of any pro-inflammatory cytokines could be detected. It should be noted that the multiplex kit was compared to a classical ELISA with similar results (data not shown).

**[0684]** Next, the dose of Z13Mad5Anaxxa (SEQ ID NO: 28) was increased to 10 nmol. However, again no cytokines were detected following s.c. administration. In contrast to s.c. administration, a transient increase of IL-6 and TNF $\alpha$  was observed at 1.5 h after i.v. administration. However, this slight increase disappeared after 3 h (Fig. 58). Subcutaneous injection of 10 nmol of Z13Mad5Anaxxa in mice did not induce any cytokines release up to 6 h after treatment (data not shown).

**Example 41: Effects of a complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist on homing of T cells to the MC-38 tumor site (not falling within the claims)**

**falling within the claims)**

**[0685]** In order to assess the effects of a complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist on homing of T cells to the tumor site, mice were vaccinated with cell penetrating peptide, different antigens and a TLR peptide agonist in the MC-38 tumor model. On day 27, mice were euthanized and FACS staining was performed to monitor the neoantigen-specific immune response in the TILs (tumor-infiltrating lymphocytes).

**[0686]** C57BL/6 mice (four mice per group, female, 7 week old) were implanted s.c. with  $2 \times 10^5$  MC-38 tumor cells in the left flank (day 0). After tumor implantation, mice of the groups "Z13Mad12Anaxa" were vaccinated at days 3, day 10 and 17 subcutaneously with 2 nmol of Z13Mad12Anaxa (cf. Example 36; SEQ ID NO: 69) at the tail base.

**[0687]** As shown in Figures 59, 60 and 61, neoantigen-specific T cells accumulate at the tumor site in vaccinated mice. The percentage of multimer-positive cells was significantly increased in mice vaccinated with Z13Mad12Anaxa. The lowest percentage of multimer-positive cells was found in control mice.

**Example 42: Human dendritic cell activation by human constructs not falling within the claims)**

**[0688]** The goal of this study was to investigate the capacity of different complexes comprising different human antigenic cargoes to induce maturation of human dendritic cells. To this end, each of the constructs ATP110 (SEQ ID NO: 72), ATP112 (SEQ ID NO: 74), ATP115 (SEQ ID NO: 77), ATP117 (SEQ ID NO: 79), ATP118 (SEQ ID NO: 80), ATP119 (SEQ ID NO: 81), ATP120 (SEQ ID NO: 82), ATP122 (SEQ ID NO: 84), ATP123 (SEQ ID NO: 85), and ATP125 (SEQ ID NO: 87) was tested. The biological indicator used for the evaluation of DC activation was the Activation Index which indicates the percentage of activation based on the expression intensity of four membrane antigens: HLA-DR, CD80, CD83 and CD86.

**[0689]** After incubation over night with 300nM or 600nM of each of the above-mentioned constructs (ATP110 (SEQ ID NO: 72), ATP112 (SEQ ID NO: 74), ATP115 (SEQ ID NO: 77), ATP117 (SEQ ID NO: 79), ATP118 (SEQ ID NO: 80), ATP119 (SEQ ID NO: 81), ATP120 (SEQ ID NO: 82), ATP122 (SEQ ID NO: 84), ATP123 (SEQ ID NO: 85), and ATP125 (SEQ ID NO: 87)), activation markers expression (CD86, CD80, CD83 and HLA-DR) was assessed on the human dendritic cells by FACS (Figures 62 - 71). Same buffer volumes of each construct were used as negative controls.

**[0690]** Results are shown in Fig. 62 - 71 with all constructs tested showing dendritic cell maturation shown by the up-regulation of CD86, HLADR and CD83.

**Example 43: Effects of combination of a PD1 inhibitor and a complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist on tumor growth and survival rate in a colon carcinoma model (not falling within the claims)**

[0691] In order to assess the effects of combination of a PD1 inhibitor and a complex comprising a cell penetrating peptide, different antigens and a TLR peptide agonist in treating colorectal cancer, the MC-38 tumor model was used. MC-38 is a colon carcinoma cell line.

[0692] To this end, C57BL/6 mice (thirteen to fourteen mice per group, female, 7 week old) were implanted s.c. with  $2 \times 10^5$  MC-38 tumor cells in the left flank (day 0). After tumor implantation, mice of the groups "Z13Mad12Anaxa" and "Z13Mad12Anaxa + anti-PD1" were vaccinated at days 3, 10 and 17 subcutaneously with 2 nmol of Z13Mad12Anaxa (cf. Example 36; SEQ ID NO: 69) at the tail base. 200 µg of anti-PD1 antibody RMP1-14 (BioXcell, West Lebanon, NH, USA) were administered i.p. on each of days 6, 10, 13, 17, 20, 24 and 27 to mice of groups "anti-PD1" and "Z13Mad12Anaxa + anti-PD1". At days 10 and 17, when both, Z13Mad12Anaxa and antibody, were administered, the antibody was administered i.p. just after s.c. administration of Z13Mad12Anaxa. Tumor size was measured with a caliper.

[0693] As shown in Figures 72 and 73, treatment with the PD1 inhibitor alone or with Z13Mad12Anaxa alone resulted in significantly reduced tumor volume (Fig. 72A) and increased survival (Fig. 72B), as compared to the control group. However, the combination of both, the PD1 inhibitor and Z13Mad12Anaxa, resulted in the most pronounced improvement, namely in strongly decreased tumor volume and strongly increased survival rates. Of note, in the "Z13Mad12Anaxa + aPD1" group only three mice developed tumors (whereas 10 mice remained tumor-free), whereas in the "aPD1" group and in the "Z13Mad12Anaxa" group eight and ten mice, respectively, developed tumors. In the control group all mice developed tumors. These data show that a combination of both, anti-PD1 therapy and Z13Mad12Anaxa vaccination, is more efficient than anti-PD1 therapy alone or Z13Mad12Anaxa vaccination alone.

[0694] As shown in Figure 73, the number of tumor-free mice in the "Z13Mad12Anaxa + aPD1" group (10 mice out of 13 mice) is larger than the sum of the numbers of tumor-free mice in the "aPD1" group (6 mice out of 14 mice) and in the "Z13Mad12Anaxa" group (3 mice out of 13 mice) together. These results thus indicate a synergistic effect of anti-PD1 therapy and Z13Mad12Anaxa vaccination.

**Example 44: Immune response elicited in mice after vaccination with ATP128**

[0695] Another human construct, ATP128 (SEQ ID NO: 89), comprising a cell penetrating peptide, epitopes of three antigens (Survivin, CEA and ASCL2) and a TLR peptide agonist was designed. In particular, "ATP128" (SEQ ID NO: 89) is a fusion protein comprising the cell-

penetrating peptide "Z13" (SEQ ID NO: 6), epitopes of three the antigens survivin, CEA and ASCL2, and a sequence variant of the TLR peptide agonist "Anaxxa" (namely, a TLR peptide agonist according to SEQ ID NO: 71). In the following, the amino acid sequence of ATP128 is shown:

KRYKNRVASRKSRKFQQLQHYREAAKSSENDRLRLLLKNRTLTFNVRNDARAYVSGIQ  
 NSVSANRSDPVTLDVLPDSSYLSGANLNLSCHSASPQYSWRINGIPQQHTQVLFIAKITPNNN  
 GTYACFVSNLATGRNNNSIVKSITVSASCTSPGLSAAPTLPPAWQPFLKDHRISTFKNWPFLEGSA  
 VKKQFEELTLGEFLKLDRERAAVARRNERERNRVKLVLGFQALRQHVPHGGASKKLSKVETLR  
 SAVEYIRALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGPSEGALSPAERELLDFSSWLGYST  
 VHEILSKLSLEGDHSTPPSAYGSVKPYTNFDE

(SEQ ID NO: 89)

**[0696]** Naive C57BL/6 mice (5 mice per group, female, 7-week-old) were vaccinated by subcutaneous injection of 4 nmol of ATP128 (SEQ ID NO: 89). Control mice received vehicle (vaccine buffer). Seven days after treatment mice were bled, and ex vivo Elispot assay was performed on blood cells stimulated with mouse dendritic cells loaded with ATP128 in order to quantify IFN- $\gamma$  producing vaccine-specific T cells.

**[0697]** The results are shown in Figure 74. Mice vaccinated with ATP128 showed a higher ATP128-specific immune response compared to naive mice.

**[0698]** The results obtained show that ATP128 is efficacious for eliciting immune response in mice.

**Example 45: Human dendritic cell activation by ATP128 construct**

**[0699]** The goal of this study was to investigate the capacity of ATP128 (SEQ ID NO: 89) comprising human antigenic cargo to induce maturation of human dendritic cells (human DCs). To this end, ATP128 (SEQ ID NO: 89) was tested. The biological indicator used for the evaluation of DC activation was the Activation Index (Figure 75) which indicates the percentage of activation based on the expression intensity of four membrane antigens: HLA-DR, CD80, CD83 and CD86.

**[0700]** After incubation over night with 300nM of ATP128 (SEQ ID NO: 89), activation markers expression (CD86, CD80, CD83 and HLA-DR) was assessed on the human dendritic cells by FACS (Figure 75). Same buffer volume was used as negative control whereas MPLA was used as positive control.

**[0701]** Results are shown in Figure 75. The results indicate that ATP128 can potently activate human DCs.

**Example 46: Human dendritic cell activation by ATP128 construct**

**[0702]** In order to confirm that human cells are able to process and present the epitopic peptides included in ATP128 (SEQ ID NO: 89), human dendritic cells (human DCs) from 2 different donors (named donor "9" and "10") were over-night loaded with ATP128 and processed for HLA Class I and Class II/presented peptide purification, peptide elution and characterization by mass spectroscopy. Briefly, peptide pools from shock-frozen human DC samples were obtained by immune precipitation using HLA-specific antibodies, acid treatment and ultrafiltration. The HLA peptide pools were separated according to their hydrophobicity by reversed-phase chromatography and the eluting peptides were analysed in a mass spectrometer (Thermo Fisher Scientific). The data were then collected and automatically processed by analysing the mass signals of unfragmented peptides as well as fragment spectra containing peptide sequence information. False discovery rates (FDR) were determined by the Percolator algorithm (Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ (2007) Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat Methods* 4(11):923-925) based on processing against a decoy database consisting of the shuffled target database. For HLA class I, peptide lengths were limited to 8-12 aa of length. For HLA class II, peptides were limited to 12-25 aa of length. HLA annotation was performed using SYFPEITHI ([www.syfpeithi.de](http://www.syfpeithi.de)).

**[0703]** Data have shown that human DCs loaded with ATP128 are able to process and present both class I and class II epitopic peptides derived from the multiantigenic domain (Figure 76). Peptides derived from all antigens were identified in the class I pool. A higher number of class II bound peptides were identified from all the antigen portions of the vaccine.

TABLE OF SEQUENCES AND SEQ ID NUMBERS (SEQUENCE LISTING):

| SEQ ID NO    | Sequence   | Remarks  |
|--------------|--|--|
| SEQ ID NO: 1 | RQIKIYFQNRRMKWKK   | CPP: Penetratin  |
| SEQ ID NO: 2 | YGRKKRRQRRR  | CPP: TAT minimal   |
| SEQ ID NO: 3 | MMDPNSTSEDVKFTPDPYQVPFVQAFDQATRV<br>YQDLGGPSQAPLPCVLWPVLPEPLPQGQLTAY<br>HVSTAPTGWSFASAPQAPENAYQAYAAPQQLFP<br>VSDITQNQQTNQAGGEAPQPGDNSTVQTAA<br>AVVFACPGANQQQLADIGVPQPAPVAAPAR<br>RTRKPQQPESLEECDSELEIKRYKNRVASRKCRAK<br>FKQLLQHYREAAKSSENDRLRLLKQMCPSL<br>DVDSIIPRTPDVLHEDLLNF | ZEBRA amino acid sequence (natural sequence from Epstein - Barr virus (EBV)) (YP_401673) |

| SEQ ID NO     | Sequence   | Remarks              |
|---------------|--|----------------------|
| SEQ ID NO: 4  | KRYKNRVASRKCRAFKQLLQHYREVAAKSSE<br>NDRLRLLKQMC   | CPP1 (Z11)           |
| SEQ ID NO: 5  | KRYKNRVASRKCRAFKQLLQHYREVAAKSSE<br>NDRLRLLK  | CPP2 (Z12)           |
| SEQ ID NO: 6  | KRYKNRVASRKSRAFKQLLQHYREVAAKSSE<br>NDRLRLLK  | CPP3 (Z13)           |
| SEQ ID NO: 7  | KRYKNRVASRKSRAFKQLLQHYREVAAAK  | CPP4 (Z14)           |
| SEQ ID NO: 8  | KRYKNRVASRKSRAFK   | CPP5 (Z15)           |
| SEQ ID NO: 9  | QHYREVAAKSSEND   | CPP6 (Z16)           |
| SEQ ID NO: 10 | QLLQHYREVAAAK  | CPP7 (Z17)           |
| SEQ ID NO: 11 | REVAAKSSENDRLRLLK  | CPP8 (Z18)           |
| SEQ ID NO: 12 | KRYKNRVA   | CPP9 (Z19)           |
| SEQ ID NO: 13 | VASRKSRAFK   | CPP10 (Z20)          |
| SEQ ID NO: 14 | ESLKISQAVHAAHAEINEAGREVVGVAL<br>KVPRNQDWLGVPRFAKFASFEAQGALA<br>NIAVDKANLDVEQLESIINFEKLTEWTGS | MAD5 cargo           |
| SEQ           | STVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE  | TLR2 peptide agonist |

| SEQ<br>ID<br>NO        | Sequence | Remarks                     |
|------------------------|----------|-----------------------------|
| ID<br>NO:<br>15        |          | Anaxa                       |
| SEQ<br>ID<br>NO:<br>16 | DDDK     | enterokinase target site    |
| SEQ<br>ID<br>NO:<br>17 | IEDGR    | factor Xa target site       |
| SEQ<br>ID<br>NO:<br>18 | LVPRGS   | thrombin target site        |
| SEQ<br>ID<br>NO:<br>19 | ENLYFQG  | protease TEV target site    |
| SEQ<br>ID<br>NO:<br>20 | LEVLFQGP | PreScission protease target |
| SEQ<br>ID<br>NO:<br>21 | RX(R/K)R | furin target site           |
| SEQ<br>ID<br>NO:<br>22 | GGGG     | peptidic linker             |
| SEQ<br>ID<br>NO:<br>23 | GGGG     | peptidic linker             |
| SEQ<br>ID<br>NO:<br>24 | EQLE     | peptidic linker             |
| SEQ<br>ID<br>NO:<br>25 | TEWT     | peptidic linker             |

| SEQ ID NO     | Sequence   | Remarks      |
|---------------|--|--------------|
| SEQ ID NO: 26 | MHHHHHHHNIDRPKGGLAFTDVVDVDSIKAWESPQGQVSRYRVTYSSPEDGIRELFPADGEDDTAELQGLRPGEYTVSVVALHDDMESQPLIGIQSTKRYKNRVASRKSRAKFQLLQHYREVAAAKSSENDRLRLLLKESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGS | EDAZ13Mad5   |
| SEQ ID NO: 27 | MHHHHHHSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAEKRYKNRVASRKSRAFKKQLQHYREVAAAKSSENDRLRLLLKESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGS  | AnaxaZ13Mad5 |
| SEQ ID NO: 28 | MHHHHHHKRYKNRVASRKSRAFKQLLQHYREVAAAKSSENDRLRLLLKESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGSSTVHEILCKLSELGDHSTPPSAYGSVKPYTNFDAE  | Z13Mad5Anaxa |
| SEQ ID NO: 29 | MHHHHHHKRYKNRVASRKSRAFKQLLQHYREVAAAKSSENDRLRLLLKESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGS   | Z13Mad5      |
| SEQ ID NO: 30 | MHHHHHHESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGS  | Mad5         |
| SEQ ID NO: 31 | MHHHHHHHNIDRPKGGLAFTDVVDVDSIKAWESPQGQVSRYRVTYSSPEDGIRELFPADGEDDTAELQGLRPGEYTVSVVALHDDMESQPLIGIQSTESLKISQAVHAAHAEINEAGREVVGVGALKVPRNQDWLGVPRFAKFASFEAQGALANIAVDKANLDVEQLESIINFEKLTWTGS  | EdaMad5      |

| SEQ ID NO     | Sequence   | Remarks         |
|---------------|--|-----------------|
| SEQ ID NO: 32 | MHHHHHHESLKISQAVHAAHAEINEAG<br>REVVGVGALKVPRNQDWLGVPRFAKFAS<br>FEAQGALANIAVDKANLDVEQLESIINFEK<br>LTEWTGSSTVHEILCKLSLEGDHSTPPSAY<br>GSVKPYTNFDAE  | Mad5Anaxa       |
| SEQ ID NO: 33 | MHHHHHHKRYKNRASRKSRACKQQL<br>QHYREAAAKESLKISQAVHAAHAEINE<br>AGREVVGVGALKVPRNQDWLGVPRFA<br>KFASFEAQGALANIAVDKANLDVEQLESI<br>INFEKLTEWTGSSTVHEILCKLSLEGDHST<br>PPSAYGSVKPYTNFDAE   | Z14 Mad5Anaxa   |
| SEQ ID NO: 34 | MHHHHHHREVAAKSSENDRLRLLLKES<br>LKISQAVHAAHAEINEAGREVGVGALKV<br>PRNQDWLGVPRFAKFASFEAQGALANIA<br>VDKANLDVEQLESIINFEKLTEWTGSSTVH<br>EILCKLSLEGDHSTPPSAYGSVKPYTNFDA<br>E   | Z18 Mad5Anaxa   |
| SEQ ID NO: 35 | SIINFEKL   | SIINFEKL OVACD8 |
| SEQ ID NO: 36 | ISQAVHAAHAEINEAGR  | OVACD4 peptide  |
| SEQ ID NO: 37 | MHHHHHHNIDRPKGIAFTDVDVDSIKIA<br>WESPQGQVSRYRVTYSSPEDGIRELFPAP<br>DGEDDTAELQGLRPGEYTVSVVALHDD<br>MESQPLIGIQSTKRYKNRASRKSRACKQ<br>LLQHYREAAAKESLKISQAVHAAHAEIN<br>EAGREVGVGALKVPRNQDWLGVPRFA<br>KFASFEAQGALANIAVDKANLDVEQLESI<br>NFEKLTEWTGS | EDAZ14Mad5      |
| SEQ ID NO: 38 | MHHHHHHNIDRPKGIAFTDVDVDSIKIA<br>WESPQGQVSRYRVTYSSPEDGIRELFPAP<br>DGEDDTAELQGLRPGEYTVSVVALHDD<br>MESQPLIGIQSTREVAAKSSENDRLRLL<br>KESLKISQAVHAAHAEINEAGREVGVGA<br>LKVRNQDWLGVPRFAKFASFEAQGALA<br>NIAVDKANLDVEQLESIINFEKLTEWTGS               | EDAZ18Mad5      |

| SEQ ID NO     | Sequence  | Remarks       |
|---------------|---|---------------|
|               |   |               |
| SEQ ID NO: 39 | KRYKNRVASRKSRAFKQLLQHYREVAAA<br>KSSENDRLRLLLKVTYHPSYAYHQFERRA<br>ILNRLVQFIKDRISVVQALVLTSTVHEILCK<br>LSLEGDHSTPPSAYGSVKPYTN FDAE   | Z13Mad8Anaxa  |
| SEQ ID NO: 40 | KRYKNRVASRKSRAFKQLLQHYREVAAA<br>KSSENDRLRLLLKNYRIATFKNWPFLLEDCA<br>MEELTVSEFLKLDQRSTVHEILCKLSLEGD<br>HSTPPSAYGSVKPYTNFDAE   | Z13Mad11Anaxa |
| SEQ ID NO: 41 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKHELASMTNMELMSSIVSTVHEILCKLS<br>LEGDHSTPPSAYGSVKPYTNFDAE   | Z13Mad9Anaxa  |
| SEQ ID NO: 42 | HLELASMTNMELMSSIV   | Mad9          |
| SEQ ID NO: 43 | VTYHPSYAYHQFERRAILN   | Mad8          |
| SEQ ID NO: 44 | NYRIATFKNWPFLLEDCAEELTVSEFLKLD  | Mad11         |
| SEQ ID NO: 45 | NIDRPKGALFTDVVDVDSIKIAWESPQGQVSRYR<br>VTYSSPEDGIRELFPAPDGEDDTAELQGLRPGSEY<br>TVSVVALHDDMESQPLIGIQST   | EDA           |
| SEQ ID NO: 46 | RKKRRQRRRRVKRISQAVHAAHAEINEAGRVK<br>RKVPRNQDWLRVKRASFEAQGALANIAVDKAR<br>VKRSIINFEKLRVKRSTVHEILCKLSLEGDHSTPPSA<br>YGSVKPYTNFDAE  | TatFMad5Anaxa |
| SEQ ID NO: 47 | MAPPQVLAFGLLLAAATATFAAAQEECVCENYK<br>LAVNCFVNNNRQCQCTSVAQNTVICSKLAAK<br>CLVMKAEMNGSKLGRRAKPEGALQNNNDGLYD<br>PDCDESGLFKAKQCNGTSMCWCVNTAGVRRT<br>DKDTEITCSERVRTYWIIIELKHKAEEKPYDSKSLR | EpCAM         |



| SEQ ID NO:    | Sequence  | Remarks          |
|---------------|---|------------------|
| SEQ ID NO: 51 | TAPPAHGVTS  | MUC-1 epitope    |
| SEQ ID NO: 52 | MGAPTLPPAWQPFLKDHRISTFKNWPFLEGAC<br>TPERMAEAGFIHCPTENEPEPDLAQCFFCFKELEGW<br>EPDDDPPIEEHHKKHSSGCAFLSVKKQFEELTLGEFL<br>KLDRERAKNKIAKETNNKKFEETAKKVRRRAIEQ<br>LAAMD  | survivin         |
| SEQ ID NO: 53 | RISTFKNWPF  | survivin epitope |
| SEQ ID NO: 54 | MESPSAPPHRWCIPWQRLLL TASLLTFWNPPPTA<br>KLTIESPFNVAEGKEVLLLVHNLPQHLFGYSWY<br>KGERVDGNRQIIGYVIGTQQATPGPAYSGREIY<br>PNASLIQNIIQNDTGFYTLHVIKSDLVNEEATG<br>QFRVYPELPKPSISSLNSKPVEDKDAVAFTCPEP<br>QDATYLWWVNNQSLPVSPRLQLSNGNRTTLF<br>NVTRNDTASYKCETQNPVSARRSDSVILNVLYC<br>PDAPTIPLNTSYRSGENLNSCHAASNPPAQYS<br>WFVNGTFQQSTQELFIPNITVNNSGSYTCQAH<br>NSDTGLNRTTVTTITVYAEPPKPFITSNNSNPVED<br>EDAVALTCEPEIQNTTYLWWVNNQSLPVSPRLQ<br>LSNDNRTLTLSSVTRNDVGPYECGIQNKLSVDH<br>SDPVILNVLYGPDDPTISPSYTYRPGVNLSLSCH<br>AASNPPAQYSWLDGNIQQHTQELFISNITEKNS<br>GLYTCQANNSASGHSRTTVKTITVSAELPKPSISS<br>NNSKPVEDKDAVAFTCPEAQNTTYLWWVNG<br>QSLPVSPRLQLSNGNRTLTFNVRTNDRAYVC<br>GIQNSVSANRSDPVTLDVLYGPDTPIISPPDSSYL<br>SGANLNLSCHSASNPSQYSWRINGIPQQHTQ<br>VLFIAKTPNNNGTYACFVSNLATGRNNNSIVKSIT<br>VSASGTSPGLSAGATVGIMIGVLVGL | CEA              |
| SEQ ID NO: 55 | YLSGANLNLS  | CEA epitope      |
| SEQ ID NO: 56 | SWRINGIPQQ  | CEA epitope      |
| SEQ ID NO:    | MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYD<br>PTIEDSYRKQVVIDGETCLLDILDAGQEEYSAM<br>PDDQWVADTCEGELQVVAIKNITKEEDQHLLIVDPOIK  | Kirsten Ras      |

| SEQ ID NO | Sequence   | Remarks             |
|-----------|--|---------------------|
| 57        | RDQYVIRTGEGCPLCVFATINNTKSPEDIPPTTREQIK<br>RVKDSEDVPMVLVGNKCDLPSRTVDTKQAQDL<br>ARSYGIPFIETSAKTRQRVEDAFYTLVREIRQYRLK<br>KISKEEKTPGCVKIKKCIIM   |                     |
| 58        | VVVGAGGVG  | Kirsten Ras epitope |
| 59        | MPLEQRSQHCKPEEGLEARGEALGLVGAQAPAT<br>EEQEAAASSSTLVEVTLGEVPAAESPDPPQSPQGA<br>SSLPTTMNYPLWSQSYEDSSNQEEEGPSTFPDLES<br>EFQAALSRKVAELVHFLLKRYRAREPVTKAEMLGS<br>VVGNWQYFFPVIFSKAFSSLQLVFGIELMEVDIG<br>HLYIFATCLGLSYDGLLGDNQIMPKAGLLIVLAI<br>AREGDCAPEEKIWEELSLEVFEGRGREDISILGDPKK<br>LLTQHFVQENYLEYRQVPGSDPACYEFLWGPR<br>LVETSYVKVLLHHMVKISGGPHIS<br>YPPLHEWVLREGE   | MAGE-A3             |
| 60        | KVAELVHFL  | MAGE-A3 epitope     |
| 61        | MAFVCLAIGCLYTFLISTTFGCTSSSDTEIKVNPPQ<br>DFFIVDPGTYLGYLYLQWQPPLSLDHFKECTVEYE<br>LKYRNIGSETWKTITKNLHYKDCFDLNKGIEAKI<br>HTLLPWQCTNGSEVQSSWAETTYWISPQGIPET<br>KVQDMDCVYYNWQYLLCSWKGPGIGVLLDTNY<br>NLFYWYEGLDHALQCVDYIKADGQNIGCRFPY<br>LEASDYKDFYICVNGSENKPIRSSYFTFQLQNIV<br>KPLPPVYLTFTRESSCEIKLKWSIPLGPIPARCFDYEI<br>EIREDDTTLVATVNETYTLKTTNETRQLCFV<br>SKVNIYCSDDGIWSEWSDKQCWEGEDLSKKTLL<br>RFWLPFGFILILVIFVTGLLRLKPNTYPKMIPEFFCD<br>T | IL13Ralpha2         |
| 62        | LPFGFIL  | IL13Ralpha2 epitope |
| 63        | LFRAAQLANDVVLQIMEHLELASMTNMELMSSI<br>VVISASIIIVFNLLLEG   | <u>Mad12</u>        |
|           | LVQFIKDRISVVQA   | gp70CD4 peptide     |

| SEQ ID NO:    | Sequence  | Remarks         |
|---------------|---|-----------------|
| NO: 64        |   |                 |
| SEQ ID NO: 65 | SPSYVYHQF   | gp70CD8 peptide |
| SEQ ID NO: 66 | ASMTNMELM   | adpgk peptide   |
| SEQ ID NO: 67 | ATKNWPFL  | survivin20-28   |
| SEQ ID NO: 68 | TVSEFLKL  | survivin97-104  |
| SEQ ID NO: 69 | KRYKNRVASRKSRAFKFQLLQHYREVAAKSSENDRLRLLLKLFRRAQLANDVVLQIMEHLEASMTNMELMSSIVVISASIIVFNLLLEGSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE  | Z13Mad12Anaxa   |
| SEQ ID NO: 70 | MELAALCRWGLLLALLPPGAASTQVCTGTDMLKRLPASPETHLDMLRHLYQGCQVQGNLELYPRTNASLSQLQDIQEYQGYVLIHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLDNRSRACHPCSPMCKGSRCWGESEDQSLRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRTFGASCVTACPYNLSTDVGSCTLVCPHNQEVTaedGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSDLPSVFQNLQVIRGRIHNNGAYSLTLQGLGISWGLRSRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARGHCWPGPTQCVNCSQFLRGQECVECRVLQGLPREYVNARHCLPCHECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRLLLQETELVEPLTPSGAMPNQAQMRLKETELRKVKVLGSGAFGTVYKGIWPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKG | Her2/neu        |

| SEQ ID NO     | Sequence   | Remarks   |
|---------------|--|---|
|               | LARLLDIDETEYHADGGKVPKWMALESILRRRF<br>HQSDVWSYGVTVWELMTFGAKPYDGIPAREIP<br>DLLEKGERLPQPPPICTIDVYIMIMVKCWMDSECR<br>PRFRELVSEFSRMARDPQRFVVIQNEDELGPASPL<br>DSTFYRSLLEDDDMGDLVDAEELYLPQQGFFCP<br>DPAPGAGGMVHHRHRSSTRSGGGDLTLGEP<br>SEEAPRSPLAPSEGAGSDVFDGDLGMAAKGL<br>QSLPTHDPSPPLQRYSEDPVTPLSETDGYVAPLT<br>CSPQPEYVNQPDVRPQPPSPREGPLPAARPAGA<br>TLERPKTLSPGKNGVVKDVFAGGAVENPEYLTP<br>QGGAAPQPHPPPAFSPAfdnlyWDQDPPER<br>GAPPSTFKGTPTAENPEYLGLDVPV |   |
| SEQ ID NO: 71 | STVHEILSKLSLEGDHSTPPSAYGSVKPYTNFdae  | TLR peptide agonist<br>"Anaxa" sequence variant |
| SEQ ID NO: 72 | KRYKNRVARSKSRAKFQQLQHYREVAAKSSEN<br>DRLRLLKAPPQVLAFLLLAAATAYVDEKAPEFS<br>MQGLKAGVIAVIVVSTVHEILCKLSLEGDHSTPPS<br>AYGSVKPYTNFdae   | ATP110  |
| SEQ ID NO: 73 | KRYKNRVARSKSRAKFQQLQHYREVAAKSSEN<br>DRLRLLKRTLTFNVRTNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNsIVKSITVSASGTSPGLSAGATVGMIGVLVG<br>VALILGDPKKLLTQHFVQENYLEYRQVPGSDPAS<br>YEFLWGPRALVETSYVKVALSRKVAELVHFLLKY<br>RAREPVTKAEMLGSVAPPQVLAFLLLAAATAY<br>VDEKAPEFSMQGLKAGVIAVIVVSTVHEILCKSL<br>EGDHSTPPSAYGSVKPYTNFdae  | ATP111  |
| SEQ ID NO: 74 | KRYKNRVARSKSRAKFQQLQHYREVAAKSSEN<br>DRLRLLKRTLTFNVRTNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNsIVKSITVSASGTSPGLSLGDPKKLLTQHFVQ<br>ENYLEYRQVPGSDPASYEFLWGPRALVETSYVKV<br>ALSRKVAELVHFLLKYRAREPVTKAEMLGSVVAP<br>PQVLAFGLLLAAATAYVDEKAPEFSMQGLKAGV<br>AVIVVSTVHEILCKLSLEGDHSTPPSAYGSVKPY<br>NFdae  | ATP112  |
| SEQ ID NO: 75 | KRYKNRVARSKSRAKFQQLQHYREVAAKSSEN<br>DRLRLLKRTLTFNVRTNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG   | ATP113  |

| SEQ ID NO     | Sequence  | Remarks |
|---------------|---|---------|
|               | RNNSIVKSITVSASGTSPGLSLGDPKKLLTQHFVQ<br>ENYLEYRQVPGSDPASYEFLWGPRALVETSYVKV<br>ALSRKVAELVHFLLKYRAREPVTKAEMLGSVVAP<br>PQVLAFGLLLAAATAYVDEKAPEFSMQGLKAGVI<br>AVIVVAPGSTAPPAHGVTSAAPDTRPAPGSTAPPA<br>HGVTSAAPDRPALGSTAPPVHNVTSSTVHEILCKL<br>SLEGDHSTPPSAYGSVKPYTNFDAE  |         |
| SEQ ID NO: 76 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSAPTLPPAWQPFKD<br>HRISTFKNWPFLGSAVKKQFEELTLGEFLKLDRE<br>RAPPQVLAFLGLLLAAATAYVDEKAPEFSMQGLK<br>AGVIAVIVVAPGSTAPPAHGVTSAAPDTRPAPGST<br>APPAHGVTSAAPDRPALGSTAPPVHNVTSSTVHEI<br>LCKLSLEGDHSTPPSAYGSVKPYTNFDAE                            | ATP114  |
| SEQ ID NO: 77 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSEYKLVVVGAVVG<br>KSALTAPPQVLAFLGLLLAAATAYVDEKAPEFSMQ<br>GLKAGVIAVIVVAPGSTAPPAHGVTSAAPDTRPAP<br>GSTAPPAHGVTSAAPDRPALGSTAPPVHNVTSS<br>VHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE  | ATP115  |
| SEQ ID NO: 78 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSAPTLPPAWQPFKD<br>HRISTFKNWPFLGSAVKKQFEELTLGEFLKLDRE<br>RAPPQVLAFLGLLLAAATAYVDEKAPEFSMQGLK<br>AGVIAVIVVLDGPKLLTQHFVQENYLEYRQVP<br>GSDPASYEFLWGPRALVETSYVKVALSRKVAELV<br>HFLLKYRAREPVTKAEMLGSVVSTVHEILCKLSE<br>GDHSTPPSAYGSVKPYTNFDAE | ATP116  |
| SEQ ID NO: 79 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVSA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLFIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSAPPQVLAFLGLLLAA<br>ATAYVDEKAPEFSMQGLKAGVIAVIVVAPGSTAP<br>PAHGVTSAAPDTRPAPGSTAPPAHGVTSAAPDRPA<br>LGSTAPPVHNVTSSTVHEILCKLSLEGDHSTPPSA<br>YGSVKPYTNFDAE  | ATP117  |

