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(54) **Title:** ARTIFICIAL ANTIGEN PRESENTING CELLS THAT CONSTITUTIVELY EXPRESS AN ANTIGEN ALONG WITH A HLA-CLASS II MOLECULE

(57) **Abstract:** CD4 T cell help is essential to promote robust cytotoxic T cell responses and could be harnessed to improve outcomes of cancer immunotherapy. To induce CD4+ T cell responses, the inventors have developed artificial antigen presenting cells (AAPCs) that derived from a mouse fibroblast cell line genetically modified to express a single human leukocyte antigen class II molecule (HLA-DR), the human CD80 costimulation as well as CD54 and CD58 adhesion molecules and that constitutively express an antigen (Ag) in peptide or protein forms in different compartments involved in the major histocompatibility complex class II Ag presentation pathway. In particular, the inventors show that the AAPC expressing the Ag peptide in the endoplasmic reticulum (ER) or protein at the plasma membrane were more potent than Epstein-Barr virus (EBV)-transformed B cells to present epitopes to specific CD4+ T-cells. Interestingly, AAPC targeting the Ag peptide in the ER was more efficient than peptide-pulsed AAPC or autologous APC to amplify memory Ag-specific CD4+ T cells that harbor a Th1 profile and express granzyme B. So, the AAPC system is a reliable and standardized tool to generate a high number of Ag-specific CD4+ with effector functions useful for a cancer adoptive immunotherapy. Thus, the present invention relates to an artificial antigen presenting cell that constitutively expresses an antigen along with a HLA-class II molecule and uses thereof in particular for amplifying and/or activating a population of antigen-specific CD4+ T cells.

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ARTIFICIAL ANTIGEN PRESENTING CELLS THAT CONSTITUTIVELY EXPRESS AN ANTIGEN ALONG WITH A HLA-CLASS II MOLECULE

FIELD OF THE INVENTION:

The present invention is in the field of immunology. In particular, the present invention relates to an artificial antigen presenting cell that constitutively expresses an antigen along with a HLA-class II molecule and uses thereof in particular for amplifying and/or activating a population of antigen-specific CD4+ T cells.

BACKGROUND OF THE INVENTION:

Adoptive cell immunotherapy (ACI) strategies, using human functional Ag-specific T lymphocytes (TLs), represent an emerging therapeutic approach to treat cancer but also chronic viral infections. Even if cytotoxic CD8+ TLs (CTLs) are the main effectors of the anti-tumor immune response, CD4+ helper T cells (Th) are crucial to support indirectly the CD8+ responses.[1] CD40 signaling on dendritic cells (DCs) *via* an interaction with activated Th cells expressing CD40-L increased Ag presentation and costimulatory capacity of DCs and promote efficient CTL priming.[2] IL-2 secretion by Th cells and induction of IL-15 production by DCs is critical for memory CTL responses.[3,4] Recently, it was shown that CD4+ T cells up-regulated CD70 on DCs which in turn interact with CD27 on CTLs delivering a help program that amplify CTL responses and down-regulated exhaustion molecules such as Programmed Death-1 (PD-1).[5] In addition, CD4+ TLs can act directly against tumor cells without contribution of CD8+ T cells. CD4+ T cells including Th1, Th9 and Th17 can act directly against tumor by secreting effector cytokines and/or displaying cytotoxic activity against major histocompatibility complex class II (MHC-II) positive tumors.[6–8]

The use of CD4+ TLs in preliminary clinical trials of ACI has shown promising results. Infusion of *in vitro* expanded autologous CD4+ T-cell clones specific for the New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO-1) Ag led to durable remission in a patient with metastatic melanoma.[9] Moreover, MHC-II-restricted neoantigens were found in many cancers and represent interesting targets for clinical applications.[10–12] A disease stabilization was observed in a patient with metastatic cholangiosarcoma after ACI with Th1 tumor infiltrating lymphocytes (TILs) specific for a neoantigen deriving from the erbb2 interacting protein (ERBB2IP).[13]

However, the main limiting step for an effective ACI is the generation of a sufficient number of Ag-specific TLs by antigen presenting cells (APCs). *In vitro*, preparation of such

APCs, like DCs, is time consuming and can raises concerns about their quantity, maintenance of their phenotype and functionality. To bypass these difficulties, our group has developed artificial antigen presenting cells (AAPCs) derived from mouse fibroblast NIH/3T3 and expressing a single human leukocyte antigen class II (HLA-II) molecule (HLA-DR1), the co-stimulatory molecule CD80 (B7.1) and the two adhesion molecules CD54 (intercellular adhesion molecule-1, ICAM-1) and CD58 (lymphocyte function-associated antigen-3, LFA-3).[14] We have shown that these AAPCs (named $AAPC^{DR}$) have strong abilities to present exogenous peptide or epitope from whole Ag that have been uptaken. $AAPC^{DR}$ exhibit lower ability to prime specific CD4+ T cells than autologous peripheral blood mononuclear cells (PBMCs), but had better ability to re-stimulate functional memory Ag-specific Th1 cells than autologous APCs.

To optimize and standardize $AAPC^{DR}$ model, induction of endogenous and constitutive expression of the Ag could be an interesting approach. Nevertheless, the cell compartments to target for Ag expression should be accurately investigated. In the classical MHC-II Ag presentation pathway, MHC-II molecules α and β chains are assembled within the endoplasmic reticulum (ER) and associated with the invariant chain (Ii). This heterotrimer migrate, *via* the Golgi apparatus, to the MHC-II compartment (MIIC). In this specialized endolysosomal compartment, Ii is sequentially degraded by proteases and only the class II invariant chain peptide (CLIP) remains in the MHC-II molecule groove. CLIP peptides are then replaced by peptides of higher affinity due to the HLA-DM chaperone protein. These peptides are mainly originated from exogenous Ags that are endocytosed by APCs and degraded by proteases but could arise from endogenous peptide from autophagosomes. Then, peptide/MHC-II complexes will migrate to the APC plasma membrane.[15–17]

SUMMARY OF THE INVENTION:

As defined by the claims, the present invention relates to an artificial antigen presenting cell that constitutively expresses an antigen along with a HLA-class II molecule and uses thereof in particular for amplifying and/or activating a population of antigen-specific CD4+ T cells.

DETAILED DESCRIPTION OF THE INVENTION:

CD4 T cell help is essential to promote robust cytotoxic T cell responses and could be harnessed to improve outcomes of cancer immunotherapy. To induce CD4+ T cell responses, the inventors have previously developed artificial antigen presenting cells (AAPCs) that derived from a mouse fibroblast cell line genetically modified to express a single human leukocyte antigen class II molecule (HLA-DR), the human CD80 costimulation as well as CD54 and

CD58 adhesion molecules. In this study, the model was optimized with novel AAPCs HLA-DR1+ ($AAPC^{DR1}$) that constitutively express an antigen (Ag) in peptide or protein forms in different compartments involved in the major histocompatibility complex class II Ag presentation pathway. The inventors found that $AAPC^{DR1}$ expressing the Ag peptide in the endoplasmic reticulum (ER) or protein at the plasma membrane were more potent than Epstein-Barr virus (EBV)-transformed B cells to present epitopes to specific CD4+ T-cells. In addition, these two Ag-encoding AAPC cell lines prime specific CD4+ T cells but at lower level than autologous peripheral blood mononuclear cells (PBMCs) used as CPA. Interestingly, $AAPC^{DR1}$ targeting the Ag peptide in the ER was more efficient than peptide-pulsed $AAPC^{DR1}$ or autologous APC to amplify memory Ag-specific CD4+ T cells that harbor a Th1 profile and express granzyme B. So, our AAPC system is a reliable and standardized tool to generate a high number of Ag-specific CD4+ with effector functions useful for a cancer adoptive immunotherapy.

A) Artificial antigen presenting cells of the present invention:

Thus the first object of the present invention relates to an artificial antigen presenting cell consisting of a eucaryotic cell that is genetically modified to stably express a single MHC class II molecule, at least one accessory molecule and at least one antigen.

As used herein, the term "antigen-presenting cell" or "APC" refers to a class of cells capable of presenting antigen to T lymphocytes which recognize antigen when it is associated with a major histocompatibility complex molecule. APCs elicit a T cell response to a specific antigen by processing the antigen into a form that is capable of associating with a major histocompatibility complex molecule on the surface of the APC.

As used herein, an "artificial antigen presenting cell" or "AAPC" refers to a eucaryotic cell that has been genetically engineered to function as a professional APC for one or more selected antigens.

According to the present invention, the eucaryotic cell does not express naturally MHC molecules and in particular class II molecules. In some embodiments, the eucaryotic cell is naturally deficient for the expression of a MHC molecule. In some embodiments, the eucaryotic cell was previously genetically modified so as to not express a MHC molecule (e.g. by using CRISPR-cas9). In some embodiments, the eucaryotic cell is naturally deficient for the expression of a molecule that is involved in the formation and transport of the MHC class II molecules. In some embodiments, the eucaryotic cell was previously genetically modified so as to not express molecule that is involved in the formation and transport of the MHC class II

molecules. Typically, the molecule that is involved in the formation and transport of the MHC class II molecules is the invariant molecule also named as “li” or “CD74”.

In some embodiments, the eucaryotic cell is not a cell deriving from the hematopoietic lineage and can be human, murine, rodentia, insect, or any other mammalian cells. When the artificial antigen presenting cell is used for activation and/or amplifying human specific CD4+ T cells, the eucaryotic cell is preferably selected from a different species. In some embodiments, the eucaryotic cell is preferably a murine cell. In some embodiments, the cells are fibroblasts, and more particularly murine fibroblast (e.g. NIH/3T3 mouse fibroblasts).

As used herein, the term "MHC Class II" or "Class II" refers to the human Major Histocompatibility Complex Class II proteins, binding peptides or genes. The human MHC region, also referred to as HLA, is found on chromosome six and includes the Class I region and the Class II region. Within the MHC Class II region are found the DP, DQ and DR subregions for Class II α chain and β chain genes (i.e., DPα, DPβ, DQα, DQβ, DRα, and DRβ).

As used herein, the term "MHC Class II molecule" means a covalently or non-covalently joined complex of an MHC Class II α chain and an MHC Class II β chain. MHC class II molecules bind peptides in an intracellular processing compartment and present these peptides on the surface of antigen presenting cells to T cells. As used herein, the term "MHC Class II α chain" means a naturally occurring polypeptide, or one encoded by an artificially mutated α gene, essentially corresponding to at least the α1 and α2 extracellular domains of one of the gene products of an MHC Class II α gene. As the C-terminal transmembrane and cytoplasmic portions of the α chain are not necessary for antigenic peptide binding in the present invention, they may be omitted while retaining biological activity. As used herein, the term "MHC Class II β chain" means a naturally occurring polypeptide, or one encoded by an artificially mutated β gene, essentially corresponding to at least the β1 and β2 extracellular domain of one of the gene products of an MHC Class II β. As the C- terminal transmembrane and cytoplasmic portions of the β chain are not necessary for antigenic peptide binding in the present invention, they may be omitted while retaining biological activity.

In some embodiments, the MHC class II molecule is selected from the group consisting of HLA-DQ molecules, HLA-DP molecules and HLA-DR molecules. In some embodiments, the MHC class II molecule is selected from the group consisting of HLA-DR1, HLA-DR15 and HLA-DR51 molecules.

In some embodiments, the at least one accessory molecule is selected from the group consisting of co-stimulatory molecules and adhesion molecules.

The term “co-stimulatory molecule” is used herein in accordance with its art recognized meaning in immune T cell activation. Specifically, a “co-stimulatory molecule” refers to a group of immune cell surface receptor/ligands which engage between T cells and antigen presenting cells and generate a stimulatory signal in T cells which combines with the stimulatory signal (i.e., “co-stimulation”) in T cells that results from T cell receptor (“TCR”) recognition of antigen on antigen presenting cells.

In some embodiments, the co-stimulatory molecule is CD80. As used herein the term “CD80” has its general meaning in the art and refers to B7-1 molecule which is a protein found on activated B cells and monocytes that provides a costimulatory signal necessary for T cell activation and survival. It is the ligand for two different proteins on the T cell surface: CD28 (T cell activation and survival) and CTLA-4 (T cell inhibition) (Peach, R J; Bajorath J, Naemura J, Leytze G, Greene J, Aruffo A, Linsley P S (Sep 1995). "Both extracellular immunoglobin-like domains of CD80 contain residues critical for binding T cell surface receptors CTLA-4 and CD28". J. Biol. Chem. (UNITED STATES) 270 (36): 21181–7; Stamper, C C; Zhang Y, Tobin J F, Erbe D V, Ikemizu S, Davis S J, Stahl M L, Seehra J, Somers W S, Mosyak L (Mar 2001). "Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses". Nature (England) 410 (6828): 608–11).

As used herein, the term "adhesion molecule" refers to a molecule on the surface of a cell whose primary, or predominant, function is to increase the strength or avidity of the interaction of the cell with another cell (e.g., the interaction between a T cell and an artificial antigen presenting cell of the present invention). Examples of families of adhesion molecules include integrins and selectins.

In some embodiment, the adhesion molecule is CD54. As used herein the term “CD54” has its general meaning in the art and refers to ICAM-1 (Intercellular Adhesion Molecule 1) also known as CD54 (Cluster of Differentiation 54) (Carlson M, Nakamura Y, Payson R, O'Connell P, Leppert M, Lathrop GM, Lalouel JM, White R (May 1988). "Isolation and mapping of a polymorphic DNA sequence (pMCT108.2) on chromosome 18 D18S24". Nucleic Acids Res. 16 (9): 4188.; Katz FE, Parkar M, Stanley K, Murray LJ, Clark EA, Greaves MF (January 1985). "Chromosome mapping of cell membrane antigens expressed on activated B cells". Eur. J. Immunol. 15 (1): 103–6). ICAM-1 is a member of the immunoglobulin superfamily, the superfamily of proteins including antibodies and T-cell receptors. ICAM-1 is a transmembrane protein possessing an amino-terminus extracellular domain, a single transmembrane domain, and a carboxy-terminus cytoplasmic domain. The structure of ICAM-

1 is characterized by heavy glycosylation, and the protein's extracellular domain is composed of multiple loops created by disulfide bridges within the protein.

In some embodiment, the adhesion molecule is CD58. As used herein the term "CD58" has its general meaning in the art and refers to lymphocyte function-associated antigen 3 (LFA-3) which is a cell adhesion molecule expressed on Antigen Presenting Cells (APC), particularly macrophages (Barbosa JA, Mentzer SJ, Kamarck ME, Hart J, Biro PA, Strominger JL, Burakoff SJ (April 1986). "Gene mapping and somatic cell hybrid analysis of the role of human lymphocyte function-associated antigen-3 (LFA-3) in CTL-target cell interactions". J. Immunol. 136 (8): 3085–91.; Wallich R, Brenner C, Brand Y, Roux M, Reister M, Meuer S (15 March 1998). "Gene structure, promoter characterization, and basis for alternative mRNA splicing of the human CD58 gene". J. Immunol. 160 (6): 2862–71).

In some embodiments, the eucaryotic cell is genetically modified to stably express the CD80, CD54 and CD58 molecules.

As used herein, the term "antigen" ("Ag") refers to a whole protein or peptide capable of eliciting a T-cell response. The skilled person in the art will be able to select the appropriate antigen, depending on the desired T-cell stimulation.

In some embodiments, the eucaryotic cell is genetically modified to stably express the antigen in a particular compartment of the cell. In some embodiments, when the antigen is peptide, it is particularly advantageous to express said peptide is in the endoplasmic reticulum (ER). In some embodiments, when the antigen is a protein it is particularly advantageous to express said protein in the endosomes or in the membrane (e.g. a transmembrane protein). Thus the skilled person will thus select the antigen with the appropriate way of expression.

