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(71) Applicant (for all designated States except US):

JULIUS-MAXIMILIANS-UNIVERSITÄT
WÜRZBURG [DE/DE]; Sanderring 2, 97070 Würzburg
(DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BECKER, Jürgen

[DE/DE]; Huttenstrasse 26, 97072 Würzburg (DE).
MÜLLER (nee Hofmeister), Valeska [DE/DE]; Hein-
rich-Böll-Strasse 41, 97276 Margetshöchheim (DE).
SCHRAMA, David [NL/DE]; Jahnstrasse 5a, 97204
Höchstberg (DE).

(74) Agent: ELBEL, Michaela; Rothkopf Theobald Elbel, Al-
theimer Eck 13, 80331 Munich (DE).

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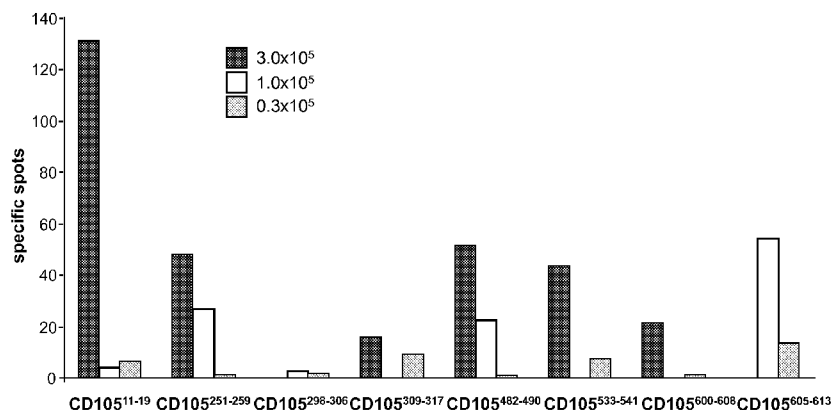


Fig. 4

(57) **Abstract:** The present invention concerns artificial peptides derived from endoglin (CD105) for the treatment of cancer. Furthermore, the invention relates to polypeptides comprising the artificial peptide, nucleic acids encoding the artificial peptide or polypeptide, antibodies against the artificial peptide and vaccines comprising at least one substance selected from the group consisting of a peptide, a nucleic acid and an antibody for the treatment of cancer. The present invention also concerns a method for preparing a vaccine and the use of a peptide, polypeptide, nucleic acid or antibody for the manufacture of a drug and/or a vaccine for the treatment and/or prevention of cancer or an immunological disorder.

Endoglin peptides, vaccines and methods for preparing the same

Field of the invention

The present invention concerns the technical field of artificial peptides. Especially, the invention relates to polypeptides derived from endoglin (CD105) for the treatment of cancer, polypeptides comprising the artificial peptide, nucleic acids encoding the artificial peptide or polypeptide, antibodies against the artificial peptide and vaccines for the treatment of cancer. The present invention also concerns a method for preparing a vaccine and the use of a peptide, polypeptide, nucleic acid or antibody according to the invention for the manufacture of a drug and/or a vaccine for the treatment and/or prevention of cancer or an immunological disorder.

Background of the invention

It is estimated that in the U.S. more than 1,400,000 and in Europe about 3,200,000 new cancer cases would be diagnosed each year, and more than half a million people in the U.S. and about 1,700,000 people in Europe would die from cancer each year. While early and localized diseases may effectively be treated by radical excision metastatic cancer is fatal in most cases. Nevertheless, some patients show spontaneous regression of both primary tumors and metastases. This event is largely attributed to adaptive immune responses, and the presence of cytotoxic T cells infiltrating the tumor is associated with a better clinical prognosis (Kondratiev et al., 2004). In addition, the increased tumor incidence in immune suppressed individuals indicates that cancer, at least in part, can be controlled by the immune system (Adami et al., 2003). Therefore, efforts are being made to stimulate patient's immune effector cells to recognize and destroy cancer cells.

The majority of current vaccination strategies is based on tumor-associated antigens (TAAs). But clinical efficacy of these immunotherapies is limited, because neoplastic cells may be subject of several immune evasion mechanisms, e.g. downregulation of antigen expression or presentation of the respective antigen. The presentation of

TAA-derived peptides to cytotoxic T cells can be lost by mutation, deletion or silencing of the TAA gene itself or by defects in the antigen presentation pathway, e.g. loss of major histocompatibility complex class I heavy chains, β 2-microglobulin, TAP or tapasin expression. Therefore, antigens contributing to survival of tumor cells seem to be more suitable therapeutic targets than neoplastic antigens themselves. However, even such contributing antigens may be subject to immune evasion due to impaired processing and cell surface expression.

Carcinogenesis is a process depending on genetic and epigenetic alterations accumulated in transforming cells. Nevertheless, many steps necessary for tumor progression, e.g. proliferation, invasion, angiogenesis, and metastasis are influenced by microenvironmental factors, such as growth factors, angiogenic factors, cytokines, and proteolytic enzymes. During transformation these factors are provided by normal cells, i.e. fibroblasts, endothelial, and immunocompetent cells due to reciprocal interactions between neoplastic and adjacent normal cells. Indeed, the microenvironment influences the stromal cells in such a way that they rather promote than inhibit tumor progression by allowing vasculogenesis and angiogenesis, recruitment of reactive stromal fibroblasts, lymphoid and phagocytic infiltrates, secretion of peptide mediators, and proteolytic enzymes, as well as production of a modified extracellular matrix. Tumor stromal cells may serve as targets for immune intervention, and several antigens have been identified, which are induced or upregulated on the tumor stroma.

Endothelial cells are possible targets for immunotherapy. Advantageously, they are highly accessible to antibodies or lytic effector cells. The immunological destruction of tumor blood vessels should lead to a widespread necrosis in solid tumors, hence inflicting its effect upon tumor cells. On tumor endothelial cells numerous molecules are expressed, like growth factor receptors, such as VEGF receptors, cell surface antigens with enzymatic activity, such as prostate-specific membrane antigens, and functionally uncharacterized antigens, such as tumor endothelial markers. Therefore, there is a wide range of endothelial antigens that may be targets for immunotherapy.

An endothelial marker scrutinized for its role in vascular targeting is CD105, also called endoglin (Hofmeister et al., 2008). CD105 is upregulated on activated endothelium by TGF- β 2 and hypoxia and has been reported to be expressed on tumor endothelial cells of a plethora of solid tumors. Expression of CD105 in these tumors is correlated with vascular density and most importantly, poor prognosis (Dales et al., 2004).

CD105 provides a restricted binding affinity for leukocyte antigen and a restricted capacity to form stable peptide-major histocompatibility complex (MHC) interactions. However, these abilities are critical for immunogenicity and probability to induce production of cytotoxic T cell, which is again responsible for the success of immunotherapies by CD105.

Therefore, there is a need for improved antigens that efficiently induce immune reactions, while undesired side effects and risk of therapy resistance are reduced.

The solution to this problem is achieved by the embodiments of the present invention characterized by the claims, and described further below.

Summary of the invention

It is an object of the present invention to provide an artificial peptide for the treatment of cancer, the peptide is derived from endoglin and comprises a nonapeptide, which inhibits 50 % of binding of a reference peptide to human leukocyte antigen A2 (HLA-A2) in a competitive binding assay at a concentration below 5 μ M of the nonapeptide, wherein the nonapeptide has a leucine or methionine at amino acid position 2 and/or a valine or leucine at amino acid position 9.

Another aspect of the invention is to provide an artificial peptide for the treatment of cancer, the peptide is derived from human endoglin and binds specifically to human leukocyte antigen A2 (HLA-A2), wherein the peptide is selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 16.

In addition, the present invention is directed to a polypeptide for the treatment of cancer, the polypeptide comprises an artificial peptide according to the invention.

Furthermore, the invention concerns a nucleic acid for the treatment of cancer, the nucleic acid encodes at least one peptide or polypeptide according to the invention.

Moreover, the invention relates to an antibody against a peptide or a polypeptide according to the invention for the treatment of cancer.

Another object of the present invention is to provide a vaccine comprising at least one substance selected from the group consisting of a peptide, a nucleic acid and an antibody according to the invention.

The invention is also directed to a method for preparing a vaccine comprising the steps of:

- a) providing the peptide, the polypeptide or the antibody according to the invention;
- b) admixing an adjuvant to the peptide, polypeptide or antibody.

The invention relates also to the use of at least one peptide, polypeptide, nucleic acid or antibody according to the invention for the manufacture of a drug and/or a vaccine for the treatment and/or prevention of cancer or an immunological disorder.