| SEQ ID NO     | Sequence   | Remarks |
|---------------|--|---------|
|               |  |         |
| SEQ ID NO: 80 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSAPPQVLAFGLLAA<br>ATAYVDEKAPEFSMQGLKAGVIAVIVVSTVHEIL<br>CKLSLEGDHSTPPSAYGSVKPYTNFDAE  | ATP118  |
| SEQ ID NO: 81 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKLGDPKLLTQHFVQENYLEYRQVPGS<br>DPASYEFLWGPRALVETSYVKVALSRKVAELVHF<br>LLKYRAREPVTKAEMLGSVVAPTLPPAWQPFLK<br>DHRISTFKNWPFLEGSAVKKQFEELTLGEFLKDR<br>ERAPGSTAPPAGVTSAPDTRPAPGSTAPPAG<br>VTSAPDRPALGSTAPPVHNNTSSTVHEILCKLSLE<br>GDHSTPPSAYGSVKPYTNFDAE   | ATP119  |
| SEQ ID NO: 82 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKRRTLTLFNVTRNDARAYVSGIQNSVA<br>NRSDPVTDPSSYLSGANLNLSHSASPQYSWRIN<br>GIPQQHTQVLIAKITPNNNGTYACFVSNLATG<br>RNNSIVKSITVSASGTSPGLSSTVHEILCKLSLEG<br>HSTPPSAYGSVKPYTNFDAE   | ATP120  |
| SEQ ID NO: 83 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTLFNVTRNDARAYVSGIQNSVS<br>ANRSDPVTLDVLPDSSYLSGANLNLSHSASPQY<br>SWRINGIPQQHTQVLIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAGATVGIMI<br>GVLVGVALIAPGSTAPPAGVTSAPDTRPAPGST<br>APPAHGVTsapdrpalgstappvhnntsappqv<br>LAFGLLAAATALIYYVDEKAPEFSMQGLKAGVIA<br>VIVVSTVHEILCKLSLEGDHSTPPSAYGSVKPYTN<br>FDAE | ATP121  |
| SEQ ID NO: 84 | KRYKNRVARSKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTLFNVTRNDARAYVSGIQNSVS<br>ANRSDPVTLDVLPDSSYLSGANLNLSHSASPQY<br>SWRINGIPQQHTQVLIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAAPGSTAPP<br>AHGVTsapdtrpapgstappahgvtsapdrpal<br>gstappvhnntsappqvlafgllaaataliyyv<br>DEKAPEFSMQGLKAGVIAVIVVSTVHEILCKLSLE<br>GDHSTPPSAYGSVKPYTNFDAE                     | ATP122  |

| SEQ ID NO     | Sequence   | Remarks |
|---------------|--|---------|
| SEQ ID NO: 85 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTNFNVRTNNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAAPGSTAPP<br>AHGVTsapDTRPAPGSTAPPAGVTsapDRPAL<br>GSTAPPVHNVTsAPPQVLAFGLLAAATALIYYV<br>DEKAPEFSMQGLKAGVIAVIVVSTVHEILCKLSLE<br>GDHSTPPSAYGSVKPYTNFdae  | ATP123  |
| SEQ ID NO: 86 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTNFNVRTNNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFFLEGSAVKQFEELTLGEF<br>LKLDRERAPGSTAPPAGVTsapDTRPAPGSTAP<br>PAHGVTSAPDRPALGSTAPPVHNVTsAPPQVLA<br>FGLLAAATALIYYVDEKAPEFSMQGLKAGVIAVI<br>VVSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFD<br>AE  | ATP124  |
| SEQ ID NO: 87 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTNFNVRTNNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFFLEGSAVKQFEELTLGEF<br>LKLDRERAPGSTAPPAGVTsapDTRPAPGSTAP<br>PAHGVTSAPDRPALGSTAPPVHNVTsAPPQVLA<br>FGLLAAATALIYYVDEKAPEFSMQGLKAGVIAVI<br>VVSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFD<br>AE  | ATP125  |
| SEQ ID NO: 88 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTLTNFNVRTNNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASGTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFFLEGSAVKQFEELTLGEF<br>LKLDRERAPPQVLAFGLLAAATALIYYVDEKAPE<br>FSMQGLKAGVIAVIVVAAVARRNERERNRVKL<br>NLGFQALRQHVPHGGASKLKVETLRSAVEYIR<br>ALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGP<br>SEGALSPAERELLDFSSWLGGYSTVHEILCKLSLEG<br>DHSTPPSAYGSVKPYTNFdae | ATP127  |
| SEQ           |  | ATP128  |

| SEQ ID NO     | Sequence   | Remarks       |
|---------------|--|---------------|
| ID NO: 89     | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTTLFNVTRNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASCTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFLGSAVKKQFEELTLGEF<br>LKLDRERAARRNERERNRVKLVLNLGFQALRQ<br>HVPHGGASKKLSKVETLRSAYEYIRALQRLLAEH<br>DAVRNALAGGLRPQAVRPSAPRGPSEGALSPA<br>RELLDFSSWLGGYSTVHEILSKLSLEGDHSTPPSA<br>YGSVKPYTNFDAE                                      |               |
| SEQ ID NO: 90 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTTLFNVTRNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASCTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFLGSAVKKQFEELTLGEF<br>LKLDRERAARRNERERNRVKLVLNLGF<br>QALRQHVPHGGASKKLSKVETLRSAYEYIRALQR<br>LLAEHDARVNALAGGLRPQAVRPSAPRGPSEG<br>LSPAERELLDFSSWLGGYSTVHEILSKLSLEGDHST<br>PPSAYGSVKPYTNFDAE                                      | ATP129        |
| SEQ ID NO: 91 | KRYKNRVASRKSRAFKQLLQHYREVAAKSSEN<br>DRLRLLLKNRTTLFNVTRNDARAYVSGIQNSVS<br>ANRSDPVTLVLPDSSYLSGANLNLSCHSASPQY<br>SWRINGIPQQHTQVLFIAKITPNNNGTYACFVS<br>NLATGRNNNSIVKSITVSASCTSPGLSAAPTLPPAW<br>QFPLKDHRISTFKNWPFLGSAVKKQFEELTLGEF<br>LKLDRERAARRNERERNRVKLVLNLGF<br>QALRQHVPHGGASKKLSKVETLRSAYEYIRALQR<br>LLAEHDARVNALAGGLRPQAVRPSAPRGP<br>PVAASPRASSSPRGGSSEPGSPRSAYSSDDSGS<br>EGALSPAERELLDFSSWLGGYSTVHEILSKLSLEG<br>DHSTPPSAYGSVKPYTNFDAE | ATP130        |
| SEQ ID NO: 92 | MDGGTLPRSAPPAPPVGCAARRPASPELLRC<br>SRRRRPATAETGGGAAAVARRNERERNRVKL<br>LGFQALRQHVPHGGASKKLSKVETLRSAYEYIR<br>LQRLLAEHDAVRNALAGGLRPQAVRPSAPRGP<br>CTTPVAASPRASSSPRGGSSEPGSPRSAYSSDD<br>SGCEGALSPAERELLDFSSWLGGY   | ASCL2         |
| SEQ ID NO: 93 | SAVEYIRALQ   | ASCL2 epitope |
| SEQ           | ERELLDFSSW   | ASCL2 epitope |

| SEQ ID NO     | Sequence   | Remarks                      |
|---------------|--|------------------------------|
| ID NO: 94     |  |                              |
| SEQ ID NO: 95 | APTLPPAWQPFLKDHRISTFKNWPFLEGSAVKK<br>QFEELTGEFLKLDRER  | Survivin fragment            |
| SEQ ID NO: 96 | NRTLTLFNVTRNDARAYVSGIQNSVSANRSDPV<br>TLDVLPDSSYLSGANLNLSCHSASPQYSWRINGI<br>PQQHTQVLIAKITPNNNGTYACFVSNLATGRN<br>NSIVKSITVSASGTSPGLSA  | CEA fragment                 |
| SEQ ID NO: 97 | AAVARRNERERNRVKLVNLGFQALRQHVPHGG<br>ASKKLSKVETLRSAYEYRALQRLLAEHDAVRNAL<br>AGGLRPQAVRPSAPRGPSEGALSPAERELLDSS<br>WLGGY   | ASCL2 fragment               |
| SEQ ID NO: 98 | NRTLTLFNVTRNDARAYVSGIQNSVSANRSDPV<br>TLDVLPDSSYLSGANLNLSCHSASPQYSWRINGI<br>PQQHTQVLIAKITPNNNGTYACFVSNLATGRN<br>NSIVKSITVSASGTSPGLSAAPTLPPAWQPFLKDHR<br>ISTFKNWPFLEGSAVKKQFEELTGEFLKLDRER<br>AAVARRNERERNRVKLVNLGFQALRQHVPHGG<br>ASKKLSKVETLRSAYEYRALQRLLAEHDAVRNAL<br>AGGLRPQAVRPSAPRGPSEGALSPAERELLDSS<br>WLGGY | antigenic cargo of<br>ATP128 |

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## P A T E N T K R A V

## 1. Kompleks der omfatter:

- a) et cellepenetrerende peptid;
- b) et antigen eller en antigen epitop; og
- c) en TLR-peptidagonist,

5 hvor bestanddelene a)-c) er forbundet kovalent,  
til anvendelse i forebyggelsen og/eller behandlingen af kolorektal cancer,  
hvor komplekset omfatter en epitop af ASCL2,  
hvor TLR-peptidagonisten er en TLR2-peptidagonist og/eller en TLR4-  
10 peptidagonist, og

komplekset er et polypeptid eller et protein, især et rekombinant polypeptid  
eller et rekombinant protein.

## 2. Kompleks til anvendelse ifølge krav 1, hvor det cellepenetrerende peptid

15 (i) har en længde af aminosyresekvensen af det nævnte peptid på i alt 5 til 50 aminosyrer, fortrinsvis på i alt 10 til 45 aminosyrer, mere fortrinsvis på i alt 15 til 45 aminosyrer; og/eller

20 (ii) har en aminosyresekvens der omfatter et fragment af det minimale domæne af ZEBRA, det nævnte minimale domæne strækker sig fra syrerest 170 til syrerest 220 af ZEBRA aminosyresekvens ifølge SEQ ID NO: 3, hvor, eventuelt, 1, 2, 3, 4, eller 5 aminosyre  
er blevet erstattet, slettet, og/eller tilføjet uden at ophæve det nævnte peptids cellepenetrerende evne, fragmentet af det minimale domæne af ZEBRA har en længde på i alt 5 til 50 aminosyrer, fortrinsvis på i alt 10 til 45 aminosyrer, mere fortrinsvis på i alt 15 til 45 aminosyre.

25 3. Kompleks til anvendelse ifølge krav 2, hvor det cellepenetrerende peptid har en aminosyresekvens, der omfatter en Ser (S) ved den tilsvarende position 189 af ZEBRA aminosyresekvensen ifølge SEQ ID NO: 3.

30 4. Kompleks til anvendelse ifølge krav 2 eller 3, hvor det cellepenetrerende peptid har en aminosyresekvens, der omfatter en aminosyresekvens valgt fra gruppen bestående af en aminosyresekvens ifølge SEQ ID NO: 4-13, eller sekvensvarianter deraf uden at ophæve det nævnte peptids cellepenetrerende evne, der deler mindst 90% sekvensidentitet uden at ophæve det nævnte peptids cellepenetrerende evne.

5. Kompleks til anvendelse ifølge et hvilket som helst af kravene 1 til 4, hvor antigenet eller den antigen epitop omfatter eller består af en tumourepitop, fortrinsvis af en kolorektal cancer epitop.

6. Kompleks til anvendelse ifølge krav 5, hvor tumourepitopen er en epitop af et antigen valgt fra gruppen bestående af, EpCAM, HER-2, MUC-1, TOMM34, RNF 43, KOC1, VEGFR, PHCG, survivin, CEA, TGFpR2, p53, KRas, OGT, CASP5, COA-1, MAGE, SART og IL13Ralpha2.

7. Kompleks til anvendelse ifølge et hvilet som helst af kravene 1 – 6, hvor komplekset omfatter

10 (i) en epitop af survivin;  
(ii) en epitop af CEA; og  
(iii) en epitop af ASCL2.

8. Kompleks til anvendelse ifølge krav 7, hvor komplekset omfatter i N- til C-terminalretning:

15 (i) en epitop af CEA;  
(ii) en epitop af survivin; og  
(iii) en epitop af ASCL2.

9. Kompleks til anvendelse ifølge krav 8, hvor komplekset omfatter i N- til C-terminalretningen:

20 (i) et peptid der har en aminosyresekvence ifølge SEQ ID NO: 54, eller et fragment der af, der har en længde på mindst 10 aminosyre, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet;

25 (ii) et peptid der har en aminosyresekvence ifølge SEQ ID NO: 52, eller et fragment der af, der har en længde på mindst 10 aminosyre, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet; og

(iii) et peptid der har en aminosyresekvence ifølge SEQ ID NO: 92, eller et fragment der af, der har en længde på mindst 10 aminosyre, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.

30 10. Kompleks til anvendelse ifølge et hvilket som helst af kravene 7 – 9, hvor komplekset omfatter et peptid der har en aminosyresekvence ifølge SEQ ID NO: 96 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet; et peptid der har

en aminosyresekvens ifølge SEQ ID NO: 95 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet; og et peptid der har en aminosyresekvens ifølge SEQ ID NO: 97 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.

11. Kompleks til anvendelse ifølge et hvilket som helst af kravene 7 – 10, hvor kom-  
5 plekset omfatter et peptid der har en aminosyresekvens ifølge SEQ ID NO: 98 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.

12. Kompleks til anvendelse ifølge et hvilket som helst af kravene 1 til 11, hvor tu-  
mourepitopen er en epitop af et neoantigen, fortrinsvis en epitop af et kolorektal cancer-  
specifikt neoantigen.

10 13. Kompleks til anvendelse ifølge et hvilket som helst af kravene 1-12 der omfatter,  
fortrinvis i N- til C-terminalretning, følgende bestanddele:

(i) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 6 eller en funktionel se-  
kvensvariant der af, der har mindst 90% sekvensidentitet;

15 (ii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 96 eller en funktionel  
sekvensvariant der af, der har mindst 90% sekvensidentitet;

(iii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 95 eller en funktionel  
sekvensvariant der af, der har mindst 90% sekvensidentitet;

(iv) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 97 eller en funktionel  
sekvensvariant der af, der har mindst 90% sekvensidentitet; og

20 (v) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 71 eller en funktionel  
sekvensvariant der af, der har mindst 90% sekvensidentitet.

14. Kompleks til anvendelse ifølge et hvilket som helst af kravene 1 til 13, hvor det  
nævnte kompleks omfatter eller består af en aminosyresekvens ifølge til et hvilket som  
helst af SEQ ID NOs: 88 – 91; eller det nævnte kompleks omfatter eller består af en ami-  
25 nosyresekvens, der deler mindst 90%, mindst 95% eller mindst 98% sekvensidentitet med  
et hvilket som helst af SEQ ID NOs: 88 – 91.

15. Kompleks til anvendelse ifølge et hvilket som helst af kravene 1 til 14, hvor det  
nævnte kompleks omfatter eller består af et polypeptid, der har en aminosyresekvens  
ifølge SEQ ID NO: 89, eller en funktionel sekvensidentitet der har mindst 80% sekvens-  
30 identitet.

16. Kompleks der omfatter:

a) et cellepenetrerende peptid;  
b) mindst tre antigen epitoper; og  
c) en TLR-peptidagonist,  
hvor bestanddelene a)-c) er forbundet kovalent,  
5 hvor TLR-peptidagonist er en TLR2-peptidagonist og/eller en TLR4-peptidagonist, og  
hvor de mindst tre antigen epitoper omfatter (i) en epitop af survivn, (ii) en epitop af CEA, og (iii) en epitop af ASCL2.

17. Kompleks ifølge krav 16, hvor komplekset omfatter i N- til C-terminalretning:  
10 (i) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 54 eller et fragment der af, der har en længde på mindst 10 aminosyrer, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.  
(ii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 52 eller et fragment der af, der har en længde på mindst 10 aminosyrer, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.  
15 (iii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 92 eller et fragment der af, der har en længde på mindst 10 aminosyrer, eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.

18. Kompleks ifølge kravene 16 eller 17, hvor komplekset omfatter et peptid der har en aminosyresekvens ifølge SEQ ID NO: 98 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet.  
20

19. Kompleks ifølge et hvilket som helst af kravene 16 – 18, hvor komplekset er et rekombinant polypeptid eller et rekombinant protein.

20. Kompleks ifølge et hvilket som helst af kravene 16 – 19, hvor det cellepenetrerende peptid er som defineret i et hvilket som helst af kravene 2-4.

21. Kompleks ifølge et hvilket som helst af kravene 16 – 20 der omfatter, fortrinsvis N- til C-terminalretningen, følgende bestanddele:  
25 (i) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 6 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet;  
(ii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 96 eller en funktionel

sekvensvariant der af, der har mindst 90% sekvensidentitet;

(iii) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 95 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet;

(iv) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 97 eller en funktionel

5 sekvensvariant der af, der har mindst 90% sekvensidentitet; og

(v) et peptid der har en aminosyresekvens ifølge SEQ ID NO: 71 eller en funktionel sekvensvariant der af, der har mindst 90% sekvensidentitet,

hvor bestanddelene (i) – (v) er eventuelt forbundet til en linker eller en spacer.

22. Kompleks ifølge et hvilket som helst af kravene 16 – 21, hvor komplekset omfatter eller består af et polypeptid, der har en aminosyresekvens ifølge SEQ ID NO: 89, eller 10 en funktionel sekvensvariant der af, der har mindst 80% sekvensidentitet.

23. Nukleinsyre der koder for komplekset ifølge et hvilket som helst af kravene 16 til 22, hvor komplekset er et polypeptid eller et protein.

24. Vektor der omfatter nukleinsyren som defineret i krav 23.

15 25. Værtscelle der omfatter vektoren som defineret i krav 24.

26. Celle der er ladet med et kompleks som defineret i et hvilket som helst af kravene 1 til 22, fortrinsvis hvor den nævnte celle er et antigen præsenterende celle, mere fortrinsvis en dendritcelle.

27. Sammensætning der omfatter:

20 (i) et kompleks som defineret i et hvilket som helst af kravene 1 til 22;  
(ii) en nukleinsyre som defineret i krav 23;  
(iii) en vektor som defineret i krav 24;  
(iv) en værtscelle som defineret i krav 25; og/eller  
(v) en celle der er ladet med et kompleks som defineret i krav 26.

25 28. Vaccine der omfatter:

(i) et kompleks som defineret i et hvilket som helst af kravene 1 til 22;  
(ii) en nukleinsyre som defineret i krav 23;  
(iii) en vektor som defineret i krav 24;  
(iv) en værtscelle som defineret i krav 25; og/eller  
30 (v) en celle der er ladet med et kompleks som defineret i krav 26.

29. Farmaceutisk sammensætning der omfatter komplekset som defineret i et hvil-

ket som helst af kravene 1 til 22 eller cellen som defineret i krav 26, og en farmaceutisk acceptabel bærer.

30. Kombination af:

- (i) et kompleks som defineret i et hvilket som helst af kravene 1 til 22; og
- 5 (ii) et kemoterapeutisk middel, et målrettet lægemiddel og/eller et immunterapeutisk middel, så som en immun-checkpoint-modulator.

31. (i) nukleinsyre ifølge krav 23;

(ii) vektoren ifølge krav 24;

(iii) værtscelle ifølge krav 25;

10 (iv) celle der er ladet ifølge krav 26;

(v) sammensætning ifølge krav 27;

(vi) vaccinen ifølge krav 28;

eller

(vii) det farmaceutisk sammensætning ifølge krav 29 til anvendelse i forebyggelsen

15 og/eller behandlingen af kolorektal cancer.

32. Kombination af

- (i) et kompleks som defineret i et hvilket som helst af kravene 1 til 22; og
- (ii) et kemoterapeutisk middel, et målrettet lægemiddel og/eller et immunterapeutisk middel, så som en immun-checkpoint-modulator, til anvendelse i forebyggelsen
- 20 og/eller behandlingen af kolorektal cancer.