As used herein, the term "endoplasmic reticulum" or "ER" has its general meaning in the art and refers to a eukaryotic cell organelle that forms an interconnected network of tubules, vesicles, and cisternae within cells that is involved in the production of phospholipids and proteins, among other functions. The ER facilitates proper protein folding and quality control (QC) for protein processing. During translation, proteins are translocated into the ER lumen after recognition of the signal sequence.

As used herein, the term "endosome" has its general meaning in the art and refers to a membrane-bound compartment inside eukaryotic cells. It is a compartment of the endocytic membrane transport pathway originating from the trans Golgi membrane.

As used herein, the term "signal peptide" or "leader sequence" has its general meaning in the art and refers to a short amino acid sequence that directs a newly synthesized protein through the endoplasmic reticulum, golgi or endosomes. The signal peptide is a short peptide,

commonly 5-30 amino acids long, present at the N- terminus of the majority of newly synthesized proteins. Signal peptides commonly contain a core sequence which is a long stretch of hydrophobic amino acids that has a tendency to form a single alpha-helix. The signal peptide may begin with a short positively charged stretch of amino acids, which helps to enforce proper topology of the peptide during translocation. At the end of the signal peptide there is typically a stretch of amino acids that is recognized and cleaved by signal peptidase. The hydrophobic amino acids in the core may, for example be: Alanine (A); Valine (V); Isoleucine (I); Leucine (L); Methionine (M); Phenylalanine (P); Tyrosine (Y); or Tryptophan (W). Signal sequences can be detected or predicted using software techniques (see for example, http://www.predisi.de/). A very large number of signal sequences are known, and are available in databases. For example, http://www.signalpeptide.de lists 2109 confirmed mammalian signal peptides in its database.

In some embodiments, the signal peptide is the signal peptide of the T cell surface glycoprotein CD8 alpha chain. In particular, use of said signal peptide is particularly advantageous for expressing a peptide in the endoplasmic reticulum. In some embodiments, the signal peptide has the amino acid sequence as forth in SEQ ID NO: 1.

SEQ ID NO:1

MALPVTALLLPLALLLHASQA

Accordingly, in some embodiments, the eucaryotic cell is genetically modified to stably express the antigen (e.g. peptide) fused to the signal sequence. In some embodiments, the antigen (e.g. peptide) is fused directly at its N-terminal end to the signal sequence. As used herein, the term “directly” means that the first amino acid at the N-terminal end of the antigen (e.g. peptide) is fused to the last amino acid at the C-terminal end of the signal peptide.

According to the present invention when the antigen is a protein, the eucaryotic cell is genetically modified to stably express a protein (i.e. the antigen) at the plasma membrane.

As used herein, the term “plasma membrane” means the phospholipid bilayer structure that comprises the external boundary of a cell. Plasma membranes have an external or extracellular face and an internal or intracellular face. Each face has unique features and components (i.e. proteins) associated with it that give it its unique properties. In addition, adherent cells exhibit polarity, meaning the aspect of the cell that is attached to the basement membrane, called the basal face, has different features than the aspect of the cell that is adluminal, called the apical face.

In some embodiments, the protein comprises a domain that will anchored the protein to the plasma membrane. In some embodiments, the protein comprises a transmembrane domain, i.e. a protein domain which traverses the cell membrane.

In some embodiments, the protein is a transmembrane protein. As used herein, the term "transmembrane protein" is a type of membrane protein spanning the entirety of the plasma membrane to which it is permanently attached in nature. That is, in nature, transmembrane proteins span from one side of a membrane through to the other side of the membrane.

In some embodiments, the antigen is a viral antigen. Examples of viral antigens include but are not limited to influenza viral Antigens (e.g. hemagglutinin (HA) protein, matrix 2 (M2) protein, neuraminidase), respiratory syncitial virus (RSV) Antigens (e.g. fusion protein, attachment glycoprotein), polio, papillomaviral (e.g. human papilloma virus (HPV), such as an E6 protein, E7 protein, L1 protein and L2 protein), Herpes simplex, rabies virus and flavivirus viral Ags (e.g. Dengue viral Ags, West Nile viral Ags), hepatitis viral Ags including Ags from HBV and HCV, human immunodeficiency virus (HIV) Ags (e.g. gag, pol or nef), herpesvirus (such as cytomegalovirus and Epstein-Barr virus) Ags (e.g. pp65, IE1, EBNA-1, BZLF-1) and adenovirus Ags.

In some embodiments, the antigen is a bacterial antigen. Examples of bacterial Ags include but are not limited to those from Streptococcus pneumonia, *Haemophilus influenza, Staphylococcus aureus, Clostridium difficile and enteric gram-negative pathogens including Escherichia, Salmonella, Shigella, Yersinia, Klebsiella, Pseudomonas, Enterobacter, Serratia, Proteus, B. anthracis, C. tetani, B. pertussis, S. pyogenes, S. aureus, N. meningitidi*s and *Haemophilus influenzae type b*.

In some embodiments, the antigen is a fungal or protozoal antigen. Examples include but are not limited to those from *Candida spp., Aspergillus spp., Crytococcus neoformans, Coccidiodes spp., Histoplasma capsulatum, Pneumocystis carinii, Paracoccidiodes brasiliensis, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae*.

In some embodiments, the antigen is a tumor-associated Antigen (TAA). Examples of TAAs include, without limitation, melanoma-associated Ags (Melan-A/MART-1, MAGE-1, MAGE-3, TRP-2, melanosomal membrane glycoprotein gp100, gp75 and MUC-1 (mucin-1) associated with melanoma); CEA (carcinoembryonic Antigen) which can be associated, e.g., with ovarian, melanoma or colon cancers; folate receptor alpha expressed by ovarian carcinoma; free human chorionic gonadotropin beta (hCGP) subunit expressed by many different tumors, including but not limited to ovarian tumors, testicular tumors and myeloma;

HER-2/neu associated with breast cancer; NY-ESO-1 of metastatic carcinomas, encephalomyelitis antigen HuD associated with small-cell lung cancer; tyrosine hydroxylase associated with neuroblastoma; prostate-specific antigen (PSA) associated with prostate cancer; CA125 associated with ovarian cancer; and the idiotypic determinants of a B-cell lymphoma that can generate tumor-specific immunity (attributed to idiotype-specific humoral immune response). Moreover, Ags of human T cell leukemia virus type 1 have been shown to induce specific cytotoxic T cell responses and anti-tumor immunity against the virus-induced human adult T-cell leukemia (ATL).

In some embodiments, the antigen is an auto-antigen. As used herein, the term "auto-antigen" means any self-antigen arising from the own body tissues which is mistakenly recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors. Examples of auto-antigens include but are not limited to preproinsulin (PPI), glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), islet-specific glucose-6-phosphatase catalytic-subunit-related protein (IGRP), zinc transporter 8 (ZnT8) and chromogranin A for T1D; myeloperoxydase and proteinase 3 for granulomatosis with polyangiitis; myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) in multiple sclerosis; and gliadins in celiac disease

In some embodiments, the antigen is an allergen. As used herein, the term "allergen" generally refers to an antigen or antigenic portion of a molecule, usually a protein, which elicits an allergic response upon exposure to a subject. Typically the subject is allergic to the allergen as indicated, for instance, by the wheal and flare test or any method known in the art. A molecule is said to be an allergen even if only a small subset of subjects exhibit an allergic immune response upon exposure to the molecule.

The AAPC of the present invention may be prepared according to any well-known method in the art. For general guidance regarding the preparation of artificial antigen-presenting cells according to the invention, the skilled would refer to the international patent application WO 2001094944; Latouche JB, Sadelain M. Induction of human cytotoxic T lymphocytes by artificial antigenpresenting cells. Nat Biotechnol 2000;18:405–9. Briefly, the AAPCs of the present are typically produced ex vivo by the insertion of one or more recombinant or synthetic nucleic acid sequences (genes) encoding the molecules of interest, such that the molecules are expressed in effective amounts in the recipient subject cell. Accordingly, genetic modification of the eucaryotic cell can be accomplished at any point during their maintenance by transducing a substantially homogeneous cell composition with a recombinant DNA construct. The nucleic

acid sequences can be obtained by conventional methods well known to those skilled in the art. Typically, said nucleic acid is a DNA or RNA molecule. Useful nucleic acid molecules for constructing the AAPCs of the present invention (e.g., selected antigens, MHC molecules, adhesion molecules, costimulatory molecules, etc.) are cloned into a vector before they are introduced into the eucaryotic cell and optionally are passage in cells other than AAPCs to generate useable quantities of these nucleic acids. As used herein, the terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a eucaryotic cell, so as to transform the cell and promote expression (e.g. transcription and translation) of the introduced sequence. Suitable vectors for the invention may be plasmid or viral vectors, including baculoviruses, adenoviruses, poxviruses, adenoassociated viruses (AAV), and retrovirus vectors (Price et al, 1987, Proc. Natl. Acad. Sci. USA, 84:156-160) such as the MMLV based replication incompetent vector pMV-7 (Kirschmeier et al., 1988, DNA, 7:219-225), as well as human and yeast modified chromosomes (HACs and YACs). Plasmid expression vectors include plasmids including pBR322, pUC or Bluescript.TM. (Stratagene, San Diego, Calif). Typically retroviral vectors are used for introducing the nucleic acid of interest into the eucaryotic cell. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse- transcription (Coffin, 1990, in Fields et al., Ceds, Virology, Raven Press, New York, pp. 1437- 1500). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the eucaryotic cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the subject cell genome (Coffin, supra). Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, 1990, Blood 76:271). Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.), 1989, Greene Publishing Associates, Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus cell lines include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types,

including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al., 1985, Science, 230:1395- 1398; Danos, et al., 1988, Proc. Natl. Acad. Sci. USA, 85:6460-6464; Wilson et al., 1988, Proc. Natl. Acad. Sci. USA, 85:3014-3018; Armentano et al., 1990, Proc. Natl. Acad. Sci. USA, 87:6141-6145; Huber et al, 1991, Proc. Natl. Acad. Sci. USA, 88:8039-8043; Ferry et al., 1991, Proc. Natl. Acad. Sci. USA, 88:8377-8381; Chowdhury et al., 1991, Science, 254:1802-1805; van Beusechem et al., 1992, Proc. Natl. Acad. Sci. USA, 89:7640-7644; Kay et al., 1992, Human Gene Therapy, 3:641-647; Dai et al., 1992, Proc. Natl. Acad. Sci. USA, 89:10892-10895; Hwu et al., 1993, J. Immunol., 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the subject genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell. In some embodiments, gamma-retroviral vector is used (Tobias Maetzig, Melanie Galla, Christopher Baum, and Axel Schambach Gammaretroviral Vectors: Biology, Technology and Application. Viruses. Jun 2011; 3(6): 677–713.). In particular a Gamma-retrovirus-derived SFG vector as described in EXAMPLE is used.

In some embodiments, a plurality of vectors are employed, each vector encoding one molecule of interest (i.e. the MHC class II molecule, the accessory molecule or the antigen). In some embodiments, the expression of the exogenous nucleic acid is under the control of a promoter. Examples of promoters and enhancers used in the expression vector for animal cell include early promoter and enhancer of SV40 (Mizukami T. et al. 1987), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana Y et al. 1987), promoter (Mason JO et al. 1985) and enhancer (Gillies SD et al. 1983) of immunoglobulin chain and the like.

In some embodiments, a vector comprising a nucleic acid sequence encoding for the signal peptide operatively linked to a nucleic acid sequence encoding for the antigen (e.g. peptide) is used to stably express the antigen peptide into the endoplasmic reticulum of the eucaryotic cell. In some embodiments, the nucleic acid sequence encoding for the signal peptide consists of the nucleic acid sequence as set forth in SEQ ID NO: 2.

SEQ ID NO: 2

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCATCGCAAGCA

As used herein, the terms "operably linked", or "operatively linked" are used interchangeably herein, and refer to the functional relationship of the nucleic acid sequences

and indicates that two or more DNA segments are joined together such that they function in concert for their intended purposes.

Typically, the expression vectors comprise one or more regulatory elements to drive and/or enhance expression of upstream or downstream nucleic acids. These regulatory sequences are selected on the basis of the cells (e.g., types of AAPCs) to be used for expression, and are operatively linked to a nucleic acid sequence to be expressed. The term "regulatory elements" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory elements are described, for example, in Goeddel; 1990, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA. Regulatory elements include those which direct expression of a nucleotide sequence in many types of subject cells as well as those which direct expression of the nucleotide sequence only in certain subject cells (e.g., tissue-specific regulatory sequences). Regulatory elements also include those which direct constitutive expression of an operatively linked nucleic acid sequence and those which direct inducible expression of the nucleic acid sequence. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, i.e., promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad range of cells in which they can activate and/or modulate transcription while others are functional only in a limited subset of cell types (See e.g., Voss et al., 1986, Trends Biochem. Sci., 11:287; and Maniatis et al., supra, for reviews). For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many eucaryotic species and has been widely used for the expression of proteins in mammalian cells (Dijkema et al, 1985, EMBO J. 4:761). Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor lα gene (Uetsuki et al., 1989, J. Biol. Chem., 264:5791; Kim et al., 1990, Gene, 91:217; and Mizushima, et al., 1990, Nagata, Nuc. Acids. Res., 18:5322) and the long terminal repeats of the Rous sarcoma virus (Gorman et al., 1982, Proc. Natl. Acad. Sci. USA, 79:6777) and the human cytomegalovirus (Boshart et al., 1985, Cell, 41:521). Suitable promoters which may be employed include, but are not limited to, TRAP promoters, adenoviral promoters, such as the adenoviral major late promoter; the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter, heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the

Herpes Simplex thymidine kinase promoter; retroviral LTRs; ITRs; the β- actin promoter; and human growth hormone promoters. The promoter also may be the native promoter that controls the nucleic acid encoding the polypeptide and the sequences of native promoters may be found in the art (see Agrawal et al., 2000, J. Hematother. Stem Cell Res., 795- 812; Cournoyer et al., 1993, Annu. Rev. Immunol., 11:297-329; van de Stolpe et al., 1996, J. Mol. Med., 74:13-33; Herrmann, 1995, J. Mol. Med., 73:157-63). A variety of enhancer sequences can also be used in the instant invention including but not limited to: Immunoglobulin Heavy Chain enhancer; Immunoglobulin Light Chain enhancer; T- Cell Receptor enhancer; HLA DQα and DQβ enhancers ; β-Interferon enhancer; interleukin-2 enhancer; Interleukin-2 Receptor enhancer;; MHC Class II HLA-DRα enhancer ; β-Actin enhancer; Muscle Creatine Kinase enhancer; Prealbumin (Transthyretin) enhancer; Elastase I enhancer; Metallothionein enhancer; Collagenase enhancer; Albumin Gene enhancer; α-Fetoprotein enhancer; β-Globin enhancer; c-fos enhancer; c-HA-ras enhancer; Insulin enhancer; Neural Cell Adhesion Molecule (NCAM) enhancer; α1-Antitrypsin enhancer; H2B (TH2B) Histone enhancer; Mouse or Type I Collagen enhancer; Glucose- Regulated Proteins (GRP94 and GRP78) enhancer; Rat Growth Hormone enhancer; Human Serum Amyloid A (SAA) enhancer; Troponin I (TN I) enhancer; Platelet-Derived Growth Factor enhancer; Duchenne Muscular Dystrophy enhancer; SV40 Polyoma enhancer; Retrovirusal enhancer; Papilloma Virus enhancer; Hepatitis B Virus enhancer; Human Immunodeficiency enhancer; Cytomegalovirus enhancer; and Gibbon Ape Leukemia Virus enhancer.