Brief description of the figures

Fig. 1 shows a selection of HLA-A2 restricted CD105 derived peptides. An amino acid sequence of the human CD105 L- and S-isoform is shown in single letter code. The positions of the peptides binding to HLA-A2 are underlined.

Fig. 2 depicts binding of CD105 derived peptides to HLA-A2 tested by a competitive binding assay. IC_{50} value specifies the peptide concentration sufficient for 50% inhibition of binding of a reference peptide.

Fig. 3 shows affinity optimized CD105 derived peptides. Anchor amino acids were exchanged to enhance affinity of nonamer peptides to HLA-A2, wherein leucine or methionine is localized at anchor amino acid position 2 and valine or leucine is localized at anchor amino acid position 9.

In the diagram of Fig. 4 T cells specific for CD105 derived peptides are detected by IFN- γ ELISPOT. 3×10^5 , 1×10^5 or 0.3×10^5 peripheral blood lymphocytes of a melanoma patient were prestimulated *in vitro* by CD105 derived peptides, and reactivities to the respective peptides were analyzed by IFN- γ ELISPOT. Results are shown as specific spots:

$$\text{specific spots} = [\text{mean spots}_{\text{with peptide}} - \text{mean spots}_{\text{without peptide}}].$$

Fig. 5 depicts responses of melanoma patients' peripheral blood lymphocytes to CD105 derived epitopes. IFN- γ responses of peripheral blood lymphocytes of melanoma patients upon stimulation with selected parental and modified CD105 peptides were measured in ELISPOT. An upward arrow means enhanced IFN- γ response, a downward arrow means reduced IFN- γ response, and a rhomb means unchanged IFN- γ response to modified peptides in comparison to parental peptides.

Fig. 6 shows immunogenic effects of CD105 derived peptides in HLA-A2/k^b transgenic mice. Vaccination of transgenic mice with or without (K) parental or modified CD105 peptides was followed by *in vitro* analysis of IFN- γ responses in ELISPOT. The legend is the same as in Fig. 5.

In Fig. 7 murine CD105 is displayed. Amino acid positions that are homologue to human HLA-A2 restricted CD105 peptides are underlined. Amino acids identical to human CD105 peptides are depicted in bold.

In Fig. 8 HLA-A2 restricted human CD105 derived parental peptides (left column) and affinity improved human CD105 derived peptides (right column) are shown. Amino acids identical to the amino acids in the corresponding position of the parental murine CD105 peptide are depicted in bold.

Fig. 9 shows the IFN- γ responses of peripheral blood lymphocytes of a melanoma patient before and after vaccination with CD105 derived peptides. For vaccination, the patient received separately 100 μ g CD105₆₀₀₋₆₀₈ (608V) and CD105₆₀₅₋₆₁₃ (613V) each in 700 μ l H₂O with 700 μ l montanide. 3×10^5 , 1×10^5 or 0.3×10^5 PBL were prestimulated, i.e. amplified *in vitro* with the indicated CD105 peptides and their reactivity to the respective peptides was analysed after 1 week of culturing by IFN- γ ELISPOT. Data are shown as specific spots [mean spots + peptide – mean spots – peptide]. The results in Fig. 9 show an induction of a CD105 specific immune response in a melanoma patient in a compassionate use setting by vaccination with CD105 epitopes.

Fig. 10 shows also the IFN- γ responses of peripheral blood lymphocytes of the same melanoma patient as in Fig. 9 before and after vaccination with CD105 derived peptides. The reactivity of 1×10^6 and 0.5×10^6 PBL was measured directly after blood withdrawal *ex vivo*, i.e. without prior stimulation/amplification. Again, a CD105 specific immune response was induced in a melanoma patient in a compassionate use setting by vaccination with CD105 epitopes.

Detailed description of the invention

It is an object of the present invention to provide an artificial peptide for the treatment of cancer, the peptide is derived from endoglin comprising a nonapeptide, which inhibits 50 % of binding of a reference peptide to human leukocyte antigen A2 in a competitive binding assay at a concentration below 5 μ M of the nonapeptide, wherein the nonapeptide has a leucine or methionine at amino acid position 2 and/or a valine or leucine at amino acid position 9.

The term "peptide" as used herein refers to an x-mer that comprises multiple amino acids linked by peptide bonds regardless of length and conformation. Therefore, the term "peptide" includes chains of several amino acids, oligomers, polymers as well as polypeptides. Non-amino acid units, like steroids or carbohydrates, linked to the polypeptide can also be included.

The term "nonapeptide" as used herein refers to a nonamer including nine amino acids including functional variants, e.g. glycosylation, modification and additionally attached residues.

The term "artificial peptide" as used herein refers to a peptide that is derived from a natural, parental peptide or polypeptide by e.g. modifying, selecting or isolating specific sequences; expression of a mutated nucleic acid; altering amino acid sequence; adding or removing residues; and exchanging, inserting and/or deleting peptide domains, multiple amino acids or single amino acids. Artificial peptides are based on CD105. Preferred modifications are exchanges of at least one sequence of SEQ ID NO: 1 to SEQ ID NO: 10 against SEQ ID NO: 11 to SEQ ID NO: 16. Other preferred modifications are exchanges of single amino acids at position 2 and/or 9 in the context of a nonapeptide.

The term "endoglin" as used herein refers to CD105, which is a 95 kD cell surface polypeptide assembled as a homodimer, which is composed of a signal sequence, an extracellular domain, a transmembrane domain and a cytoplasmic domain. There exists two isoforms of endoglin, L-endoglin and S-endoglin, differing in their C-terminal intracellular domain. CD105 functions as an accessory receptor for kinase receptor complexes of the transforming growth factor (TGF)- β superfamily. Thereby, CD105 modulates TGF- β signaling, i.e. it antagonizes the inhibitory effects of TGF- β 1, and CD105 is upregulated on activated endothelium via TGF- β 2. In addition, CD105 has an anti-apoptotic effect under hypoxic conditions.

CD105 is expressed as tumor stroma associated antigen. Its expression is not restricted to tumor endothelial cells (TEC). It is present on activated monocytes, differentiated macrophages, early B cells, erythroid precursor cells, fibroblasts,

follicular dendritic cells, melanocytes, heart smooth muscle cells, and trophoblasts. However, only on TEC CD105 is expressed in significant amounts that are relevant for immune recognition. CD105 is not confined to one tumor type, but has been reported to be expressed on TEC for a broad spectrum of solid tumors, e.g. breast, prostate, gastric, colorectal, renal and cervical tumors, and endometrial carcinoma, as well as for melanoma, and glioblastoma. However, binding affinity of CD105 is limited. In contrast to CD105, peptide epitopes selectively isolated from CD105 show high affinity to HLA-A and are beneficial targets for therapeutic interventions.

The term “human leukocyte antigen A2” (HLA-A2) as used herein refers to an HLA-A serotype that identifies the gene products of many HLA-A2 alleles, including HLA-A*0201, *0202, *0203, *0206, and *0207 gene products. HLA-A belongs to the superfamily of immunoglobulins with a domain like structure, i.e. it is an MHC class I receptor consisting of an HLA-A heavy chain and β 2-microglobulin. The heavy chain anchors the HLA complex in the membrane. HLA class I antigens are expressed on nearly all cells. HLA class I presents small allo-, auto- and inter-species antigens generated by cellular processing to the immune system, which surveys for non-self peptides. By interaction of HLA-A with T-cell receptors T-cell immune reactions are induced and regulated. As in most mammalian populations, MHC class I molecules are extremely variable in their primary structure, and HLA is ranked among the genes in humans with the fastest evolving coding sequence. Many diseases, like multiple sclerosis, schizophrenia or myasthenia gravis are associated with different HLA alleles.

Binding affinity of peptides according to the invention was controlled by a competitive binding assay. This assay is based on binding of the peptide to be tested and a reference peptide, e.g. a fluorescein-labeled reference peptide, to empty, acid-stripped HLA-A2-antigens expressed on cells. Reduction of binding of the reference peptide by competitive binding of different concentrations of the tested peptide to the cells, e.g. HLA-A2 positive cells JY, was analyzed by flow cytometry. The IC_{50} value specifies the peptide concentration sufficient for 50% inhibition of binding of the reference peptide, wherein $IC_{50} < 5 \mu M$ means high affinity, $5 \mu M \leq IC_{50} \leq 15 \mu M$

means intermediate affinity, $15 \mu\text{M} < \text{IC}_{50} \leq 100 \mu\text{M}$ means low affinity, and $\text{IC}_{50} > 100 \mu\text{M}$ means no binding.