33. Kombination til anvendelse ifølge krav 32, hvor immun-checkpoint-modulatoren er en PD-1 hæmmer.

# DRAWINGS

Drawing

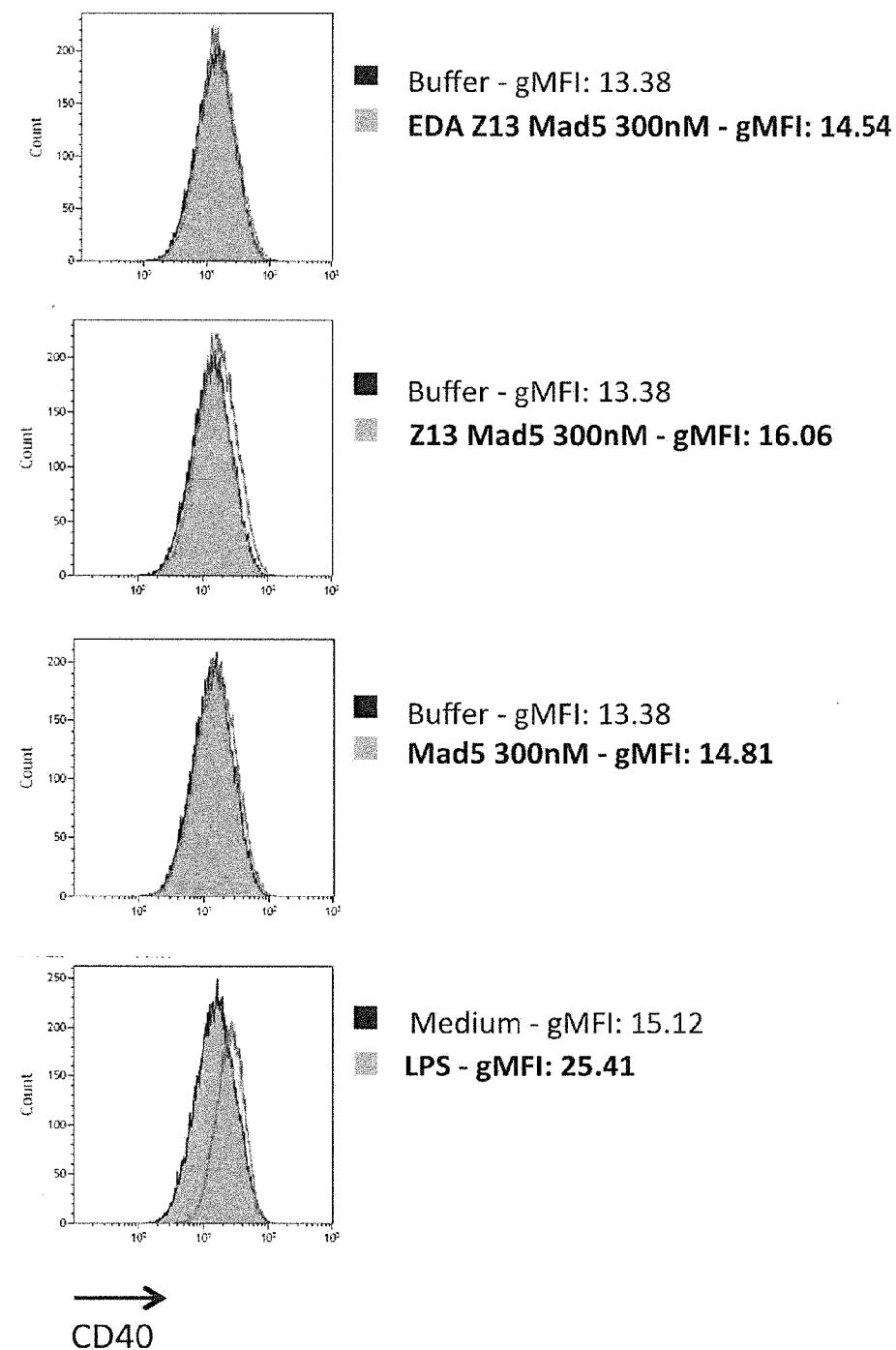


Fig. 1

DK/EP 3515476 T3

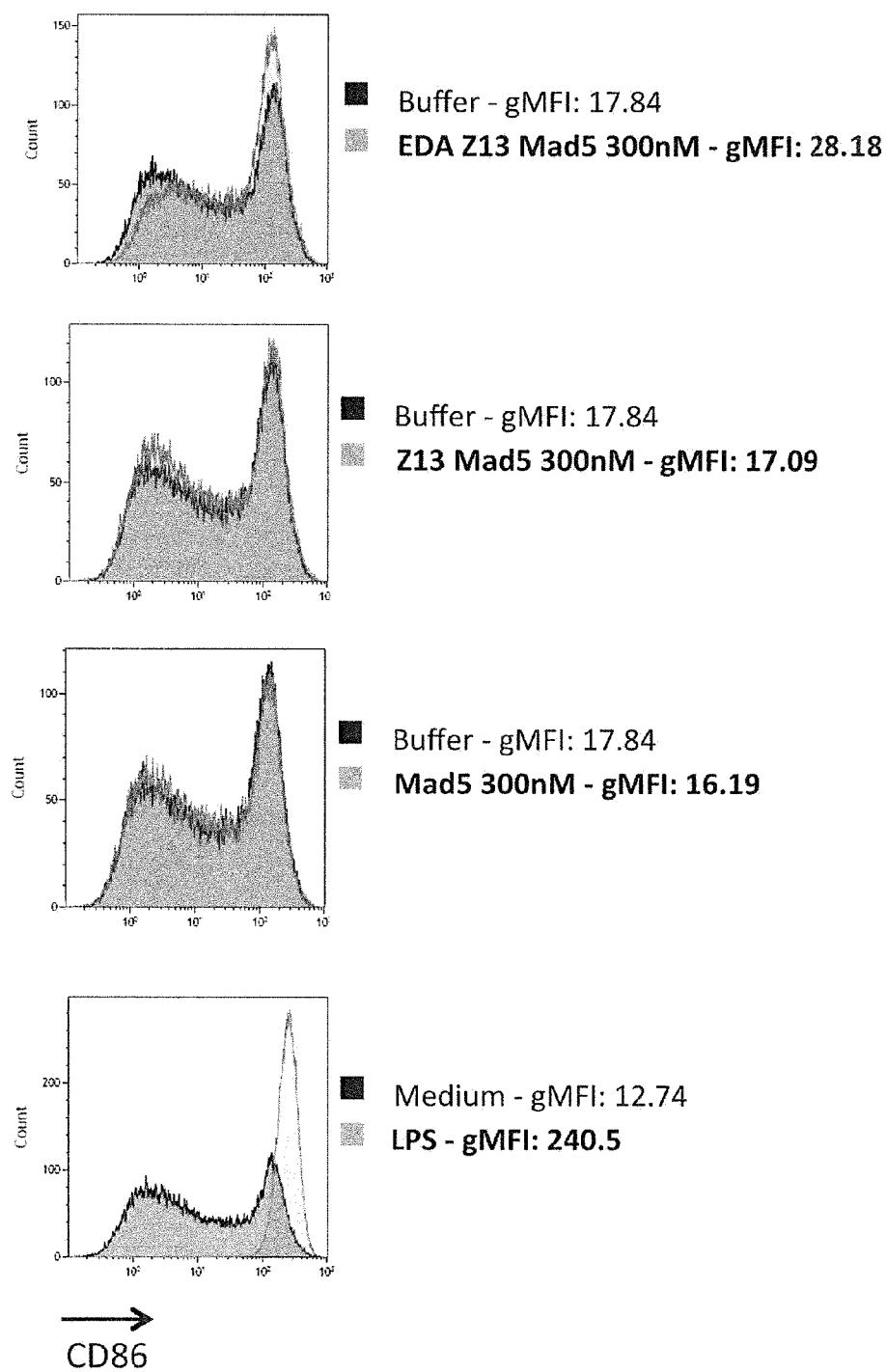


Fig. 2

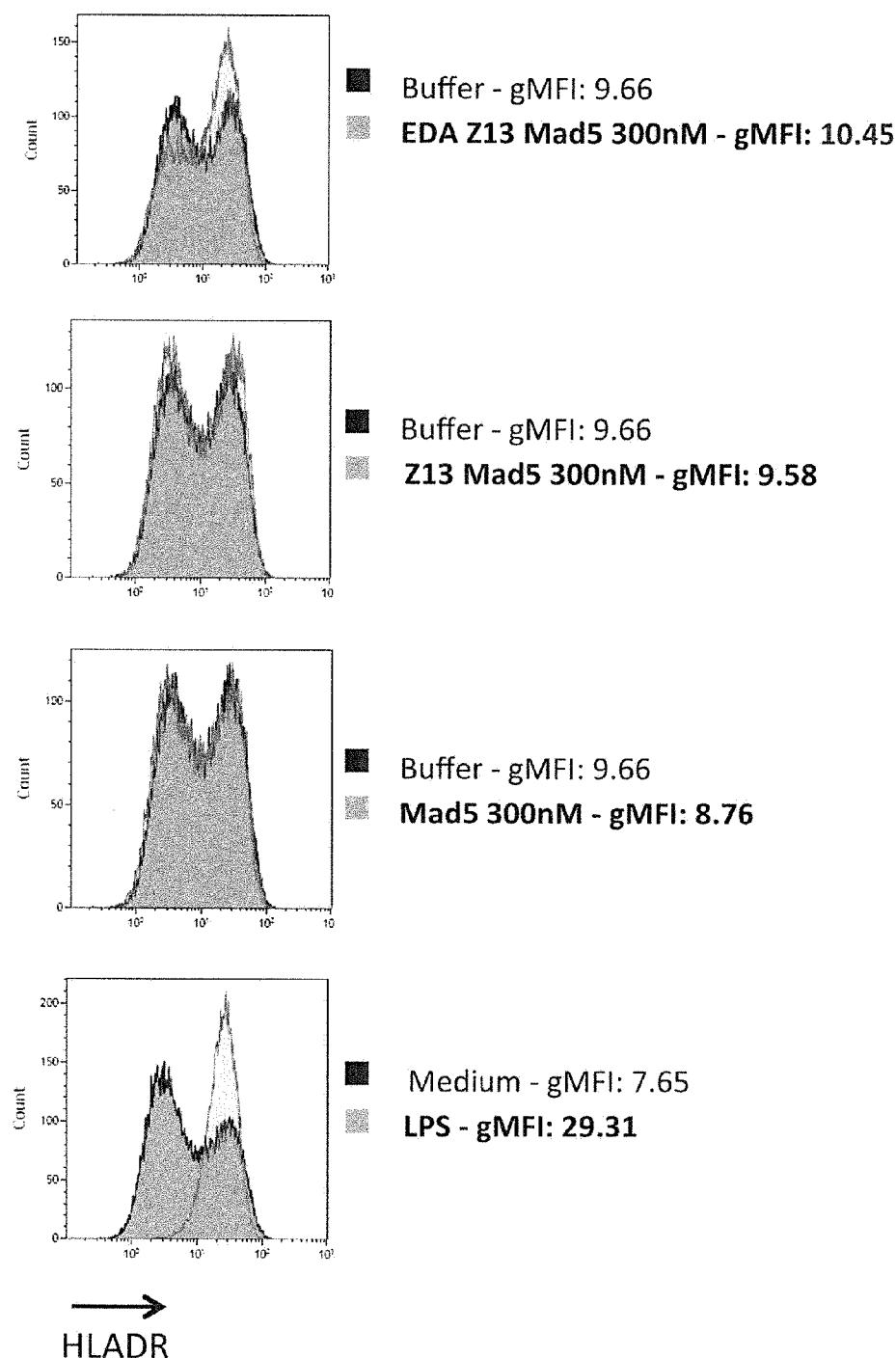


Fig. 3

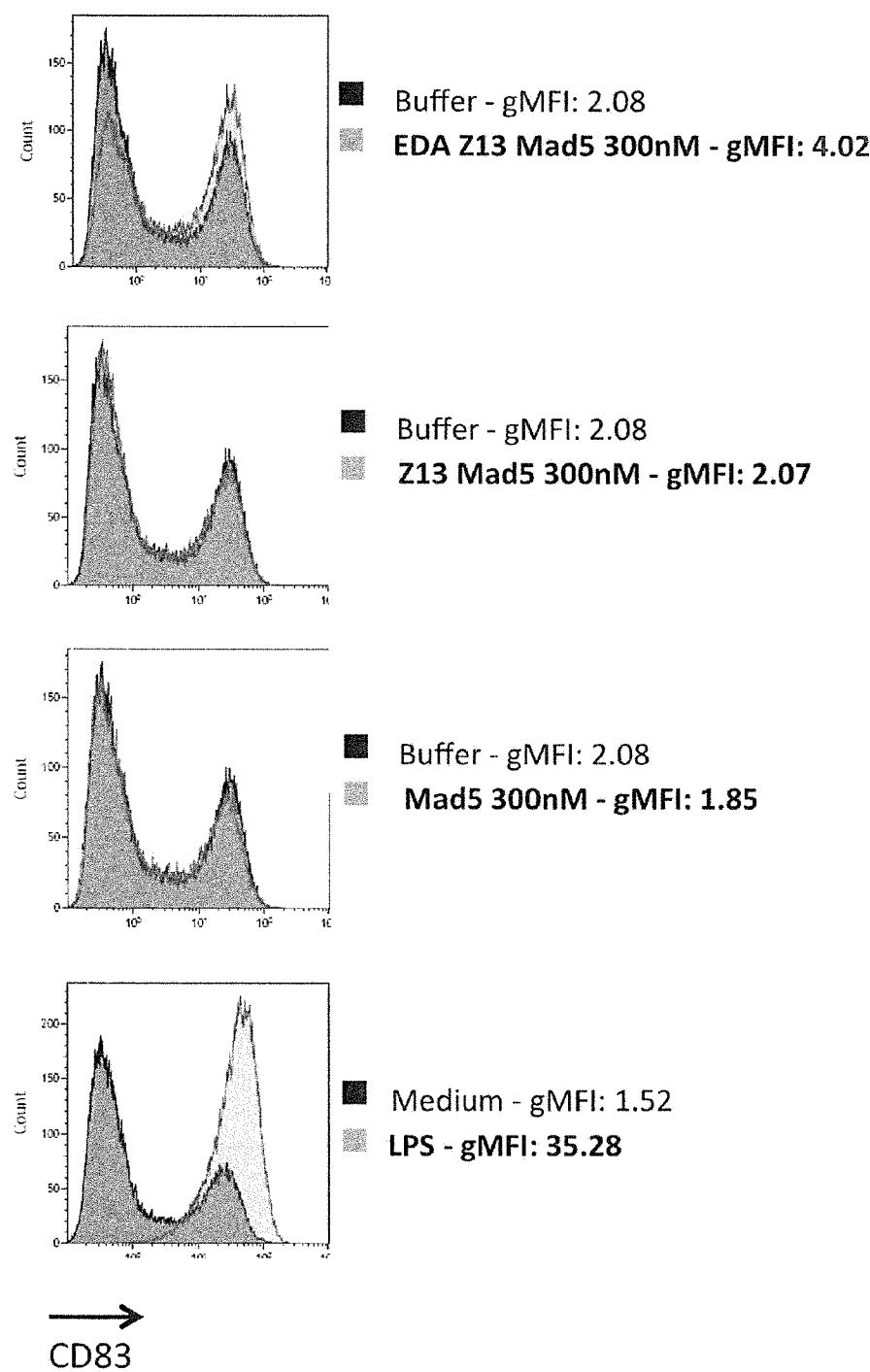


Fig. 4

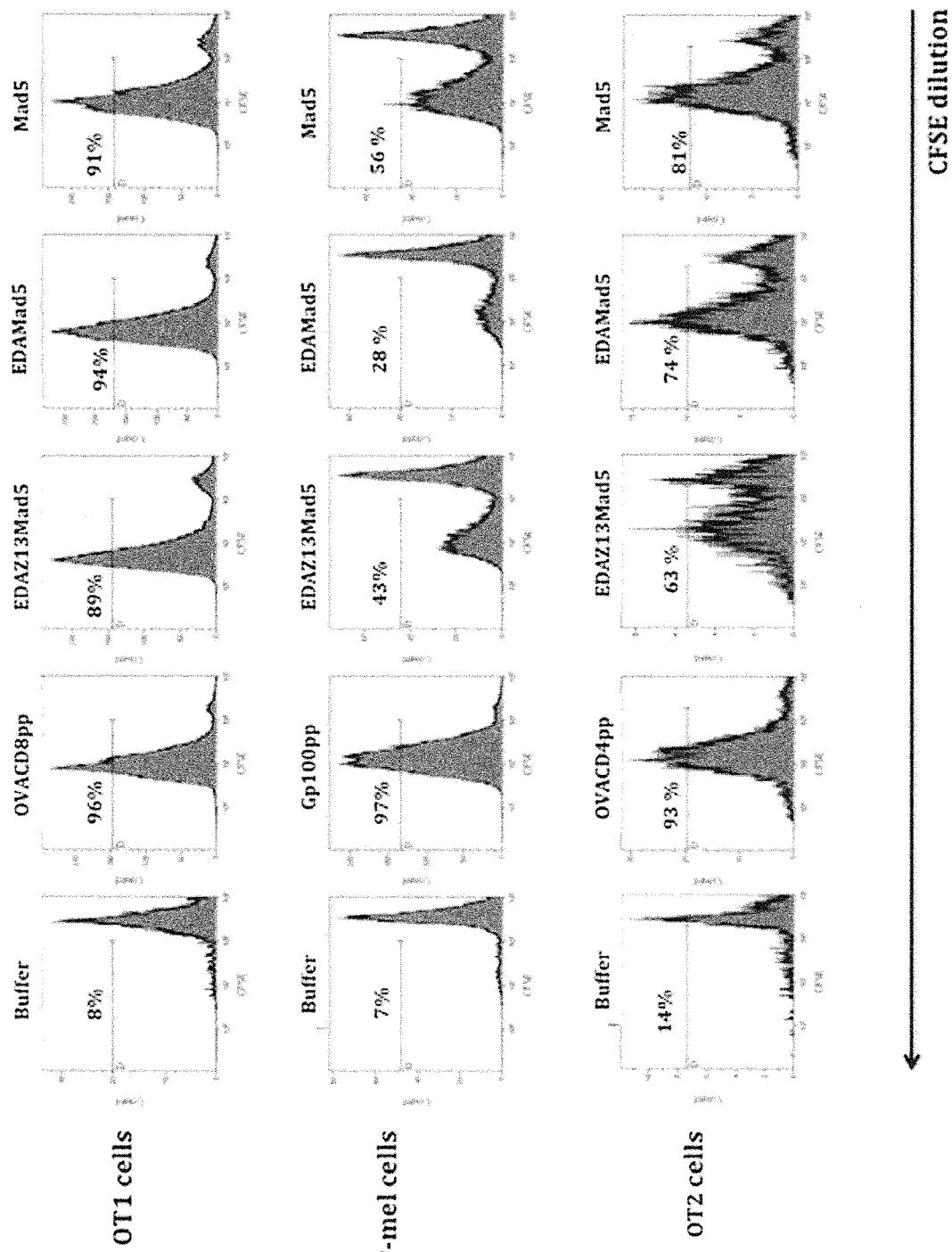
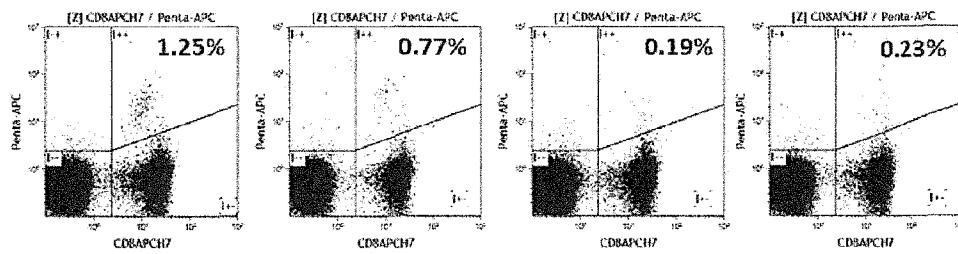


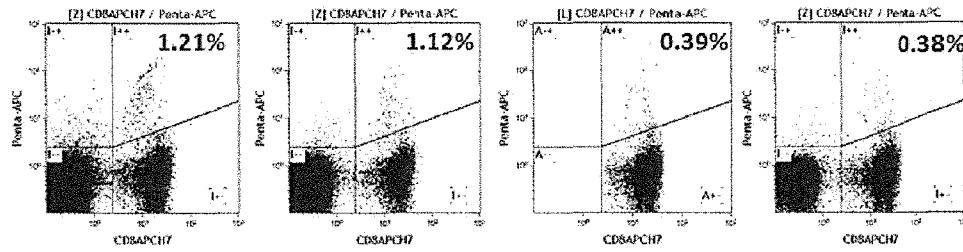
Fig. 5

2 nmol of protein injected

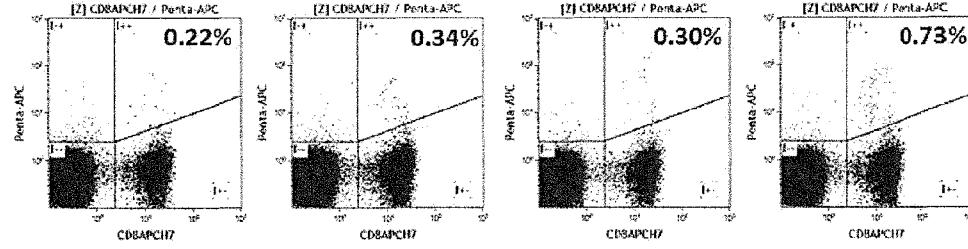
EDA-MAD5



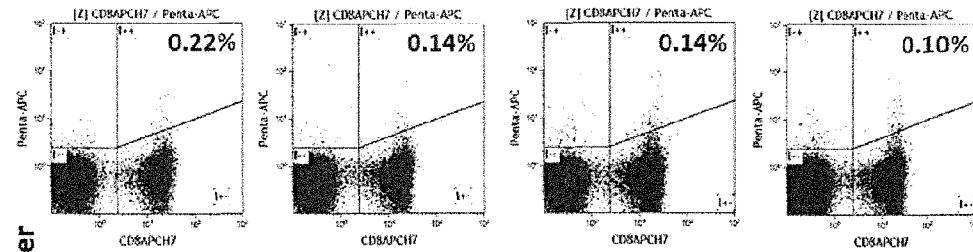
EDA-Z13-MAD5



MAD5+MPLA



Naive

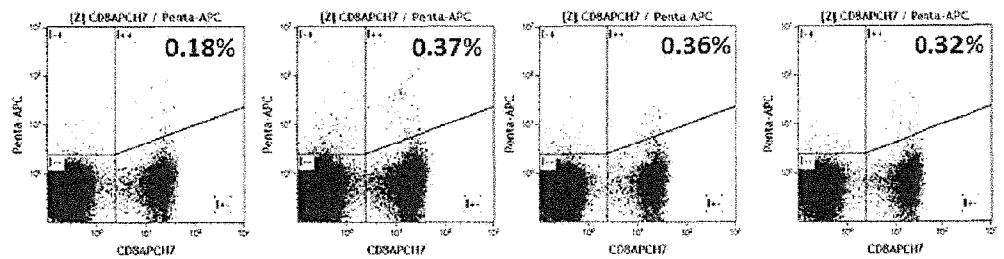


Pentamer  
CD8

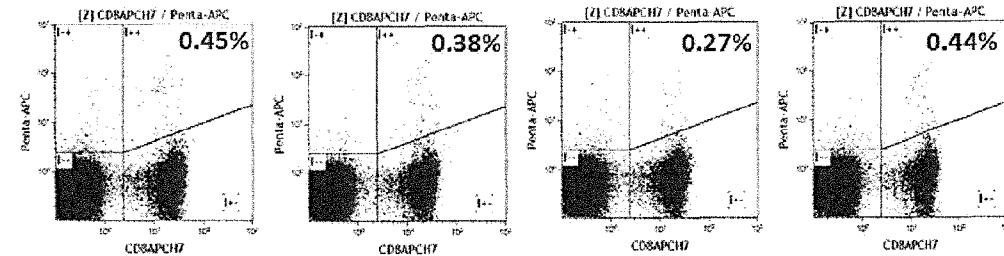
Fig. 6

10 nmol of protein injected

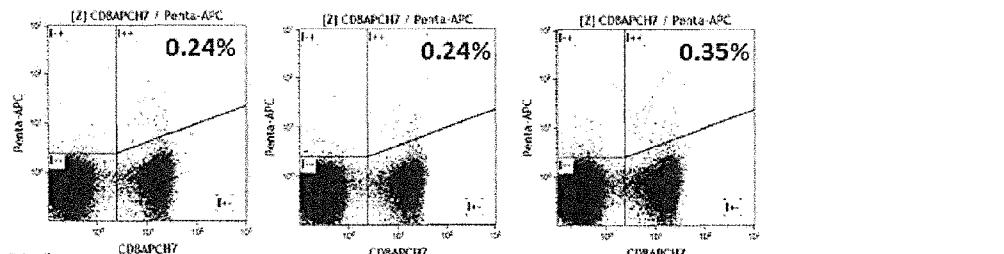
EDA-MAD5



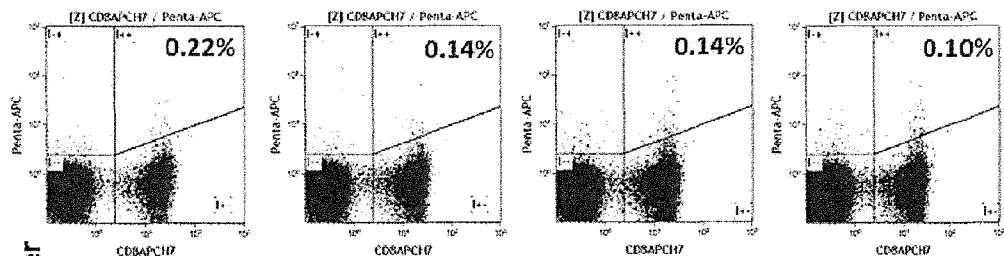
EDA-Z13-MAD5



MAD5+MPLA



Naive



Pentanamer  
CD8

Fig. 7

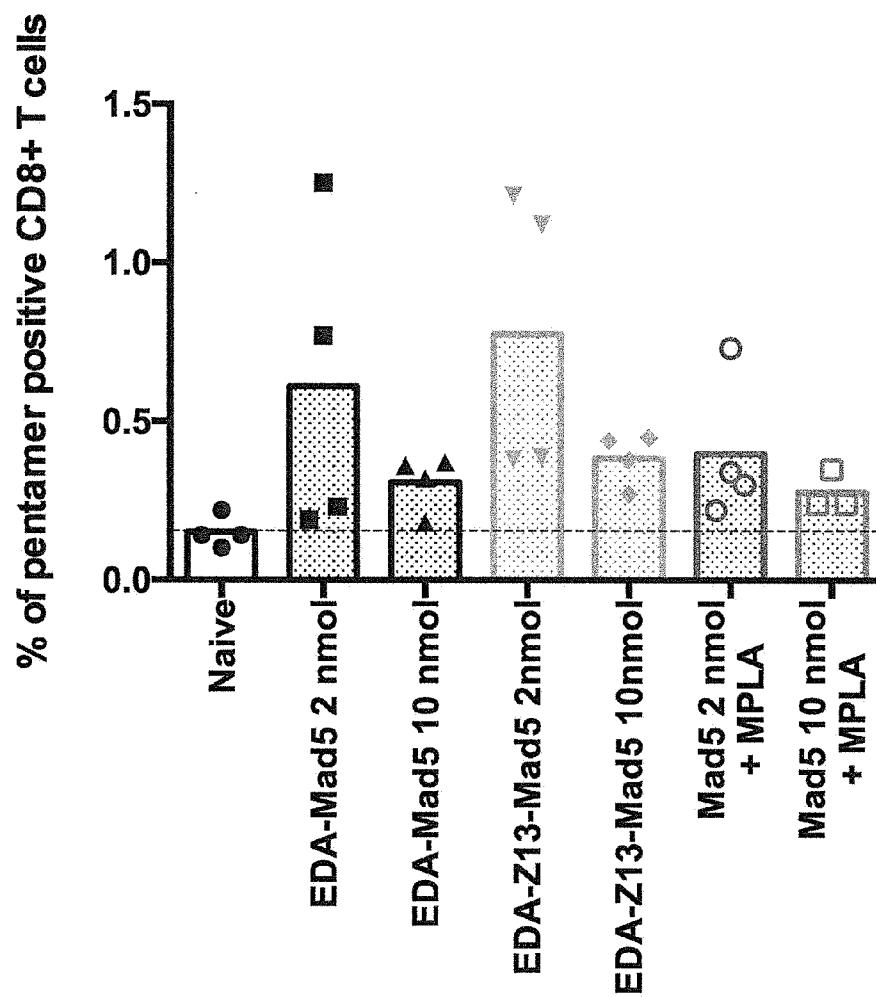


Fig. 8

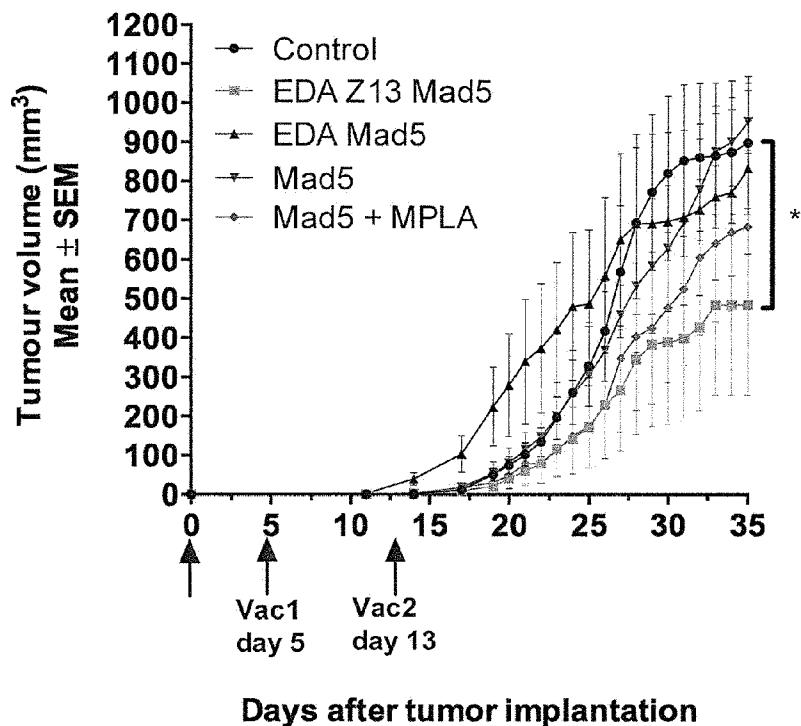


Fig. 9

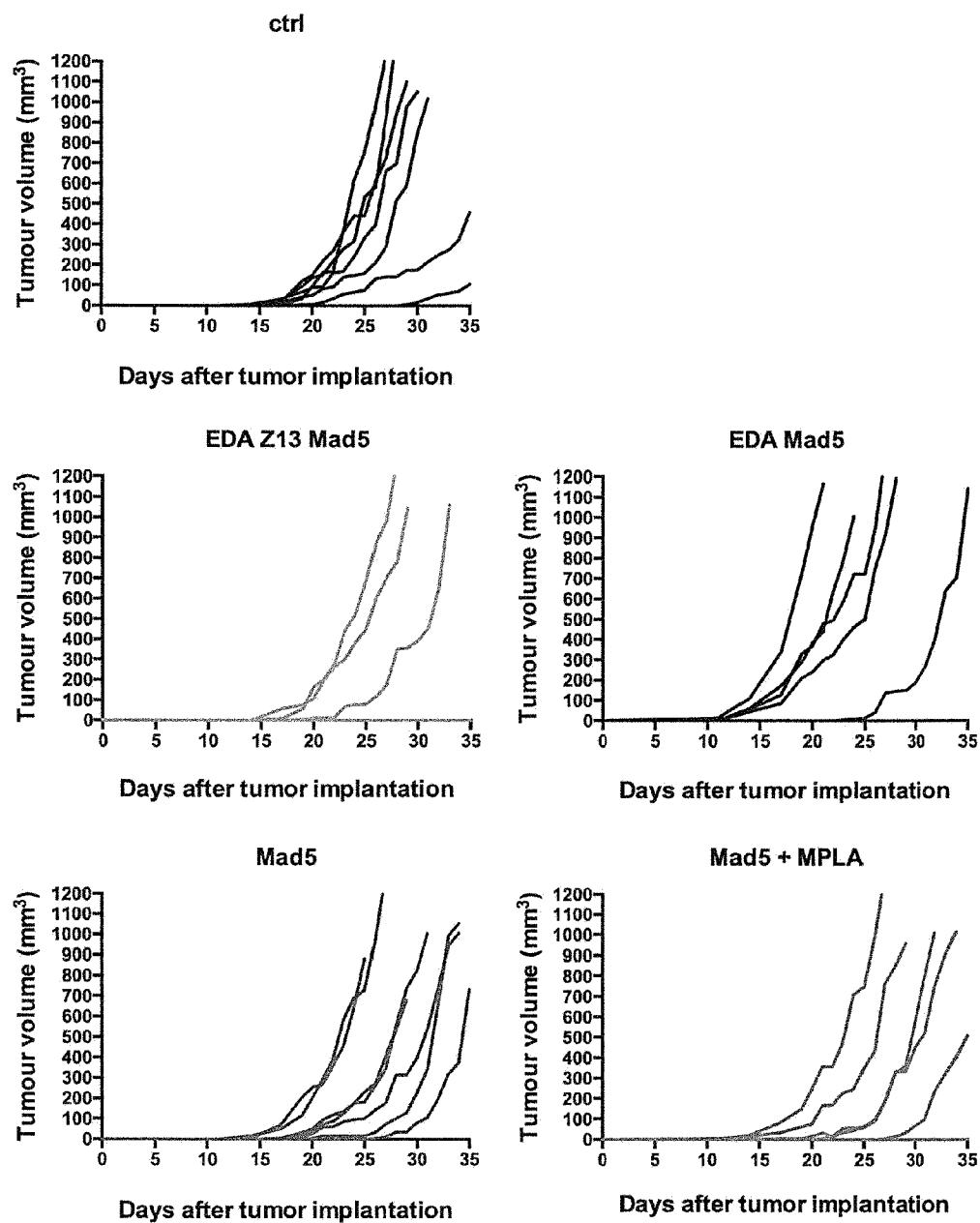
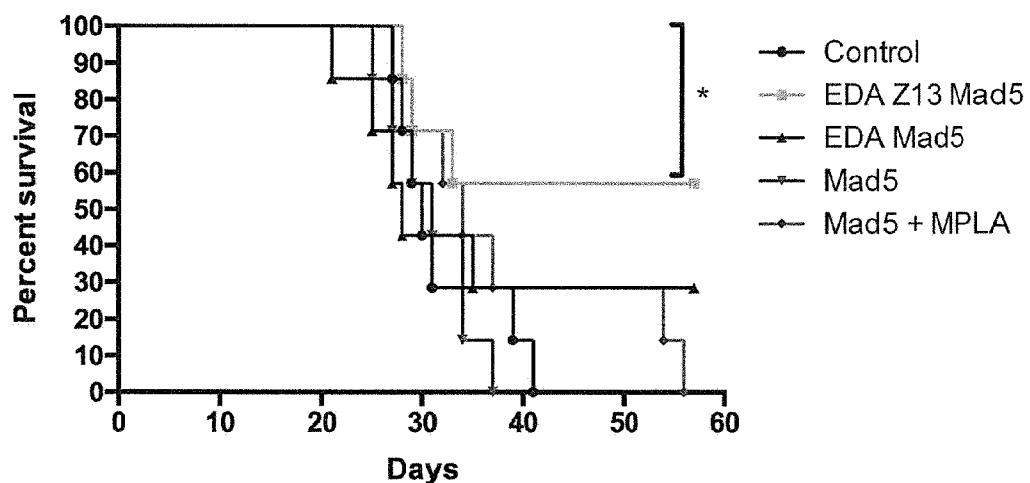


Fig. 10

A



B

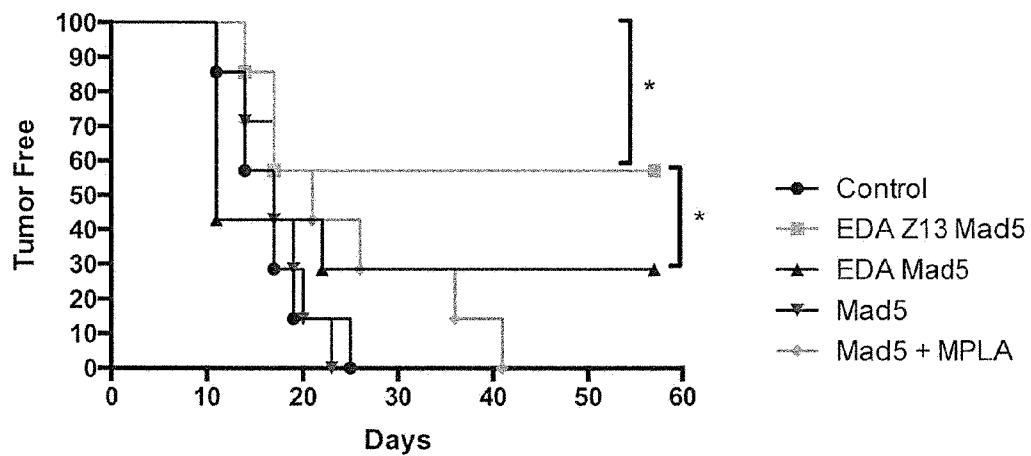


Fig. 11

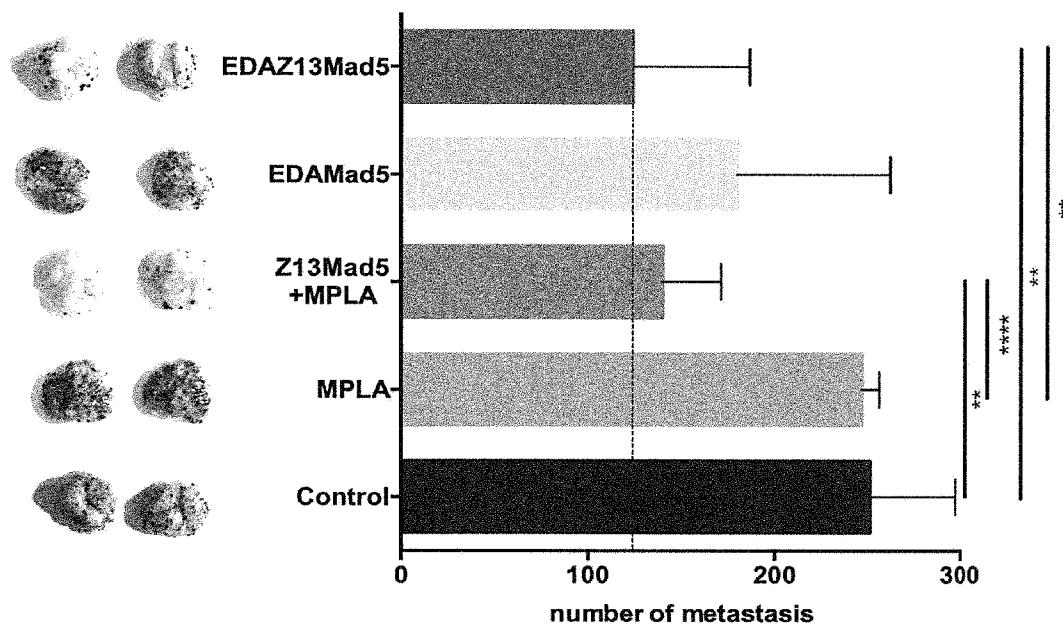


Fig. 12

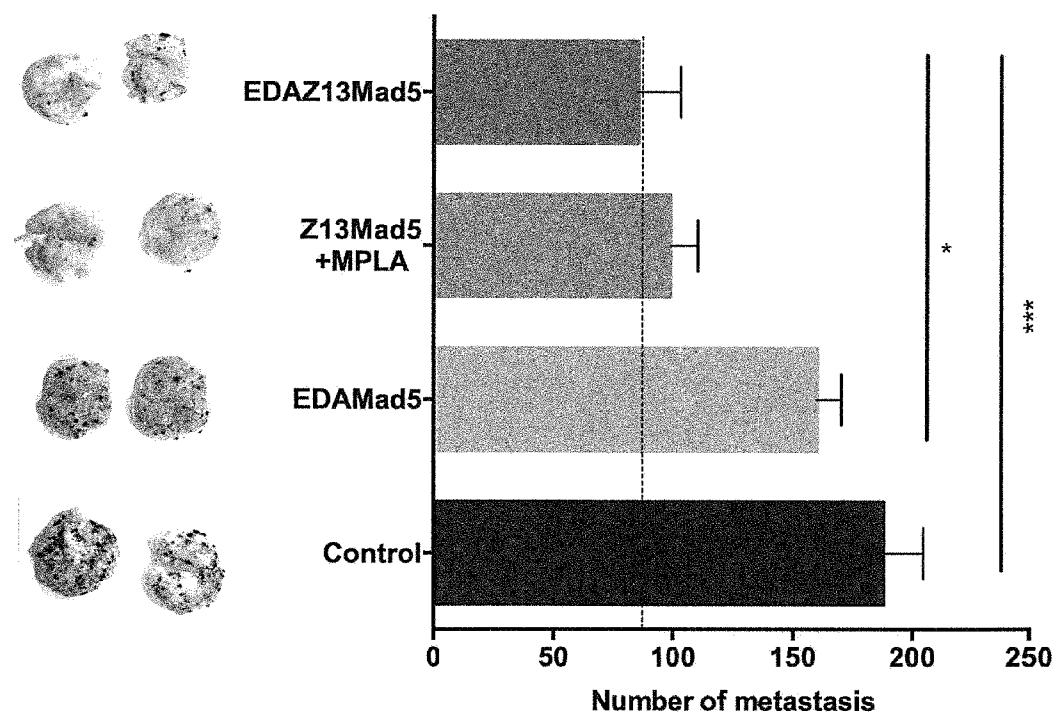
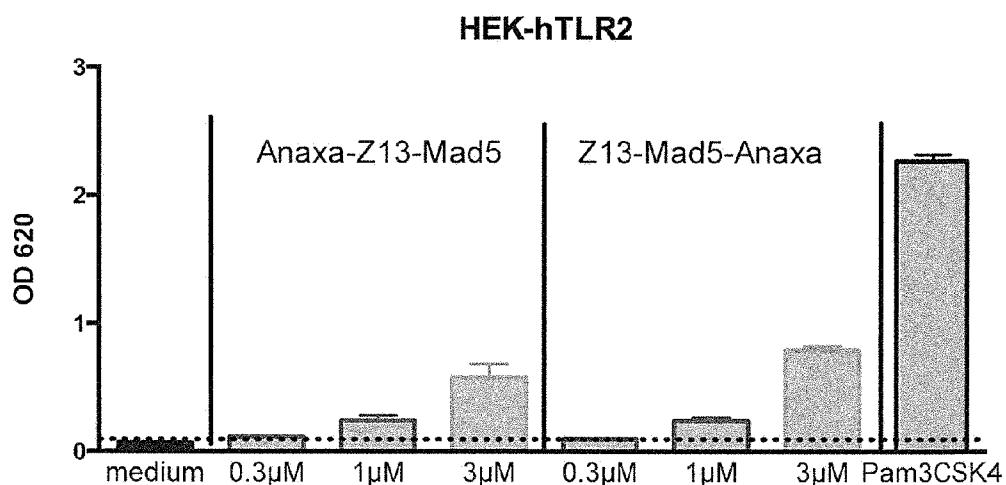