The artificial antigen presenting cell of the present invention may find applications and uses in a wide number of contexts.

B) Methods of activating and/or amplifying a population of antigen-specific CD4+ T cells:

A further object of the present invention relates to a method of activating and/or amplifying a population of antigen-specific CD4+ T cells comprising the steps of coculturing a population of a T cells with the population of artificial antigen presenting cells of the present invention.

In some embodiments, the population of AAPCs is irradiated by any conventional method well known in the art before incubation with the population of CD4 T cells.

In some embodiments, the population of CD4+ T cells is substantially purified by magnetic bead purification systems such as those available in the art, e.g., Miltenyi beads (Myltenyi Biotec) and Dynabead systems (Dynal Biotech) or with cell sorting procedures, such as FACS-based methods, or other appropriate cell sorting devices and methodologies. In some

embodiments, the population of CD4+ T cells is purified from a PBMC sample. The term "PBMC" or "peripheral blood mononuclear cells" or "unfractionated PBMC", as used herein, refers to whole PBMC, i.e. to a population of white blood cells having a round nucleus, which has not been enriched for a given sub-population. Cord blood mononuclear cells are further included in this definition. Typically, the PBMC sample according to the invention has not been subjected to a selection step to contain only adherent PBMC (which consist essentially of >90% monocytes) or non-adherent PBMC (which contain T cells, B cells, natural killer (NK) cells, NK T cells and DC precursors). A PBMC sample according to the invention therefore contains lymphocytes (B cells, T cells, NK cells, NKT cells), monocytes, and precursors thereof. Typically, these cells can be extracted from whole blood using Ficoll, a hydrophilic polysaccharide that separates layers of blood, with the PBMC forming a cell ring under a layer of plasma. Additionally, PBMC can be extracted from whole blood using a hypotonic lysis buffer which will preferentially lyse red blood cells.

The population of CD4+ T cells and the population of AAPC are cocultured for a time sufficient to activate and enrich for a desired population of activated memory CD4+ T cells. For instance, the T cells and the AAPCs of the present invention are contacted for 5, 6, 7, 8, 9, 10, 11, or 12, days. Typically the T cells and the AAPCs of the present invention are contacted for at least 7 days. Any culture medium suitable for growth, survival and differentiation of T cells is used for the coculturing step. Typically, it consists of a base medium containing nutrients (a source of carbon, aminoacids), a pH buffer and salts, which can be supplemented with serum of human or other origin and/or growth factors and/or antibiotics to various cytokines could be added. Typically, the base medium can be RPMI 1640, DMEM, IMDM, X-VIVO or AIM-V medium, all of which are commercially available standard media.

In particular, the method of the present invention is particularly suitable for activating and/or amplifying a population of antigen-specific memory CD4+ T cells.

As used herein the term "memory CD4+ T cell" has its general meaning in the art and to a subset of CD4+ T cells that are specific to the antigen they first encountered and can be called upon during the secondary immune response. Typically, memory CD4+ T cells are characterized by the expression at their cell surface of CD45RO.

In some embodiments, the method further comprises the step of isolating the population of antigen-specific CD4+ T cells. Methods for isolating the population of antigen-specific CD4+ T cells are conventional to the skilled person. In some embodiments, the method may use Class II multimers. With this procedure, Ag-reactive T cells recognizing specific peptide epitopes are detected, using either commercially available reagents (e.g., ProImmune MHC

Class I Pentamers, Class II Ultimers; or Immudex MHC Dextramers) or in-house generated ones, e.g., from the NIH Tetramer Facility at Emory University, USA; from Dr. S. Buus, University of Copenhagen, Denmark [Leisner et al., *PLoSOne* 3:e1678, 2008], from Dr. G.T. Nepom, Benaroya Research Institute, Seattle, USA [Novak et al., *J.Clin.Invest.* 104:R63, 1999]. In some embodiments, the method is based on the detection of the upregulation of activation markers. With this procedure, Antigen-specific T helper cell responses are detected by their differential expression of activation markers exposed on the membrane following Ag-recognition. In some embodiments, the method may consist in a cytokine capture assay. This system developed by Miltenyi Biotech is a valid alternative to the ELISpot to visualize Antigen-specific T helper cells according to their cytokine response. In some embodiments, the method may consist of a CD154 assay. This procedure has been described in detail [Chattopadhyay et al., *Nat.Med.* 11:1113, 2005; Frentsch et al., *Nat.Med.* 11: 1118, 2005]. It is limited to detection of Ag-specific CD4+ T cells. In some embodiments, the method may consist in a CFSE dilution assay. This procedure detects Antigen-specific T helper cells according to their proliferation following Ag recognition [Mannering et al., *J.Immunol.Methods* 283:173, 2003]. Other methods suitable for detecting cell proliferation (e.g. BrdU incorporation, Ki67 expression) may also be used. Besides being suitable for detecting the population of antigen-specific CD4+ T cells, said methods allows the direct sorting and/or cloning of the T cells of interest.

In some embodiments, the method of the present invention is particularly suitable for generating antigen-specific memory Th1, Th2 or Th17 cells. The term "T helper cell" ("Th cell") refers to a subset of lymphocytes which complete maturation in the thymus and have various roles in the immune system, including the identification of specific foreign antigens in the body and the activation and deactivation of other immune cells. By this, T helper cells are involved in almost all adaptive immune responses. Mature Th cells are believed to always express the surface protein CD4 and are therefore also termed CD4+ T cells. As used herein, the term “Th1 cell” and “Th2 cell” mean a type-1 helper T cell and a type-2 helper T cell, respectively. For instance Th1 cells produce high levels of the proinflammatory cytokine IFNγ. Polarization in said T cell subset can be carried out by any conventional method well known in the art that typically consists in incubation the T cells with at least one cytokine (e.g. IL-12 for Th1 cells). As used herein, the term “Th17 cells” has its general meaning in the art and refers to a subset of T helper cells producing interleukin 17 (IL-17). “A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage”. Nat. Med. 13 (2): 139-145, 2007). The term “IL-17” has its general meaning in the art and refers to the interleukin-17A protein. Typically, Th17 cells are characterized by classical expression of

Th cell markers at their cell surface such as CD4, and by the expression of IL-17. Typically, as referenced herein, a Th17 cell is a IL-17+ cell.

In some embodiments, the method of the present invention further comprise a step consisting of polarizing the antigen-specific CD4+ T cells into a population of antigen-specific T regulatory cells.

As used herein, the term 'Treg' or 'T regulatory cell' denotes a T lymphocyte endowed with a given antigen specificity imprinted by the TCR it expresses and with regulatory properties defined by the ability to suppress the response of conventional T lymphocytes or other immune cells. Such responses are known in the art and include, but are not limited to, cytotoxic activity against antigen-presenting target cells and secretion of different cytokines. Different types of Tregs exist and include, but are not limited to: inducible and thymic-derived Tregs, as characterized by different phenotypes such as CD4+CD25+/high, CD4+CD25+/highCD127-/low alone or in combination with additional markers that include, but are not limited to, FoxP3, neuropilin-1 (CD304), glucocorticoid-induced TNFR-related protein (GITR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152); T regulatory type 1 cells; T helper 3 cells. All these Tregs can be obtained either upon direct ex vivo purification or upon in vitro expansion or differentiation from the population of antigen-specific CD4+ T cells of the present invention. Examples of in vitro amplification protocols can be found in Battaglia et al., J. Immunol. 177:8338-8347 (2006), Putnam et al., Diabetes 58:652-662 (2009), Gregori et al., Blood 116:935-944 (2009). Typically, the polarization consists in incubating the antigen-specific T helper cells with an amount of at least one cytokine such as TGFbeta.

The population of antigen-specific CD4+ T cells of the present invention is particularly suitable for adoptive cell therapy in subjects in need thereof.

For example, the population of antigen-specific CD4+ T cells of the present invention are suitable for the treatment of cancer. As used herein, the term "cancer" has its general meaning in the art and includes, but is not limited to, solid tumors and blood-borne tumors. The term cancer includes diseases of the skin, tissues, organs, bone, cartilage, blood and vessels. The term "cancer" further encompasses both primary and metastatic cancers. Examples of cancers that may be treated by methods and compositions of the invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal tract, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant;

carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous; adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; Paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and roblastoma, malignant; Sertoli cell carcinoma; Leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; Ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant;

chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

In some embodiments, the population of antigen-specific CD4+ T cells of the present invention are suitable for treating subjects afflicted with, or at risk of developing, an infectious disease, including but not limited to viral, retroviral, bacterial, and protozoal infections, etc. Subjects that can be treated include immunodeficient patients afflicted with a viral infection, including but not limited to CMV, EBV, adenovirus, BK polyomavirus infections in transplant patients, etc. Typically, the subjects at risk of developing an infectious disease include patients undergoing hematopoietic stem cell transplantation using peripheral blood or CB precursors. As used herein, the term "patient undergoing hematopoietic stem cell transplantation (HSCT)" refers to a human being who has to be transplanted with HSC graft. Typically, said patient is affected with a disorder which can be cured by HSCT. In some embodiments, the patient undergoing HSCT is affected with a disorder selected from the group consisting of leukemia, lymphoma, myeloproliferative disorders, myelodysplastic syndrome (MDS), bone marrow (BM) failure syndromes, congenital immunodeficiencies, enzyme deficiencies and hemoglobinopathies. In some embodiments, the HSCT is an allogeneic HSCT. As used herein, the term "allogeneic" refers to HSC deriving from, originating in, or being members of the same species, where the members are genetically related or not. An "allogeneic transplant" refers to transfer of cells or organs from a donor to a recipient, where the recipient is the same species as the donor. Allogeneic transplantation involves infusion of donor stem cells, typically using a donor that matches the recipient's MHC. However, matched unrelated donor (MUD) transplants are also associated with a stronger graft versus host reaction, and thus result in higher mortality rates. In another embodiment, the HSCT is an autologous HSCT. As used herein, the term "autologous" refers to deriving from or originating in the same subject or

patient. An "autologous transplant" refers to collection and retransplant of a subject's own cells or organs. Autologous transplantation involves infusion of a recipient's own cells following myeloablative treatment. Autologous cell transplants minimize the risk of graft versus host disease (GVHD) and result in reduced complications. Thus, the population of antigen-specific CD4+ T cells of the present invention are particularly suitable for preventing bacterial, viral, protozoal and/or fungal infection following CB HSCT. Non-limiting examples of viral infections include Herpes simplex virus (HSV) infections, CMV infections, Varicella-zoster virus (VZV) infections, Human herpes virus 6 (HHV6) infections, EBV infections, respiratory virus infections (such as respiratory syncytial virus (RSV), parainfluenza virus, rhinovirus, and influenza virus) and adenovirus infections. Non-limiting examples of bacterial infections include Gram-negative bacteria infections such as Escherichia (e.g. Escherichia coli), Salmonella, Shigella, and other Enterobacteriaceae, Pseudomonas (e.g. Pseudomonas aeruginosa), Moraxella, Helicobacter, and Legionella infections. Non-limiting examples of protozoal infections include Giardia infections (e.g. Giardia lamblia), Entamoeba infections (e.g. Entamoeba histolytica) and Toxoplasma (e.g. Toxoplasma gondii). Non-limiting examples of fungal infections include Aspergillus infection (e.g. Aspergillus fumigatus), Candida infection (e.g. Candida albicans and non-albicans Candida) and other emerging fungal infections including Trichosporon, Alternaria, Fusarium, and Mucorales infections.

In some embodiments, the population of antigen-specific CD4+ T cells of the present invention having regulatory properties are suitable for the treatment of autoimmune diseases. As used herein, the term “autoimmune disease” refers to the presence of an autoimmune response (an immune response directed against an auto- or self-antigen) in a subject. Autoimmune diseases include diseases caused by a breakdown of self-tolerance such that the adaptive immune system, in concert with cells of the innate immune system, responds to self-antigens and mediates cell and tissue damage. In some embodiments, autoimmune diseases are characterized as being a result of, at least in part, a humoral and/or cellular immune response. Examples of autoimmune disease include, without limitation, acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/Anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticaria, axonal

and neuronal neuropathies, Behcet's disease, bullous pemphigoid, autoimmune cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogans syndrome, cold agglutinin disease, congenital heart block, coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), discoid lupus, Dressler's syndrome, endometriosis, eosinophilic fasciitis, erythema nodosum, experimental allergic encephalomyelitis, Evans syndrome, fibromyalgia, fibrosing alveolitis, giant cell arteritis (temporal arteritis), glomerulonephritis, Goodpasture's syndrome, granulomatosis with polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, hypergammaglobulinemia, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, inflammatory bowel disease, insulin-dependent diabetes (type 1), interstitial cystitis, juvenile arthritis, Kawasaki syndrome, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease (LAD), lupus (SLE), Lyme disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease (MCTD), monoclonal gammopathy of undetermined significance (MGUS), Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's), autoimmune neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS syndrome, polyarteritis nodosa, type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatica, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, psoriatic arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red cell aplasia, Raynaud’s phenomenon, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome,

subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/Giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, ulcerative colitis, undifferentiated connective tissue disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, Waldenstrom's macroglobulinemia (WM), and Wegener's granulomatosis [Granulomatosis with Polyangiitis (GPA)]. In some embodiments, the autoimmune disease is selected from the group consisting of rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus (lupus or SLE), myasthenia gravis, multiple sclerosis, scleroderma, Addison's Disease, bullous pemphigoid, pemphigus vulgaris, Guillain-Barré syndrome, Sjogren syndrome, dermatomyositis, thrombotic thrombocytopenic purpura, hypergammaglobulinemia, monoclonal gammopathy of undetermined significance (MGUS), Waldenstrom's macroglobulinemia (WM), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), Hashimoto's Encephalopathy (HE), Hashimoto's Thyroiditis, Graves' Disease, Wegener's Granulomatosis [Granulomatosis with Polyangiitis (GPA)]. In some embodiments, the autoimmune disease is type 1 diabetes.

In some embodiments, the population of antigen-specific CD4+ T cells of the present invention having regulatory properties are suitable for the treatment of allergies. As used herein, the term “allergy” generally refers to an inappropriate immune response characterized by inflammation and includes, without limitation, food allergies, respiratory allergies and other allergies causing or with the potential to cause a systemic response such as, by way of example, Quincke’s oedema and anaphylaxis. The term encompasses allergy, allergic disease, hypersensitive associated disease or respiratory disease associated with airway inflammation, such as asthma or allergic rhinitis. In some embodiments, the method of the present invention is effective in preventing, treating or alleviating one or more symptoms related to anaphylaxis, drug hypersensitivity, skin allergy, eczema, allergic rhinitis, urticaria, atopic dermatitis, dry eye disease, allergic contact allergy, food hypersensitivity, allergic conjunctivitis, insect venom allergy, bronchial asthma, allergic asthma, intrinsic asthma, occupational asthma, atopic asthma, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). Hypersensitivity associated diseases or disorders that may be treated by the method of the present invention include, but are not limited to, anaphylaxis, drug reactions, skin allergy, eczema, allergic rhinitis, urticaria, atopic dermatitis, dry eye disease [or otherwise referred to as Keratoconjunctivitis sicca (KCS), also called keratitis sicca, xerophthalmia], allergic contact allergy, food allergy, allergic conjunctivitis, insect venom allergy and respiratory diseases associated with airway inflammation, for example, IgE mediated asthma and non-IgE mediated asthma. The respiratory diseases associated with airway inflammation may include, but are not

limited to, rhinitis, allergic rhinitis, bronchial asthma, allergic (extrinsic) asthma, non-allergic (intrinsic) asthma, occupational asthma, atopic asthma, exercise induced asthma, cough-induced asthma, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD).