The present inventors surprisingly discovered that presentation of antigens by antigen presenting cells (APC) is enhanced for antigens, which possess a specific sequence motif of two or more essential amino acids, herein also called peptide anchor residues, in the context of a nonapeptide. Examples of essential amino acids of the specific sequence motif are leucine, methionine and valine. Amino acid exchanges in peptides improved binding to HLA-A2, when an amino acid at anchor position 2 was changed by leucine or methionine and/or an amino acid at anchor position 9 was changed by leucine or valine. The competitive binding assay indicates a binding affinity to HLA-A2 for CD105 derived peptides with leucine or methionine at amino acid position 2 and/or valine or leucine at amino acid position 9 by an IC_{50} value below $5 \mu\text{M}$ (Fig. 3). An important advantage is that the recognition of the peptides by T cells was not altered by these amino acid exchanges. In other words, the recognition of the amended/modified/alterd nonamer peptides of the invention by T cells is the same as with wild type/parental/unmodified peptides

Leucine or methionine at anchor position 2 and/or valine or leucine at anchor position 9 of a specific binding motif in the context of a nonapeptide not only show high binding affinity of the peptides to HLA-A2, but also high immunogenicity and induction of T cell responses demonstrated by IFN- γ ELISPOT, which measures the ability of T cells to respond to a peptide. Specific responses to CD105 derived peptides in peripheral blood of melanoma patients were significantly increased by the exchange of amino acids at amino acid position 2 against leucine and/or amino acid position 9 against valine in comparison to unchanged peptides (Fig. 5).

To analyze the immunogenicity of the peptides according to the invention *in vivo* HLA-A2/ k^b transgenic mice were vaccinated with the peptides derived from CD105 having a specific binding motif at amino acid position 2 and/or 9. HLA-A2/ k^b transgenic mice express a chimeric MHC class I antigen comprising peptide binding domains $\alpha 1$ and $\alpha 2$ of the human HLA-A2 antigen together with transmembrane, intracellular and $\alpha 3$ domains of the murine H-2 k^b antigen allowing optimal interaction

with murine T cells. In accordance to high affinity and high immune responses in melanoma patients' blood samples, high immune responses were induced by peptides with leucine at amino acid position 2 and/or valine at amino acid position 9 (Fig. 6). In comparison to parental CD105 derived peptides, immune responses were enhanced by exchanges of amino acids against leucine or methionine at anchor position 2 and/or against valine or leucine at anchor position 9.

Therefore, peptides with exchanged amino acids according to the invention optimize binding to HLA-A2 and enhance immunogenicity.

In contrast to tumor cells and adjacent neoplastic cells, tumor stromal cells are genetically stable. Therefore, peptides according to the invention are more suitable antigens for immunotherapy than antigens derived from tumor or neoplastic cells.

In a preferred embodiment of the invention the IC_{50} is equal or below 3.5 μM .

In another preferred embodiment of the invention the peptide is selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 16.

Binding affinities of peptides including SEQ ID NO: 11 to SEQ ID NO: 16 are indicated by an IC_{50} equal or below 3.5 μM (Fig. 3), and specific responses to CD105 derived peptides in peripheral blood of melanoma patients were multiplied by using peptides of SEQ ID NO: 12, 15, and 16 in comparison to control experiments and in comparison to parental peptides selected from CD105 (Fig. 5). In accordance with improved affinity and immune response, enhanced immune responses were induced in melanoma patients' blood samples by peptides of SEQ ID NO: 12, 15, and 16 in comparison to control experiments and in comparison to parental epitopes in the mouse model (Fig. 6).

In another preferred embodiment of the invention the peptide is derived from mammalian endoglin, mice endoglin, rat endoglin, guinea pig endoglin and/or primate endoglin, particularly human endoglin.

As the human peptides SEQ ID NO: 15 and 16 are also included in the murine CD105 amino acid sequence (Fig. 7), major side effects of immune responses induced by polypeptides comprising peptides SEQ ID NO: 15 and/or 16 are excluded in HLA-A2/k^b transgenic mouse model.

In a preferred embodiment of the invention the reference peptide is a fluorescein labeled peptide. In one possible embodiment the fluorescein-labeled reference peptide is (FLPSDC(FI)FPSV (JPT Peptide Technologies GmbH, Berlin, Germany). In a further preferred embodiment of the invention competitive binding assay is performed with HLA-A2 positive cells, preferably JY cells.

It is also an object of the invention to provide an artificial peptide for the treatment of cancer, the peptide is derived from human endoglin and binds specifically to human leukocyte antigen A2, wherein the artificial peptide is selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 16.

In order to use CD105 as a target for tumor therapy distinct peptides have to be isolated that are immunogenic, function as epitopes and are presented on APC. CD105 is a large polypeptide of 658 (L-endoglin) or 625 (S-endoglin) amino acids, from which specific peptides have to be chosen that possess the desired immunogenicity and capability to be presented on APC. By reverse immunology the present inventors have specifically selected peptides of SEQ ID NO: 1 to 10 from human CD105 that bind to the common MHC class I antigen HLA-A2. The selected peptides all bind to HLA, and binding affinity ranges between high affinity with an IC₅₀ value below 5 µM, intermediate affinity with an IC₅₀ equal or between 5 µM and 15 µM; and low affinity with an IC₅₀ between 15 µM and 100 µM or equal to 100 µM (Fig. 2). The peptides selected from human CD105 were further improved in affinity to HLA-A2. Peptides of SEQ ID NO: 11 to 16 indicate an IC₅₀ value below or equal to 3.5 µM, wherein amino acids at anchor position 2 of the nonapeptides of SEQ ID NO: 1 to 10 selected from CD105 were exchanged against methionine and leucine and/or amino acids at anchor position 9 were exchanged against valine or leucine (Fig. 3). Especially, peptides of SEQ ID NO: 3, 7, 8 and 11 to 16 show high binding affinity to HLA-A2.

Artificial peptides of SEQ ID NO: 1 to SEQ ID NO: 16 according to the invention not only bind specifically to HLA-A2, but also show high immunogenicity that was measured by detecting the ability of T cells to respond to a peptide according to the invention. Therefore, generation of functionally CD105 reactive T cells induced by peptides of SEQ ID NO: 1 to 16 was measured by IFN- γ ELISPOT assays. Different concentrations of peripheral blood lymphocytes of a melanoma patient were prestimulated *in vitro* with peptides of SEQ ID NO: 1 to 16 and their reactivities to the respective peptides were analyzed by IFN- γ ELISPOT. Specific immune responses to CD105 peptides in peripheral blood of melanoma patients were readily detectable for all peptides of SEQ ID NO: 1 to 16 (Figs. 4 and 5). Especially peptides of SEQ ID NO: 1, 3, 7, 8, 10, 12, 15 and 16 induce T cell activity *in vitro* very efficiently.

To analyze immunogenicity of peptides of SEQ ID NO: 1 to 16 *in vivo* HLA2/k^b transgenic mice were vaccinated with these peptides, and immune responses to peptides of SEQ ID NO: 1 to 16 are analyzed. As shown in Fig. 6 immunization of mice induced CD105 specific immune responses. Especially, peptides of SEQ ID NO: 3, 7, 8, 10, 12, 15 and 16 evoked high T cell IFN- γ responses.

Another aspect of the invention concerns a polypeptide for the treatment of cancer, the polypeptide comprises an artificial peptide according to the invention.

Furthermore, the invention is directed to a nucleic acid for the treatment of cancer, the nucleic acid encodes at least one peptide or polypeptide according to the invention.

The term "nucleic acid" as used herein refers to a macromolecule composed of monomeric nucleotides, wherein nucleic acids include amongst other deoxynucleic acids (DNA); ribonucleic acids (RNA); artificial nucleic acids, like peptide nucleic acid (PNA) and glycolic nucleic acid (GNA); single stranded nucleic acids and double stranded nucleic acids. The term "nucleic acid" also comprises recombined nucleic acid as well as vectors carrying nucleic acids and/or recombined nucleic acids. The term "vector" refers to a vehicle for transferring genetic material into a cell, wherein

plasmids, viral vectors, cloning vectors, expression vectors, transcription vectors, artificial particles and artificial chromosomes are included. The vector comprises double or single stranded nucleic acids as DNA or RNA and includes at least a transgene, a backbone and optionally a promoter and a marker.

In a preferred embodiment of the invention the nucleic acid comprises an expression system. By means of the expression system the peptides and/or polypeptides encoded by the nucleic acid can be expressed in an organism to be treated. In addition, the nucleic acid can be expressed in a system to gain therapeutic peptides, polypeptide or antibodies.

The invention also concerns an antibody against a peptide or polypeptide according to the invention for the treatment of cancer.

The term "antibody" as used herein refers to an immunoglobulin used by the immune system to identify and neutralize foreign objects. The term "antibody" includes monoclonal and polyclonal antibodies; antibodies of all known isotypes; artificial antibodies; and a functional part of an antibody.