Fig. 13

A



B

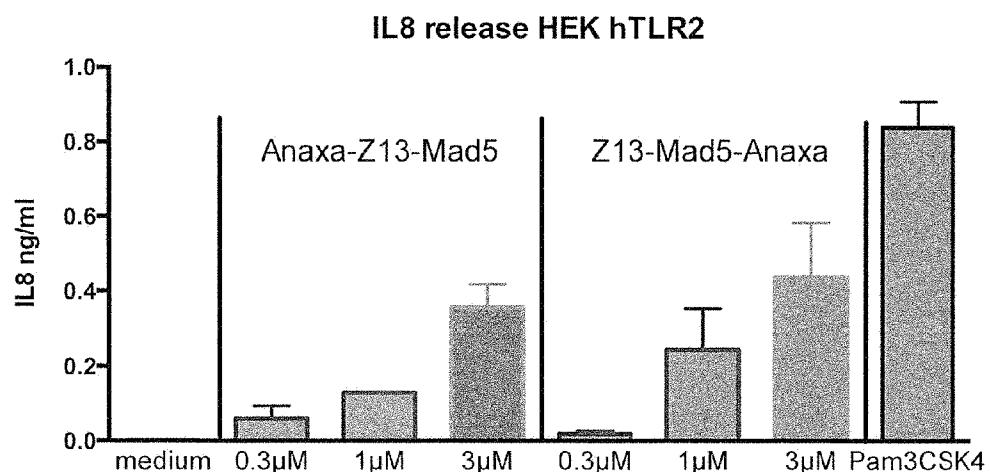


Fig. 14

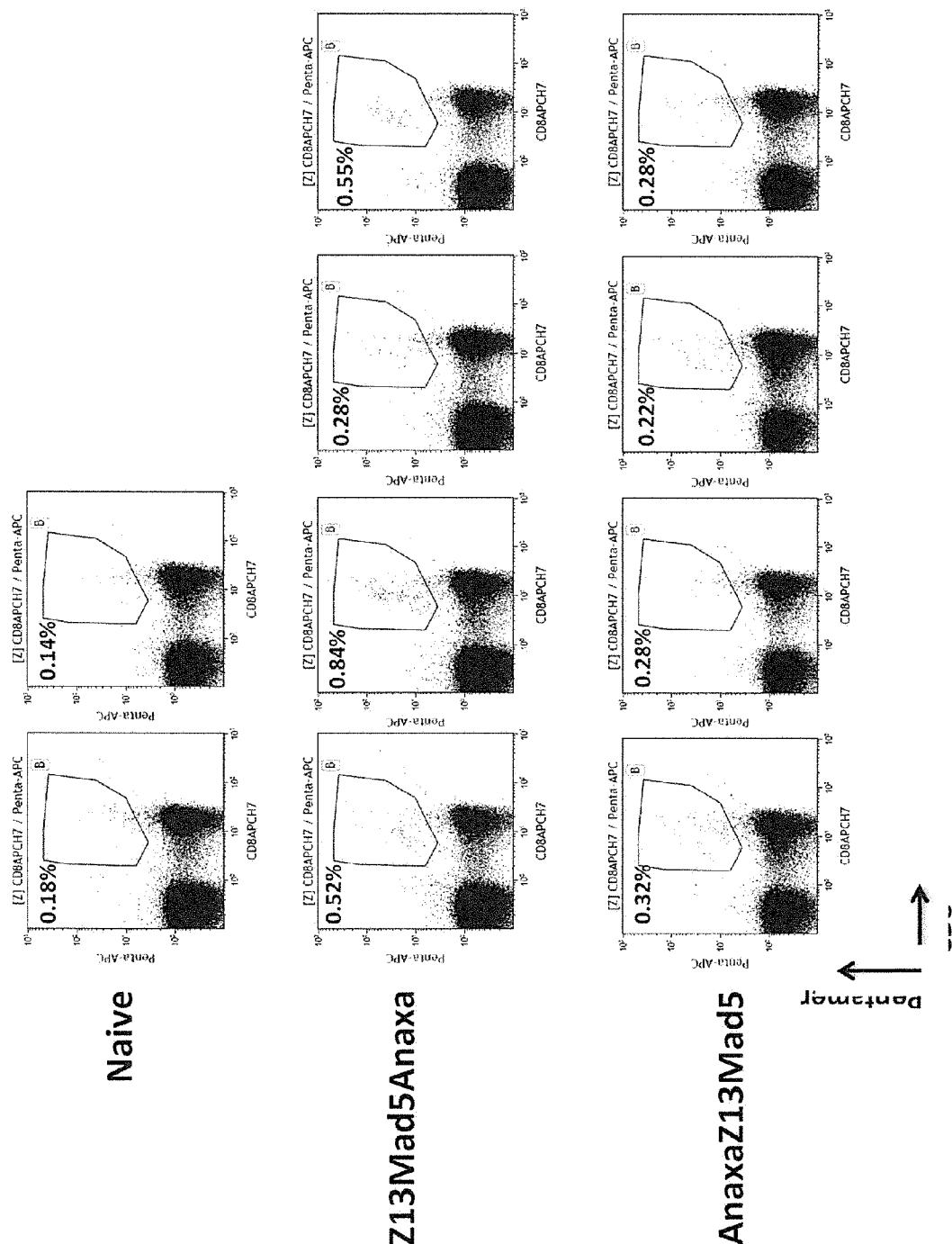


Fig. 15

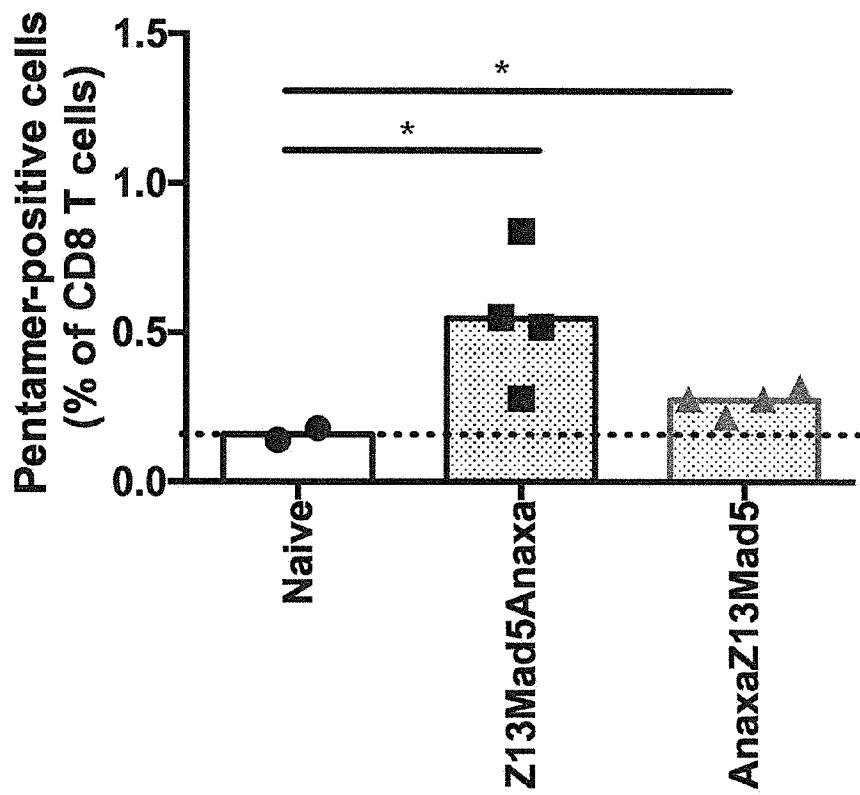


Fig. 16

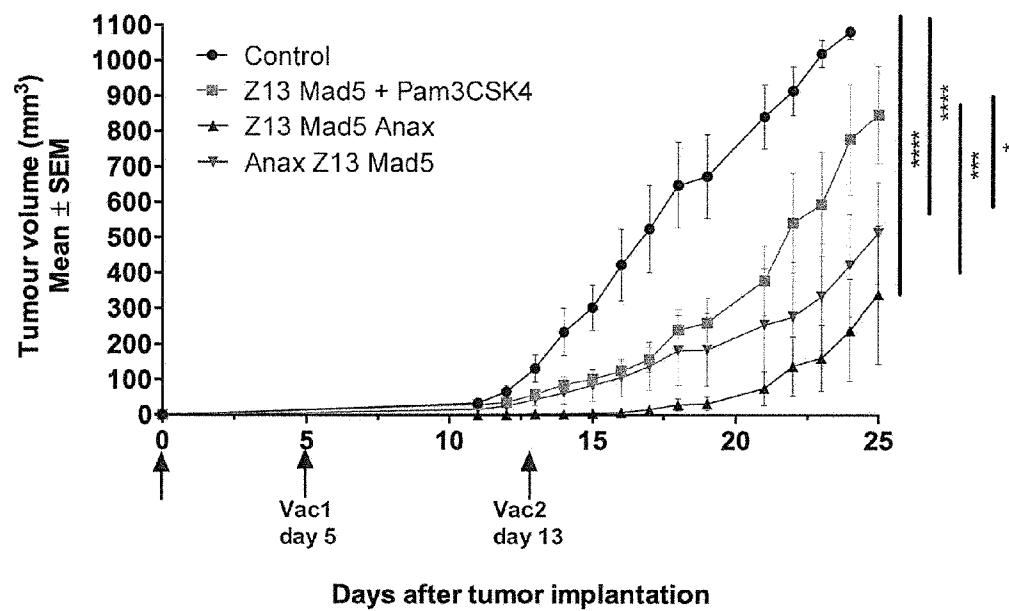


Fig. 17

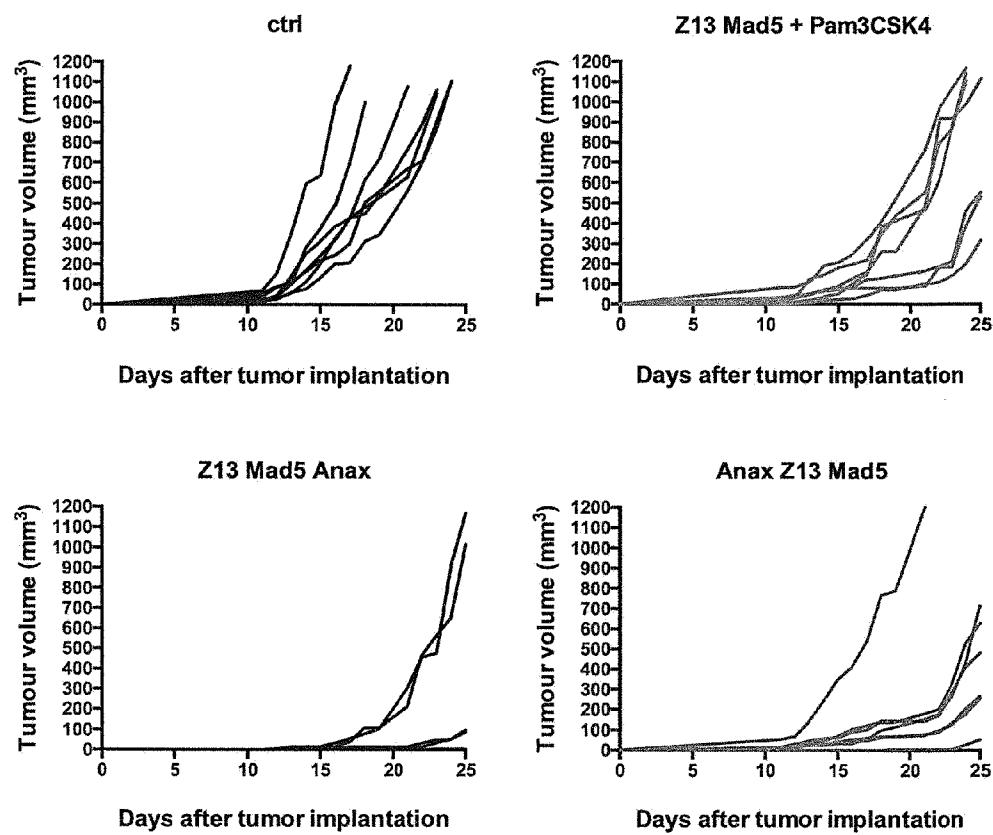


Fig. 18

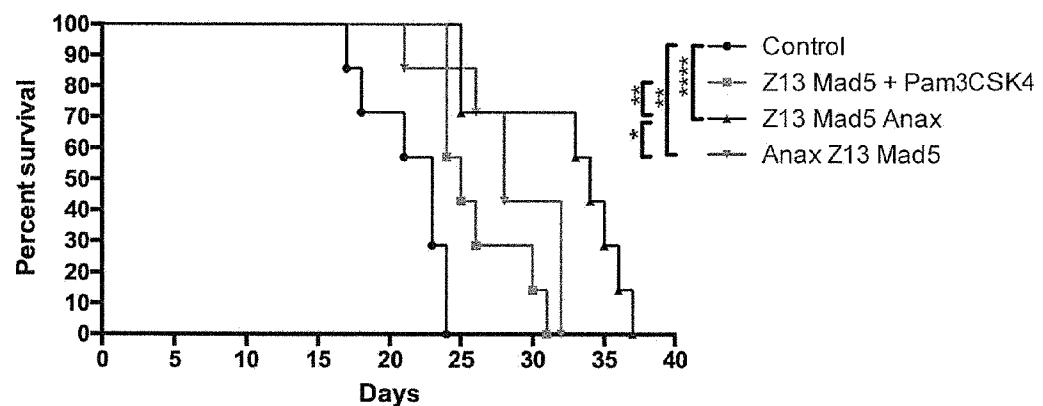


Fig. 19

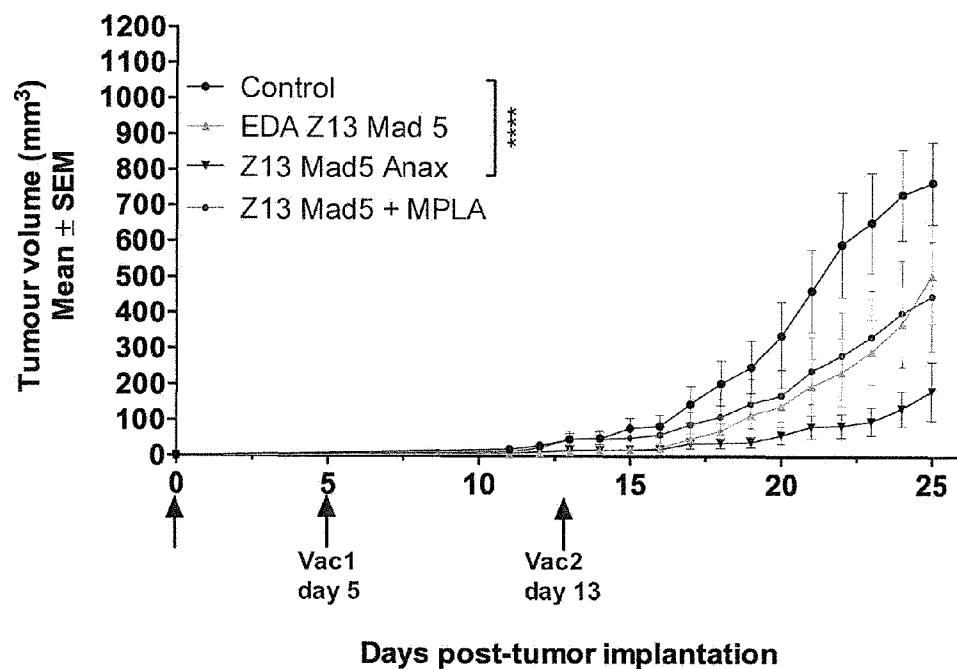


Fig. 20

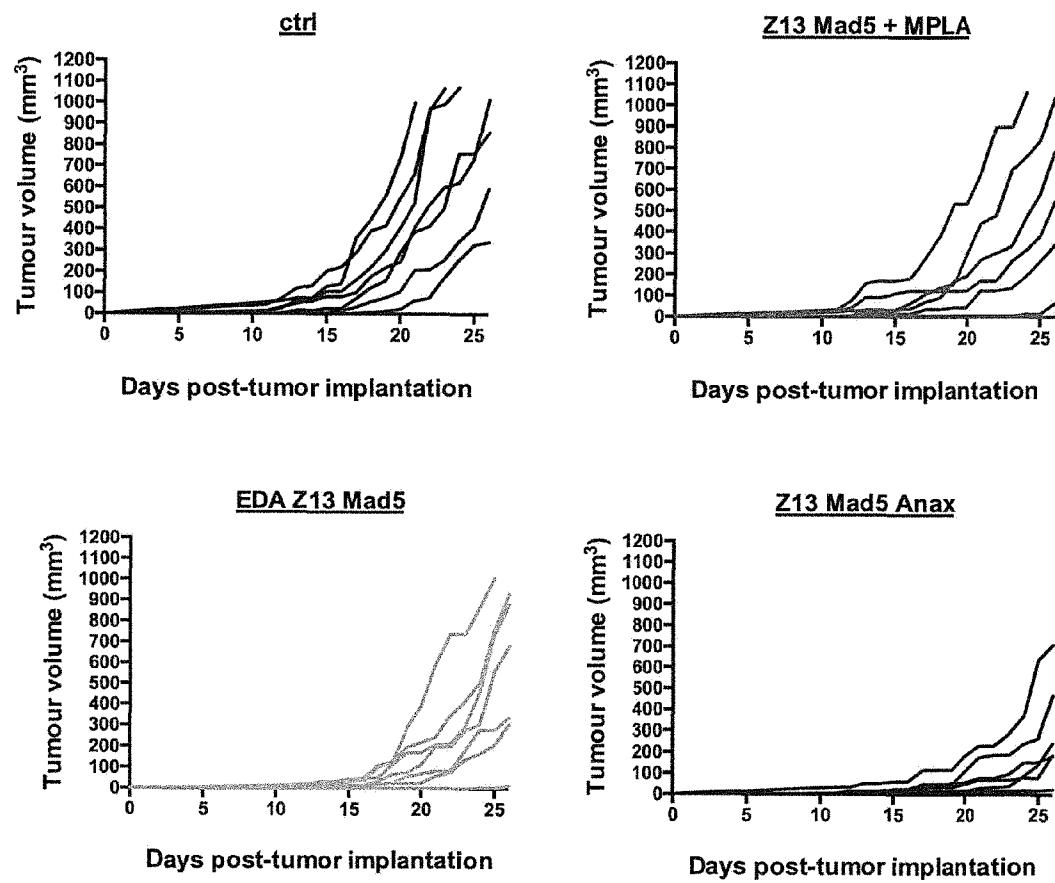


Fig. 21

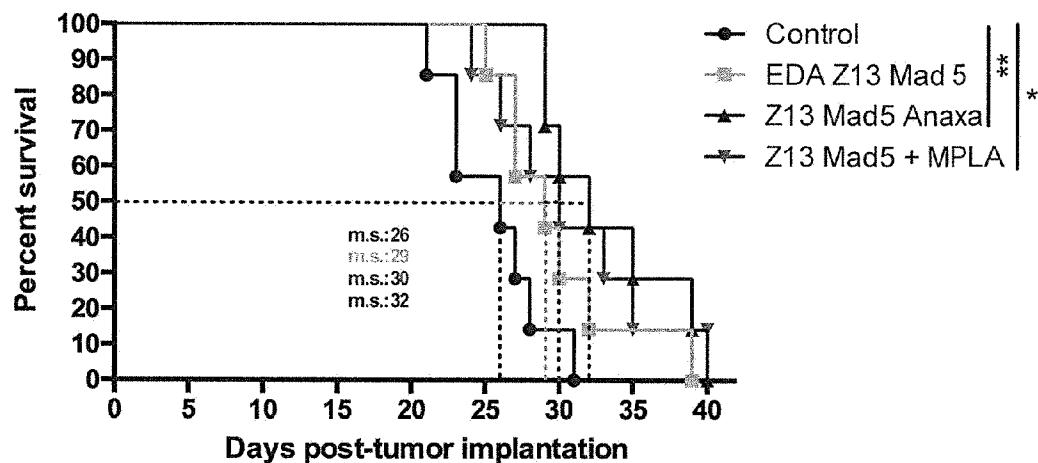


Fig. 22

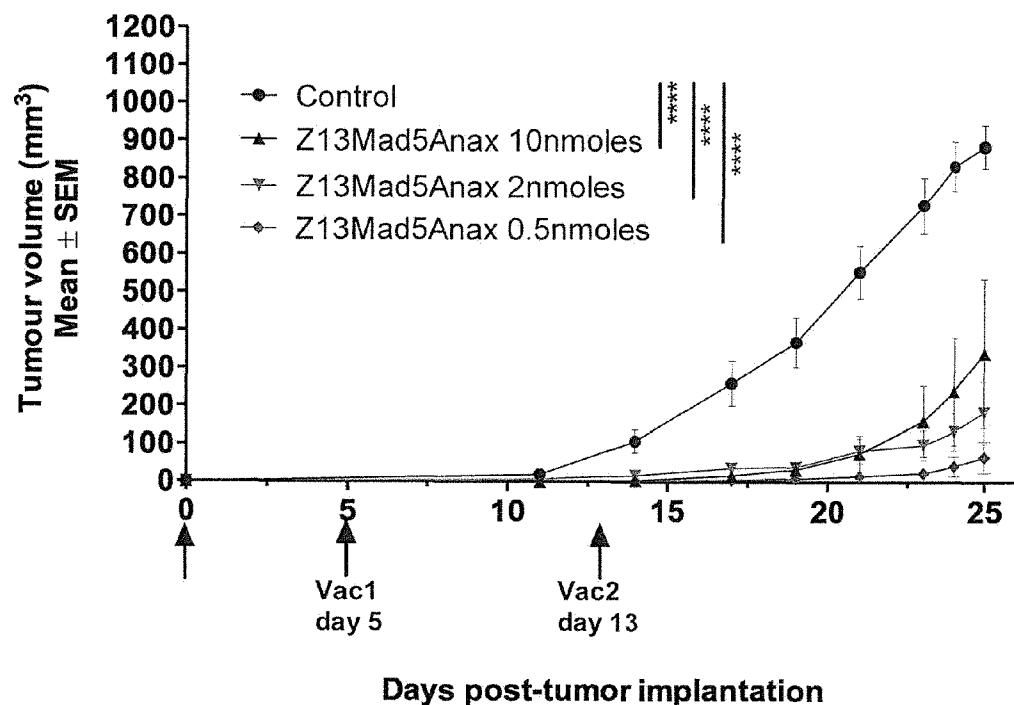
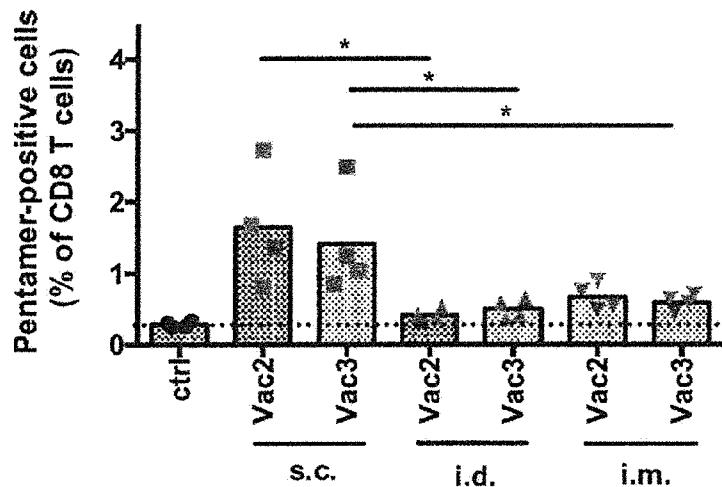


Fig. 23

A

0.5nmoles



B

2nmoles

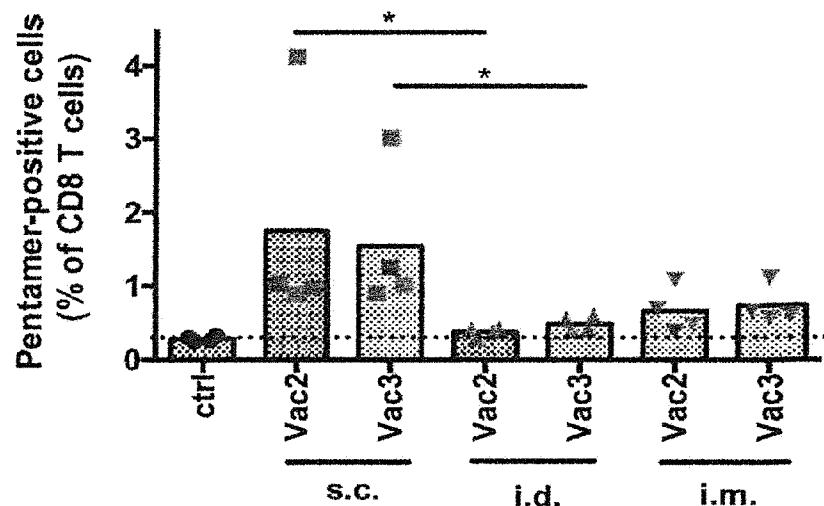
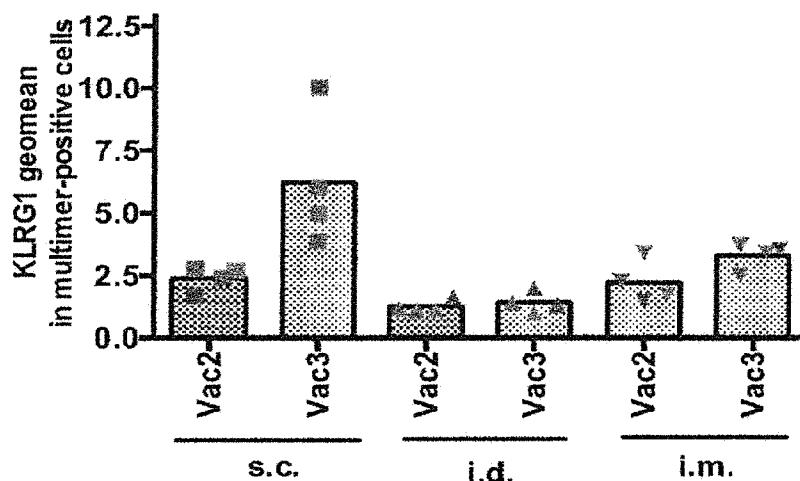


Fig. 24

A

## KLRG1



B

## PD-1

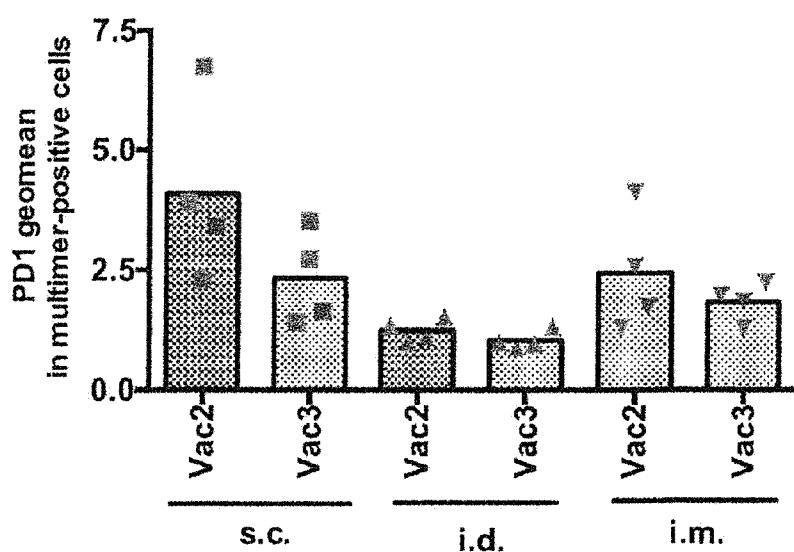


Fig. 25

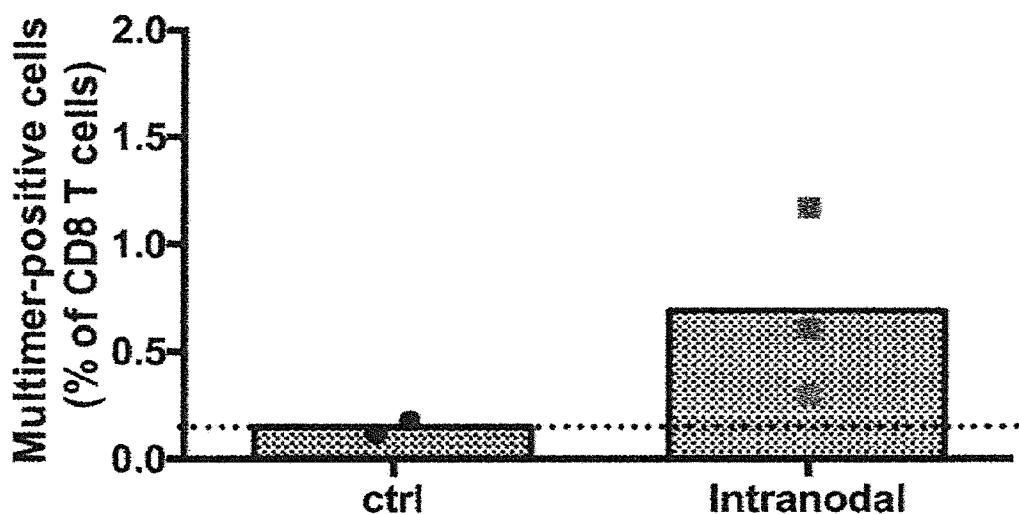
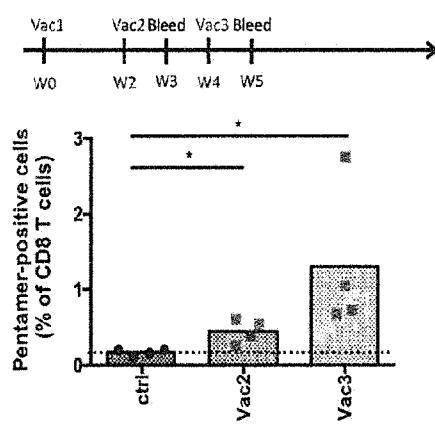
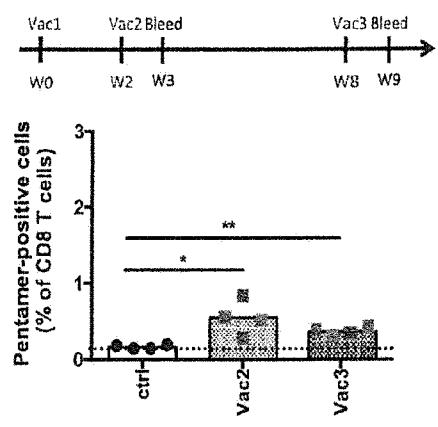