In some embodiments, the population of antigen-specific CD4+ T cells of the present invention having regulatory properties are suitable for the treatment of immune reactions against a grafted tissue or grafted hematopoietic cells or grafted blood cells. Typically the subject may have been transplanted with a graft selected from the group consisting of heart, kidney, lung, liver, pancreas, pancreatic islets, brain tissue, stomach, large intestine, small intestine, cornea, skin, trachea, bone, bone marrow, muscle, or bladder. The method of the present invention is also particularly suitable for preventing or suppressing an immune response associated with rejection of a donor tissue, cell, graft, or organ transplant by a recipient subject. Graft-related diseases or disorders include graft versus host disease (GVHD), such as associated with bone marrow transplantation, and immune disorders resulting from or associated with rejection of organ, tissue, or cell graft transplantation (e.g., tissue or cell allografts or xenografts), including e.g., grafts of skin, muscle, neurons, islets, organs, parenchymal cells of the liver, etc. Thus the method of the invention is useful for preventing Host-Versus-Graft-Disease (HVGD) and Graft-Versus-Host-Disease (GVHD). The population of antigen-specific CD4+ T cells may be administered to the subject before, during and/or after transplantation (e.g., at least one day before transplantation, at least one day after transplantation, and/or during the transplantation procedure itself). In some embodiments, the population of antigen-specific CD4+ T cells may be administered to the subject on a periodic basis before and/or after transplantation.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction

period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at regular intervals, e.g., daily, weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

The population of antigen-specific CD4+ T cells of the present invention can be utilized in methods and compositions for adoptive cell therapy in accordance with known techniques, or variations thereof that will be apparent to those skilled in the art based on the instant disclosure. See, e.g., US Patent Application Publication No. 2003/0170238 to Gruenberg et al; see also US Patent No. 4,690,915 to Rosenberg. In some embodiments, the cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "pharmaceutically acceptable" carrier) in a treatment-effective amount. Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin. A treatment-effective amount of cells in the composition is dependent on the relative representation of the antigen-specific T helper cells with the desired specificity, on the age and weight of the recipient, on the severity of the targeted condition and on the immunogenicity of the targeted Ags. These amount of cells can be as low as approximately 103/kg, preferably 5x103/kg; and as high as 107/kg, preferably 108/kg. The number of cells will depend upon the ultimate use for which the composition is intended, as will the type of cells included therein. For example, if cells that are specific for a particular Ag are desired, then the population will contain greater than 70%, generally greater than 80%, 85% and 90-95% of such cells. If frequencies of antigen-specific T cells are insufficient, T cell lines can be enriched by cell sorting using tetramers or dextramers®; or MACS® cytokine secretion

assay (Miltenyi Biotec) For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 ml or less, even 250 ml or 100 ml or less. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed the desired total amount of cells.

As used herein, the term "administering" refers to administration of the compounds as needed to achieve the desired effect. Administration may include, but is not limited to, oral, sublingual, intramuscular, subcutaneous, intravenous, transdermal, topical, parenteral, buccal, rectal, and via injection, inhalation, and implants.

C) Methods of identifying a population of CD4+ T:

A further object of the present invention relates to a method of identifying a population of CD4+ T cells within a test population of T cells comprising the steps of i) providing a population of artificial antigen presenting cells that express the antigen, ii) coculturing the population of T cells with the population of artificial antigen presenting cells of step i) and iii) detecting whether a population of CD4+ T cells is activated and/or amplified.

In some embodiments, the population of T cells is isolated from a biological sample obtained from a subject/patient.

As used herein, the term “sample” to any biological sample obtained from the purpose of evaluation in vitro. The sample is typically a tissue sample or a body fluid sample. The term “tissue sample” includes sections of tissues such as biopsy or autopsy samples and frozen sections taken for histological purposes. In some embodiments, the tissue sample is a tumor tissue sample. The term “tumor tissue sample” means any tissue tumor sample derived from the patient. Said tissue sample is obtained for the purpose of the in vitro evaluation. In some embodiments, the tumor sample may result from the tumor resected from the patient. In some embodiments, the tumor sample may result from a biopsy performed in the primary tumour of the patient or performed in metastatic sample distant from the primary tumor of the patient. Examples of body fluids are blood, serum, plasma, amniotic fluid, brain/spinal cord fluid, liquor, cerebrospinal fluid, sputum, throat and pharynx secretions and other mucous membrane secretions, synovial fluids, ascites, tear fluid, lymph fluid and urine. More particularly, the sample is a blood sample. As used herein, the term “blood sample” means a whole blood sample obtained from the patient.

For example, the method can be used to screen for the ability of a test agent (e.g. a vaccine.) to elicit a particular antigen-specific population of CD4+ T cells. This method would involve administering to the subject the test agent, obtaining sample comprising a population

of T cells and performing the method of the present invention for determining whether the antigen-specific population of CD4+ T cells can be activated and/or amplified.

In some embodiments, the method of the present invention is also particularly suitable for determining if a subject has been exposed to (or is presently exposed to) one or more particular antigens. For instance, the method of the invention can be used to see if a subject has any immunity left from previous vaccinations/immunizations. Known antigens associated with a given vaccine, for example, can be used to detect and quantitate any effector cells present in a biological sample obtained from the subject. One can also use the method of the present invention to identify the best antigen or combinations of antigens for a particular vaccine (e.g. for a particular year's influenza vaccine). In particular, the method of the present invention is also particular suitable for optimizing an antigen for use in a vaccine. The method typically involves providing a plurality of antigens that are candidates for the vaccine; screening the antigens using any of the method of the present invention; and selecting an antigen that is capable of activating and/or the antigen-specific population of CD4+ T cells.

In some embodiments, the method of the present invention may also find application in diagnosis. For instance, the method of the present invention is particularly suitable for diagnosing infectious diseases, cancer and autoimmune diseases. For instance, the method of the present invention can be used to detect one or more antigen specific T cell responses which are correlated with the disease. The method of the present invention is also particularly suitable for monitoring immune therapy. As used herein, the term “monitoring immune therapy” refers to measurement of changes in T cell responses induced in a given subject following in vivo administration of immune modulating agents. For monitoring applications, different types of situations are found, according to the type of disease. In autoimmune diseases, immune modulatory therapies can be used to blunt pathological immune responses. One strategy to accomplish this result relies on non-Ag-specific interventions based on a number of immune modulatory agents. For example, said immune modulatory agents is an immunosuppressive drug. As used herein, the term “immunosuppressive treatment” refers to any substance capable of producing an immunosuppressive effect, e.g., the prevention or diminution of the immune response and in particular the prevention or diminution of the production of Ig. Immunosuppressive drugs include, without limitation thiopurine drugs such as azathioprine (AZA) and metabolites thereof; nucleoside triphosphate inhibitors such as mycophenolic acid (Cellcept) and its derivative (Myfortic); derivatives thereof; prodrugs thereof; and combinations thereof. Other examples include but are not limited to 6-mercaptopurine ("6-MP"), cyclophosphamide, mycophenolate, prednisolone, sirolimus, dexamethasone, rapamycin,

FK506, mizoribine, azothioprine and tacrolimus. Other examples also include calcineurin inhibitors and corticosteroids. Examples of corticosteroids include flunisolide, prednisone, prednisolone, methylprednisolone, triamcinolone, deflazacort and betamethasone. corticosteroids, for example, cortisone, hydrocortisone, methylprednisolone, prednisone, prednisolone, betamethesone, beclomethasone dipropionate, budesonide, dexamethasone sodium phosphate, flunisolide, fluticasone propionate, triamcinolone acetonide, betamethasone, fluocinolone, fluocinonide, betamethasone dipropionate, betamethasone valerate, desonide, desoximetasone, fluocinolone, triamcinolone, triamcinolone acetonide, clobetasol propionate, and dexamethasone. Therefore it is of therapeutic interest to follow the immune changes induced by such intervention. Successful interventions should translate in a decrease (in the case of autoimmune diseases) or increase (in the case of cancer and infectious diseases) of the disease-related Ag-specific T cell responses. Such changes in disease-related Ag-specific T cell responses could be either quantitative or qualitative. Availability of these immune surrogate markers of clinical efficacy can be of great utility for a variety of applications. For example: better selection of patients to treat and therapeutic agents to use based on patient's immune responses; optimization and/or tailoring of therapeutic doses or administration regimens (for example, increase in doses/frequency of administration if no immune change is registered), thus improving the risk-to-benefit ratio; prognostic stratification of treated patients according to their probability to respond to treatment; decision of whether to treat patients again based on maintenance or not of the induced immune changes. In some embodiments, the method of the present invention is particularly suitable for determining whether a subject is at risk of relapse. As used herein, the term "relapse" refers to the return of signs and symptoms of a disease after a subject has enjoyed a remission after a treatment. Thus, if initially the target disease is alleviated or healed, or progression of the disease was halted or slowed down, and subsequently the disease or one or more characteristics of the disease resume, the subject is referred to as being "relapsed."

D) Methods of determining identifying and/or characterizing the T cell epitopes of an antigen:

A further object of the present invention relates to a method of determining identifying and/or characterizing the T cell epitopes of an antigen comprising the steps of i) providing a population of artificial antigen presenting cells that express the antigen, ii) coculturing the population of T cells with the population of artificial antigen presenting cells of step i), iii) detecting whether a population of CD4+ T cells is activated and/or amplified, iv) selecting the population of artificial antigen presenting cells that is capable of activating and/or amplifying

the population of CD4+ T cells, v) recovering and characterizing the peptides from the MHC class II molecules wherein said peptides represent the T cell epitopes of the antigen.

Typically, peptides are identified by the classical method of HLA-peptide complex extraction, peptide elution and subsequent peptide epitope identification by sequencing mass spectrometry. In particular, the method of the present invention comprises the steps of lyzing the population of artificial antigen presenting cells, recovering the MHC class II molecules typically in an immune affinity step, recovering the peptides from the MHC class II molecules and analyzing by mass spectrometry said peptides. In particular, the peptides are analyzed by LC-MS or LC-MS/MS that are the combination of liquid chromatography (LC) with mass spectrometry (MS). MS means you only analyze your precursor ion (as generated in the source) for example in an iontrap, a quadrupole or time-of-flight Massspec. MS/MS is the combination of two mass analyzers in one mass spectrometry instrument. The first MS filters for the precursor ion followed by a fragmentation of the precursor ion with high energy and e.g. nitrogen gas. A second mass analyzer is then filtering for the product ions, generated by the fragmentation. This is usually done in a triple quadrupole MS (QQQ) or a QTOF. The advantages of MS/MS is the increased and to can gain more structural information on the analyte (QTOF) based on the fragmentation pattern.

The method of the present invention is particularly suitable for determining protein antigenicity, identifying which antigens from an oncogene, virus, bacteria, pathogen, vaccine or other vector are actually presented by antigen presenting cells, discovering new peptide targets for cancer and infectious disease interventions, understanding the impact of protein modifications on immune responses…

For instance, the method of the present invention is thus particularly suitable for the in vitro study of the immunogenicity (or tolerogenicity) of therapeutic proteins. The term "therapeutic proteins" as used herein refers to protein or peptide compounds of any aminoacid length which are administered or are planned to be administered in vivo to human subjects to achieve a therapeutic effect. Examples of such therapeutic proteins are antibodies of different species (either in their native form or partially/fully humanized), cytokines, hormones or hormone analogues, coagulation factors, enzymes, bacterial or viral proteins. Such proteins are not limited to natural ones, but also include modified proteins or chimeric constructs, obtained for example by changing selected aminoacid sequences or by fusing portions of different proteins. In particular, there are two different therapeutic settings where evaluation of immunogenicity of therapeutic proteins is of relevance. One first therapeutic setting concerns the use of disease-associated antigens for in vivo administration, with the aim of inducing a

tolerogenic effect (e.g., in the case of autoimmune diseases) or an immunogenic effect (e.g., in the case of cancer or infectious diseases). It is important to first evaluate in vitro the potential to achieve said desired therapeutic effect. In other therapeutic settings, the aim is not to induce immunogenic responses of any kind to the administered protein, but rather to avoid such responses so to allow said protein to achieve the therapeutic effect for which it is designed. Example of such settings include, without being limited to, cytokine-based immune therapies, hormone replacement therapies and replacement therapies for coagulation factors (e.g., Factor VIII in Haemophilia A) or enzymatic deficits (e.g., beta-glucuronidase in mucopolysaccharidosis VII). In all these situations, mounting of immunogenic responses against the administered protein is not desirable, as this would be counterproductive for achieving the desired therapeutic effect (e.g., side effects such as cytokine release syndromes; or neutralization/degradation of the therapeutic protein).

Another application of the method of the present invention is its use for Ag or epitope discovery (also known as "mapping"), i.e. for screening Ags and epitopes in order to select those eliciting an Ag-specific T cell response.

E) Kits of the present invention:

Other aspects of the present invention include kits for carrying out the methods of the present invention. A kit may include the population of AAPC of the present invention and materials useful such as a device (e.g. a 96-well plate in which the AAPCs and the T cells are cocultured). Different combinations of such materials may be organized as a kit in order to aid the skilled artisan in carrying out the methods of the present invention.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1 Phenotype of the $AAPC^{DR1}$ cell lines. Expression of transduced molecules: the eight Ag-encoding $AAPC^{DR1}$ cell lines and the $AAPC^{DR1}$ cell line were stained with anti-human CD54, CD58, CD80 and HLA-DR antibodies. Fluorescence black histograms show the expression levels of transduced molecules. The white histograms represent isotype controls **(A)**. Expression of mouse MHC-II molecules: mouse splenocytes, NIH/3T3 cells and the $AAPC^{DR1}$ cell line were stained with an anti-mouse I-A/I-E antibody. Fluorescence black histograms show the expression levels of mouse "classical" MHC-II molecules (I-A/I-E; H-2A, H-2E). The white histograms represent isotype control **(B)**.

Figure 2 Peptide Ag presentation by the AAPC^{DR1} cell lines. AAPC^{DR1} or B-EBV^{DR1} cell lines loaded with HA or control peptide **(A)**, HA peptide-encoding AAPC^{DR1} cell lines **(B)** or HA protein-encoding AAPC^{DR1} cell lines were used to stimulate the HA-specific CD4+ T-cell clone **(C)**. Several ratios of stimulating cells to T cells (1 : 1, 1 : 3 and 1 : 9) were performed to discriminate the Ag presentation ability of the AAPC^{DR1} cell lines. Frequencies of activated TLs were evaluated by intracellular IFN-γ staining and FACS analysis. Results show percentages of IFN-γ+ cells among CD4+ TLs. A representative experiment from three independent experiments is shown.

Figure 3 Priming of CD4+ TLs by the AAPC^{DR1} cell lines or by autologous PBMCs. The AAPC^{DR1} cell lines expressing HA peptide in the ER or HA protein at the plasma membrane, or the AAPC^{DR1} cell line loaded with 10 µg/ml of HA peptide were used to stimulate purified CD4+ TLs for 14 days. Alternatively, autologous PBMCs were cultured with 10 µg/ml of HA peptide for the same duration. Frequency of HA-specific CD4+ effector TLs was evaluated by DR1-HA (or control DR1-CLIP) tetramer staining at day 14. Results show percentages of HA-tetramer+ cells among CD4+ TLs. A representative experiment is shown from four independent experiments with four donors **(A)** For the same primary culture condition, intracellular IFN-γ staining was also performed after reactivation by the B-EBV^{DR1} cell line loaded with 10 µg/ml of HA or control peptide. Results show percentages of IFN-γ+ cells among CD4+ TLs. A representative experiment is shown from six independent experiments with six donors **(B)**.