Moreover, the invention relates to a vaccine comprising at least one substance selected from the group consisting of a peptide, a nucleic acid and an antibody according to the invention.

The data of affinity to HLA-A2 and immunogenicity of artificial peptides according to the invention suggest that passive immunotherapy using antibodies specific for artificial peptides according to the invention either alone or conjugated with different effector molecules as well as active immunotherapy by peptides according to the invention inhibit angiogenesis, tumor growth, and metastases. Antibodies specific for parental CD105 react only weakly or not at all with quiescent endothelium and side effects were low (Hofmeister et al., 2008). This property can also be applied to the peptides according to the invention.

In a preferred embodiment of the invention the vaccine is used for an active immunization. In another preferred embodiment the vaccine is used for a passive immunization. In a third embodiment the vaccine is used for DNA vaccination.

In a further preferred embodiment of the invention the vaccine comprises an adjuvant. The adjuvant includes, but is not limited to pharmaceutically acceptable carriers; co-stimulatory substances; dendritic cells; *Bacillus Calmette-Guéri*; molecules expressed by Epstein-Barr virus immortalized B cells, like B7.1 (DC80) and B7.2 (CD 86); GM-CSF; incomplete Freund's adjuvant; and cisplatin. Active immunization combined with co-stimulatory substances, like cisplatin revealed a synergistic antitumor activity.

In addition the invention is directed to a method for preparing a vaccine comprising the steps of:

- a) providing the peptide, the polypeptide or the antibody according to the invention;
- b) admixing an adjuvant to the peptide, polypeptide or antibody.

Furthermore, the invention concerns the use of at least one peptide, polypeptide, nucleic acid or antibody according to the invention for the manufacture of a drug and/or a vaccine for the treatment and/or prevention of cancer or an immunological disorder.

Manufactured vaccines and/or drugs according to the invention are employed to treat diseases, like cancer including non-Hodgkin's lymphoma; colorectal, head, neck, cervix, renal, breast, prostate, gastric and colorectal tumors; endometrial carcinoma; melanoma; and glioblastoma cancer. Immune deficiencies, such as X-linked agammaglobulinemia and hypogammaglobulinemia resulting in partial or complete lack of antibodies can also be treated by a vaccine and/or a drug according to the invention.

Material and methods

1. Cells and cell lines

Human HLA-A*0201-positive cell lines T2, a transporter associated with antigen processing (TAP)-deficient T cell leukemia/B cell line hybrid and JY, a B-LCL were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal calve serum (FCS). After informed consent peripheral blood lymphocytes (PBL) were collected from HLA-A2-positive patients with advanced malignant melanoma. PBL from HLA-A2-positive healthy individuals were used as controls. PBL were isolated using Lymphoprep separation (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's instructions and used directly or frozen in FCS with 10% DMSO. PBL were cultured in RPMI/10% human AB serum.

2. Immunohistochemical staining

Paraffin sections were treated with pronase (Roche, Mannheim, Germany) for 20 minutes at 37°C and stained with the CD105-specific antibody SN6h (Acris Antibodies GmbH, Hiddenhausen, Germany) and the Vector VIP system (Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions.

3. Selection of CD105 nonamer peptides

Peptides derived from the full-length human CD105 polypeptide were selected using both BIMAS (Parker et al., 1994) and SYFPEITHI (Rammensee et al., 1999) peptide binding algorithms available via the internet (http://bimas.cit.nih.gov/molbio/hla_bind/) and (<http://www.syfpeithi.de/>). Residues 2 and 9 of some peptides were changed to optimize anchor amino acids.

4. Competitive binding assay for binding to HLA-A2 molecules

The binding affinity of the synthetic peptides (GeneScript Corporation, Piscataway, New Jersey, USA) to HLA-A2 molecules was measured in the competitive binding assay as described previously (Kessler et al., 2003). The assay is based on the

binding of the competitive peptide to be tested and the fluorescein-labeled reference peptide (FLPSDC(FI)FPSV, JPT Peptide Technologies GmbH, Berlin, Germany) to the acid-stripped HLA-A2 positive cell line JY. Reduction of the binding of the reference peptide was analyzed by flow cytometry. The percentage of binding inhibition of the FI-labeled reference peptide was calculated using the following formula:

$$(1 - (\text{MF}_{\text{reference + competitor peptide}} - \text{MF}_{\text{background}}) / (\text{MF}_{\text{reference peptide}} - \text{MF}_{\text{background}})) \times 100\%$$

The binding affinities of the competitor peptides are expressed as IC₅₀ values in the figures, specifying their concentration sufficient for 50% inhibition of binding of the reference peptide. IC₅₀ was calculated by nonlinear regression analysis with software CurveExpert 1.3. Peptides with an IC₅₀ < 5 µM were considered as high-affinity, with 5 µM ≤ IC₅₀ ≤ 15 µM as intermediate-affinity, with 15 µM < IC₅₀ ≤ 100 µM as low-affinity, and with IC₅₀ > 100 µM as no binders.

5. Antigen stimulation of PBL

To extend the sensitivity of the ELISPOT assay, PBL were stimulated once *in vitro* before analysis (McCutcheon et al., 1997). At day 0, PBL were thawed or freshly isolated from peripheral blood and plated in a cell concentration of 1x10⁶/ml and 5 ml/well in 6-well plates (Greiner GmbH, Frickenhausen, Germany) in X-vivo medium (Cambrex Biosciences, Verviers, Belgium) with 10% heat inactivated human AB serum in the presence of 10 µM peptide (GeneScript Corporation, Piscataway, New Jersey, USA). On day 1 and 4 40 U IL-2/ml (Proleukin, Chiron GmbH, Munich, Germany) were added to the culture. The cultured cells were tested for reactivity in ELISPOT on day 7.

6. ELISPOT assays

The human IFN-γ ELISPOT assay was used to quantify peptide epitope-specific IFN-γ-releasing effector cells as described in Berke *et al.* 2000. Nitrocellulose-bottomed

96-well plates (MultiScreen MAIP N45, Millipore GmbH, Schwalbach, Germany) were activated with 35% ethanol, washed with PBS and coated with anti-IFN- γ antibody (1-D1K, Mabtech, Hamburg, Germany). The wells were washed and blocked by X-vivo medium before adding 1×10^4 stimulator T2 cells loaded with or without 10 μ M peptide and 3×10^5 , 1×10^5 or 3×10^4 effector cells. After incubation overnight the wells were washed before addition of biotinylated secondary antibody (7-B6-1-Biotin, Mabtech, Hamburg, Germany). The plates were incubated for 2 h, washed, and streptavidine-enzyme conjugate (Streptavidin-ALP-PQ, Mabtech, Hamburg, Germany) was added. Incubation at room temperature for 1 h was followed by addition of enzyme substrate NBT/BCIP (Mabtech, Hamburg, Germany). The reaction was stopped by washing with tap water upon the appearance of dark purple spots. Spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Cellular Technology Ltd., Schwäbisch Gmünd, Germany). The peptide-specific CTL frequency was calculated from the numbers of spot-forming cells. All assays were performed in duplicates. Responders were defined as having an average number of > 25 antigen-specific spots per 10^5 cells (spots_{with peptide} - spots_{without peptide}).

7. Immunization of mice and analysis of immune responses by ELISPOT

HLA-A2/k^b transgenic mice (deposited by Linda A. Sherman at MMRCC, University of North Carolina, Chapel Hill, USA) were immunized with a mixture of 5-6 CD105 peptides (5 μ g/peptide) and incomplete Freund's adjuvant 7 times once weekly. Spleen cells were analyzed in a murine IFN- γ ELISPOT after *in vitro* stimulation. Cells were stimulated for six days with 200 U IL-2/ml and 10 μ M peptide loaded, mitomycin C treated LPS blasts, generated by stimulation of HLA-A2k^b spleen cells with 25 μ g/ml LPS (Sigma-Aldrich, Taufkirchen, Germany) and 7 μ g/ml dextran sulfate for three days. Subsequently 3×10^5 , 1×10^5 , and 0.3×10^5 spleen cells were seeded with or without 10 μ M peptide in RPMI 1640/10% FCS in duplicates in nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45, Millipore GmbH) coated with an IFN- γ specific coating antibody (Mabtech) and blocked with cell culture medium and incubated overnight. The development of the murine IFN- γ ELISPOT was done as described for the human ELISPOT, with the exception that

PBS/0.05% Tween 20 was used as washing buffer and the biotinylated anti-mouse IFN- γ antibody (Mabtech, diluted in PBS/1% BSA) as detection antibody.