Fig. 26

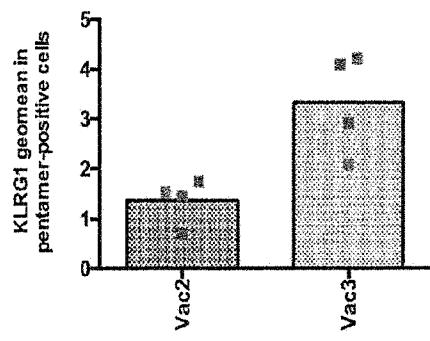
A



B



C



D

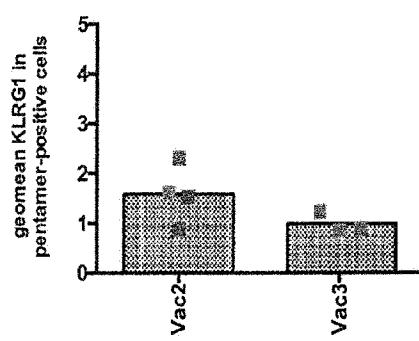


Fig. 27

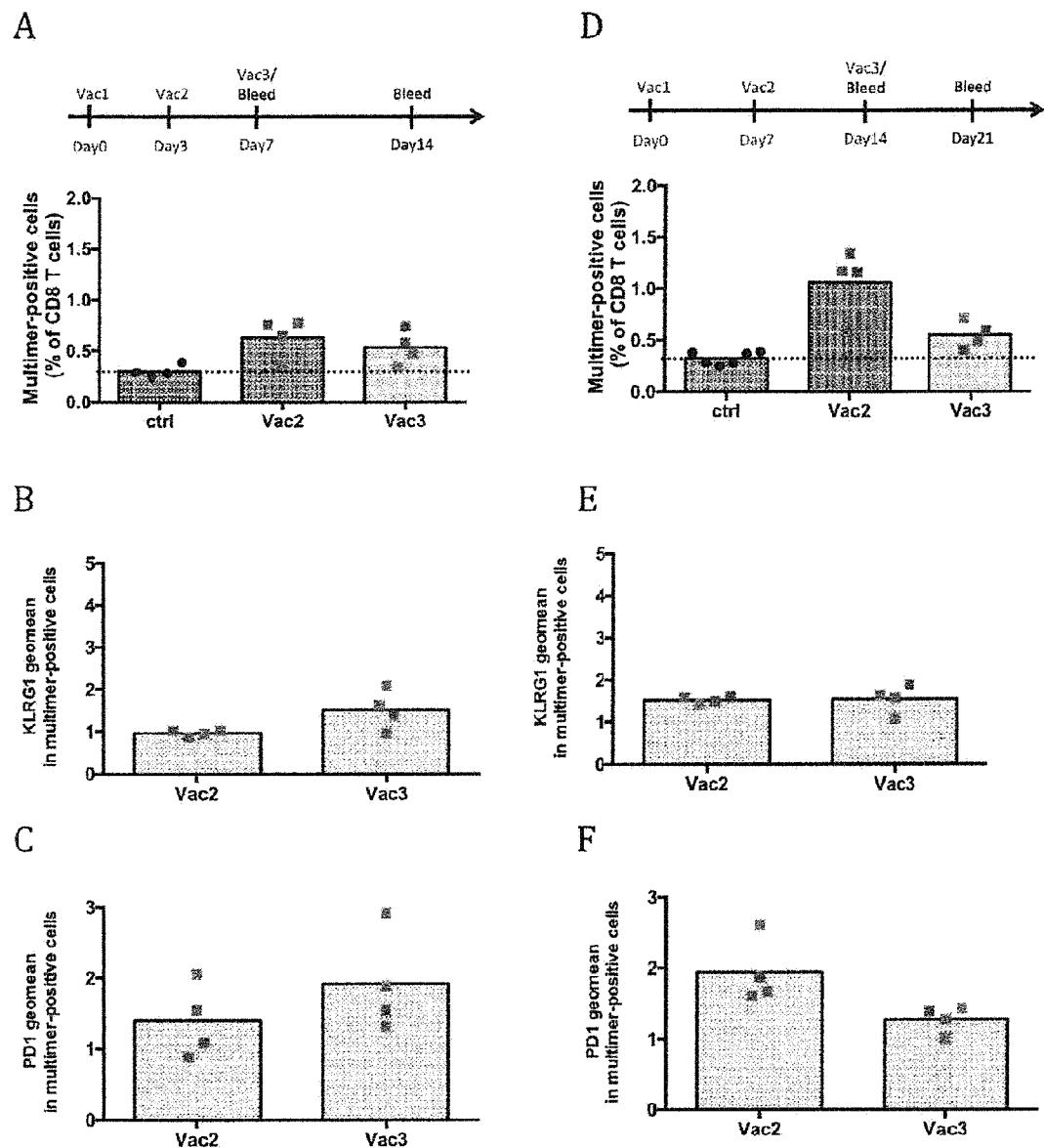


Fig. 28

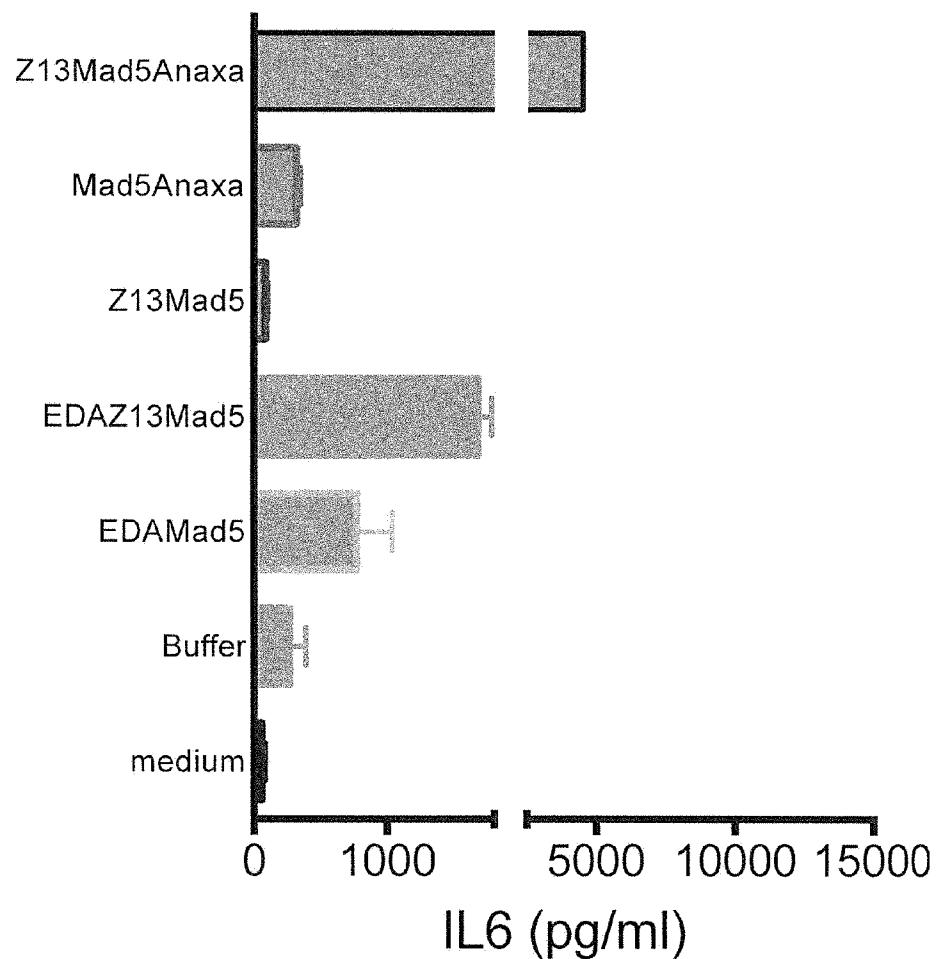


Fig. 29

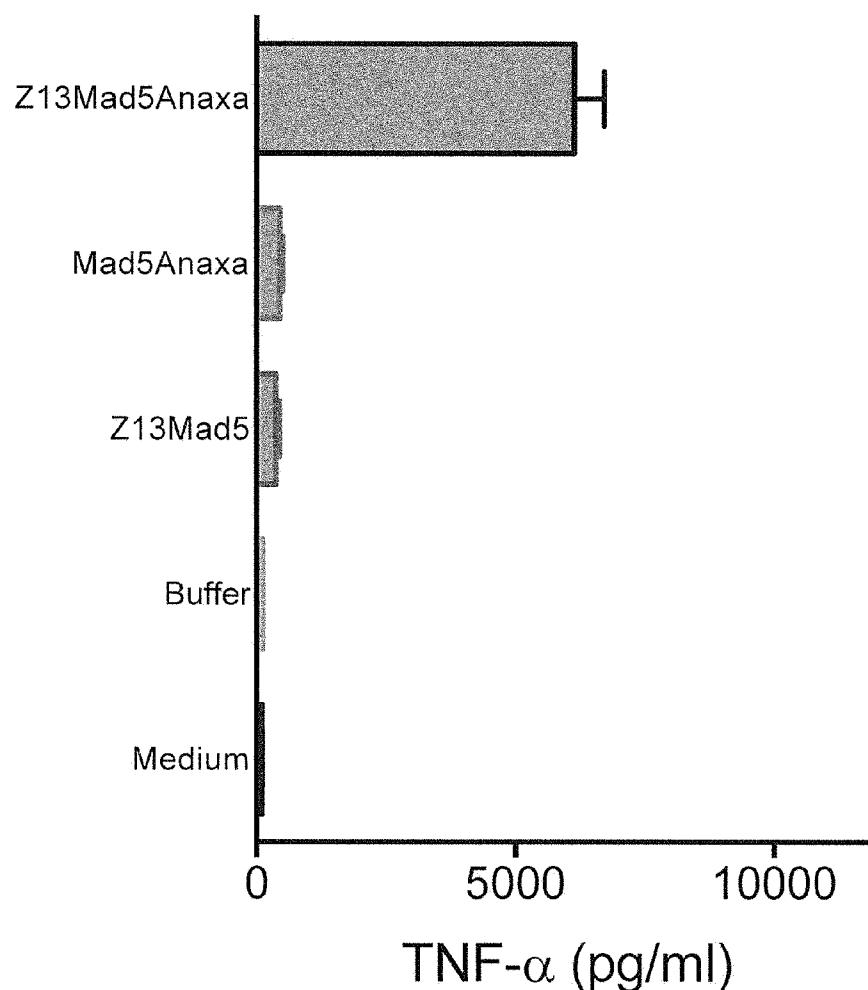


Fig. 30

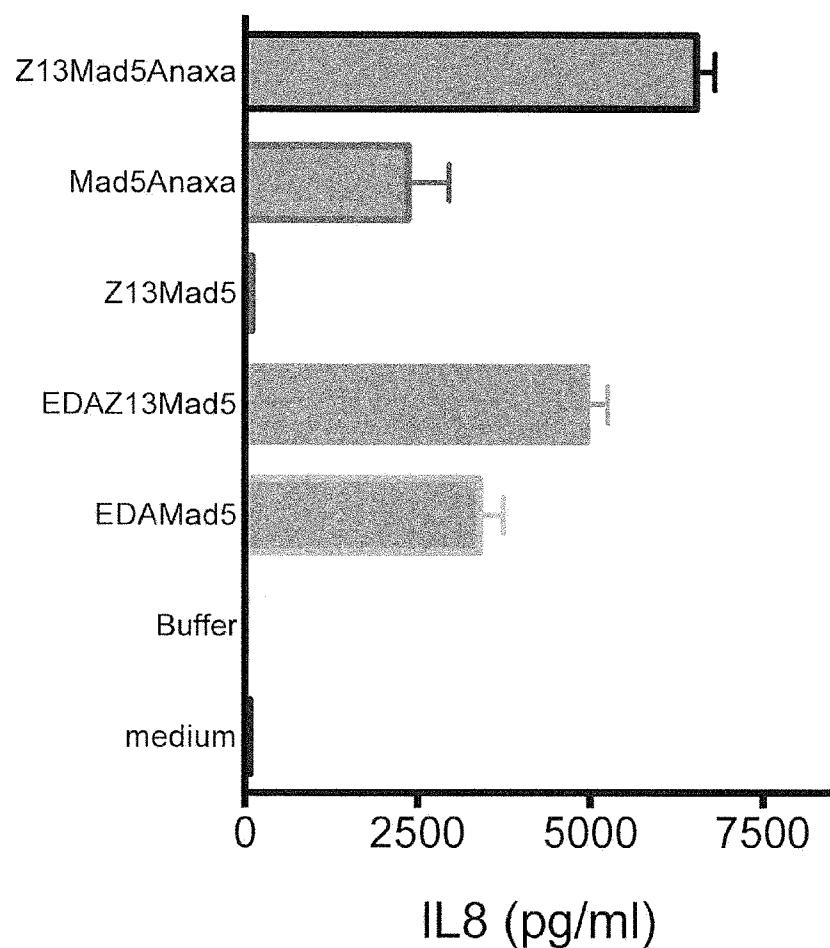


Fig. 31

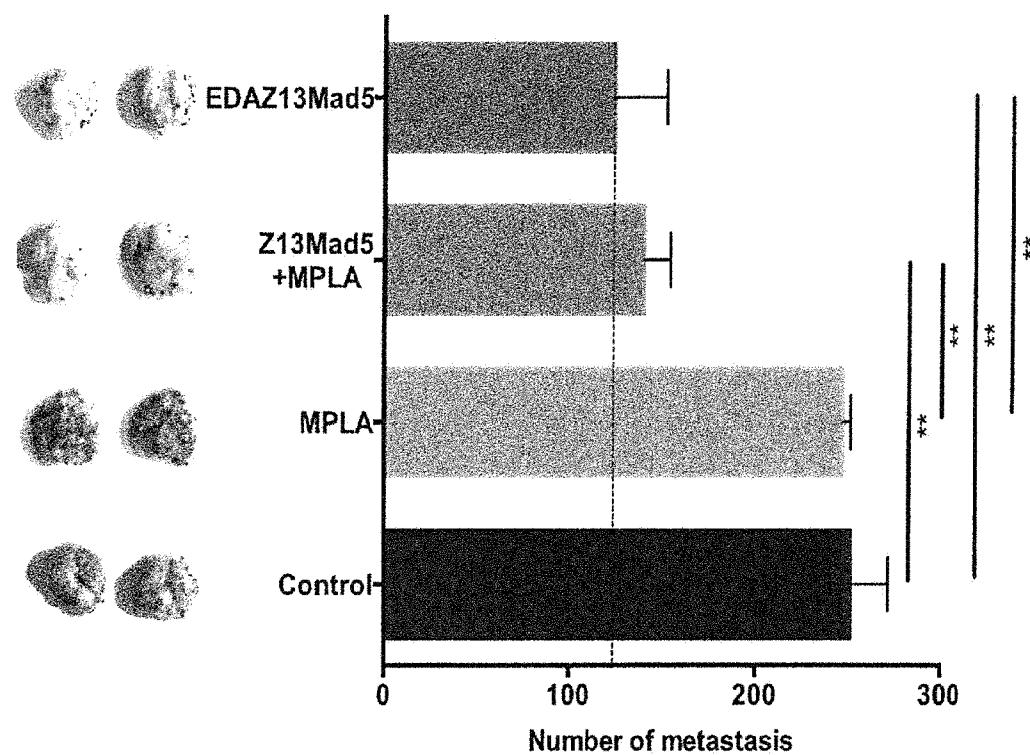


Fig. 32

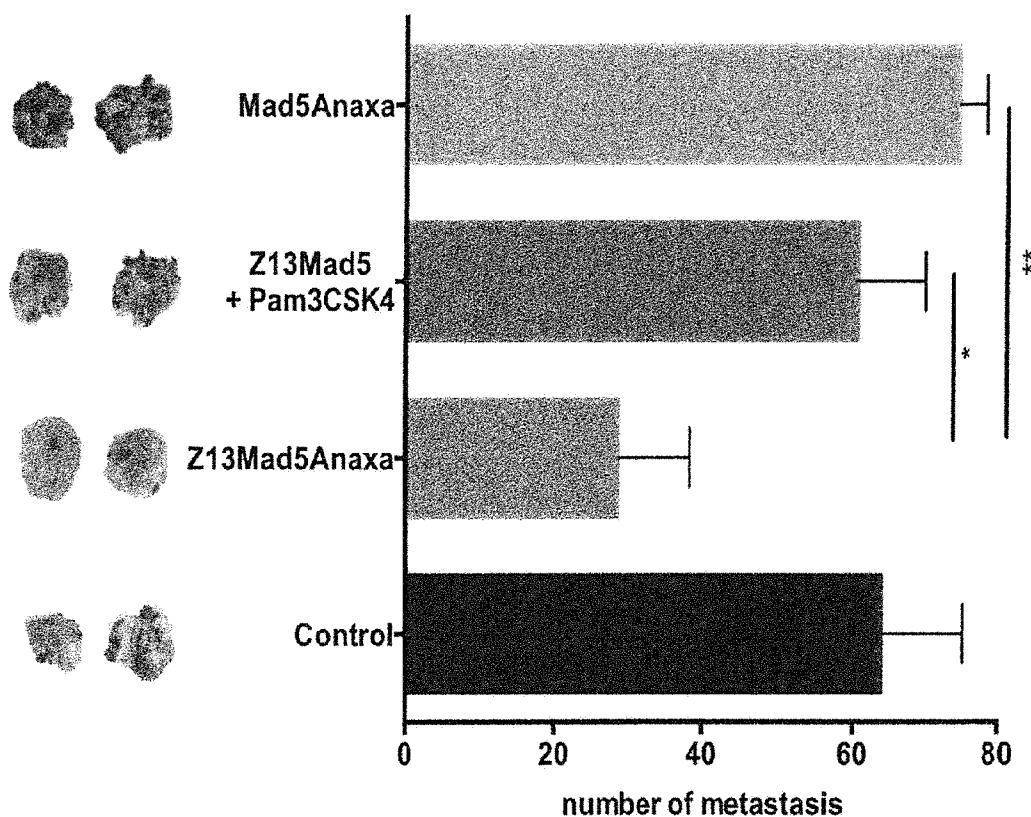


Fig. 33

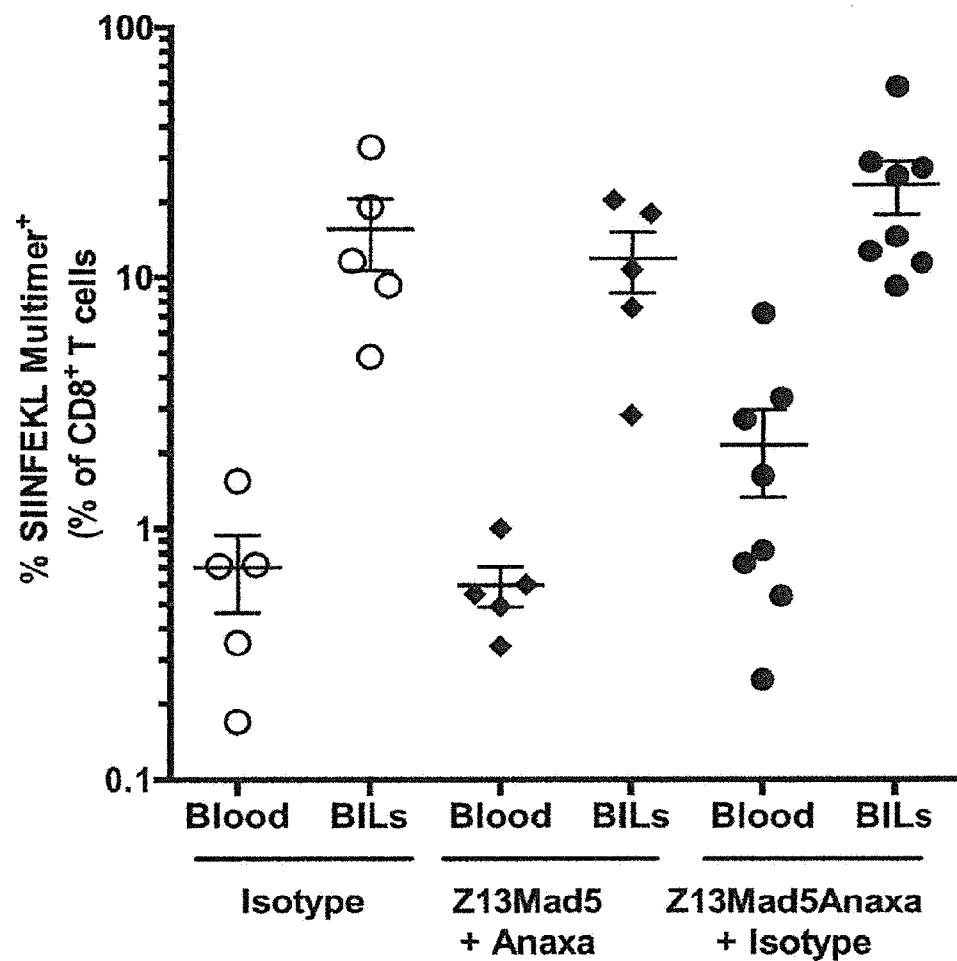


Fig. 34

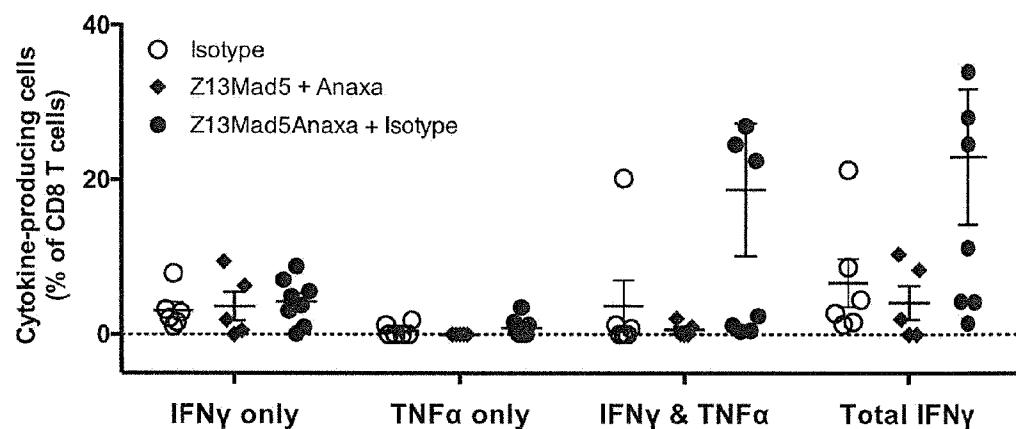


Fig. 35

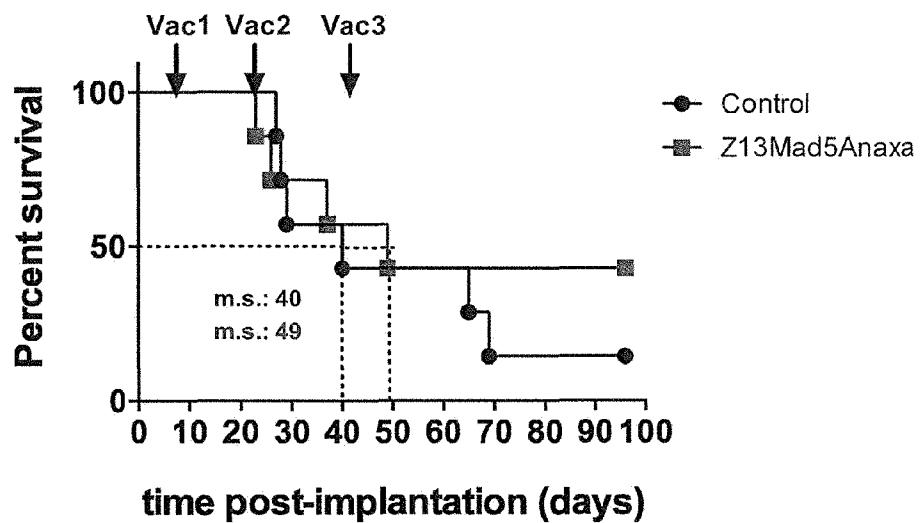
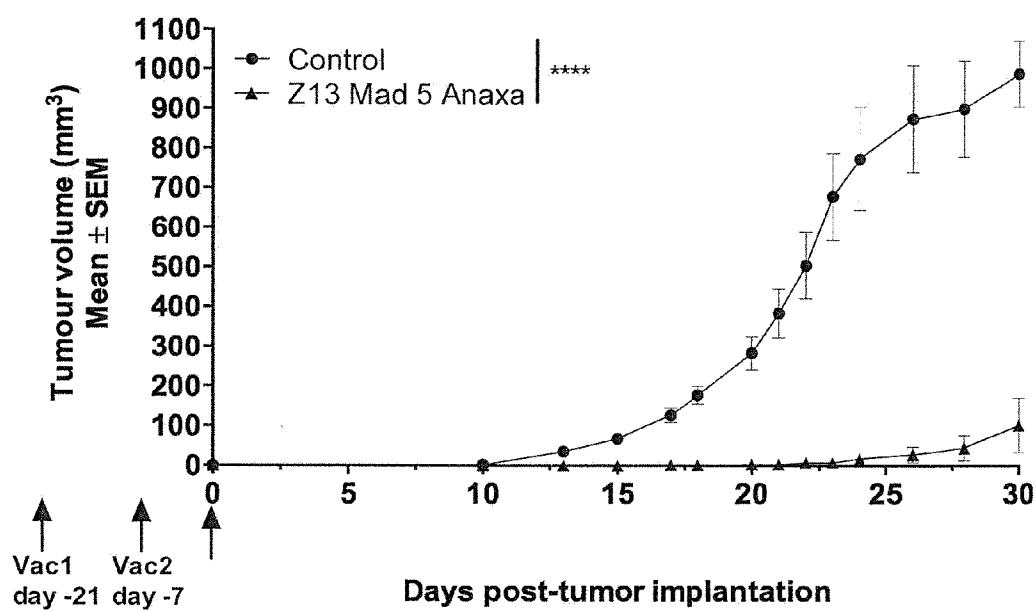


Fig. 36

A



B

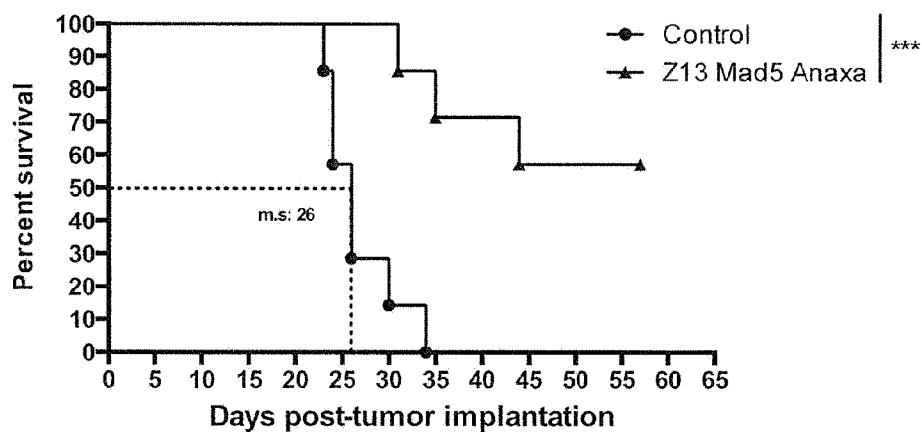
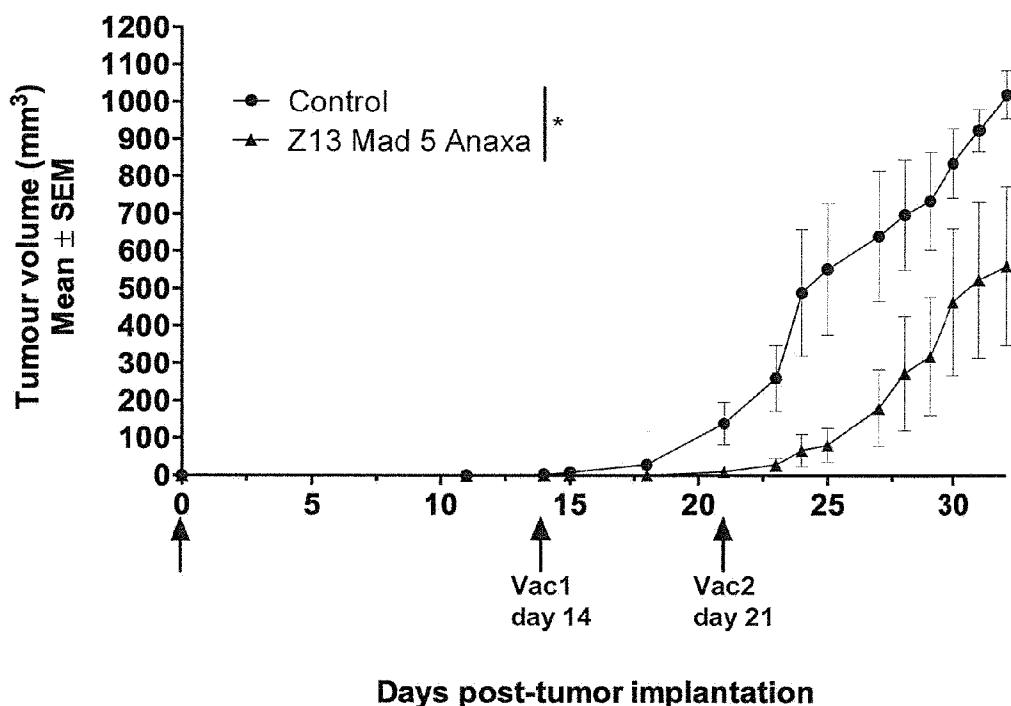


Fig. 37

A



B

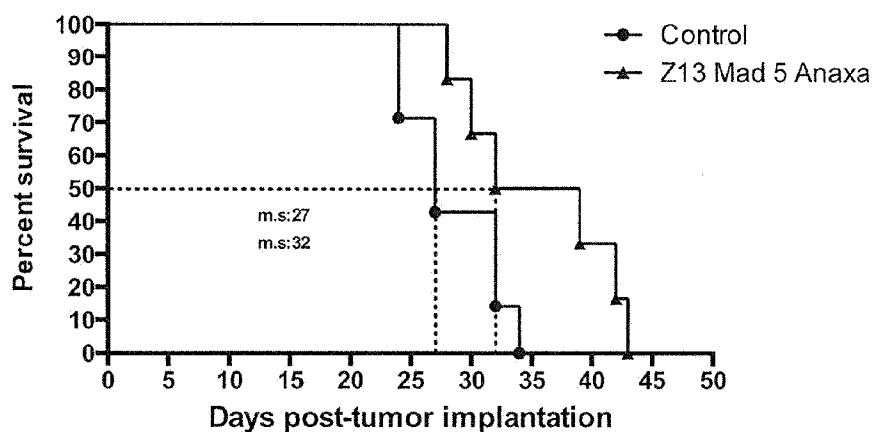
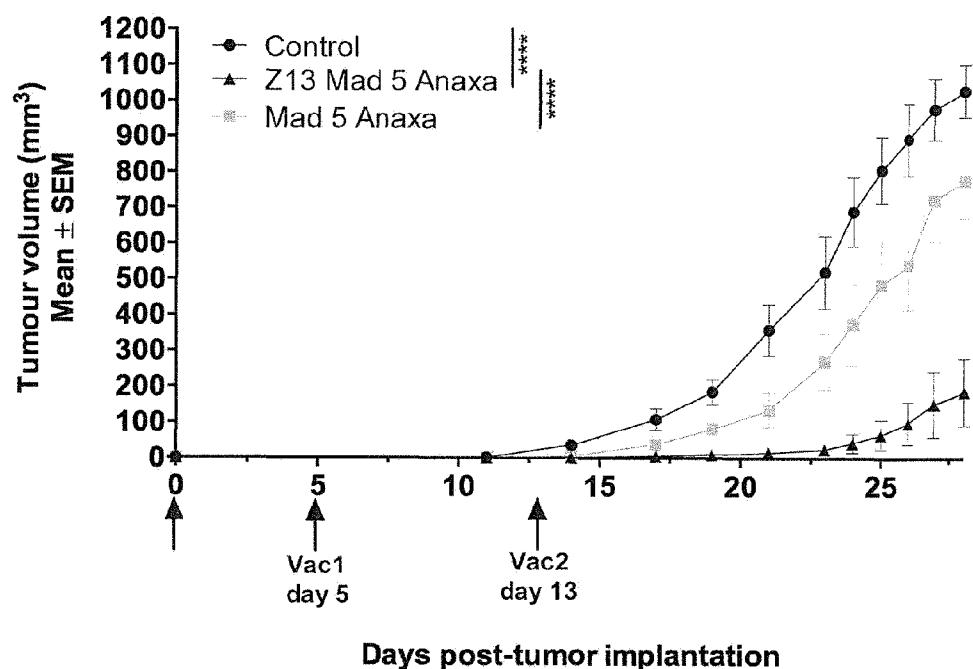


Fig. 38

A



B

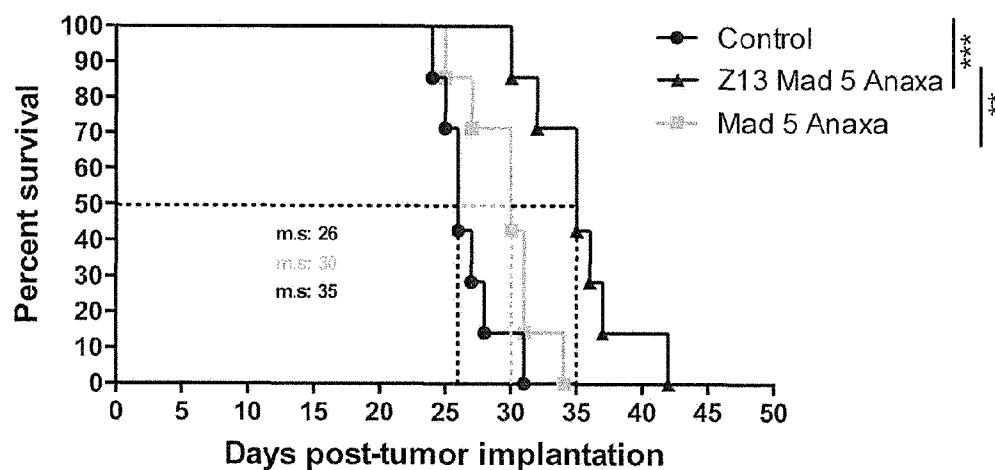
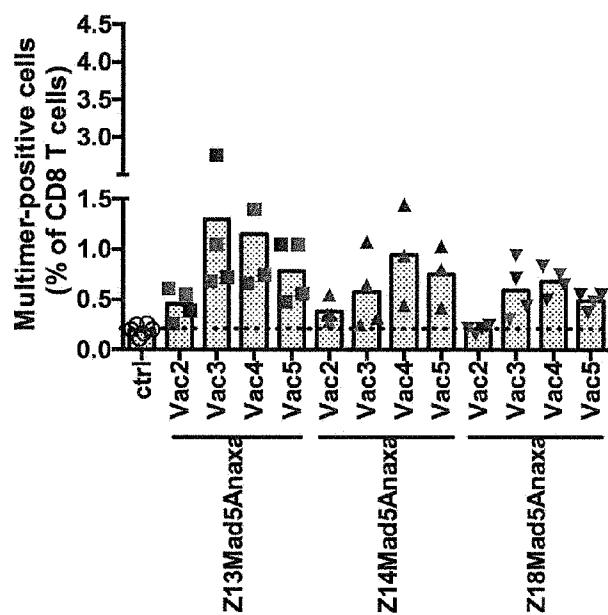