Figure 4 Re-stimulation of CD4+ TLs by the AAPC^{DR1} cell lines or by autologous PBMCs. TLs generated after primary culture of autologous PBMCs loaded with 10µg/ml of HA peptide for 14 days were re-stimulated for 7 additional days by the AAPC^{DR1} cell lines expressing HA peptide in the ER or HA protein at the plasma membrane, or by the AAPC^{DR1} cell line or autologous PBMCs loaded with 10 µg/ml of HA peptide. Frequency of HA-specific CD4+ effector TLs was evaluated by DR1-HA (or control DR1-CLIP) tetramer staining at day 21. Results show percentages of HA-tetramer+ cells among CD4+ TLs. For the same re-stimulation conditions, the naïve/memory phenotype of the generated CD4+ TLs was harvested by flow cytometry using the following antibodies: anti-human CD45RO, CD45RA, CD62L and CCR7. Results show percentages of cells subsets among CD4+ HA-tetramer+ TLs **(A)**. At days 14 and 21, the percentages and absolute numbers of HA-specific TLs were evaluated by tetramer staining. A representative experiment is shown from four independent experiments with four donors **(B)**. They were also evaluated by intracellular IFN-γ staining. A representative

experiment is shown from six independent experiments with six donors **(C)**.*P<0.05 (repeated measures of one-way ANOVA analysis followed by Dunnett's post test); ns, not significant.

Figure 5. Ag addressing strategies to target the MHC-II Ag presentation pathway. HA protein addressing strategies were developed to target the plasma membrane, the cytoplasm, endosomes, and the extracellular media. Excepted for the native HA protein which is addressed to the plasma membrane, the others approaches are based on the use of the extracellular domain of HA, coupling or not, to a specific signal peptide to reach the targeted cell compartment **(A)**. HA peptide addressing strategies were developed to target the cytoplasm, the ER, endosomes and the extracellular media. HA peptide is coupling, or not, to a specific signal peptide to reach the targeted cell compartment **(B)**. TM: Transmembrane domain, Vtg: Vitellogenin.

EXAMPLE:

Methods

Healthy donors and CD4$^+$ TLs purification

Peripheral blood from HLA-DRB1*01:01$^+$ (HLA-DR1) healthy donors of the French Blood Service (EFS Hauts de France, Caen, France) was collected in heparin tubes, after informed consent was obtained. PBMCs were isolated by density gradient centrifugation on lymphocyte separation medium (Eurobio,Courtaboeuf, France). CD4$^+$ TLs were isolated from PBMCs by negative magnetic purification, using the Dynabead Untouched Human CD4 T Cells Kit (ThermoFischer Scientific, Illkirch, France), according to the manufacturer's protocol.

Design of strategies for the targeting of Ag in MHC-II pathway compartments and construction of AAPC^{DR1} cell lines

The viral Ag of hemagglutinin (HA, Influenza A H3N2, strain A/Aichi/2/1968) used in this study is a transmembrane protein of 566 amino acids. It is consisted of a signal peptide (HA_{1-16}), an extracellular domain (HA_{17-530}) which contains the epitope $HA_{306-318}$ restricted to the HLA-DR1 context, a transmembrane domain ($HA_{531-551}$) and by a cytoplasmic domain ($HA_{552-566}$). Nine novels Ag-encoding AAPC^{DR1} were constructed. The first one was transduced with a vector encoding the whole HA protein (**Figure 5A**). For the others HA protein-encoding AAPC^{DR1}, the signal peptide, transmembrane and cytoplasmic domains of the protein were deleted to ameliorate Ag trafficking. In this condition, without signal peptide, the HA protein can be express in the cytoplasm. The extracellular domain of HA protein was coupled to the signal peptide of the glycoprotein 75 (gp75) *via* a linker to target endosomes, or to the secretion signal peptide of the vitellogenin (Vtg) in order to induce secretion of the protein.[18–20] Using the same approaches, the $HA_{306-318}$ peptide was used to target the cytoplasm, endosomes and

the extracellular media (**Figure 5B**). To target the ER, $HA_{306-318}$ peptide was coupled to the CD8α signal peptide, as we previously described.[21]

The sequences are depicted as follows:

SEQ ID NO:3 (Membrane HA protein (Membr HA prot)

CTCGAGGCCACCATGAAGACCATCATTGCCCTGAGCTACATCTTCTGCCTGGCTCTGGGCCAGGACCTG
CCCGGCAACGACAACAGCACCGCCACCCTGTGTCTGGGCCACCACGCCGTGCCTAACGGCACCCTGGTG
AAAACCATCACCGACGACCAGATCGAAGTGACCAACGCCACCGAGCTGGTGCAGAGCAGCAGCACCGGC
AAGATCTGCAACAACCCTCACAGAATCCTGGACGGCATCGACTGCACCCTGATCGACGCCCTGCTGGGC
GACCCTCACTGCGACGTGTTCCAGAACGAGACATGGGACCTGTTCGTGGAAAGAAGCAAGGCCTTCAGC
AACTGCTACCCCTACGACGTGCCCGACTACGCCAGCCTGAGAAGCCTGGTGGCCAGCAGCGGCACACTG
GAATTCATCACCGAGGGCTTCACCTGGACCGGCGTGACCCAGAACGGCGGCAGCAACGCCTGCAAGAGA
GGCCCTGGCAGCGGCTTCTTCAGCAGACTGAACTGGCTGACCAAGAGCGGCTCCACCTACCCCGTGCTG
AACGTGACCATGCCCAACAACGACAACTTCGACAAGCTGTACATCTGGGGCATCCACCACCCCAGCACC
AACCAGGAACAGACCAGCCTGTACGTGCAGGCCAGCGGCAGAGTGACCGTGTCCACCAGAAGAAGCCAG
CAGACCATCATCCCCAACATCGGCAGCAGACCCTGGGTCCGCGGCCTGAGCAGCAGAATCAGCATCTAC
TGGACCATCGTGAAGCCCGGCGACGTGCTGGTCATCAACAGCAACGGCAACCTGATCGCCCCTAGAGGC
TACTTCAAGATGAGAACCGGCAAGAGCAGCATCATGAGAAGCGACGCCCCTATCGACACCTGTATCAGC
GAGTGCATCACCCCTAACGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGTGAACAAGATCACCTAC
GGCGCCTGCCCCAAATACGTGAAGCAGAACACCCTGAAGCTGGCCACCGGCATGAGAAACGTGCCCGAG
AAGCAGACCAGAGGCCTGTTCGGCGCTATCGCCGGCTTCATCGAGAACGGCTGGGAGGGCATGATCGAC
GGGTGGTACGGCTTCAGACACCAGAACAGCGAGGGCACCGGCCAGGCCGCCGACCTGAAGTCTACACAG
GCCGCCATCGACCAGATCAACGGCAAGCTGAACAGAGTGATCGAAAAGACCAACGAGAAGTTCCACCAG
ATCGAGAAAGAATTCAGCGAGGTGGAAGGCAGAATCCAGGACCTGGAAAAGTACGTGGAAGATACCAAG
ATCGACCTGTGGTCCTACAACGCCGAGCTGCTGGTGGCCCTGGAAAACCAGCACACCATCGACCTGACC
GACAGCGAGATGAACAAGCTGTTCGAGAAAACCAGAAGGCAGCTGCGCGAGAACGCCGAGGAAATGGGC
AACGGCTGCTTCAAGATCTACCACAAGTGCGACAACGCCTGTATCGAGAGCATCAGAAACGGCACCTAC
GACCACGACGTGTACAGGGACGAGGCCCTGAACAACAGATTCCAGATCAAGGGCGTGGAACTGAAGTCC
GGCTACAAGGACTGGATTCTGTGGATCAGCTTCGCTATCAGCTGCTTCCTGCTGTGCGTGGTGCTGCTG
GGCTTCATCATGTGGGCCTGCCAGAGGGGCAACATCAG ATGCAACATCTGCATC TGAGAGGATCC

SEQ ID NO:4 Cytoplasmic HA protein (Cyto HA prot)

CTCGAGGCCACCATGCAGGACCTGCCCGGCAACGACAACAGCACCGCCACCCTGTGTCTGGGCCACCAC
GCCGTGCCTAACGGCACCCTGGTGAAAACCATCACCGACGACCAGATCGAAGTGACCAACGCCACCGAG
CTGGTGCAGAGCAGCAGCACCGGCAAGATCTGCAACAACCCTCACAGAATCCTGGACGGCATCGACTGC
ACCCTGATCGACGCCCTGCTGGGCGACCCTCACTGCGACGTGTTCCAGAACGAGACATGGGACCTGTTC
GTGGAAAGAAGCAAGGCCTTCAGCAACTGCTACCCCTACGACGTGCCCGACTACGCCAGCCTGAGAAGC
CTGGTGGCCAGCAGCGGCACACTGGAATTCATCACCGAGGGCTTCACCTGGACCGGCGTGACCCAGAAC
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SEQ ID NO:5 Endosomal HA protein (Endo HA prot)

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SEQ ID NO:6 Extracellular HA protein (Extracell HA prot)

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GCCGAGGAAATGGGCAACGGCTGCTTCAAGATCTACCACAAGTGCGACAACGCCTGTATCGAGAGCATC
AGAAACGGCACCTACGACCACGACGTGTACAGGGACGAGGCCCTGAACAACAGATTCCAGATCAAGGGC
GTGGAACTGAAGTCCGGCTACAAGGATTGGTGAGAGGATCC

SEQ ID NO:7 Cytoplasmic HA peptide (Cyto HA pept)

CTCGAGGCCACCATGCCCAAATACGTGAAGCAGAACACCCTGAAGCTGGCCACCTGAGAGGATCC

SEQ ID NO:8 Endoplasmic Reticulum HA peptide (ER HA pept)

CTCGAGGCCACCATGGCCCTGCCTGTGACAGCCCTGCTGCTGCCCCTGGCTCTGCTGCTGCACGCCTCC
CAGGCCCCTAAATACGTGAAGCAGAACACCCTGAAGCTGGCCACCTGAGAGGATCC

SEQ ID NO:9 Endosomal HA peptide (Endo HA pept)

CTCGAGGCCACCATGCCCAAATACGTGAAGCAGAACACCCTGAAGCTGGCCACCAGCGGCGGAAGCGGA
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GCCTCTTGCCTGATCAGAAGCAGATCCACCAAGAACGAGGCCAACCAGCCCCTGCTGACCGACCACTAC
CAGAGATACGCCGAGGACTACGAGGAACTGCCCAACCCCAACCACAGCATGGTCTAAGAGGATCC

SEQ ID NO:10 Extracellular HA peptide (Extracell HA pept)
CTCGAGGCCACCATGAGAGTGCTGGTGCTGGCCCTGGCCGTGGCTCTGGCTGTGGGCGATCAGAGCAAC
CTGGGCCCCAAATACGTGAAGCAGAACACCCTGAAGCTGGCCACCTGAGAGGATCC

All Ag-encoding complementary DNAs (cDNAs) were synthesized and purchased from GeneArt® (ThermoFischer Scientific). These cDNAs were optimized for Ag translation in *mus musculus* organism to favor their expression in NIH/3T3 based AAPCs. The construction of NIH/3T3-derived $AAPC^{DR1}$ was previously described.[14] The cDNAs encoding addressed Ags were subcloned into dicistronic gammaretrovirus-derived SFG vectors expressing puromycin-N-acetyltransferase. All constructs were verified by Sanger DNA sequencing. H29/293 GPG packaging cells were transfected with each vector, using the calcium chloride precipitation method.[22] $AAPC^{DR1}$ were genetically modified by sequential infections with cell-free gammaretroviral supernatants corresponding to the Ag of interest (Ag-encoding $AAPC^{DR1}$), in the presence of 8µg/ml of polybrene (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 16 h. Cells transduced by dicistronic vector were selected with 5µg/ml of puromycin (Sigma-Aldrich). $AAPC^{DR1}$ cell lines were cultured in DMEM (ThermoFischer Scientific) supplemented with 10% of decomplemented AB human serum (Sigma-Aldrich).

RNA extraction from $AAPC^{DR1}$ and mouse splenocytes followed by two-step RT-PCRs

Mouse splenocytes were kindly provided by G Riou (Inserm U1234, University of Rouen Normandy, Rouen, France). RNAs of $AAPC^{DR1}$ and mouse splenocytes were extracted from 5.10^6 cells using the NucleoSpin RNA isolation kit (Macherey-Nagel, Easton, PA, USA) according to the manufacturer's protocol. cDNAs were generated from200 ng of total using oligo (dT) and random sequence primers (Random Hexamer) and Verso™ cDNA kit (Thermo Fischer Scientific), according to the manufacturer's instructions. cDNAs were PCR-amplified obtained using primers (Eurogentec SA, Liège, Belgium).

Peptide Ags

Two peptides were used in this study. The HA_{306}PKYVKQNTLKLAT$_{318}$ (SEQ ID NO:11) peptide of H3N2 *influenza virus* restricted to the HLA-DR1 molecule presentation was used as the peptide of interest. The Myelin Basic Protein, MBP_{84}DENPVVHFFKNIVTPRTPP$_{102}$ (SEQ ID NO:12) peptide was used as a control peptide. These peptides were kindly provided by Dr J Leprince and B Lefranc (Inserm U1239, University of Rouen Normandy, Rouen, France).

T-cell clone and B-EBV^{DR1} cell lines

$CD4^+$ T-cell clone Flu-2, specific for $HA_{306-318}$ peptide, was kindly provided by Pr A Godkin (Institute of Infection and Immunity, Cardiff, UK).[23] The homozygous HLA-$DR1^+$ B-EBV cell line was a kind gift from Dr H Vié (Inserm UMR 1232, Nantes, France). Culture of the T-cell clone was performed in 96-well U bottom plates in RPMI (ThermoFischer Scientific), supplemented with 1% of Fetal Calf Serum (FCS, ThermoFischer Scientific), 2 mM of glutamine (ThermoFischer Scientific), 50 IU/ml of penicillin (ThermoFischer Scientific) and 50 µg/ml of streptomycin (ThermoFischer Scientific). Culture of HLA-$DR1^+$ B-EBV cell line was performed in RPMI supplemented with 10% of FCS (Fetal Calf Serum, ThermoFischer Scientific).