Table 1: Sequence listing

SEQ ID NO:	Sequence name	Additional remarks
1.	hCD105 11-19	parental peptide; AA position 11-19
2.	hCD105 61-69	parental peptide; AA position 61-69
3.	hCD105 251-259	parental peptide; AA position 251-259
4.	hCD105 298-306	parental peptide; AA position 298-306
5.	hCD105 309-317	parental peptide; AA position 309-317
6.	hCD105 419-427	parental peptide; AA position 419-427
7.	hCD105 482-490	parental peptide; AA position 482-490
8.	hCD105 533-541	parental peptide; AA position 533-541
9.	hCD105 600-608	parental peptide; AA position 600-608
10.	hCD105 605-613	parental peptide; AA position 605-613
11.	hCD105 251-259 (252L)	AA 252: leucine
12.	hCD105 309-317 (310L, 317V)	AA 310: leucine AA 317: valine
13.	hCD105 482-490 (483L, 490V)	AA 483: leucine AA 490: valine

14.	hCD105 533-541 (541V)	AA541: valine
15.	hCD105 600-608 (608V)	AA 608: valine
16.	hCD105 605-613 (613V)	AA 613: valine
17.	hCD105 L-isoform	parental
18.	hCD105 S-isoform	parental
19.	murine CD105	parental

AA = amino acid; L= leucine; V = valine

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Parker et al. (1994) Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152: 163-175.

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Claims

1. An artificial peptide for the treatment of cancer, the peptide is derived from endoglin and comprises a nonapeptide, which inhibits 50 % of binding of a reference peptide to human leukocyte antigen A2 in a competitive binding assay at a concentration below 5 μ M of the nonapeptide, the nonapeptide has a leucine or methionine at amino acid position 2 and/or a valine or leucine at amino acid position 9, wherein the peptide is selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 16.
2. The artificial peptide of claim 1, wherein the peptide is derived from mammalian endoglin, mice endoglin, rat endoglin, guinea pig endoglin and/or primate endoglin, particularly human endoglin.
3. The artificial peptide of claim 1 or 2, wherein the reference peptide is a fluorescein labeled peptide.
4. An artificial peptide for the treatment of cancer, the peptide is derived from human endoglin and binds specifically to human leukocyte antigen A2, wherein the peptide is selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 16.
5. A polypeptide for the treatment of cancer, the polypeptide comprises the artificial peptide of any of the preceding claims.
6. A nucleic acid for the treatment of cancer, the nucleic acid encodes at least one peptide of any of claims 1 to 4 or at least one polypeptide of claim 5.
7. An antibody against at least one peptide of any of claims 1 to 4 or at least one polypeptide of claim 5 for the treatment of cancer.

8. A vaccine comprising at least one substance selected from the group consisting of the peptide of any of claims 1 to 4, the polypeptide of claim 5, the nucleic acid of claim 6 and the antibody of claim 7.
9. A method for preparing a vaccine comprising the steps of:
 - a) providing the peptide of any of claims 1 to 4, the polypeptide of claim 5 or the antibody of claim 7;
 - b) admixing an adjuvant to the peptide, polypeptide or antibody.
10. Use of at least one peptide of any of claims 1 to 4, polypeptide of claim 5, nucleic acid of claim 6 or antibody of claim 7 for the manufacture of a drug and/or a vaccine for the treatment and/or prevention of cancer or an immunological disorder.

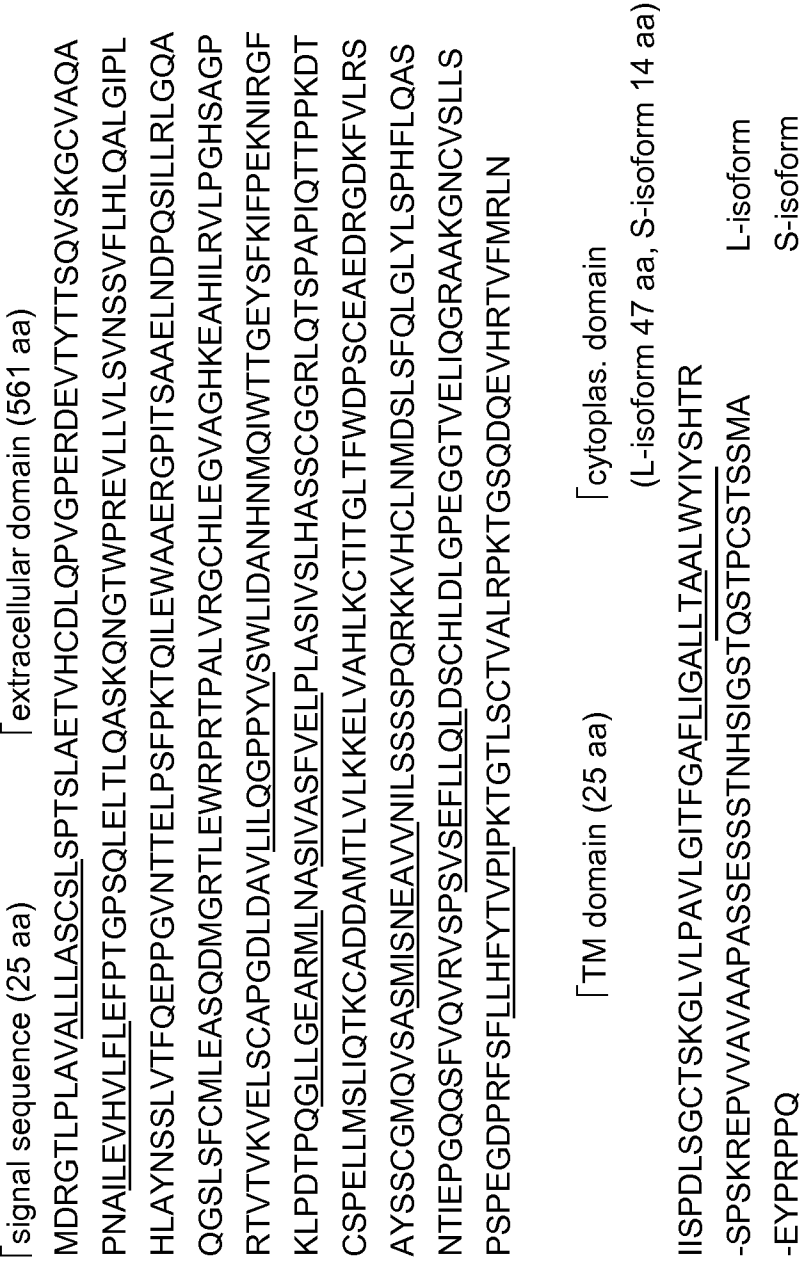


Fig. 1

peptide	IC ₅₀ (μM)	affinity
HIV Pol ⁴⁷⁶⁻⁴⁸⁴ (ILKEPVHGV)	10.25	++
hCD105 ¹¹⁻¹⁹ (ALLLASCSL)	12.19	++
hCD105 ⁶¹⁻⁶⁹ (ILEVHVLF $\overline{\text{L}}$)	22.99	+
hCD105 ²⁵¹⁻²⁵⁹ (LILQGPPYV)	2.77	+++
hCD105 ²⁹⁸⁻³⁰⁶ (GLLGEARML)	14.38	++
hCD105 ³⁰⁹⁻³¹⁷ (SIVASEVEL)	12.90	++
hCD105 ⁴¹⁹⁻⁴²⁷ (SMISNEAVV)	77.83	+
hCD105 ⁴⁸²⁻⁴⁹⁰ (SVSEFLLQL)	1.44	+++
hCD105 ⁵³³⁻⁵⁴¹ (LLHFYTVPI)	1.08	+++
hCD105 ⁶⁰⁰⁻⁶⁰⁸ (FLIGALLTA)	45.10	+
hCD105 ⁶⁰⁵⁻⁶¹³ (LLTAALWYI)	5.03	++

$\text{IC}_{50} < 5 \mu\text{M}$ high affinity
 $5 \mu\text{M} \leq \text{IC}_{50} \leq 15 \mu\text{M}$ intermediate affinity
 $15 \mu\text{M} < \text{IC}_{50} \leq 100 \mu\text{M}$ low affinity
 $\text{IC}_{50} > 100 \mu\text{M}$ no binding