Fig. 39

A

2 nmol



B

0.5 nmol

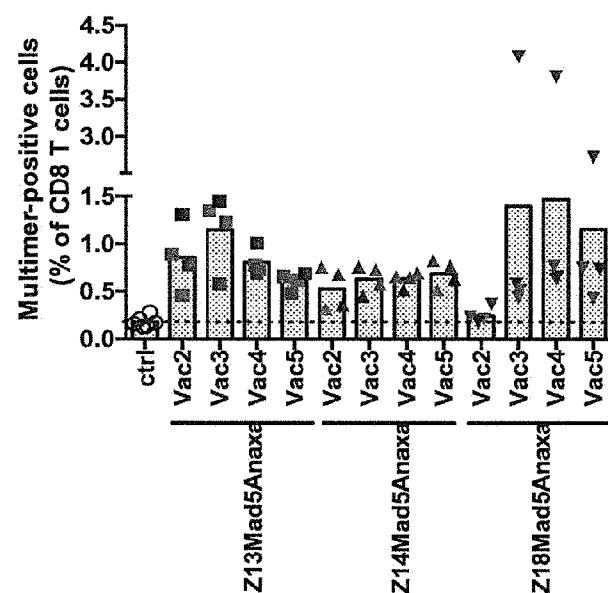


Fig. 40

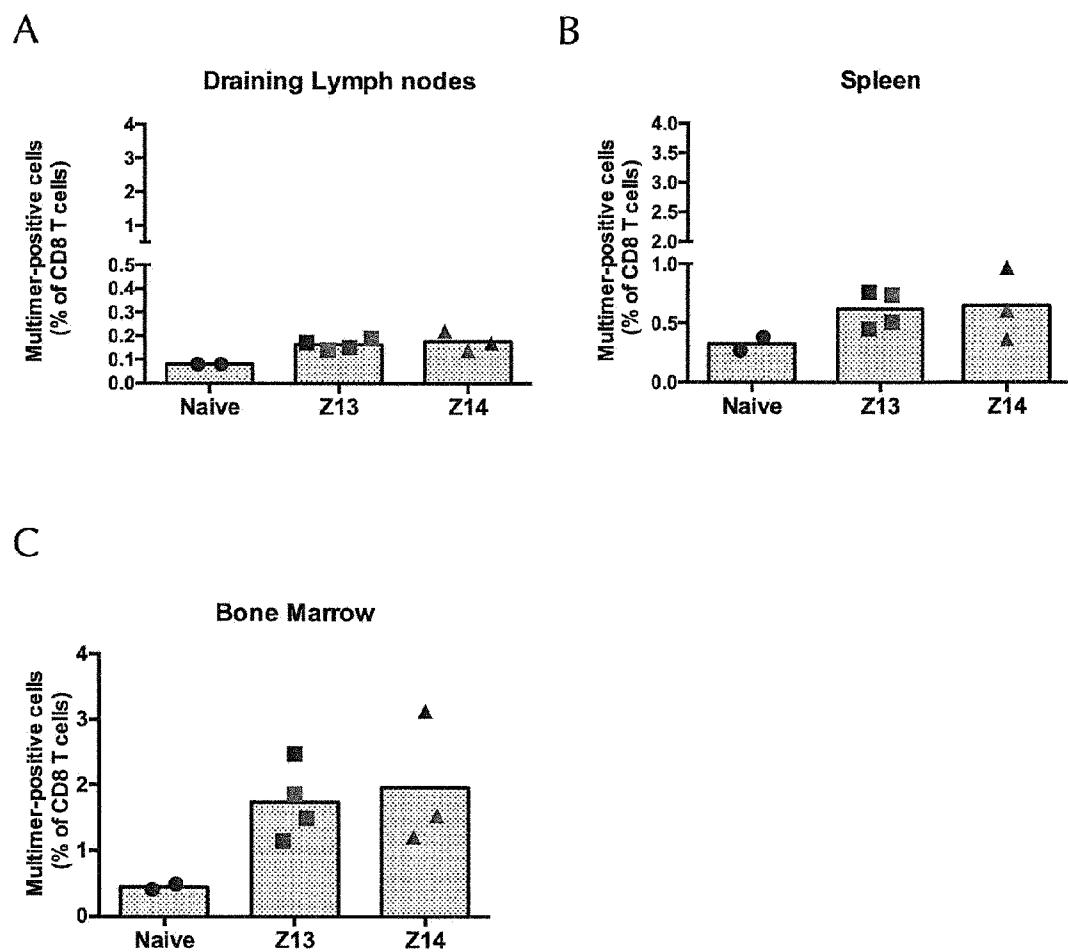
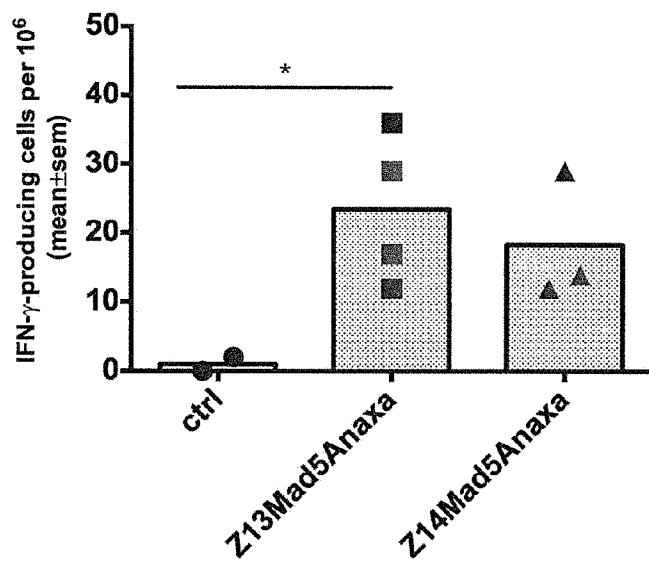


Fig. 41

A



B

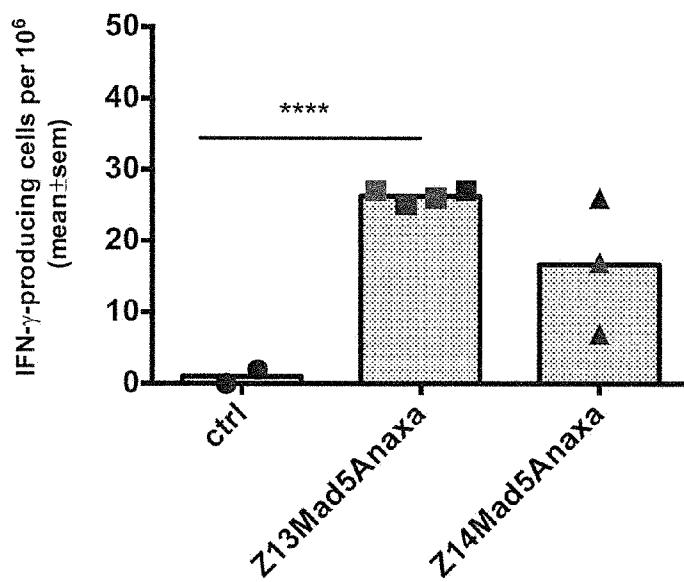


Fig. 42

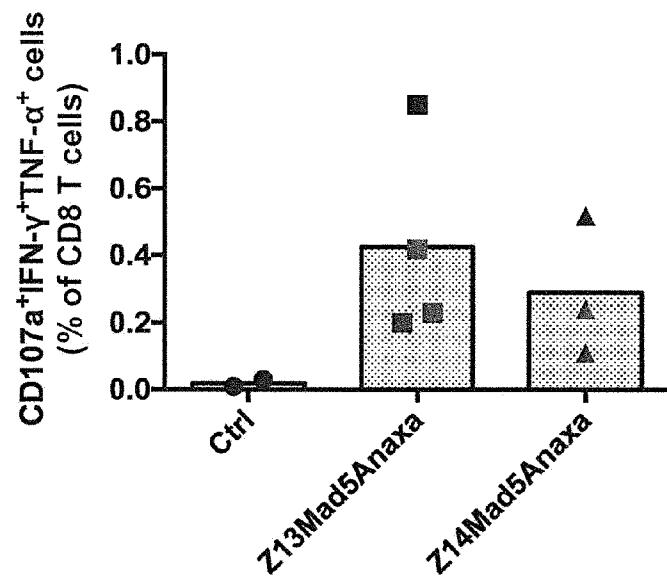
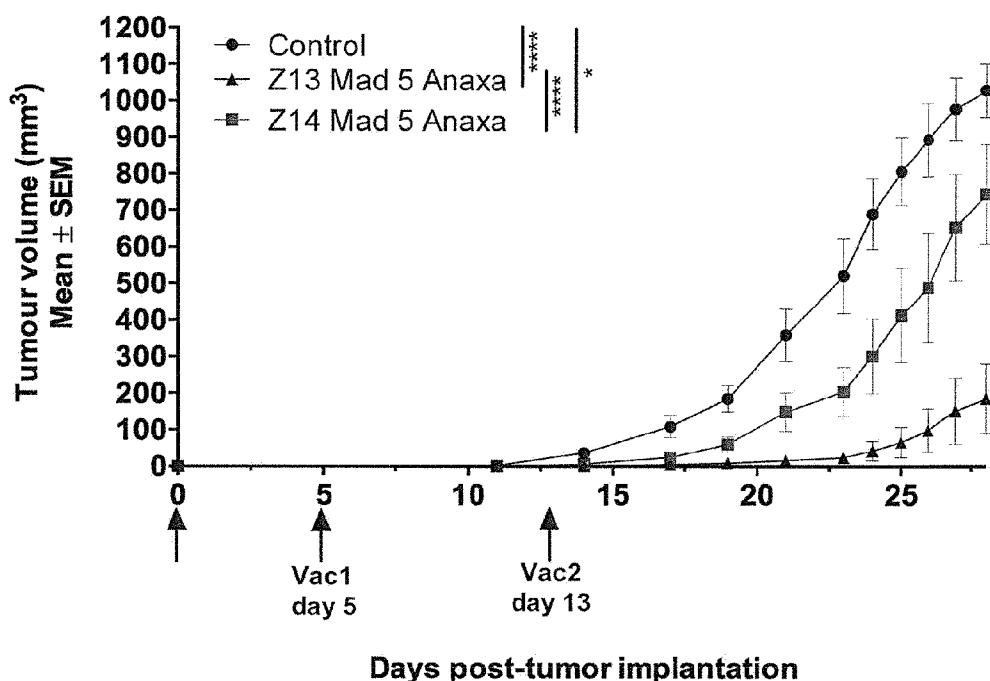


Fig. 43

A



B

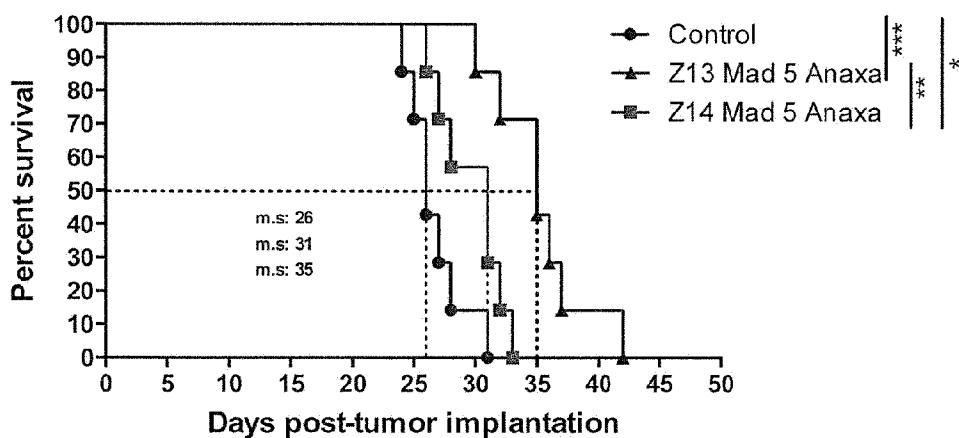
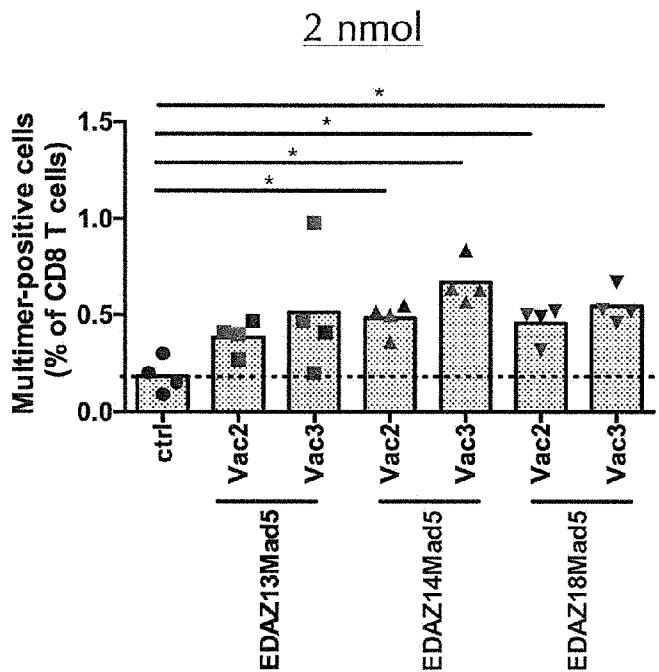


Fig. 44

A



B

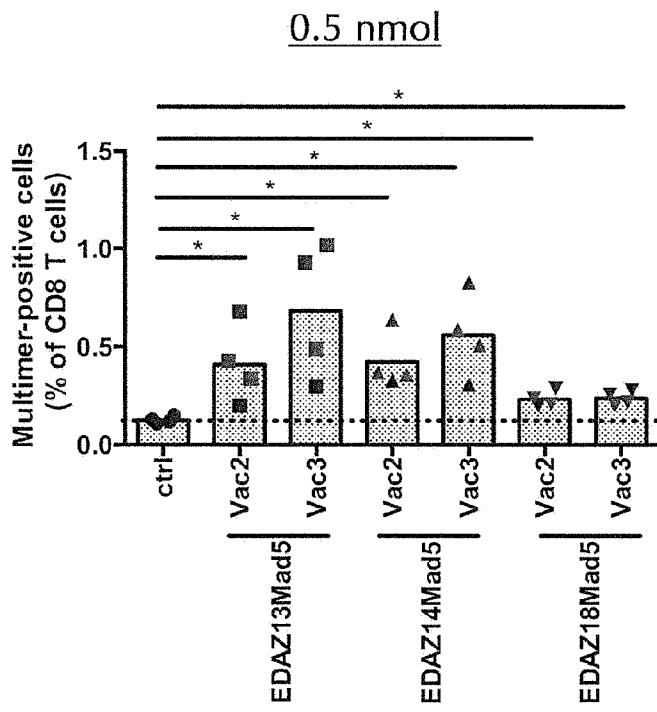
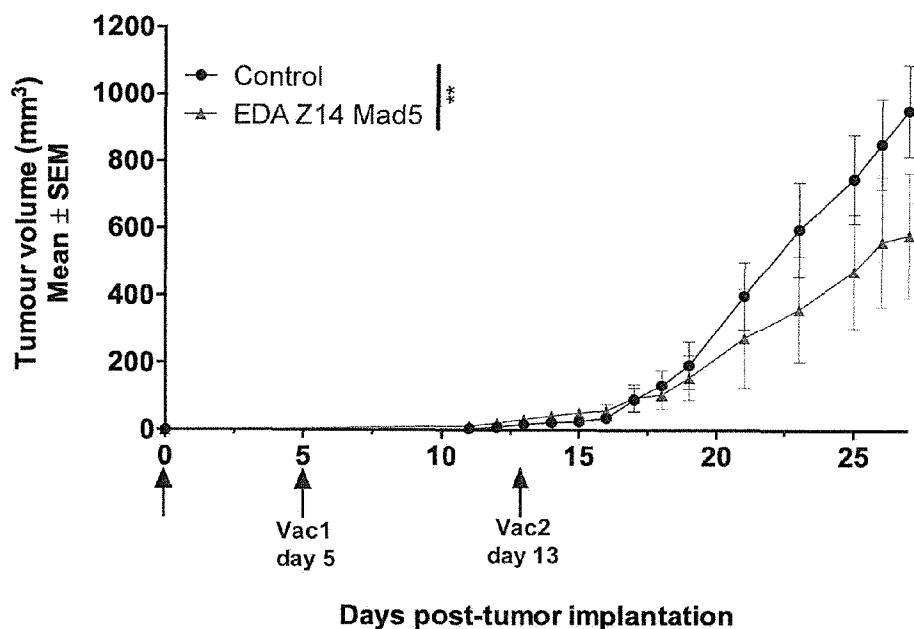


Fig. 45

A



B

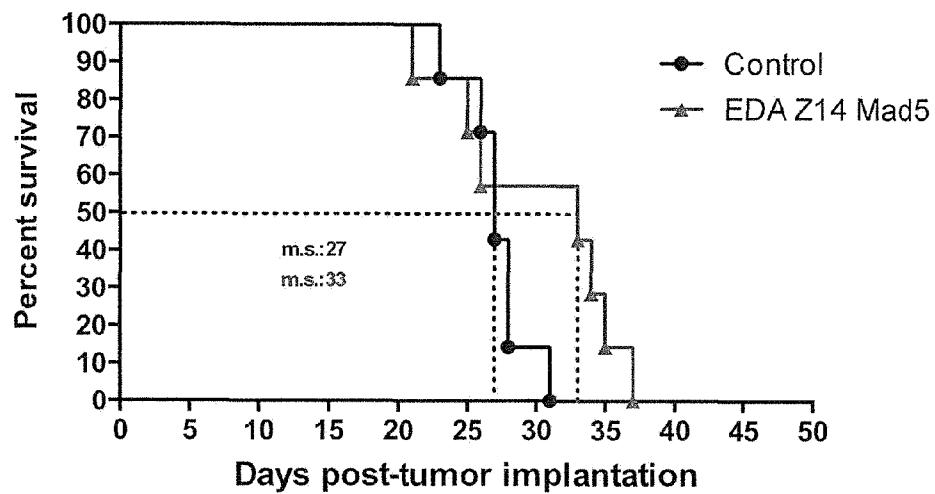
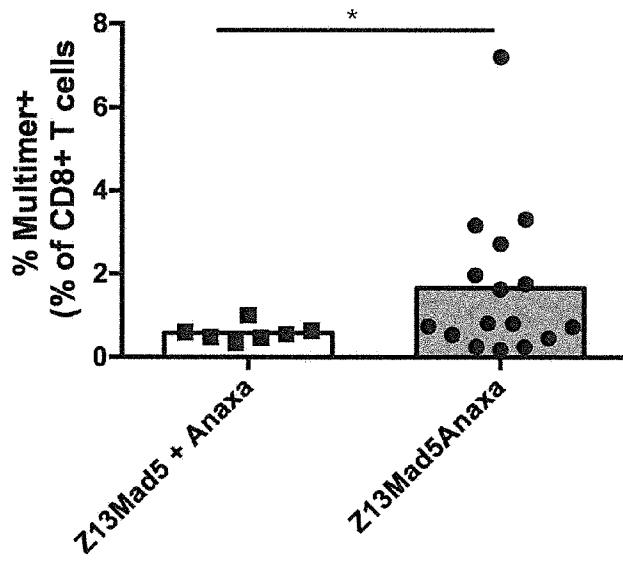


Fig. 46

A



B

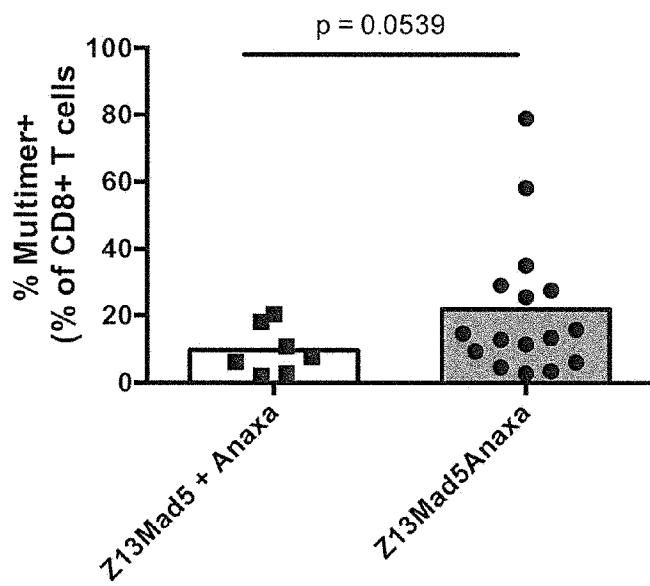


Fig. 47

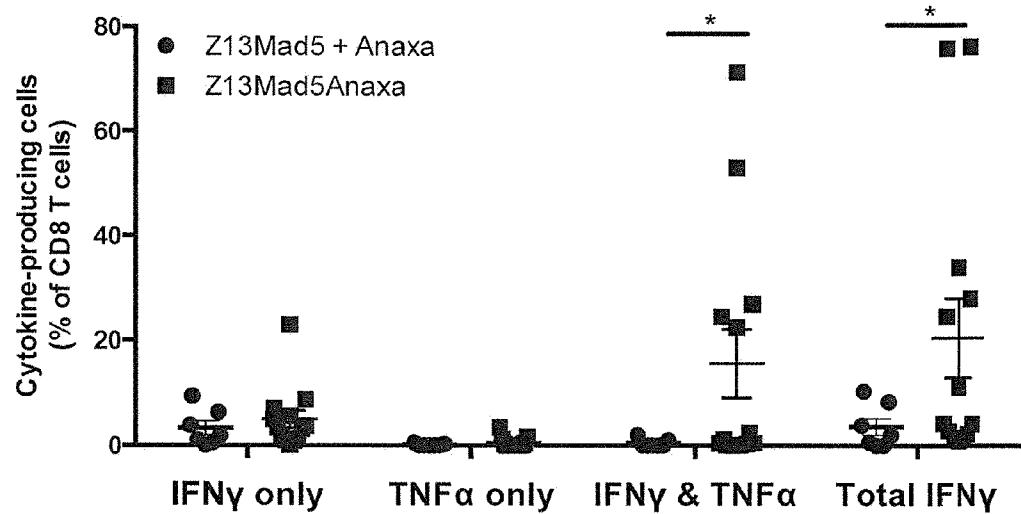
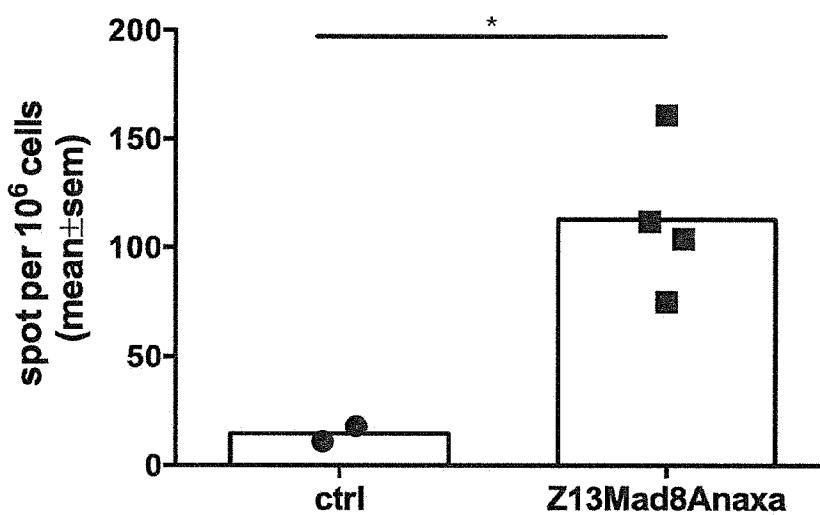


Fig. 48

A

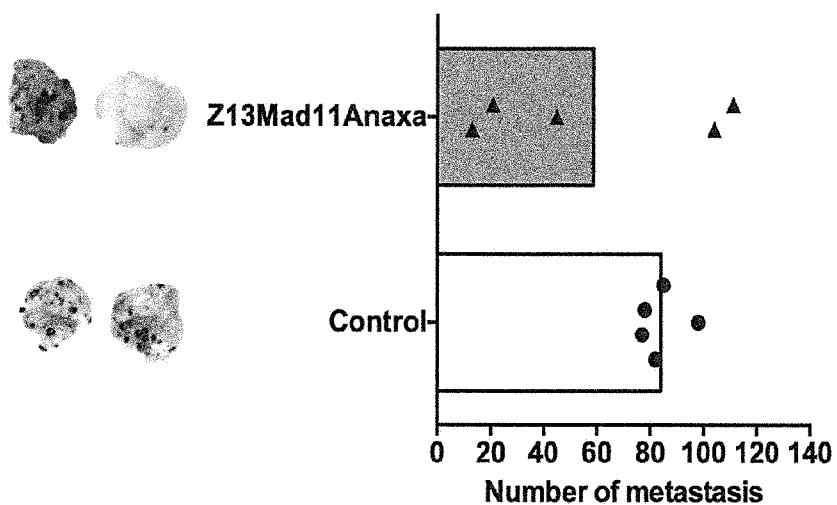


B



Fig. 49

A



B

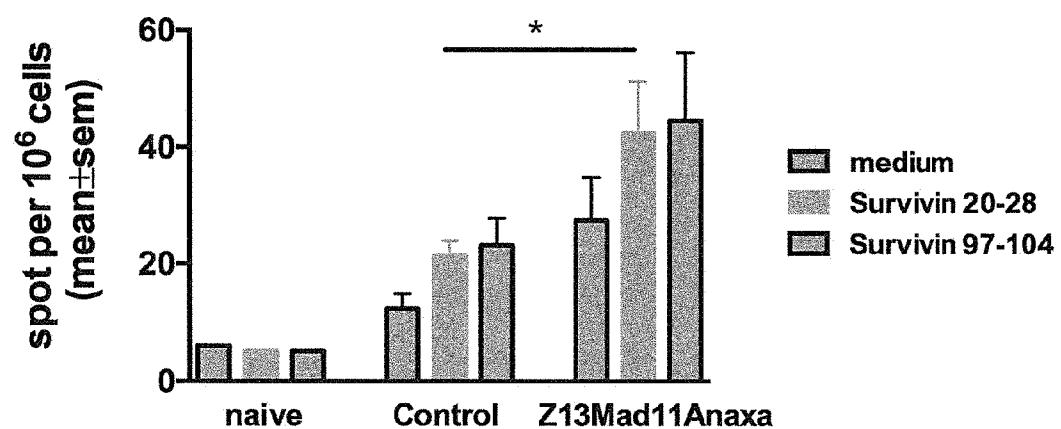


Fig. 50

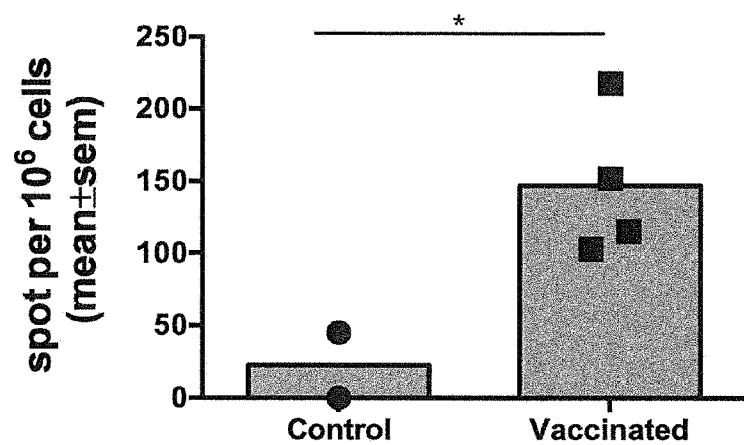


Fig. 51

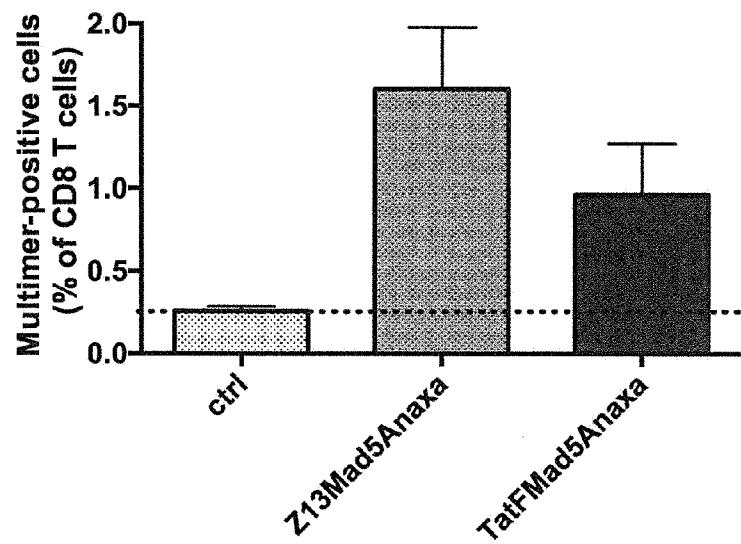


Fig. 52

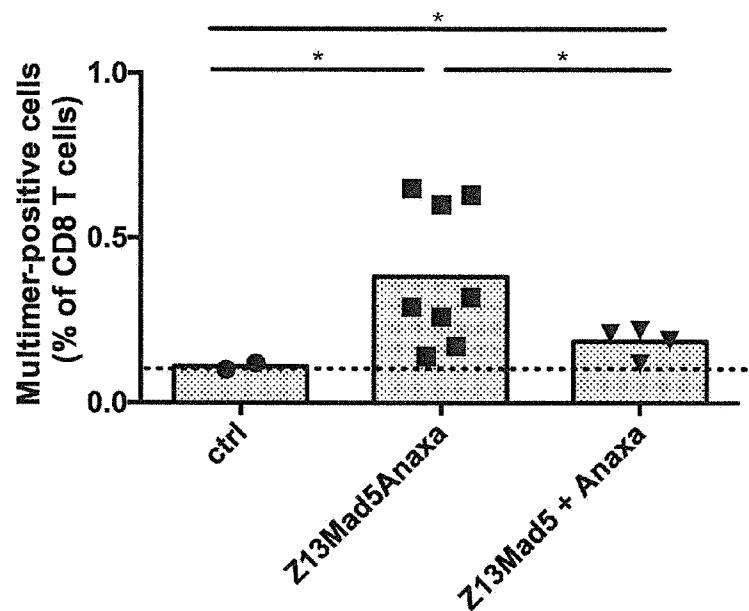


Fig. 53

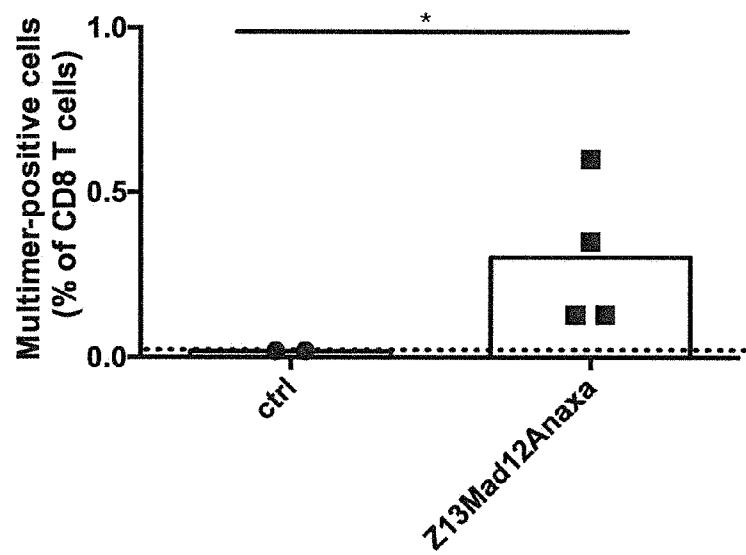


Fig. 54

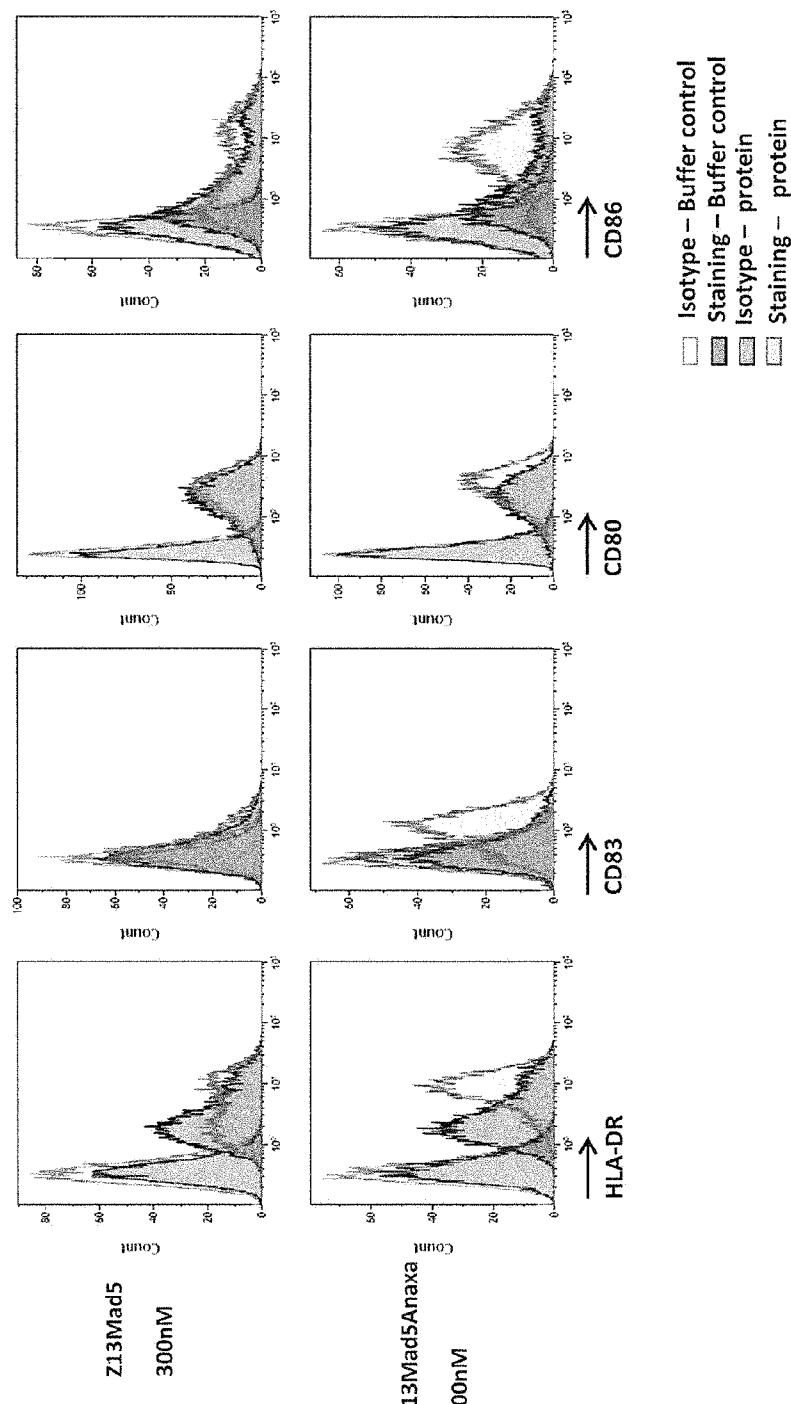


Fig. 55

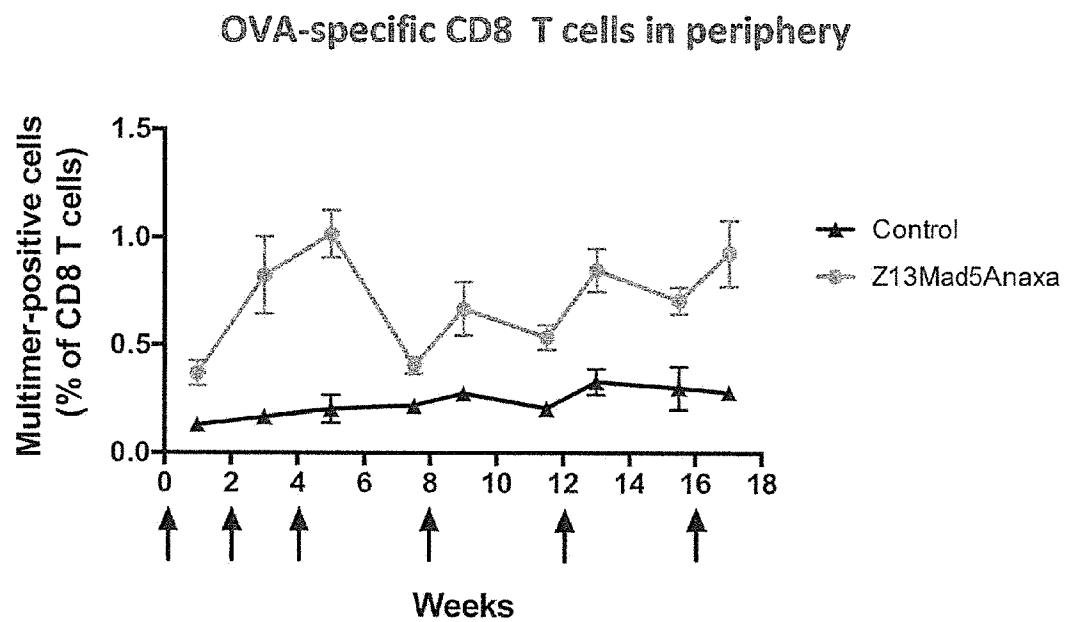
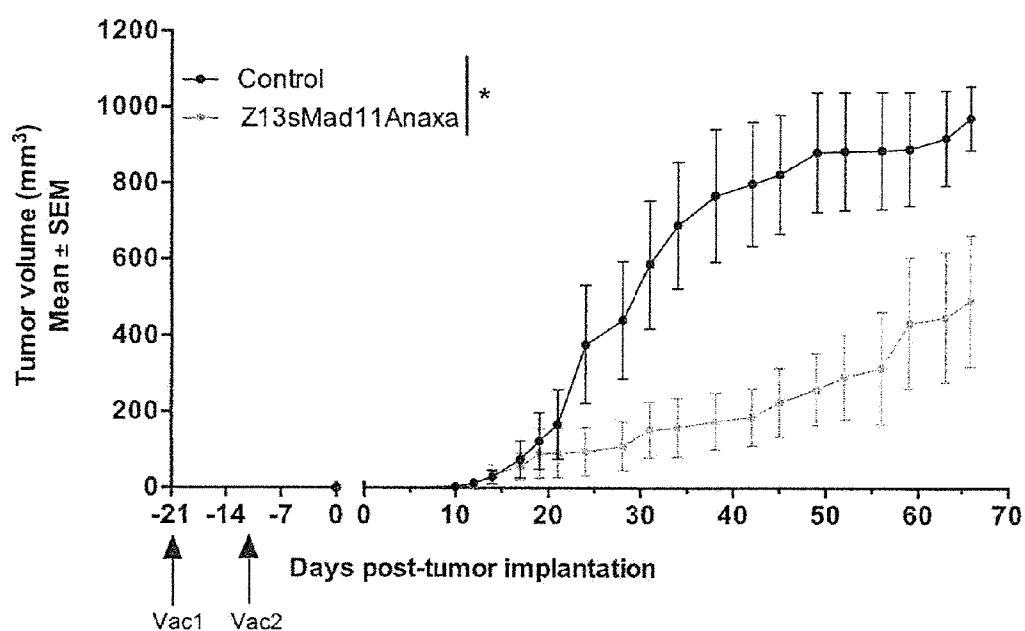