Cell membrane staining

$AAPC^{DR1}$ were stained for 20–30 min at 4°C in phosphate-buffered saline/bovine serum albumin/ethylenediamine tetraacetic acid (PBS/BSA/EDTA) buffer with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse I-A/I-E (BioLegend, London, UK), FITC-conjugated anti-human CD58 (BD Biosciences, Piscataway, NJ, USA) and FITC-conjugated anti-human CD54, CD80 and HLA-DR (all from ThermoFischer Scientific). In the same conditions, TLs were stained with the following antibodies: FITC-conjugated anti-human CD4, phycoerythrin (PE)/Cyanine 7 (Cy7)-conjugated anti-human CD45RA, peridinin chlorophyll protein complex (PerCP)/Cyanine 5.5 (Cy5.5)-conjugated anti-human CD45RO, allophycocyanin (APC)-conjugated anti-human CCR7, APC/Cy7-conjugated anti-human CD62L, PerCP/Cy5.5-conjugated anti-human PD-1 (all from ThermoFischer Scientific), APC/Cy7-conjugated anti-human CD3, brillant violet (BV)786-conjugated anti-human CD8, APC-conjugated anti-human CTLA-4, BV711-conjugated anti-human TIM-3 (all from Sony Biotechnology, Weybridge, UK) and BV421-conjugated anti-human TIGIT (BioLegend). CTLA-4 expression was also detected by intracellular staining as described below. Frequency of HA-specific $CD4^+$ TLs was assessed by tetramer staining for 30 min at room temperature with PE-conjugated HLADRB1*01:01-HA (DR1-HA, ProImmune, Oxford, UK) or control HLA-DRB1*01:01-CLIP tetramers (DR1-CLIP, a kind gift of the NIH Tetramer Core Facility, Atlanta, GA, USA). Cells were analyzed using BD LSRFortessa cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular staining

For intracellular cytokine staining, TLs were treated using the Intraprep Permeabilization Reagent kit (Beckman Coulter, Villepinte, France) according to the manufacturer's protocol. TLs were stained with the following antibodies: APC-conjugated anti-

human IFN-γ, PE-conjugated anti-human TNF-α, PE-conjugated anti-human granzyme B (all from ThermoFischer Scientific) and APC-conjugated anti-human perforin (Sony Biotechnology).

Stimulation of the T-cell clone

For evaluation of Ag presentation, $CD4^+$ T-cell clone (10^5 per well) was cultured for 6 h with $AAPC^{DR1}$ or B-EBV^{DR1} cell lines (10^5 per well for an APC : TL ratio of 1 : 1) previously loaded for 1 hour with 10 µg/ml of HA or MBP control peptide or cultured with Ag-encoding $AAPC^{DR1}$ cell lines (10^5 per well for an APC : TL ratio of 1 : 1). Brefeldin A at 10 µg/ml (Sigma-Aldrich) was added for the last 5 h of incubation before intracellular cytokine staining.

Stimulation of purified $CD4^+$ TLs by $AAPC^{DR1}$ or PBMCs

For primary stimulation experiments, irradiated (25 Gy) Ag-encoding $AAPC^{DR1}$ cell lines or irradiated $AAPC^{DR1}$ loaded for 1 hour with 10µg/ml of HA or control peptides were plated (1.5×10^5 per well) 4 hours before incubation with purified $CD4^+$ TLs from healthy subjects ($1x10^6$ per well) for 14 days. Alternatively, PBMCs (2×10^6 per well) were incubated with 10 µg/ml of HA or control peptides for the same duration. For re-stimulation experiments, effector TLs ($1x10^6$ per well) generated by primary culture of PBMCs pulsed with HA peptide were incubated with either irradiated (25 Gy) adherent Ag-encoding $AAPC^{DR1}$ cell lines (1.5×10^5 per well), or either $AAPC^{DR1}$ (1.5×10^5 per well) or autologous PBMCs (2×10^6 per well) loaded with 10 µg/ml of HA or control peptides for 7 days. Cultures were performed in 24-well plates with AIM-V CTS medium (ThermoFischer Scientific) supplemented with 2 mM of glutamine (ThermoFischer Scientific) and 5% of decomplemented human AB serum (Sigma-Aldrich). On day 4, and then every other day, 20 IU/ml of interleukin-2 (R&D Systems, Lille, France) was added.

Statistics

Prism software (GraphPad software, La Jolla, CA, USA) was used to perform repeated measures of one-way ANOVA analysis followed by Dunnett's post test.

Results

Ag-encoding $AAPC^{DR1}$ express molecules required to activate human CD4+ TLs but not mouse molecules involved in MHC-II Ag presentation pathway

$AAPC^{DR1}$ were constructed by gammaretroviral transduction of mouse fibroblasts NIH/3T3 with vectors encoding HLA-DRα, HLA-DRβ1*01:01 (HLA-DR1), CD80 co-stimulatory and CD54 and CD58 adhesion molecules. To obtain Ag-encoding $AAPC^{DR1}$ cell lines, $AAPC^{DR1}$ were additionally transduced with different constructs expressing the HA viral Ag, in the form of a protein or a peptide, and addressing the Ag to a particular cellular

compartment (**Figure 5A and 5B**). After transduction, AAPC^{DR1} and the eight Ag-encoding AAPC^{DR1} cell lines presented high expression and comparable levels of HLA-DR, CD80, CD54 and CD58 molecules (**Figure 1A**). NIH/3T3 or AAPC^{DR1} do not express the classical mouse MHC-II molecules (I-A and I-E) at their surface (**Figure 1B**) neither the mouse invariant chain nor the "non-classical" mouse MHC-II molecules (H-2M and H-2O), which are all involved in the mouse MHC-II Ag presentation pathway (**data not shown**). Taken together, these data indicates that these AAPCs are well equipped to activate human but not mouse CD4+ T cells.

Ag-encoding AAPC^{DR1} cell lines efficiently process proteins and present peptide Ags

To assess the ability of the novels Ag-encoding AAPC^{DR1} cell lines to present Ags, we used a CD4+ T-cell clone that recognizes the $HA_{306-318}$ epitope, restricted to the HLA-DR1 context. Several ratios of AAPCs to T-cell clone were used in order to compare their stimulatory ability. As previously described, AAPC^{DR1} loaded with HA peptide were very efficient to activate the specific T-cell clone, at a higher level than B-EBV^{DR1} cells (**Figure 2A**). Among the eight HA Ag-encoding AAPCs, those expressing HA peptide in the ER and those expressing HA protein at the plasma membrane are the most powerful stimulatory cells (**Figure 2B and 2C**). These both AAPCs have similar Ag presentation ability than AAPC^{DR1} loaded with HA peptide. In contrast, AAPC^{DR1} expressing HA Ag in the cytoplasm have the lowest stimulatory capacities.

Ag-encoding AAPC^{DR1} prime human CD4+ T cells at lower level than autologous APCs

Next, we selected AAPC^{DR1} expressing HA peptide in the ER and HA protein at the cell surface, and evaluated their ability to prime human CD4+ T cell response. The two HA Ag-encoding AAPC^{DR1} were able to generate HA-specific CD4+ T cell with the same efficacy than AAPC^{DR1} pulsed with HA peptide. HA-tetramer staining and IFN-γ production after reactivation with B-EBV^{DR1} cells show about 1,5 to 3% specific CD4+ T cells (**Figure 3A and 3B**). However, autologous PBMCs pulsed with HA peptide were more effective to prime CD4+ T cells, resulting in 15% of CD4+/HA-tetramer+ cells but also in 10,9% of CD4+/IFN-γ+ cells after B-EBV^{DR1} reactivation.

Ag-encoding AAPC^{DR1} expressing HA peptide in the ER are more effective to reactivate memory specific CD4+ T cell than autologous APCs

Effectors generated in primary culture by stimulating CD4+ T cells with PBMCs pulsed with HA peptide were re-stimulated by AAPCs or autologous APCs. AAPC^{DR1} pulsed with HA peptide and the two HA Ag-encoding AAPC^{DR1} generated superior frequencies of memory

specific CD4+ T cells than autologous PBMCs as assessed by tetramer or IFN-γ staining (**Figure 4A, 4B, and 4C**). In term of absolute number, $AAPC^{DR1}$ encoding HA peptide in the ER is significantly the more effective stimulating cells for amplifying memory T cells (**Figure 4B and 4C**). After re-stimulation by autologous PBMCs or by AAPCs, HA-specific CD4+ TLs were IFN-γ and TNF-α producing Th1 cells with a similar naïf/memory phenotype including a majority of effector memory T cells (T_{EM}; $CD45RO^+$, $CD62L^-$, $CCR7^-$) (**Figure 4A and 4C**). The other cells were transitional memory (T_{TM}; $CD45RO^+$, $CD62L^+$, $CCR7^-$). A contingent of CD4+ T cells produce granzyme B but not perforin after reactivation with the HA peptide, suggesting they are cytotoxic T cells (**data not shown**). Re-stimulation by autologous APCs or AAPCs increased similarly expression of immuno-regulation molecules as T cell immunoreceptor with Ig and ITIM domains (TIGIT), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), cytotoxic T lymphocyte antigen-4 (CTLA-4) and PD-1 (**data not shown**).

Discussion:

In this study, we have developed novels AAPCs that endogenously express Ag in several compartments involved in MHC II pathway. We identified $AAPC^{DR1}$ encoding HA peptide in the ER and $AAPC^{DR1}$ encoding HA membrane protein as the most effective cells to present Ag. Previously, with AAPCs expressing HLA-I molecules we have demonstrated that addressing a peptide Ag in the ER using the signal sequence of the CD8α molecule is a very reliable strategy to trigger CTL responses.[21] Similarly, the high efficiency of peptide encoding $AAPC^{DR1}$ in ER may explain by an optimal loading of HA peptide into nascent MHC-II molecule in absence of the chaperone protein Ii that blocks peptide cleft. The potent Ag presentation ability of $AAPC^{DR1}$ expressing HA protein at the cell membrane may result of the recycling of HA proteins and HLA-DR1 molecules. Indeed, it has been already demonstrated in professional APCs but also in fibroblasts that some HA epitopes can be generated into early endosomes which contain MHC-II recycled molecules and unfolding HA protein that have underwent mild proteolysis, independently of Ii and HLA-DM present in the MIIC.[24–26] Conversely, the weak Ag presentation ability of protein-encoding AAPCs targeting endosome could be linked to a high proteolysis activity that does not lead to the generation of the appropriate HA epitope. In accordance with this hypothesis, Kim et al described that the $HA_{306-318}$ epitope was highly sensitive to the digestion by cathepsins.[27] AAPCs expressing peptide or protein in extracellular media were less efficient to present Ag than AAPCs targeting ER or the plasma membrane likely because of a low level of endocytosis. AAPCs expressing HA protein

or peptide in the cytosol are the weakest stimulating cells. This may explain by a inappropriate proteasome cleavage of HA protein and/or a weak TAP (Transporter associated with Antigen Processing) transport of the HA peptide in ER due to their sequence and length that do not fit common features of HLA-I ligands.[28,29]

Although AAPCs expressing HA peptide in ER or protein in membrane as well as AAPCs loaded with HA peptide have strong Ag presentation capacity, they activate effector CD4+ T cells after primary stimulation but at lower level than autologous APCs. It is likely that endogenous expression of HA Ag is not sufficient to hamper presentation of xenopeptide competitors and so to limit non-specific CD4+ T cell responses. To prevent the access of xenopeptides in the ER, a promising approach is to induce expression of the TAP inhibitors such as UL49.5 and US6 viral proteins of $AAPC^{DR1}$ by gene transfer.[30] The xenopeptides presented by HLA-DR1 molecules can also arise from autophagy mechanisms.[31,32] Autophagy represents an important source of endogenous peptides presented on MHC-II molecules.[33] Blockade of this mechanism using specific inhibitors such as bafilomycin A1, could decrease the binding of xenopeptides on MHC-II molecules.[34] Moreover, to increase priming ability of $AAPC^{DR1}$, it will be interesting to generate an $AAPC^{DR1}$ cell line expressing both HA peptide in the ER and HA protein at the cell surface.

Interestingly, targeting approach of HA peptide in ER is an efficient strategy to obtain a higher number of Ag-specific CD4+ T cells than with AAPCs or autologous APCs loaded with HA peptide. Effector T cells harbor a strong Th1 profil with high production of IFN-γ and TNF-α, that is a relevant property for anti-tumor immunotherapy.[35] In addition, we identified a subset of CD4+ T cells that express granzyme B but not perforin. This expression was already reported as a Th1 memory cells characteristic.[36] Further studies are needed to evaluate the function of this subset. However, whatever the stimulating cells, expanded specific CD4+ T cells express immuno-regulation molecules as TIGIT, TIM-3, PD-1 and CTLA-4 that could limit their functionality *in vivo*. It was shown that CTL based ACI efficiency was improved by immune checkpoints blockades combination.[37,38] It likely that the same therapeutic approach is required for ACI replying in part on effector CD4+ T cells. AAPCs could also be modified to express appropriate costimulation signals that improve CD4+ TLs survival and functionality. For this purpose, CD70 and 4-1BBL molecules are two promising candidates. Indeed, the CD27/CD70 costimulatory signaling pathway has been reported to promotes Th1 polarization but also CD4+ TLs survival.[39] On the other hand, interaction between 4-1BB and 4-1BBL molecules favors proliferation and effector functions like cytolytic activity of activated CD4+ TLs.[40–42]

In this study we confirmed that AAPCs is a suitable tool to generate an absolute number of functional specific CD4+ TLs compatible for ACI protocols.[14] We also show that HA protein-encoding AAPC^{DR1} are efficient to process whole Ag and generate peptide epitopes. This property could be exploited to identify new T CD4+ epitopes from therapeutic interest Ags by HLA-II/peptide complexes immunoprecipitation of AAPC^{DR1}, coupled to mass spectrometry analysis.[43] In parallel, AAPC^{DR1} expressing endogenous or exogenous Ag could be a reliable tool to monitor human T CD4+ responses *in vitro*.[44] In conclusion, based on Ag-encoding AAPC^{DR1}, we have developed an *in vitro* protocol enabling a rapid and robust amplification of functional memory Th1 cells for future ACI applications.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. An artificial antigen presenting cell consisting of a eucaryotic cell that is genetically modified to stably express a single MHC class II molecule, at least one accessory molecule and at least one antigen.

2. The artificial antigen presenting cell of claim 1 wherein the eucaryotic cell is a murine fibroblast.

3. The artificial antigen presenting cell of claim 1 wherein the MHC class II molecule is selected from the group consisting of HLA-DQ molecules, HLA-DP molecules and HLA-DR molecules.

4. The artificial antigen presenting cell of claim 1 of claim 1 wherein the MHC class II molecule is selected from the group consisting of HLA-DR1, HLA-DR15 and HLA-DR51 molecules.

5. The artificial antigen presenting cell of claim 1 wherein the eucaryotic cell is genetically modified to stably express the CD80, CD54 and CD58 molecules.

6. The artificial antigen presenting cell of claim 1 wherein the eucaryotic cell is genetically modified to stably express a peptide antigen in the endoplasmic reticulum (ER).

7. The artificial antigen presenting cell of claim 1 wherein the eucaryotic cell is genetically modified to stably express a protein antigen in the endosomes or in the membrane.

8. The artificial antigen presenting cell of claim 6 wherein the eucaryotic cell is genetically modified to stably express the antigen peptide fused to a signal sequence.

9. The artificial antigen presenting cell of claim 8 wherein the signal peptide is the signal peptide of the T cell surface glycoprotein CD8 alpha chain.

10. The artificial antigen presenting cell of claim 9 wherein the signal peptide has the amino acid sequence as forth in SEQ ID NO: 1.

11. The artificial antigen presenting cell of claim 1 wherein the antigen is a viral antigen, a bacterial antigen, a fungal antigen, a protozoal antigen, a tumor associated antigen, an auto-antigen, or an allergen.

12. A method of preparing the artificial antigen presenting cell of claim 1 wherein the eucaryoticn cell is a transfected with a vector encoding for the antigen, at least one vector encoding for the accessory molecule and a vector encoding for the MHC class II molecule.

13. The method of claim 12 wherein a vector comprising a nucleic acid sequence encoding for the signal peptide operatively linked to a nucleic acid sequence encoding for the antigen peptide is used to stably express the antigen peptide into the endoplasmic reticulum of the eucaryotic cell.

14. The method of claim 13 wherein the nucleic acid sequence encoding for the signal peptide consists of the nucleic acid sequence as set forth in SEQ ID NO: 2.