+++
++
+
-

Fig. 2

peptide	IC ₅₀ (μM)	affinity	influence of modification on affinity
hCD105 ²⁵¹⁻²⁵⁹ (LILQGPPYV)	2.77	+++	
hCD105 ²⁵¹⁻²⁵⁹ (252L) (LLLQGPPYV)	2.14	+++	↑
hCD105 ³⁰⁹⁻³¹⁷ (SIVASFVEL)	12.90	++	
hCD105 ³⁰⁹⁻³¹⁷ (310L, 317V) (SLVASFVEV)	3.34	+++	↑↑
hCD105 ⁴⁸²⁻⁴⁹⁰ (SVSEFLLQL)	1.44	+++	
hCD105 ⁴⁸²⁻⁴⁹⁰ (483L, 490V) (SLSEFLLQV)	1.05	+++	↑
hCD105 ⁵³³⁻⁵⁴¹ (LLHFTYVPI)	1.08	+++	
hCD105 ⁵³³⁻⁵⁴¹ (541V) (LLHFTYVVPV)	0.46	+++	↑
hCD105 ⁶⁰⁰⁻⁶⁰⁸ (FLIGALLTA)	45.10	+	
hCD105 ⁶⁰⁰⁻⁶⁰⁸ (608V) (FLIGALLTV)	1.45	+++	↑↑
hCD105 ⁶⁰⁵⁻⁶¹³ (LLTAALWYI)	5.03	++	
hCD105 ⁶⁰⁵⁻⁶¹³ (613V) (LLTAALWYV)	2.65	+++	↑↑

IC ₅₀ < 5 μM	high affinity	+++
5 μM ≤ IC ₅₀ ≤ 15 μM	intermediate affinity	++
15 μM < IC ₅₀ ≤ 100 μM	low affinity	+
IC ₅₀ > 100 μM	no binding	-