Fig. 56

A



B

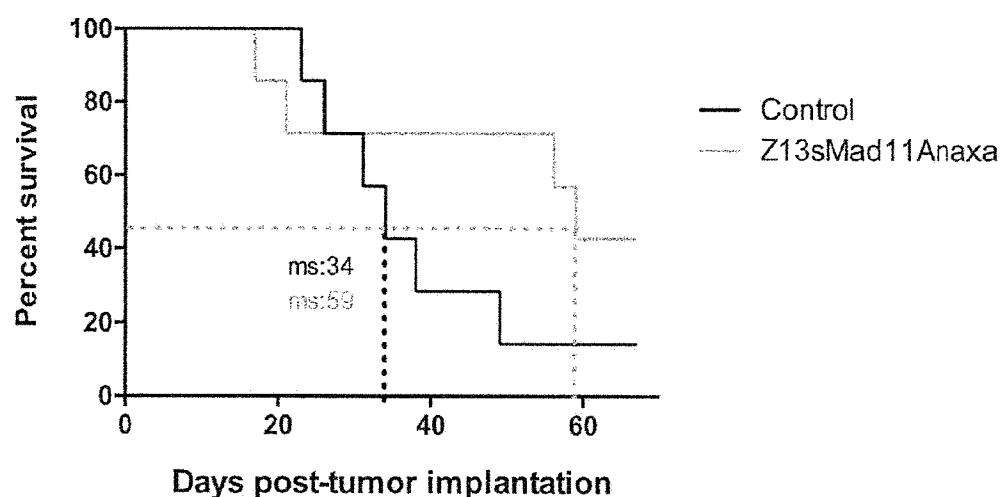


Fig. 57

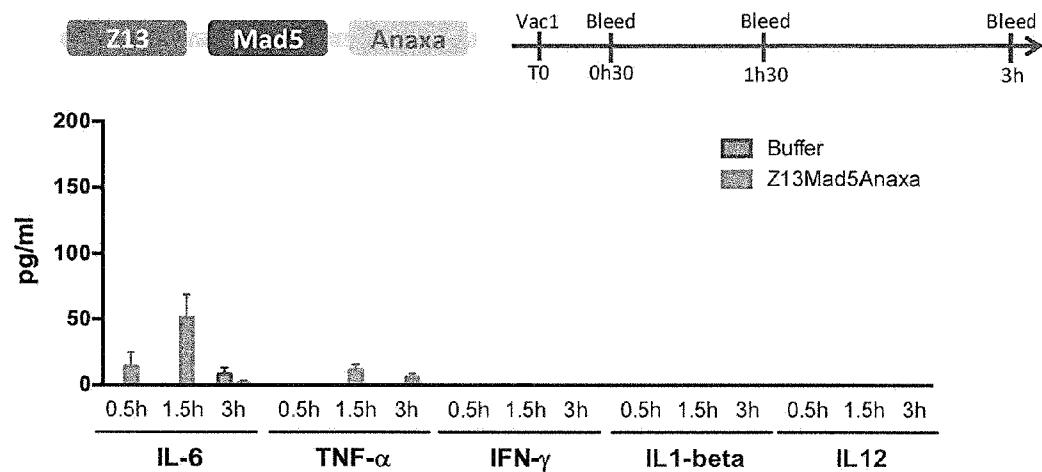


Fig. 58

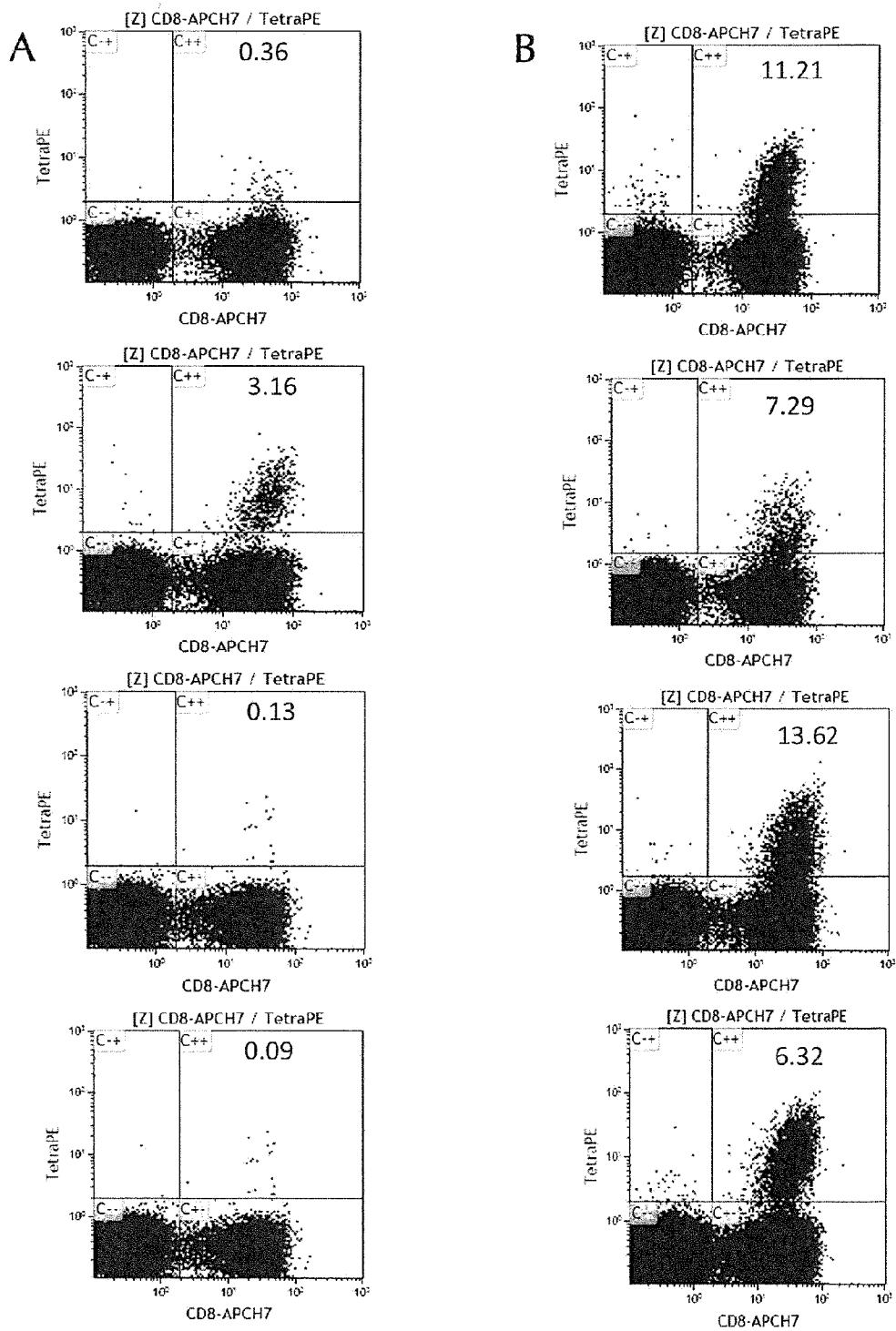


Fig. 59

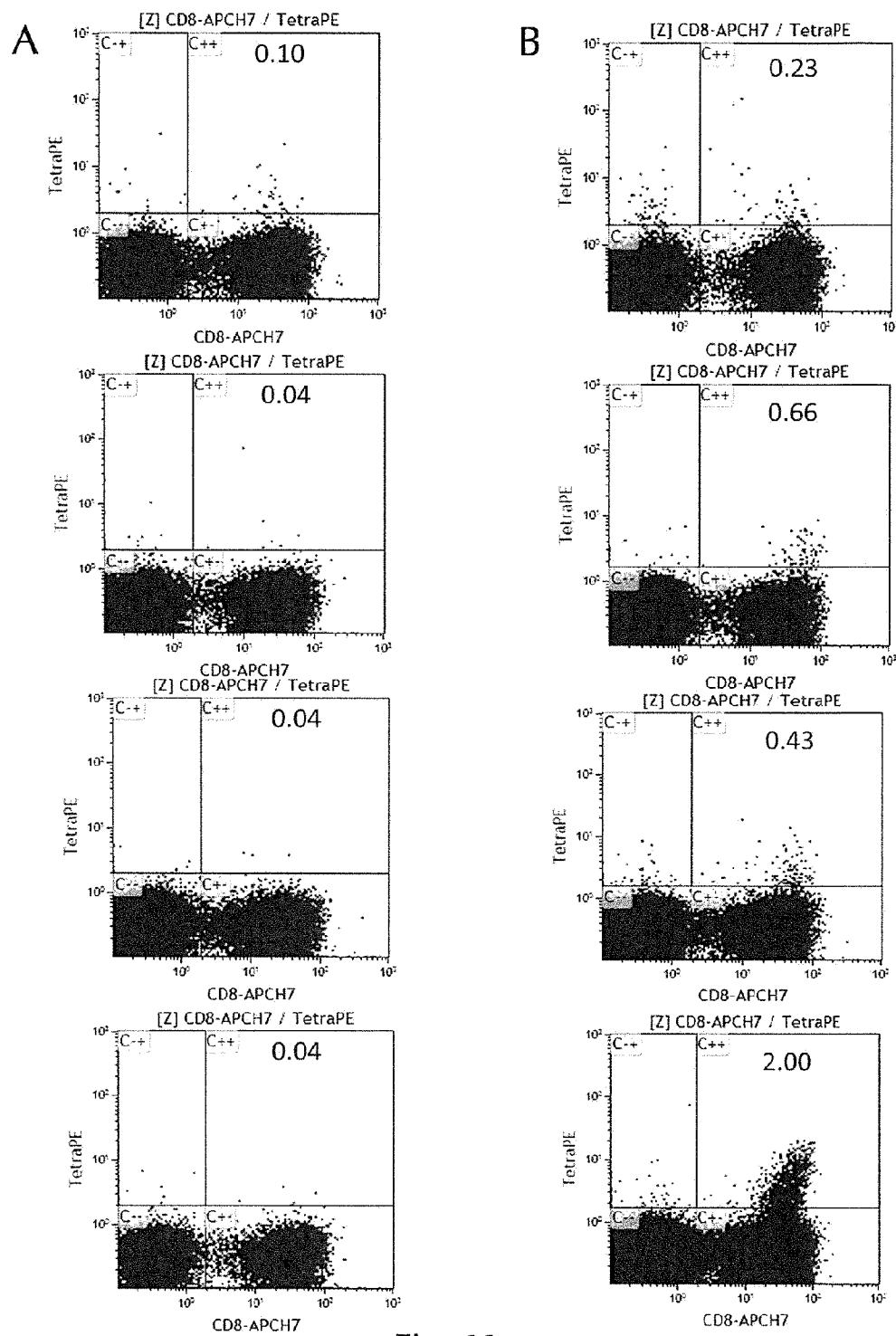
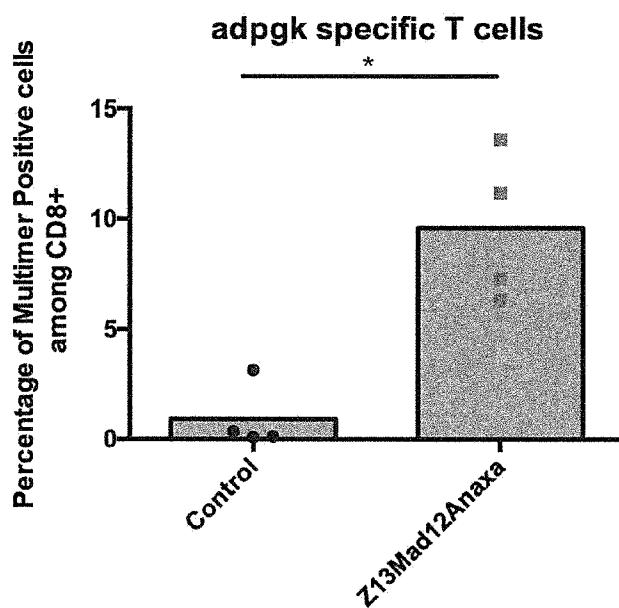