15. The method of claim 12 wherein the vector are gamma retroviral vectors

16. A method of amplifying a population of antigen-specific CD4+ T cells comprising the steps of coculturing a population of a T cells with the population of artificial antigen presenting cells of claim 1

17. The method of claim 16 wherein the population of AAPCs is irradiated before incubation with the population of CD4 T cells.

18. The method of claim 16 which further comprise a step consisting of polarizing the antigen-specific CD4+ T cells into a population of antigen-specific T regulatory cells.

19. A population of antigen-specific CD4+ T cells obtainable by the method of claim 16.

20. A method of treatment in a subject in need thereof comprising administering to the subject the population of antigen-specific CD4+ T cells of claim 19.

21. The method of claim 20 for the treatment of cancer, infectious disease, autoimmune disease, or allergy

22. The method of claim 20 for the treatment of immune reactions against a grafted tissue or grafted hematopoietic cells or grafted blood cells.

23. A method of identifying a population of CD4+ T cells within a test population of T cells comprising the steps of i) providing a population of artificial antigen presenting cells that express the antigen, ii) coculturing the population of T cells with the population of artificial antigen presenting cells of step i) and iii) detecting whether a population of CD4+ T cells is activated and/or amplified.

24. A method of determining identifying and/or characterizing the T cell epitopes of an antigen comprising the steps of i) providing a population of artificial antigen presenting cells that express the antigen, ii) coculturing the population of T cells with the population of artificial antigen presenting cells of step i), iii) detecting whether a population of CD4+ T cells is activated and/or amplified, iv) selecting the population of artificial antigen presenting cells that is capable of activating and/or amplifying the population of CD4+ T cells, v) recovering and characterizing the peptides from the MHC class II molecules wherein said peptides represent the T cell epitopes of the antigen.

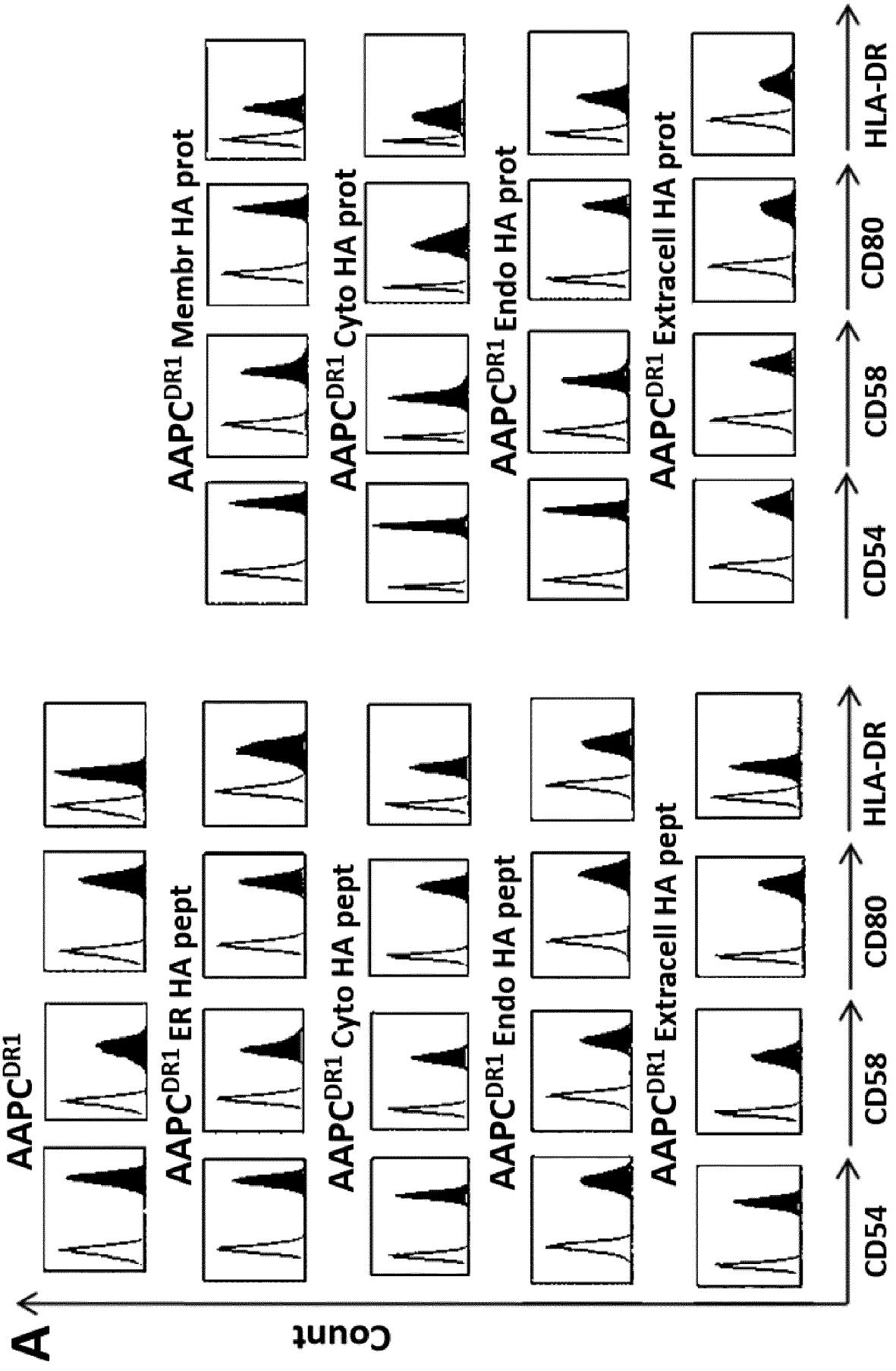

A
Count
AAPC^{DR1}
AAPC^{DR1} ER HA pept
AAPC^{DR1} Cyto HA pept
AAPC^{DR1} Endo HA pept
AAPC^{DR1} Extracell HA pept
AAPC^{DR1} Membr HA prot
AAPC^{DR1} Cyto HA prot
AAPC^{DR1} Endo HA prot
AAPC^{DR1} Extracell HA prot
CD54
CD58
CD80
HLA-DR

Figure 1A

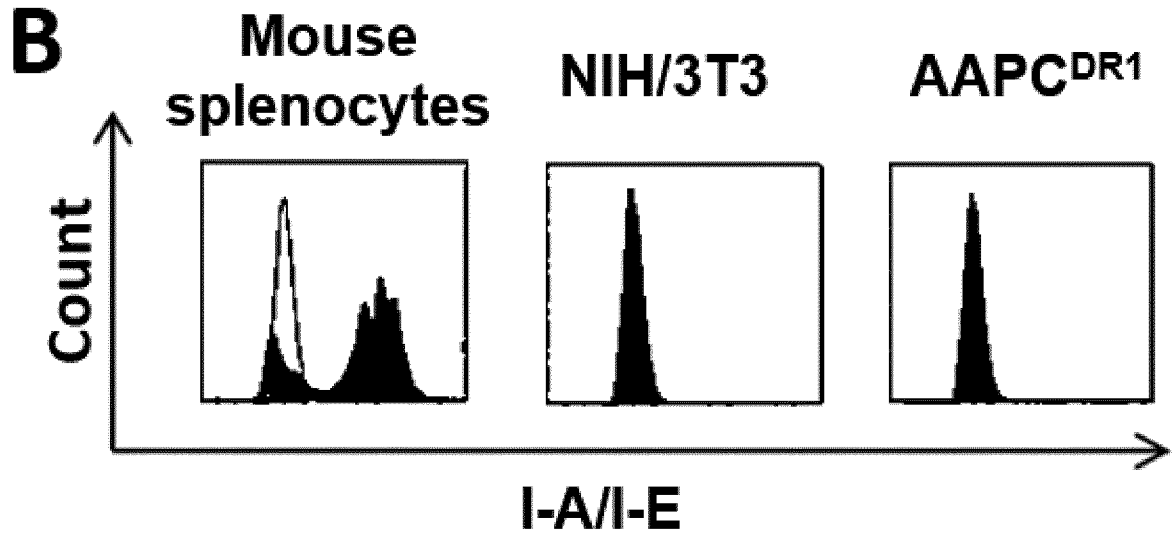


Figure 1B

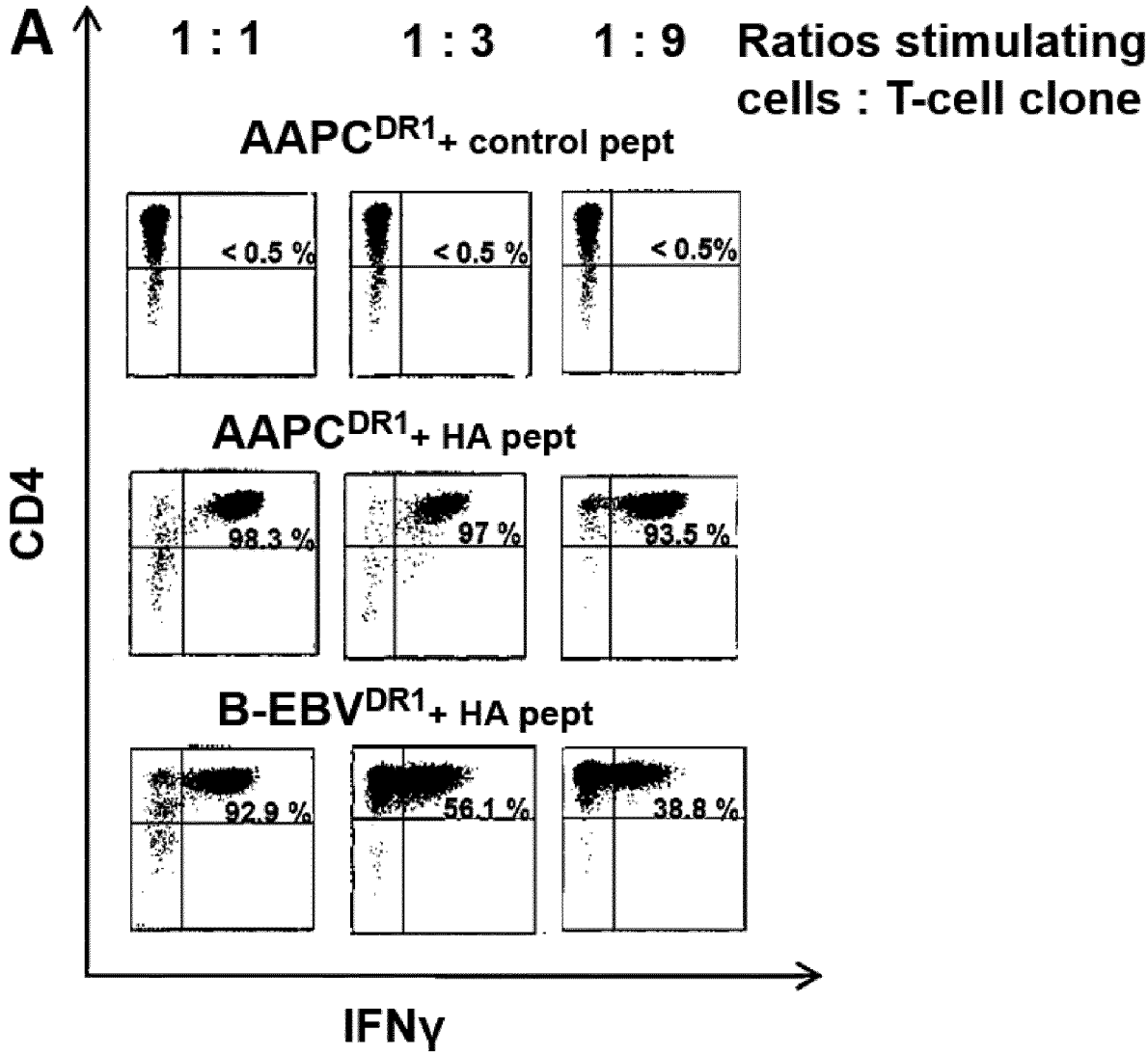


Figure 2A

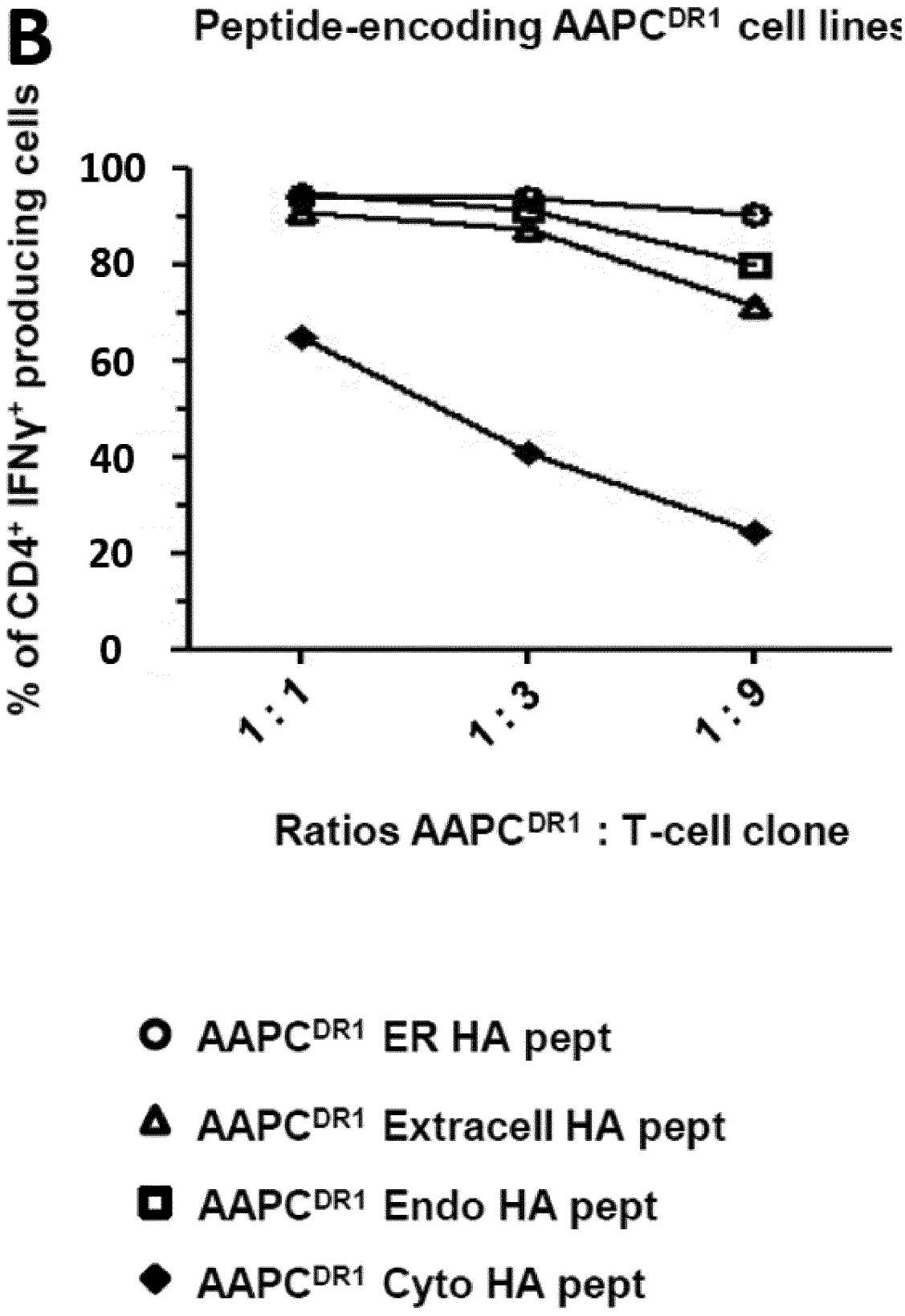

B
Peptide-encoding AAPC^{DR1} cell lines
% of CD4^{+} IFNγ^{+} producing cells
100
80
60
40
20
0
1:1
1:3
1:9
Ratios AAPC^{DR1} : T-cell clone
AAPC^{DR1} ER HA pept
AAPC^{DR1} Extracell HA pept
AAPC^{DR1} Endo HA pept
AAPC^{DR1} Cyto HA pept