Fig. 3

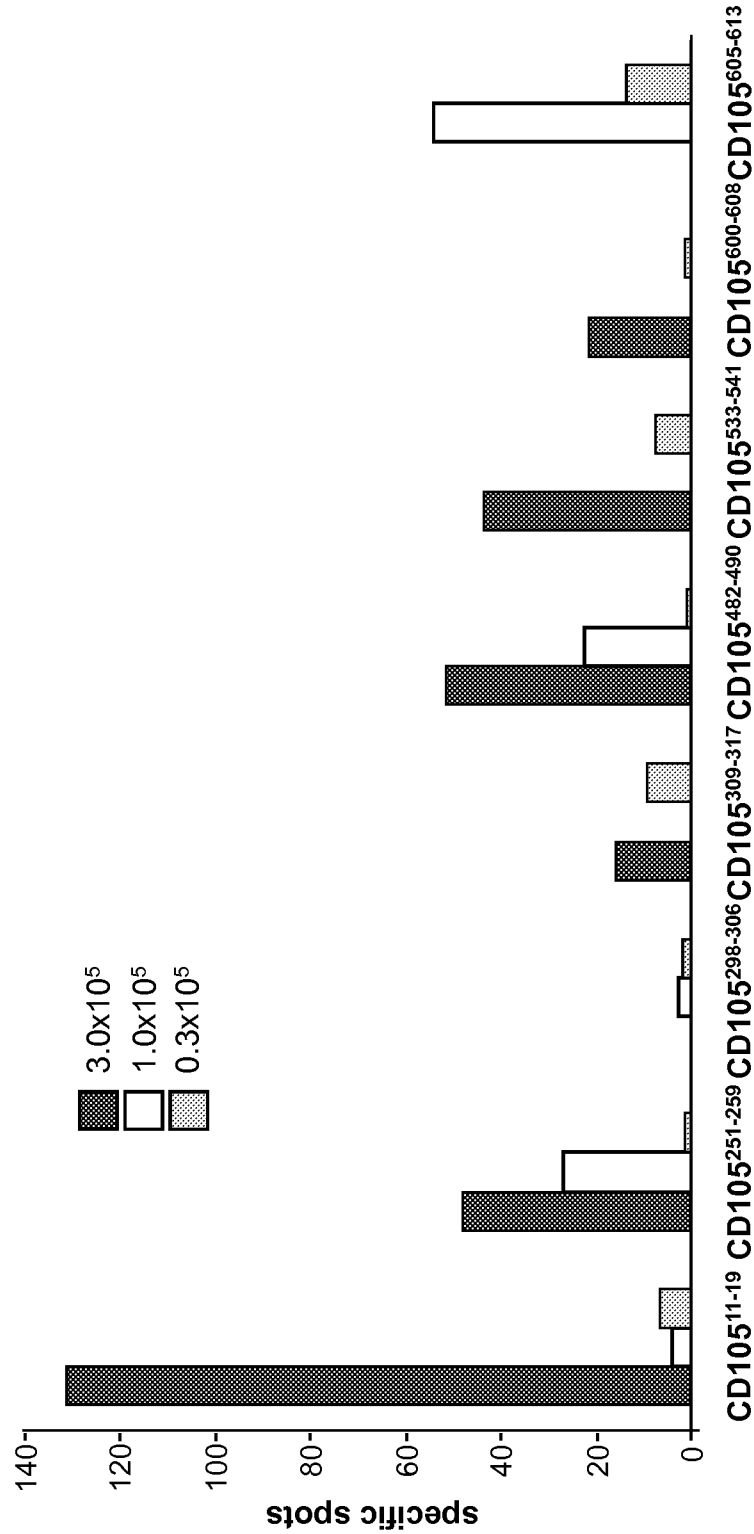


Fig. 4

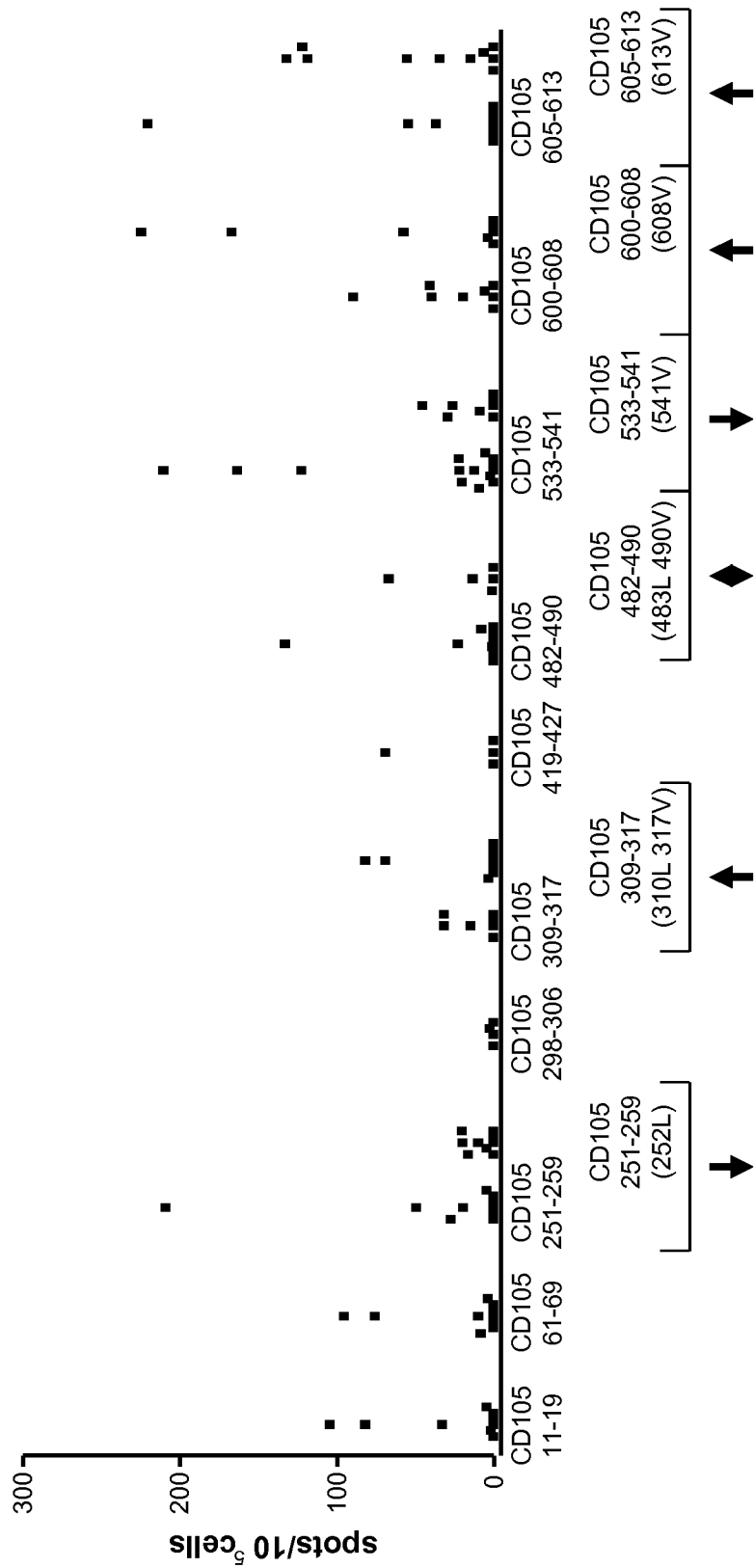


Fig. 5

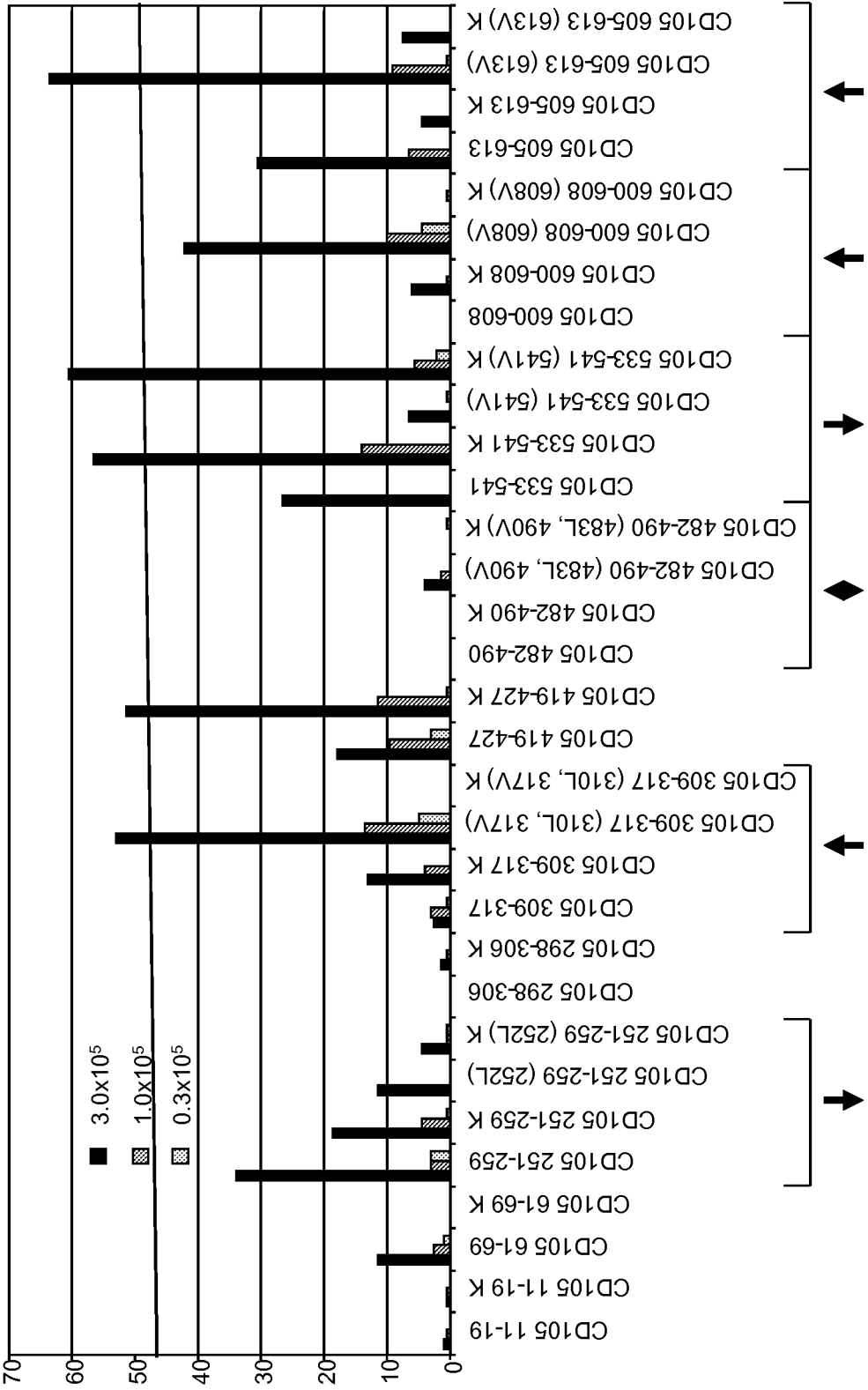


Fig. 6

[signal sequence (26 aa)] [extracellular domain (555 aa)]
 MDRGVLPLPITLLFVIYSFVPTTGLAERVGCDLPVDPTRGEVFTTTSQVSEGCVAQAANA
VREVHVLFLDFPGMLSHLELTQASKQNGTETREVLVLSNKNVFKQAPEIPLHLAYD
 SSLVIFQGGQPRVNITVLPSTSRKQILDWAATKGAITSAALDDPQSIVLQLGQDPKAPFLCL
 PEAHKDMGATLEWQPRAQTPVQSCRLEGVSGHKEAYILRILPGSEAGPRRTVTVMMEELSC
 TSGDAILILHGPPYVSWFIDINHSMQILTTGEYSVKIFPGSKVKGVELPDTTPQQGLIAEARKLN
ASIVTSFVELPLVSNVSLRASSCGGVFQTTPAPVVTTPPKDTCSPVLLMSLIQPKCGNQVMT
 LALNKKHVQTLQCTITGLTFWDSSCQAEDTDDHLVLSSAYSSCGMKVTAHVVSNEVIISFPS
 GSPPLRKKVQCIDMDSLFSQLGLYLSPHFLQASNTIELGQQAFVQSVSPLTSEVTVQLDSC
 HLDLGPEGDMVELIQSRTAKGSCVTLLSPSPEGDPFRFSFLLRVYVMVPTPTAGTLCNLALRP

 [TM domain (25 aa)] [cytoplas. domain]
 STLSQEVYKTVSMRLNVVSPDLSGKGLVLPVSLGITFGAFLIGALLTAALWYIYSHTRGPSKR
 EPVVAAPASSESSSTNHSIGSTQSTPCSTSSMA

Fig. 7

human parental CD105 peptide	human affinity improved CD105 peptide
hCD105 ¹¹⁻¹⁹ (ALLASCSL)	
hCD105 ⁶¹⁻⁶⁹ (ILEVHVLF)	
hCD105 ²⁵¹⁻²⁵⁹ (LILQPPYV)	hCD105 ²⁵¹⁻²⁵⁹ (252L) (LLQGPPYV)
hCD105 ²⁹⁸⁻³⁰⁶ (GLLGEARML)	
hCD105 ³⁰⁹⁻³¹⁷ (SIVASFVEL)	hCD105 ³⁰⁹⁻³¹⁷ (310L, 317V) (SLVASFVEV)
hCD105 ⁴¹⁹⁻⁴²⁷ (SMISNEAVV)	
hCD105 ⁴⁸²⁻⁴⁹⁰ (SVSEFLLQL)	hCD105 ⁴⁸²⁻⁴⁹⁰ (483L, 490V) (SLSEFLLQV)
hCD105 ⁵³³⁻⁵⁴¹ (LLHFTVP)	hCD105 ⁵³³⁻⁵⁴¹ (541V) (LLHFTVPV)
hCD105⁶⁰⁰⁻⁶⁰⁸ (FLIGALLTA)	hCD105 ⁶⁰⁰⁻⁶⁰⁸ (608V) (FLIGALLTV)
hCD105⁶⁰⁵⁻⁶¹³ (LITTAALWYI)	hCD105 ⁶⁰⁵⁻⁶¹³ (613V) (LLTAALWYV)