Fig. 60

A



B

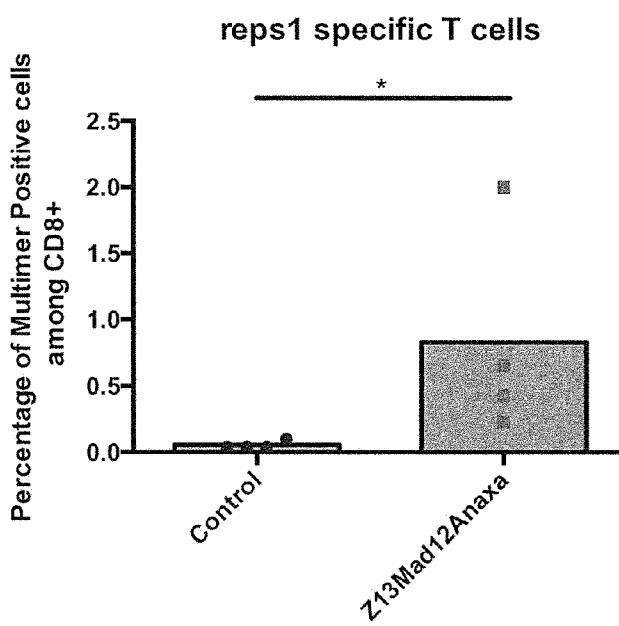


Fig. 61

## ATP110

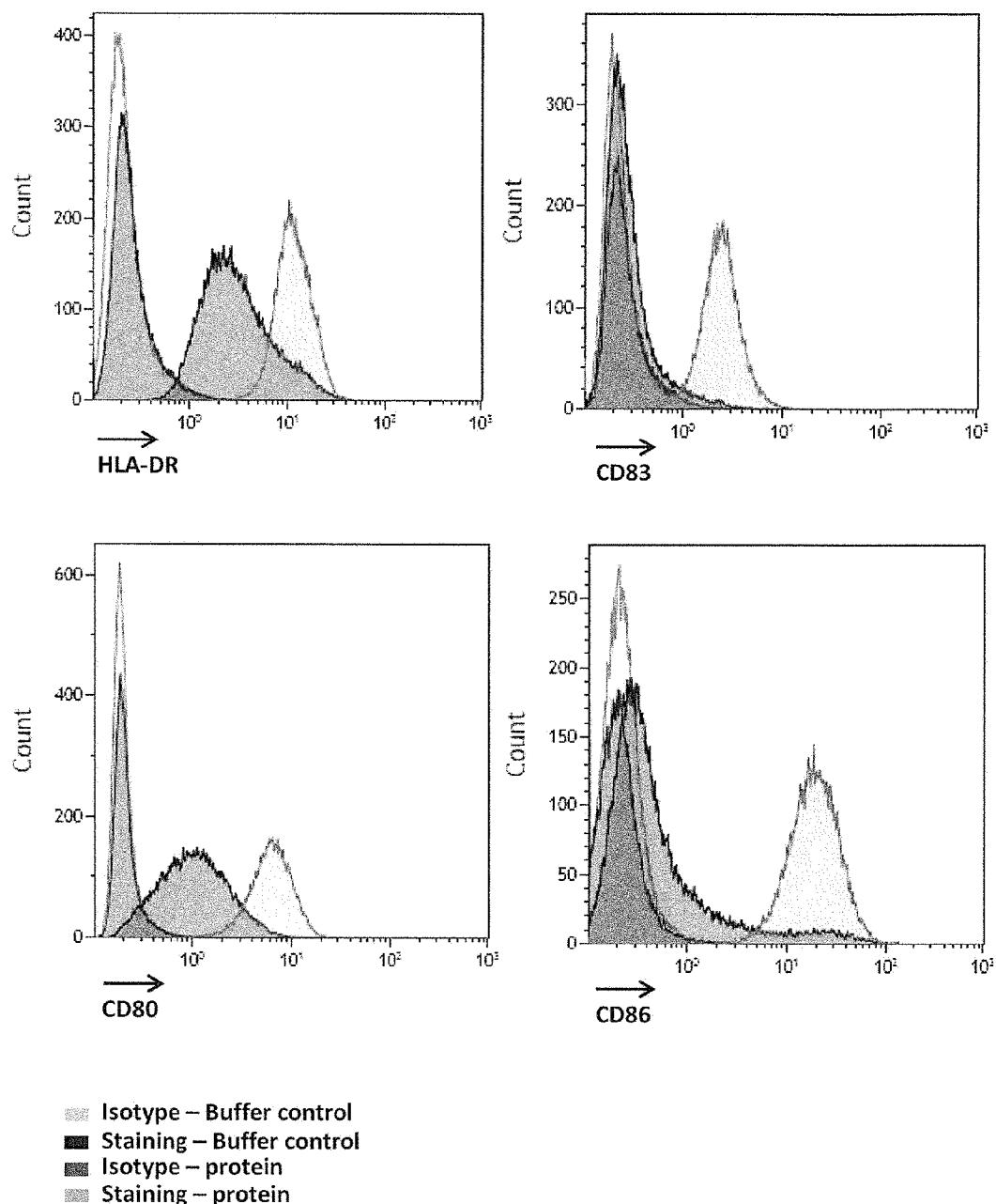


Fig. 62

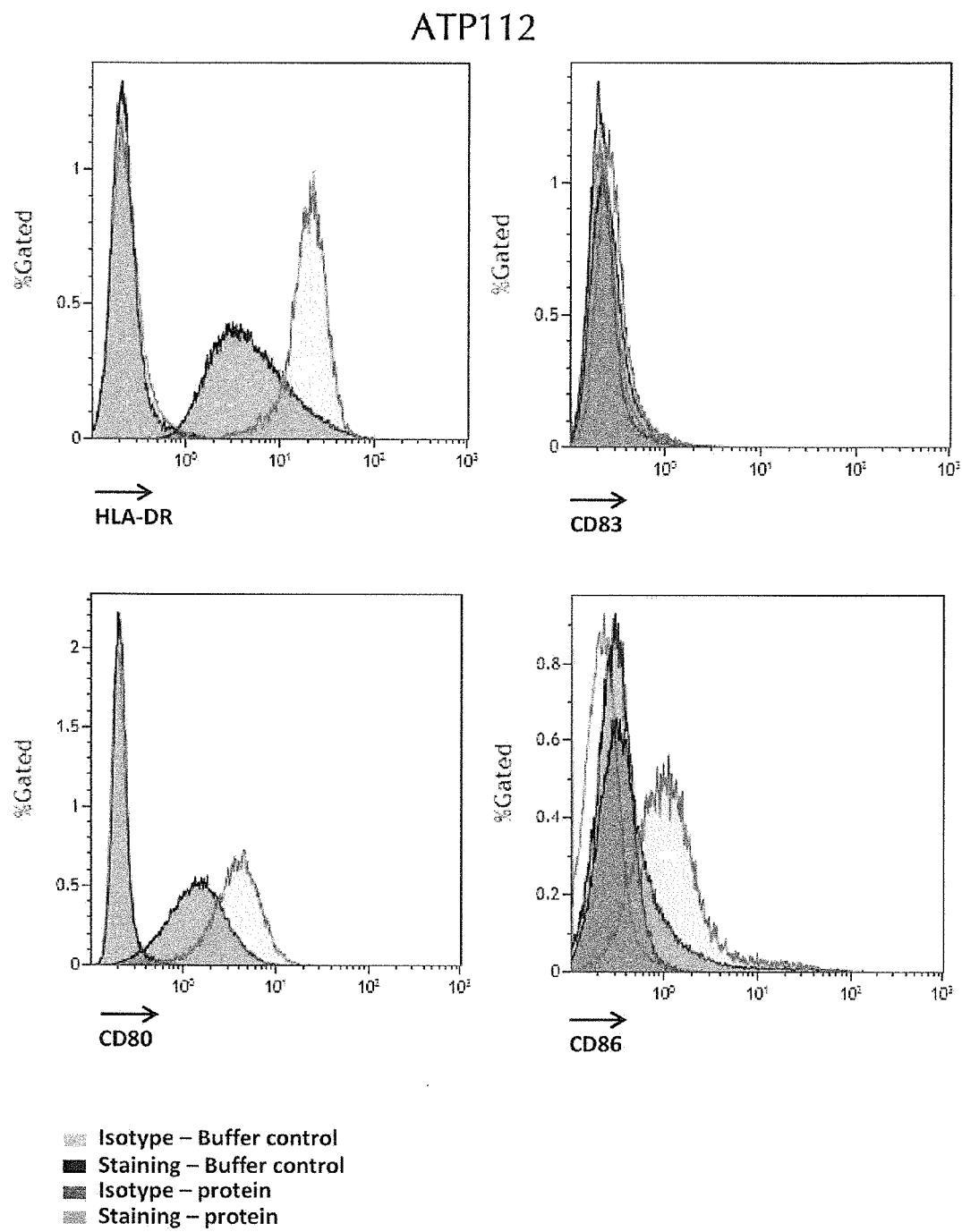


Fig. 63

## ATP115

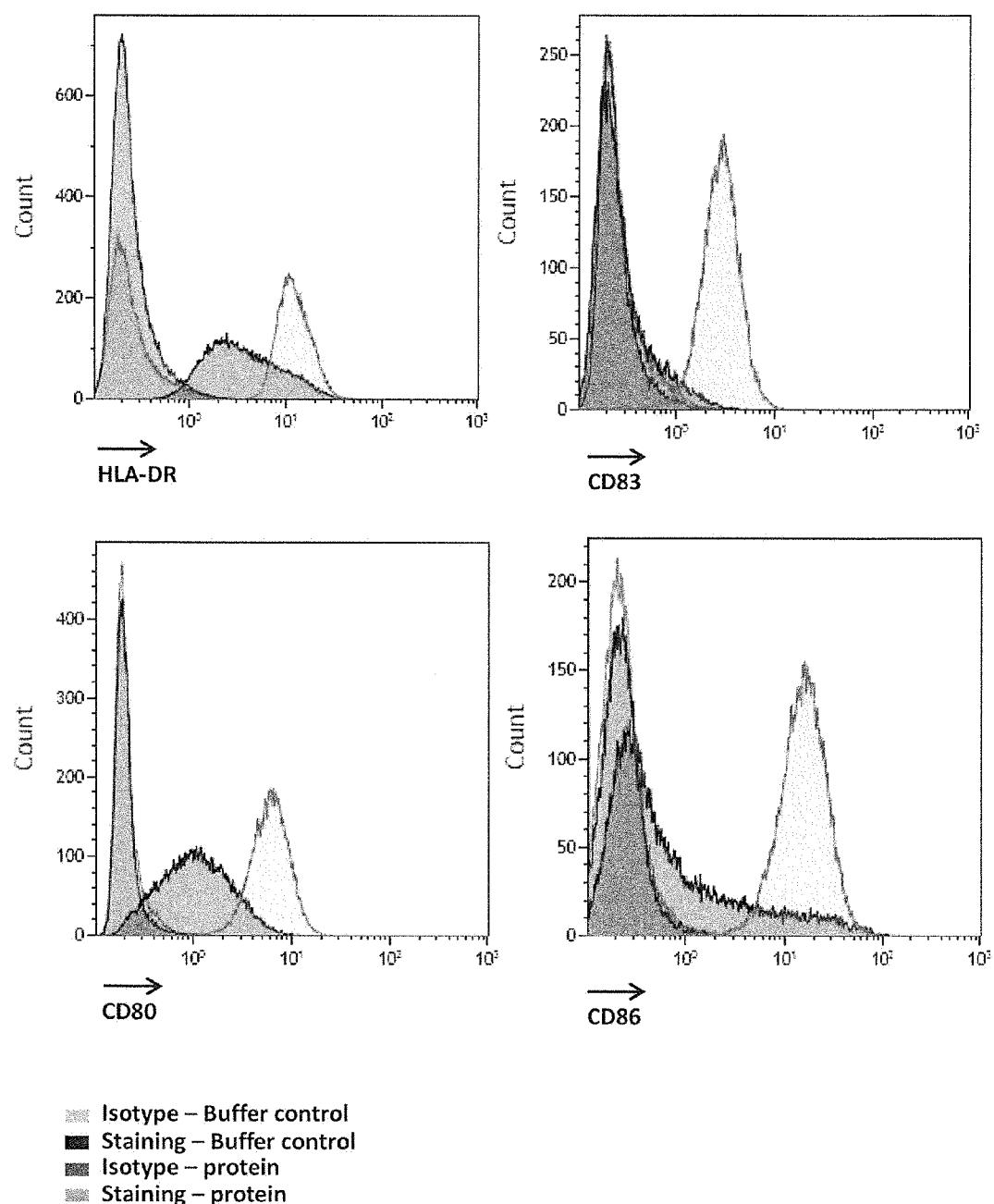


Fig. 64

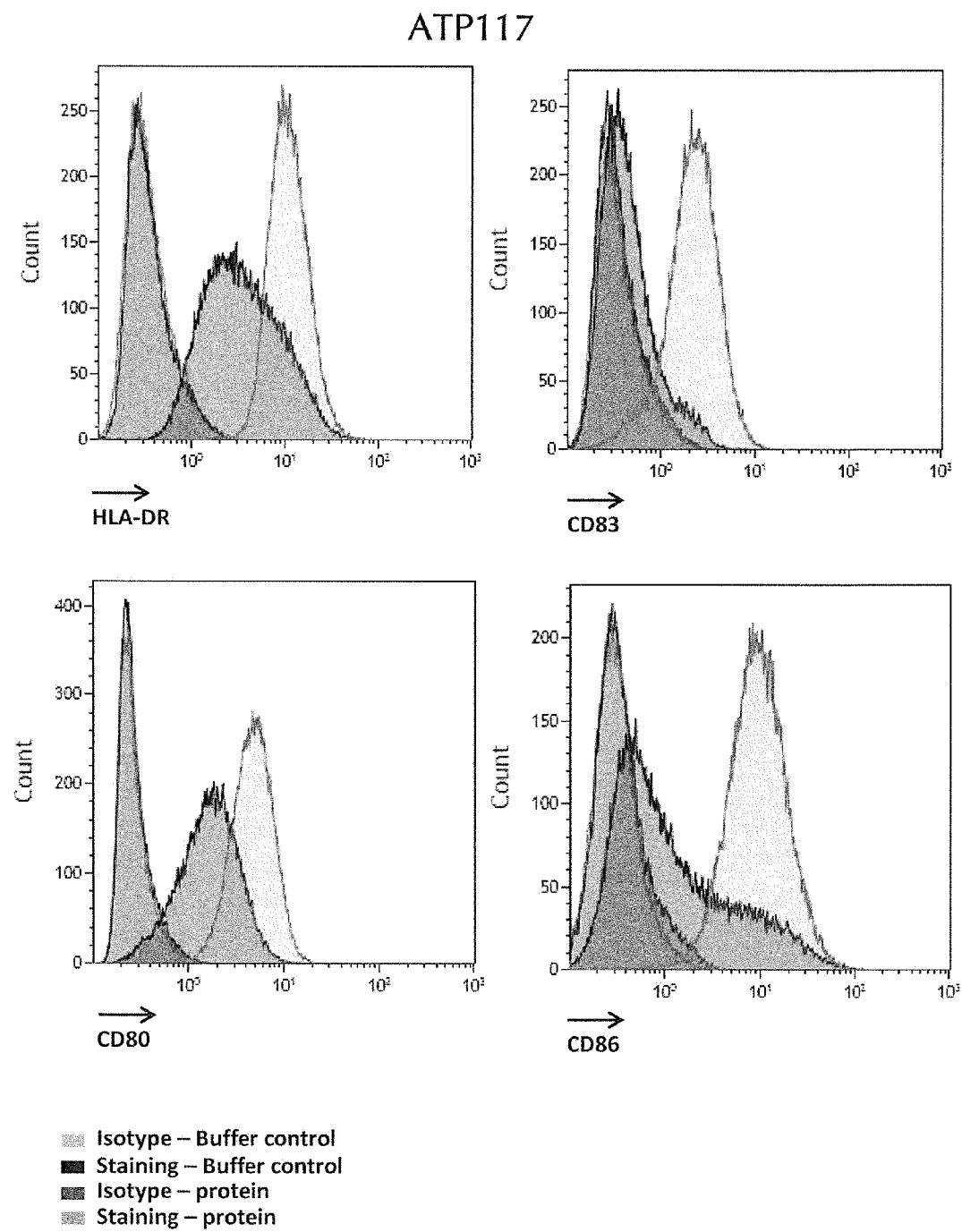


Fig. 65

## ATP118

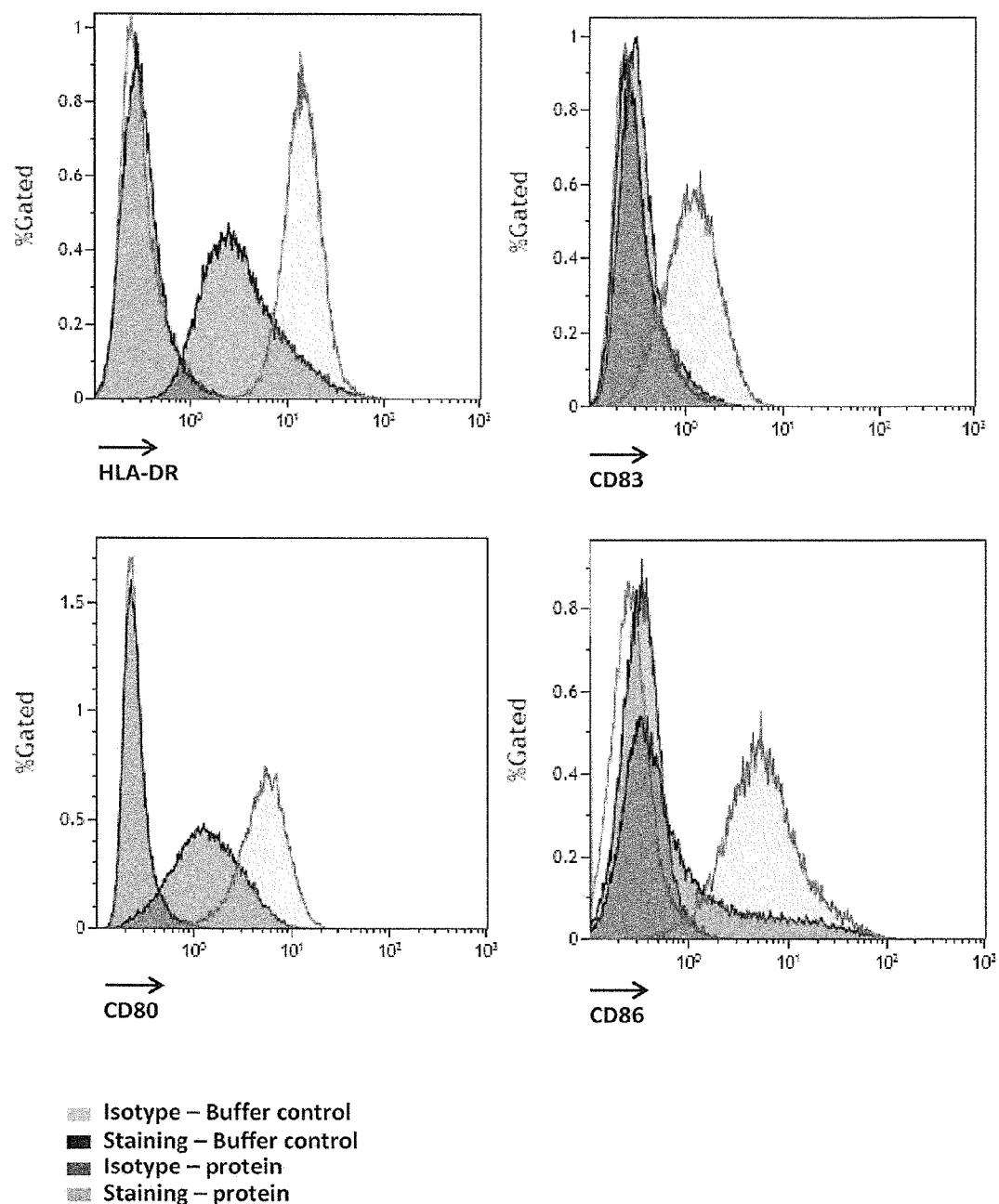


Fig. 66

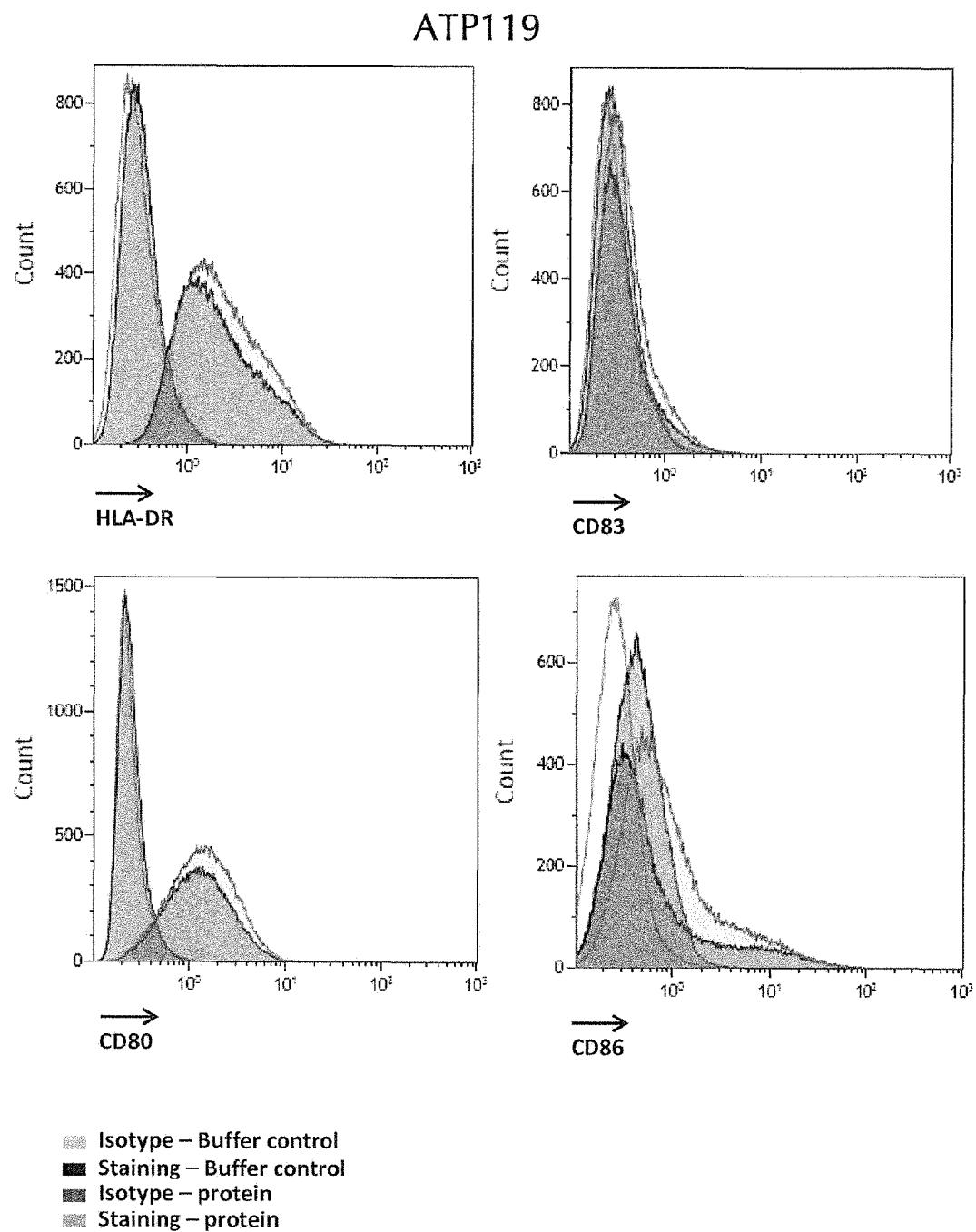


Fig. 67

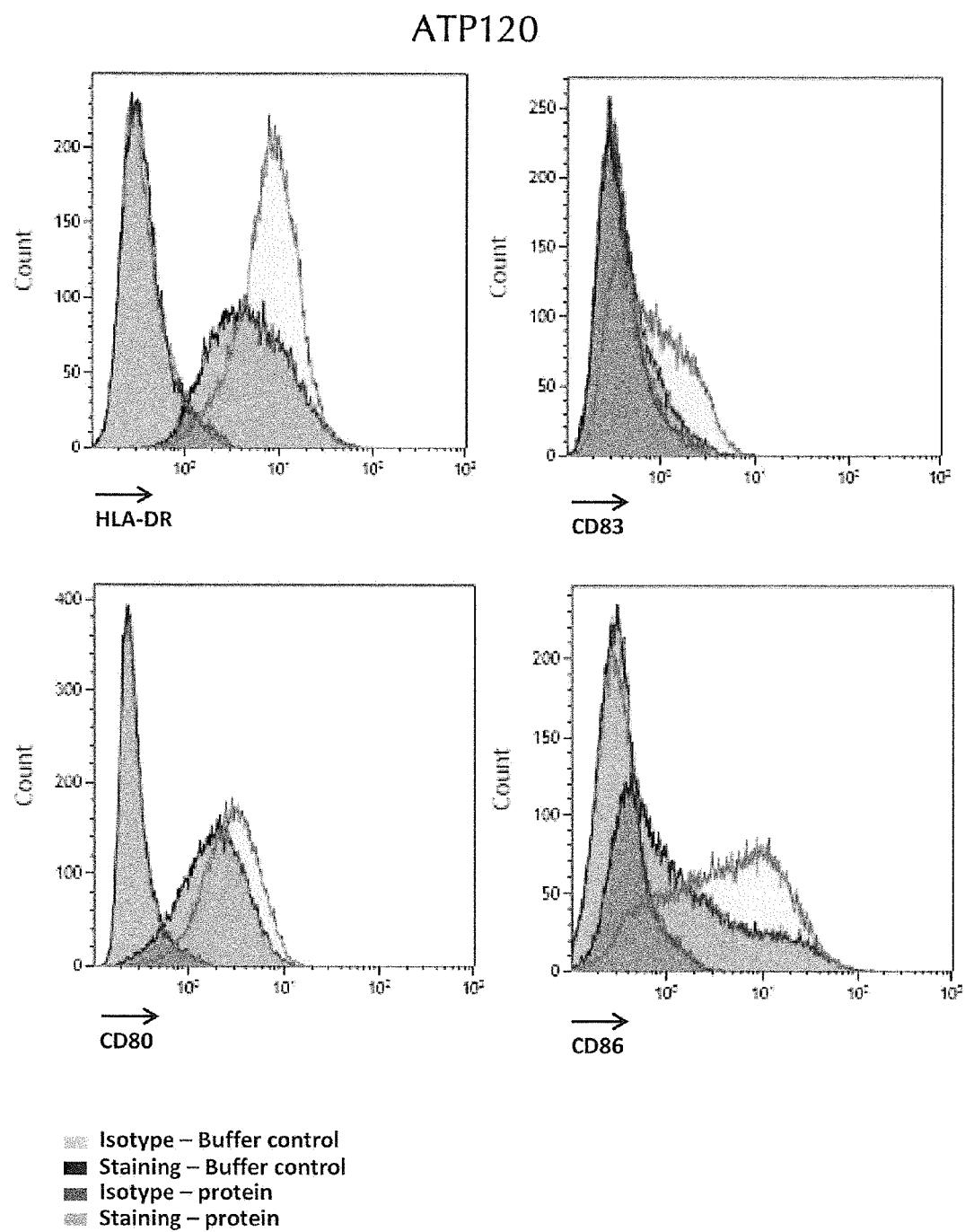


Fig. 68

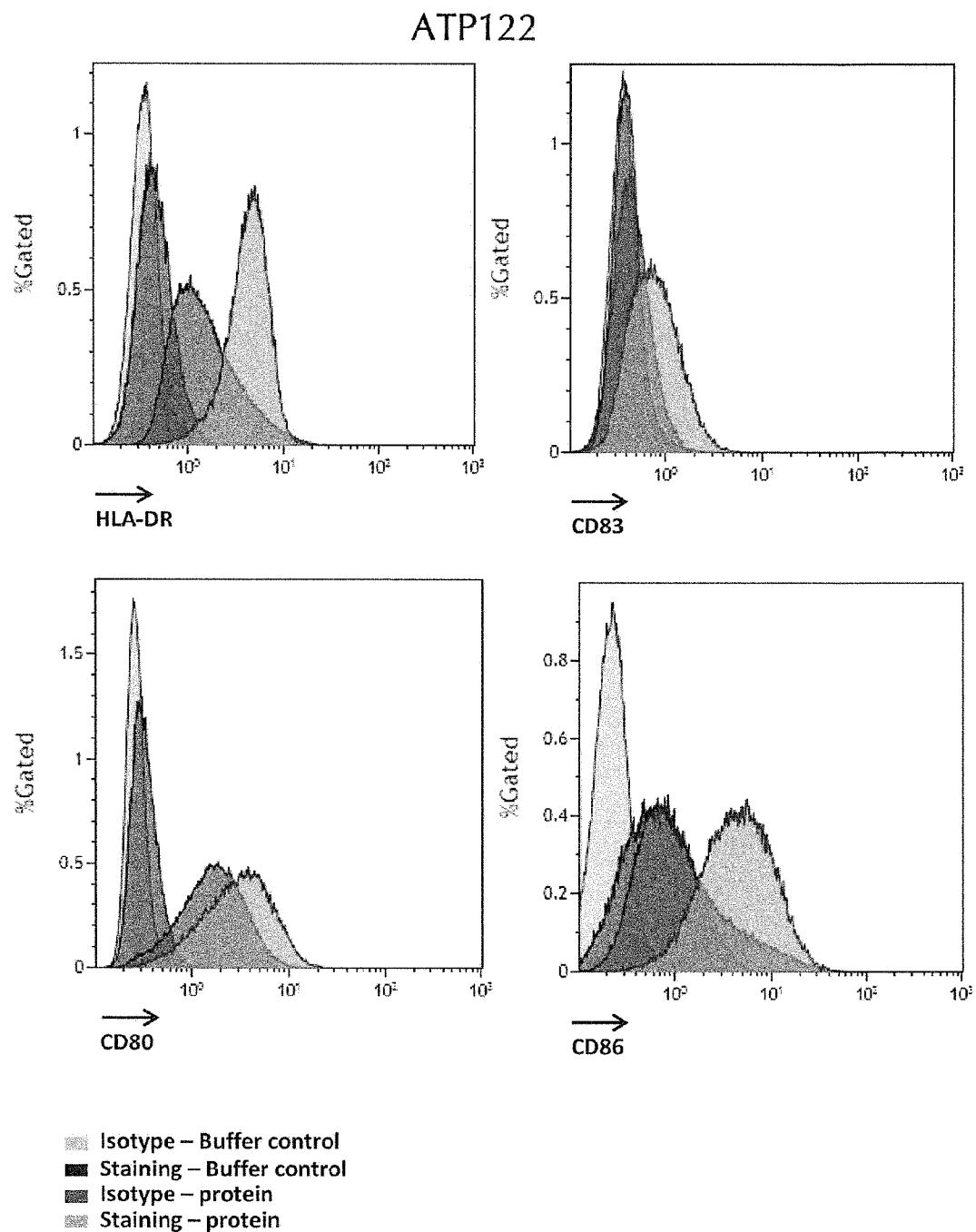


Fig. 69

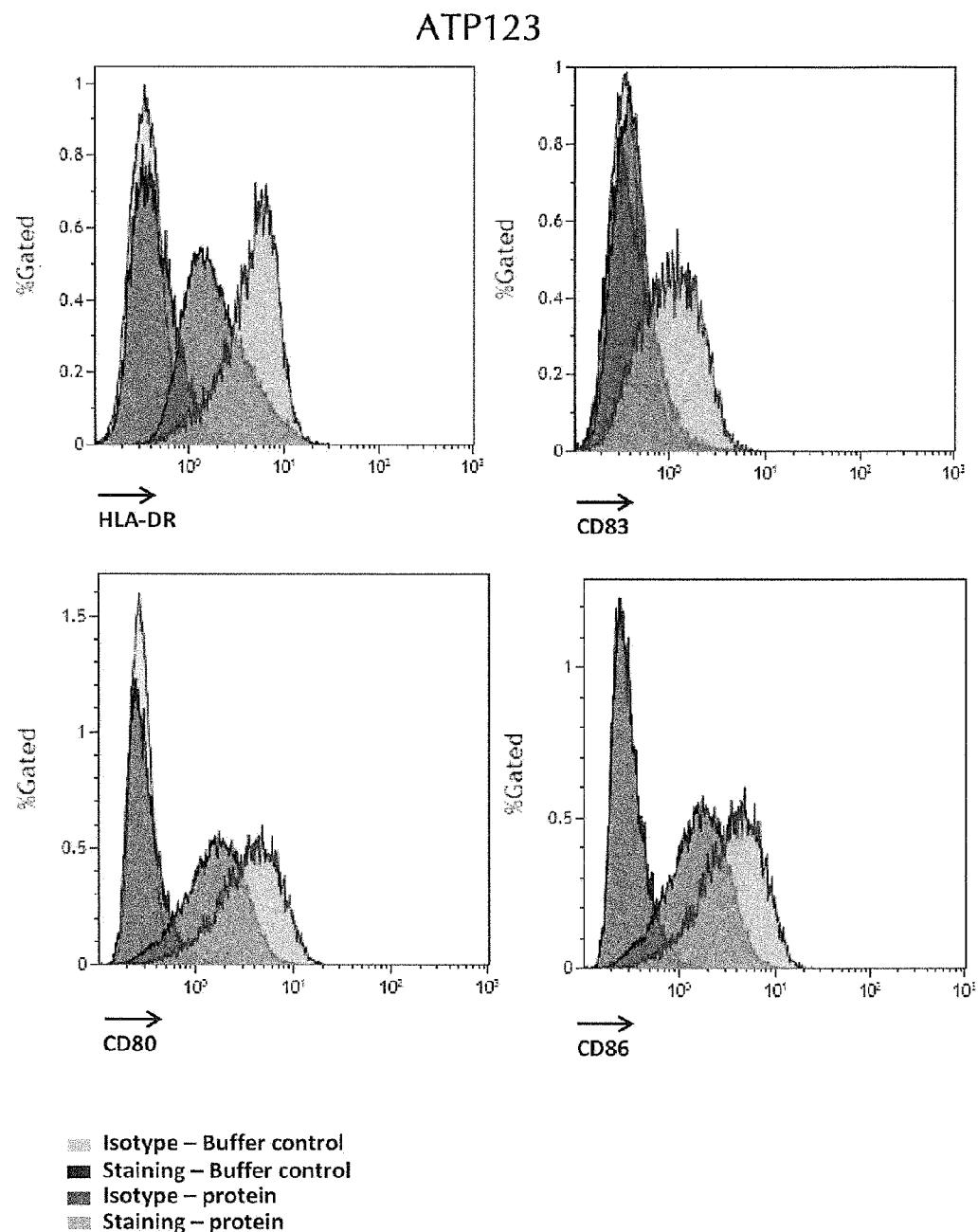


Fig. 70

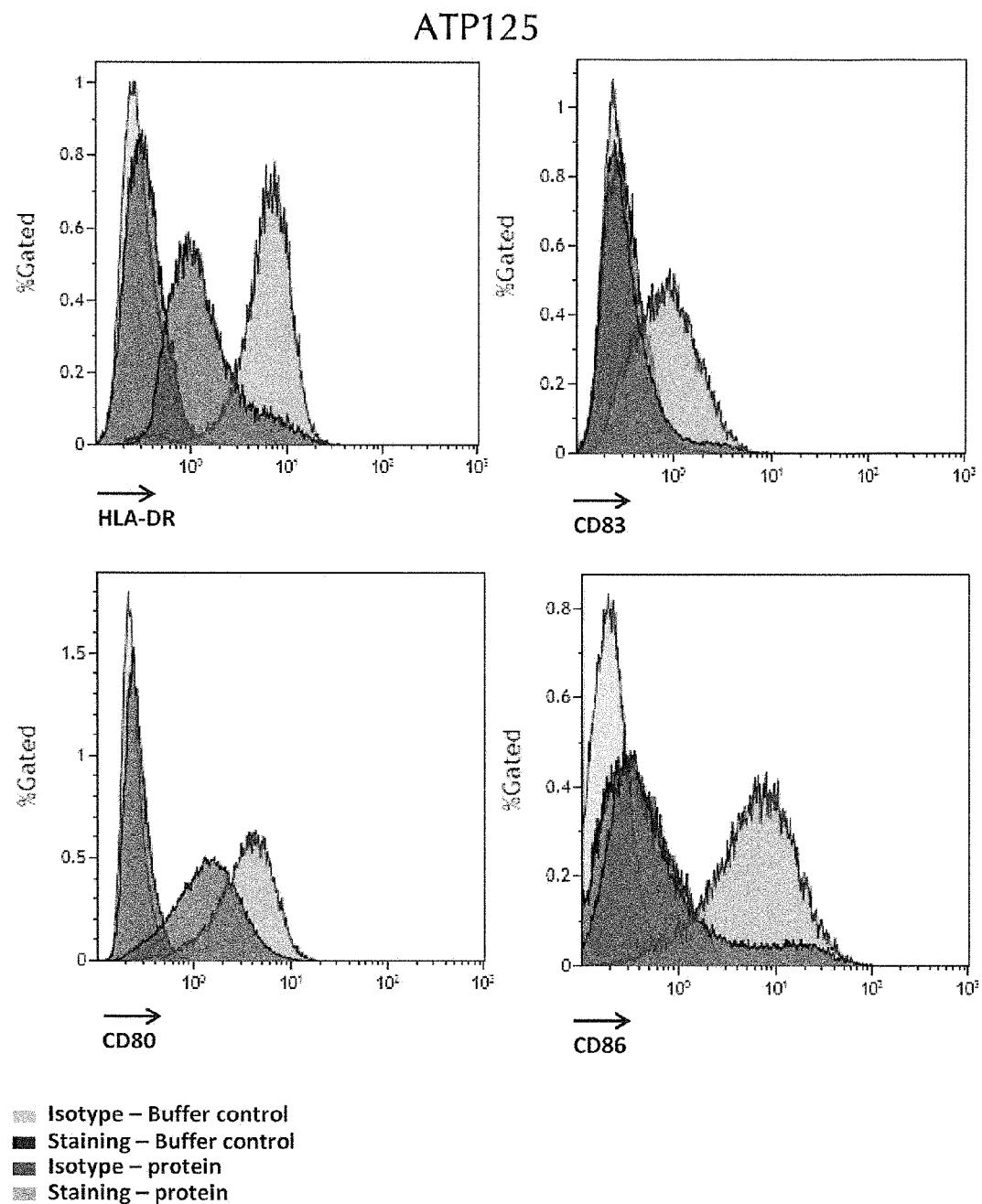
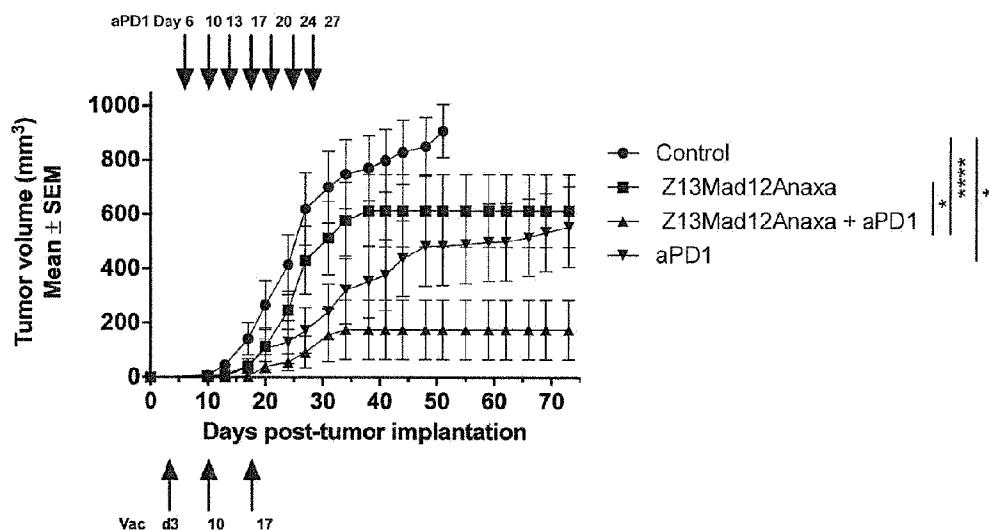


Fig. 71

A



B

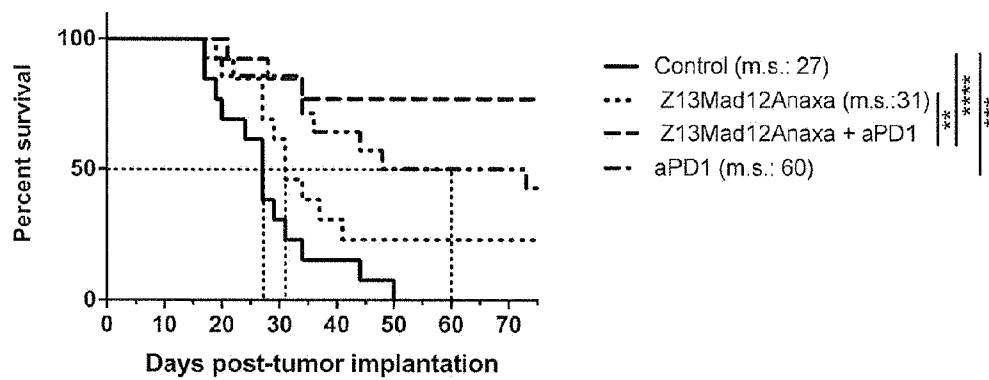


Fig. 72

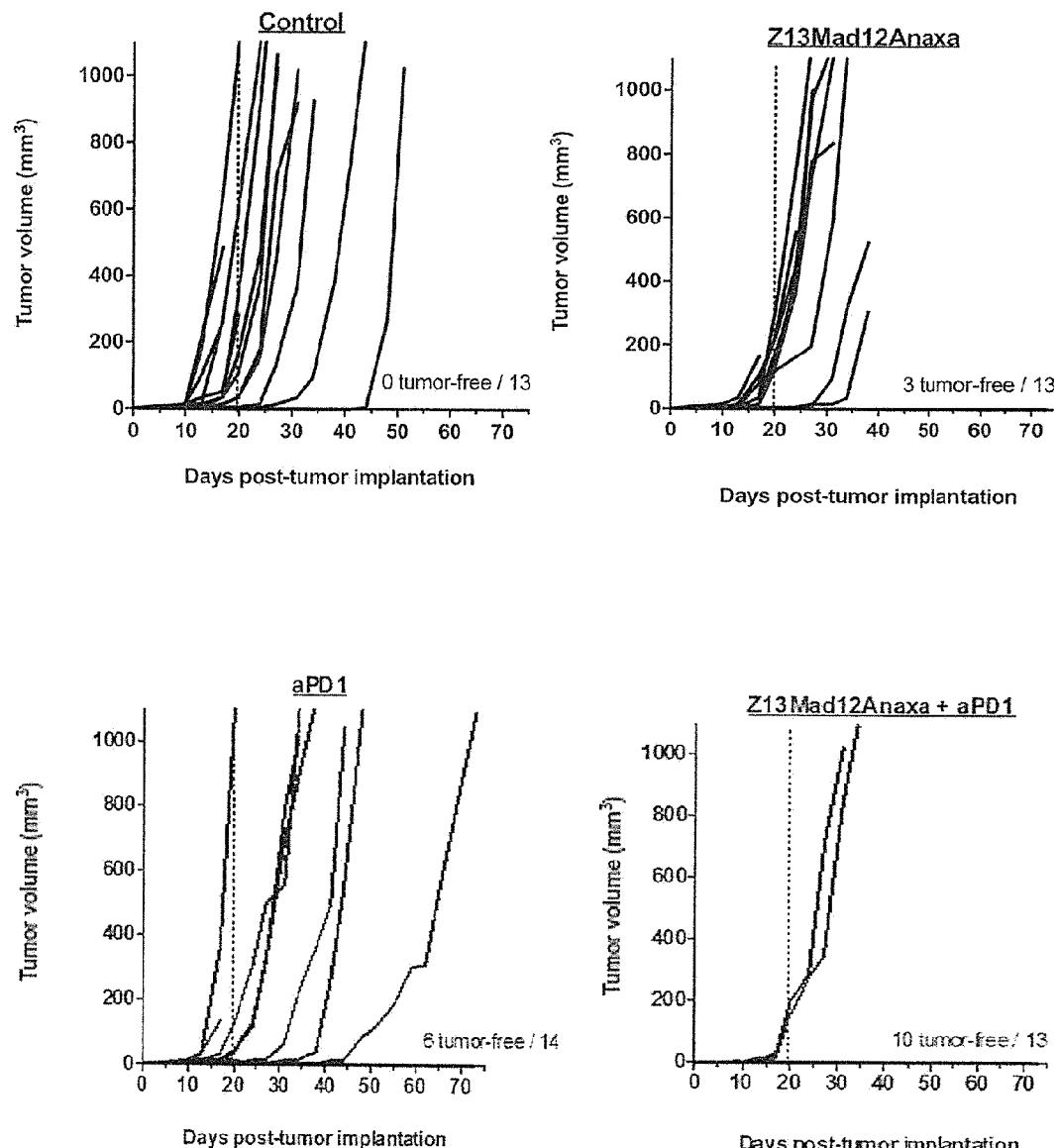


Fig. 73

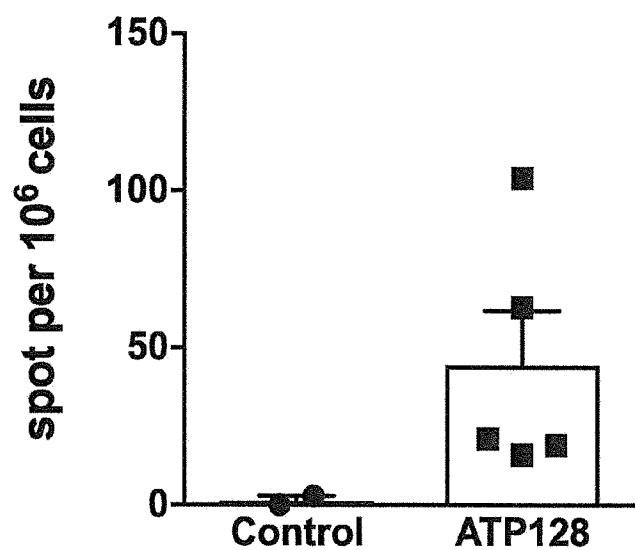


Fig. 74

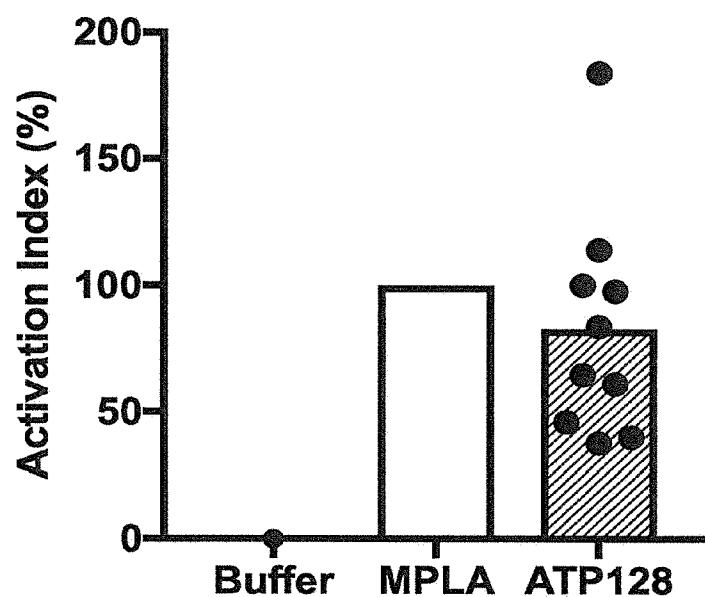
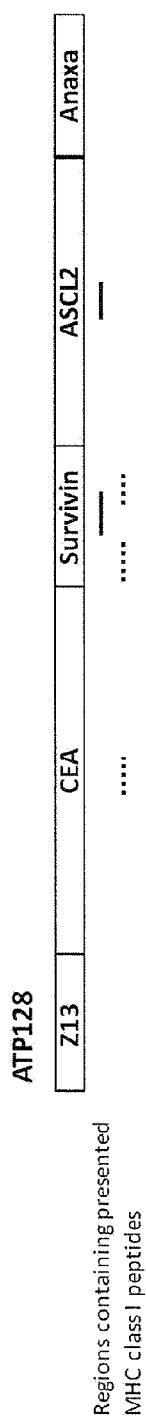


Fig. 75

A



B

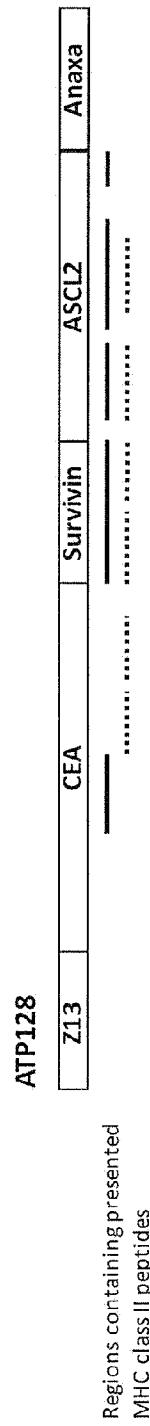


Fig. 76

C

| Donor | HLA class I haplotype                | CEA      | Survivin | ASCL2 |
|-------|--------------------------------------|----------|----------|-------|
| 9     | A*02:01, A*29:02<br>B*07:02, B*55:01 | 0        | 1        | 2     |
| 10    | A*02:01, A*31:01<br>B*35, B*40:05    | <u>1</u> | <u>1</u> | 0     |

| Donor | HLA class II haplotype                           | CEA | Survivin | ASCL2 |
|-------|--|-----|----------|-------|
| 9     | DRB1*14, DRB1*15<br>DQB1*05:03, DQB1*06:03       | 6   | 40       | 66    |
| 10    | DRB1*08:02, DRB1*14:02<br>DQB1*03:01, DQB1*03:01 | 2   | 8        | 27    |

Fig. 76 continued

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

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