Fig. 8

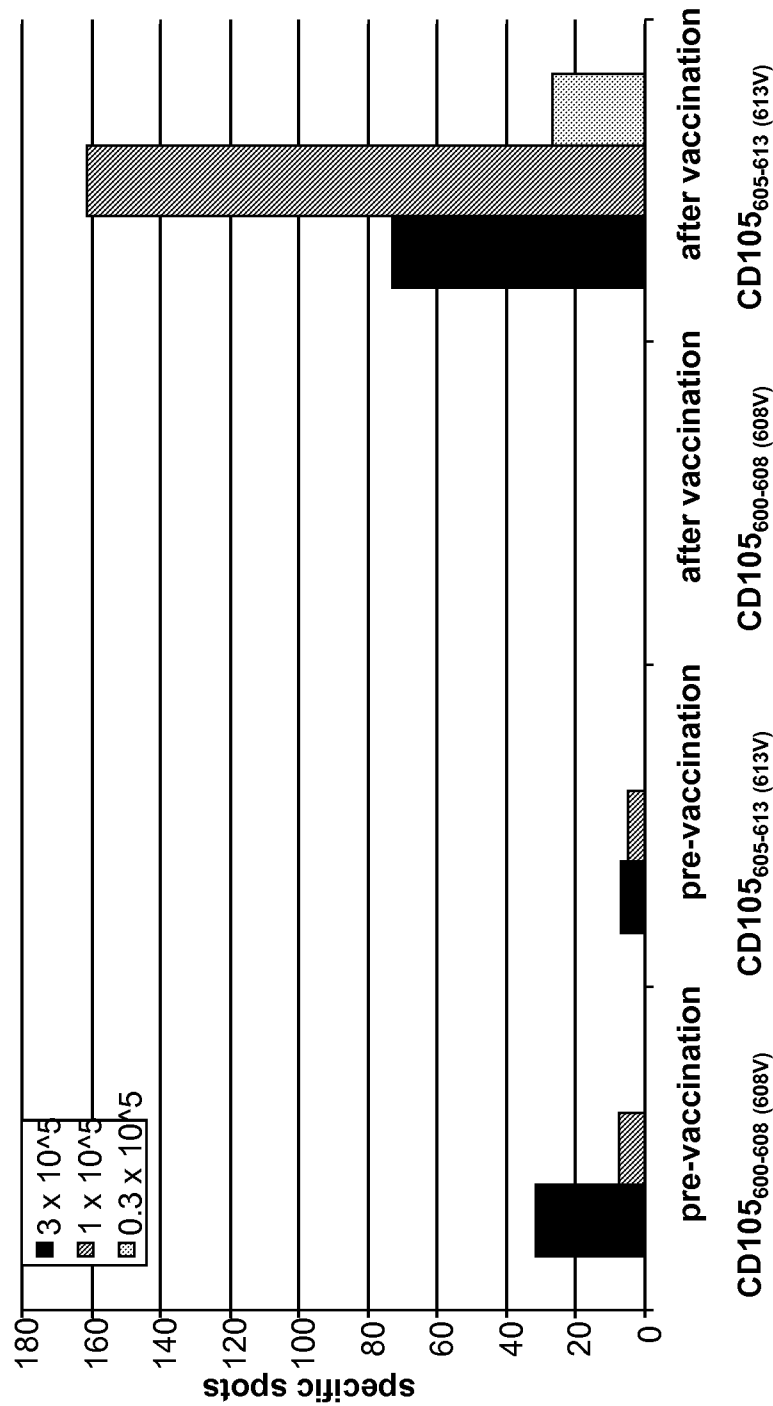


Fig. 9

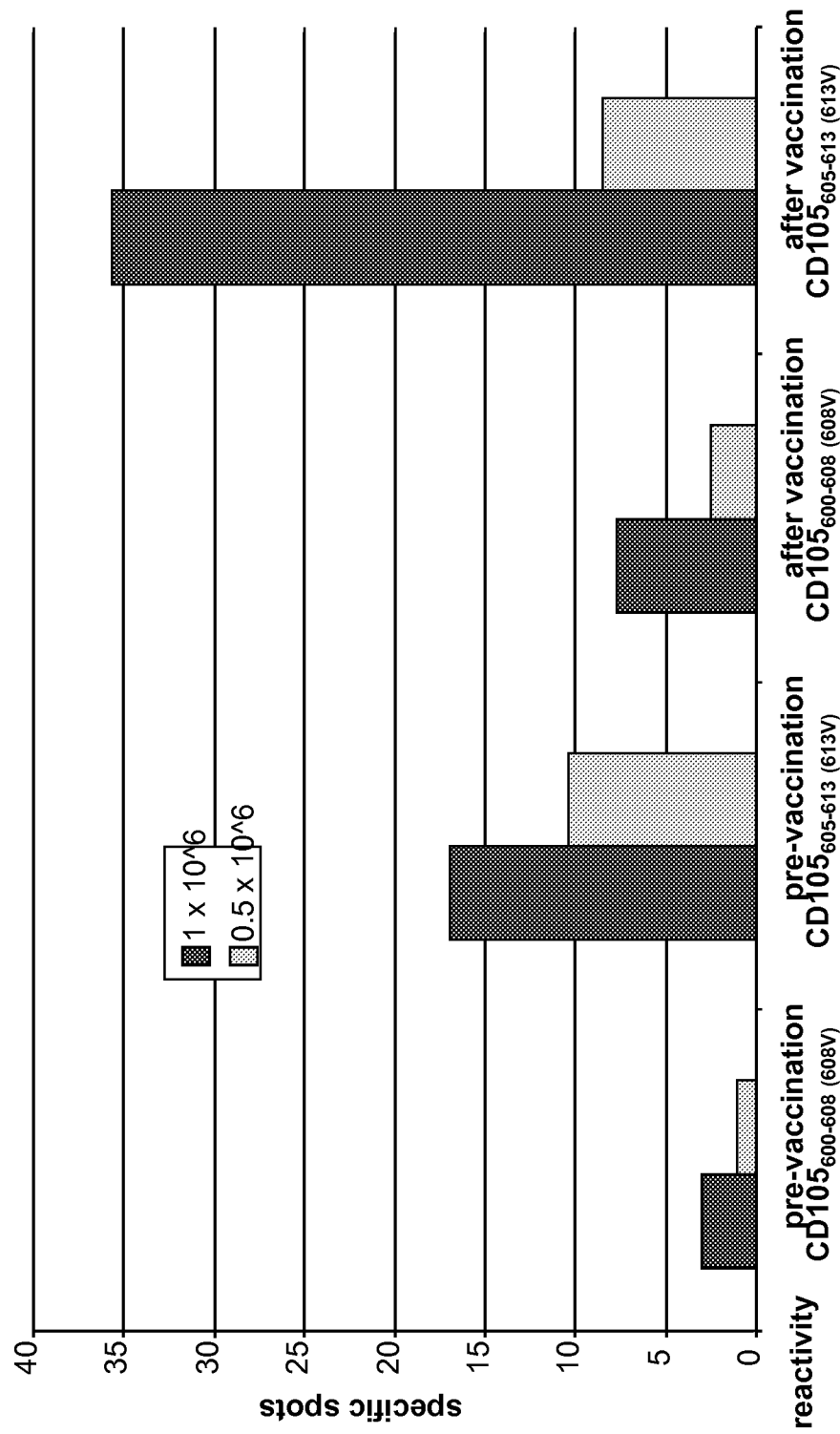


Fig. 10

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/060487

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/00 C07K16/28 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAN GUANG-HONG ET AL: "Active immunotherapy of tumors with a recombinant xenogeneic endoglin as a model antigen" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 34, no. 7, July 2004 (2004-07), pages 2012-2021, XP002508237 ISSN: 0014-2980 abstract page 2013, column 1, last paragraph - column 2, paragraph 4 page 2015, column 1, paragraph 2 - last paragraph page 2018, column 1, paragraph 2 page 2018, column 2 - page 2019, column 1, paragraph 2 ----- -/--	1-2, 5, 8-10

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

11 December 2009

Date of mailing of the international search report

18/12/2009

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
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Authorized officer

Noë, Veerle

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/060487

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAN GUANG-HONG ET AL: "Combination of recombinant xenogeneic endoglin DNA and protein vaccination enhances anti-tumor effects" IMMUNOLOGICAL INVESTIGATIONS, vol. 36, no. 4, 2007, pages 423-440, XP008099601 ISSN: 0882-0139 abstract page 425, paragraph 2 - page 426, paragraph 1 page 428, paragraphs 2,3 -----	1-2,5-6, 8-10
X	MIZUTANI N ET AL: "DNA vaccines suppress angiogenesis and protect against growth of breast cancer metastases." BREAST DISEASE 2004, vol. 20, 2004, pages 81-91, XP008099602 ISSN: 0888-6008 page 87, column 1, last paragraph - page 89, column 1, paragraph 1 -----	6,8,10
X	JIAO ET AL: "A plasmid DNA vaccine encoding the extracellular domain of porcine endoglin induces anti-tumour immune response against self-endoglin-related angiogenesis in two liver cancer models" DIGESTIVE AND LIVER DISEASE, W.B. SAUNDERS, vol. 38, no. 8, 1 August 2006 (2006-08-01), pages 578-587, XP005541415 ISSN: 1590-8658 abstract -----	6,8,10
X	US 6 190 660 B1 (SEON BEN K [US]) 20 February 2001 (2001-02-20) abstract column 4, lines 53-64 column 5, lines 5-18 column 9, lines 26-33 page 9, line 52 - page 10, line 30 column 11, lines 19-51 column 19, line 63 - column 21, last line ----- -/--	7-10

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/060487

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOFMEISTER V ET AL: "Peptide Epitopes derived from tumor stroma-associated antigens CD105 and FAP alpha for cancer immunotherapy" JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 125, no. 3, Suppl. S, September 2005 (2005-09), page A70, XP008099590 & 35TH ANNUAL MEETING OF THE EUROPEAN-SOCIETY-FOR-DERMATOLOGICAL-RESEARCH; TUBINGEN, GERMANY; SEPTEMBER 22 -24, 2005 ISSN: 0022-202X abstract -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/060487

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6190660...	B1	20-02-2001	NONE