Figure 2B

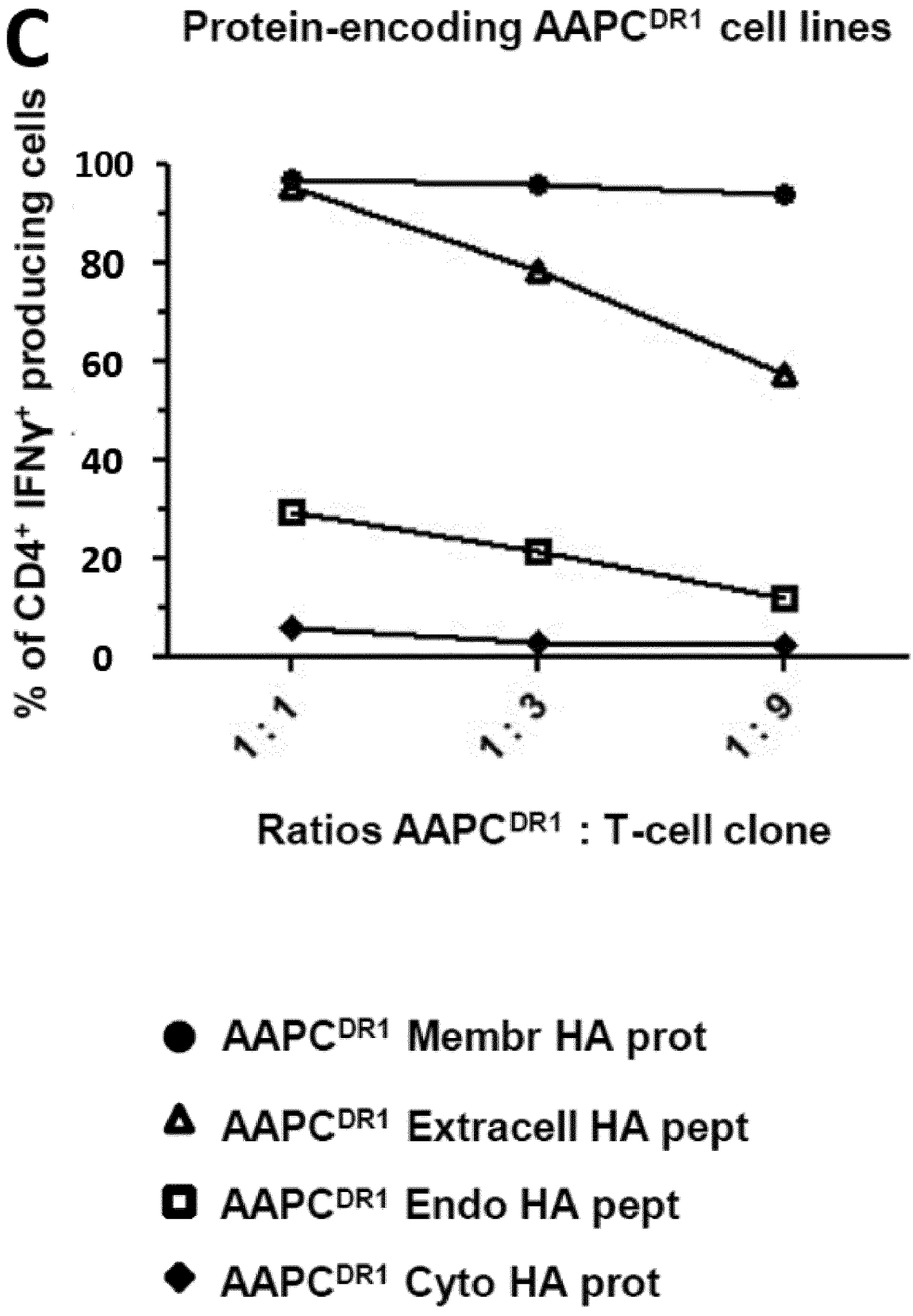

C
Protein-encoding AAPC^{DR1} cell lines
% of CD4$^+$ IFNγ$^+$ producing cells
100
80
60
40
20
0
1 : 1
1 : 3
1 : 9
Ratios AAPC^{DR1} : T-cell clone
AAPC^{DR1} Membr HA prot
AAPC^{DR1} Extracell HA pept
AAPC^{DR1} Endo HA pept
AAPC^{DR1} Cyto HA prot

Figure 2C

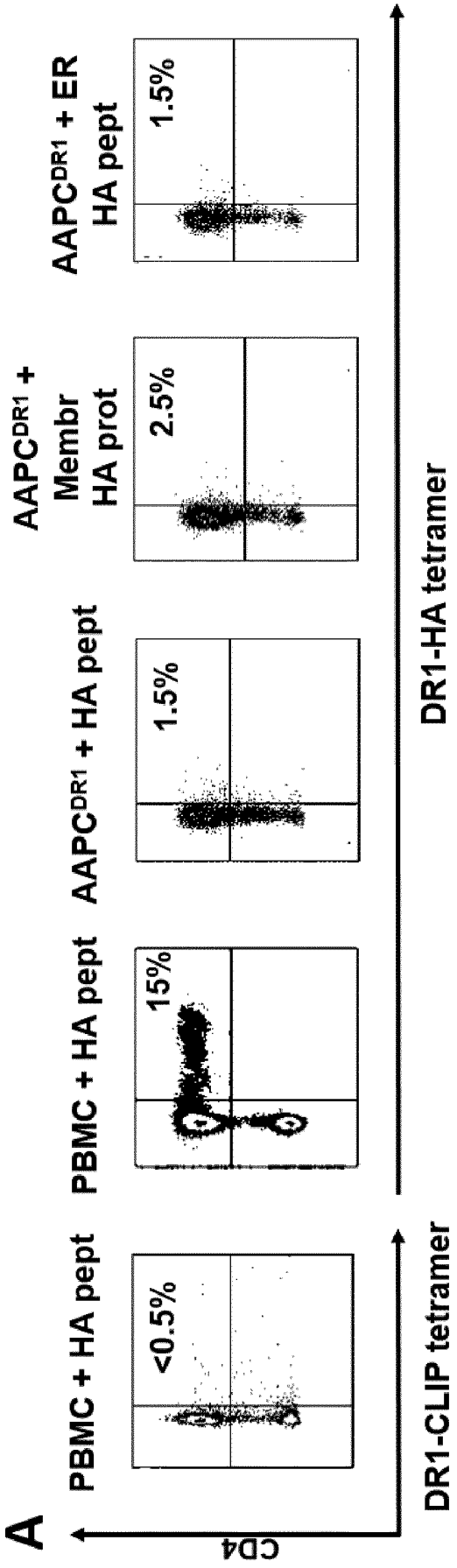

A
PBMC + HA pept
<0.5%
PBMC + HA pept
15%
AAPC^{DR1} + HA pept
1.5%
AAPC^{DR1} + Membr HA prot
2.5%
AAPC^{DR1} + ER HA pept
1.5%
CD4
DR1-CLIP tetramer
DR1-HA tetramer

Figure 3A

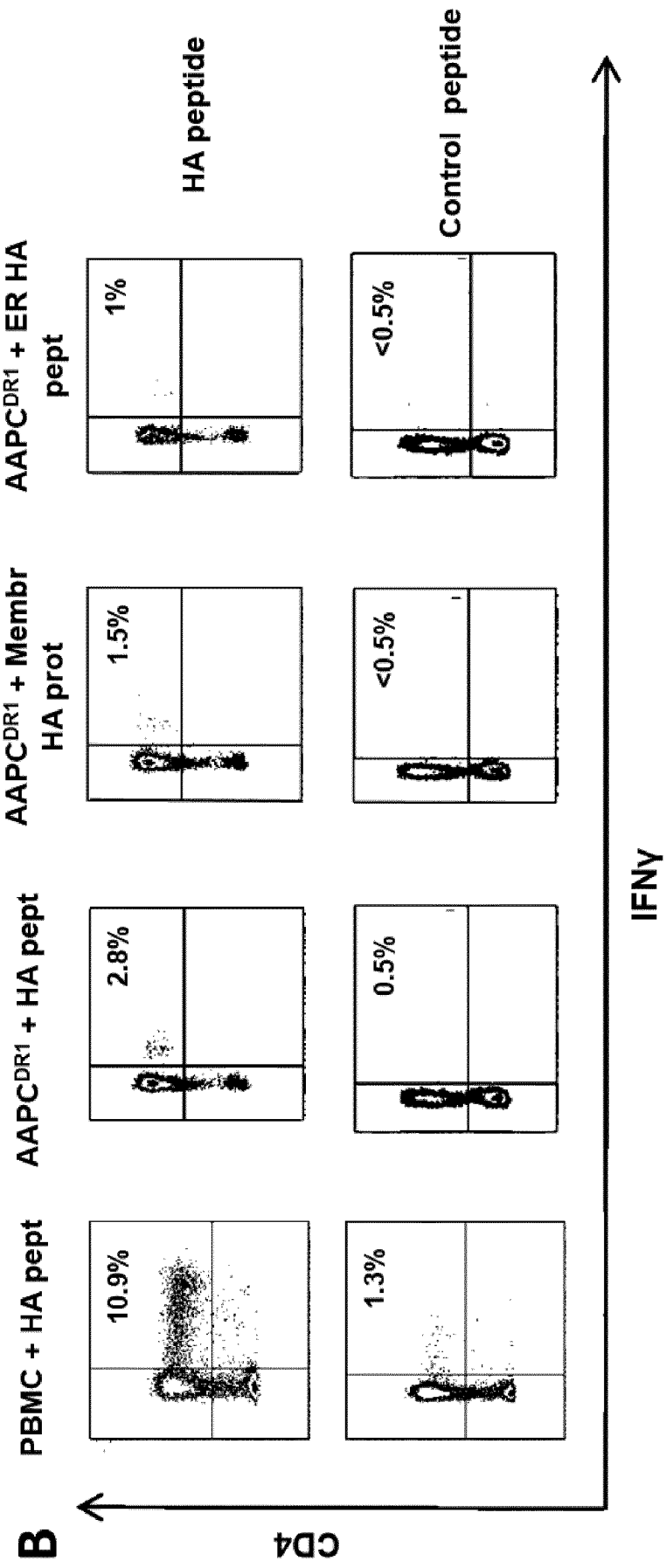

B
HA peptide
Control peptide
AAPC^{DR1} + ER HA pept
AAPC^{DR1} + Membr HA prot
AAPC^{DR1} + HA pept
PBMC + HA pept
1%
<0.5%
1.5%
<0.5%
2.8%
0.5%
10.9%
1.3%
CD4
IFNγ

Figure 3B

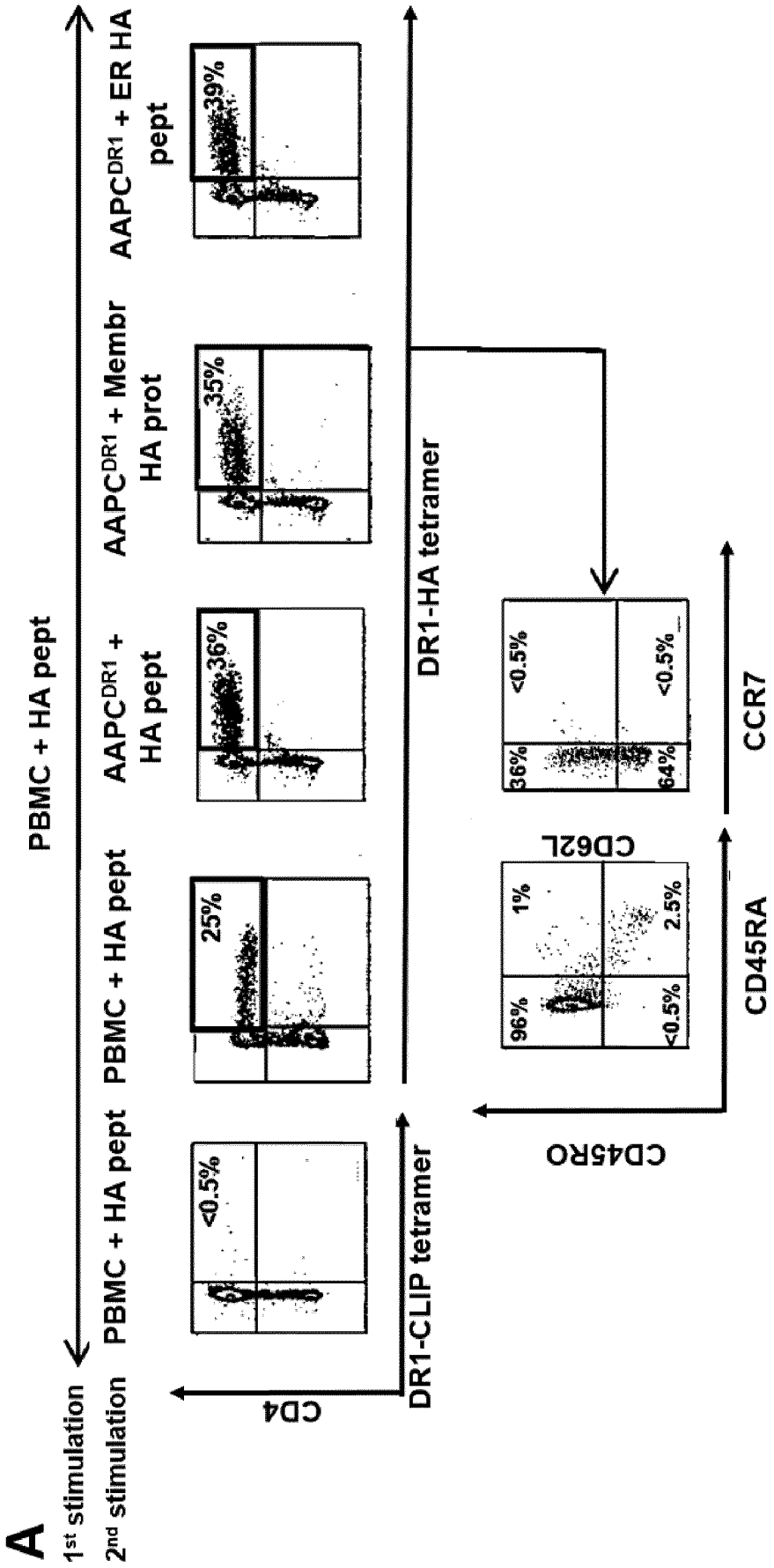

A
1st stimulation
2nd stimulation
PBMC + HA pept
PBMC + HA pept
PBMC + HA pept
PBMC + HA pept
AAPC^DR1 + HA pept
AAPC^DR1 + Membr HA prot
AAPC^DR1 + ER HA pept
DR1-CLIP tetramer
DR1-HA tetramer
CD4
CD45RO
CD62L
CD45RA
CCR7
<0.5%
25%
36%
35%
39%
96%
1%
2.5%
<0.5%
36%
64%
<0.5%

Figure 4A

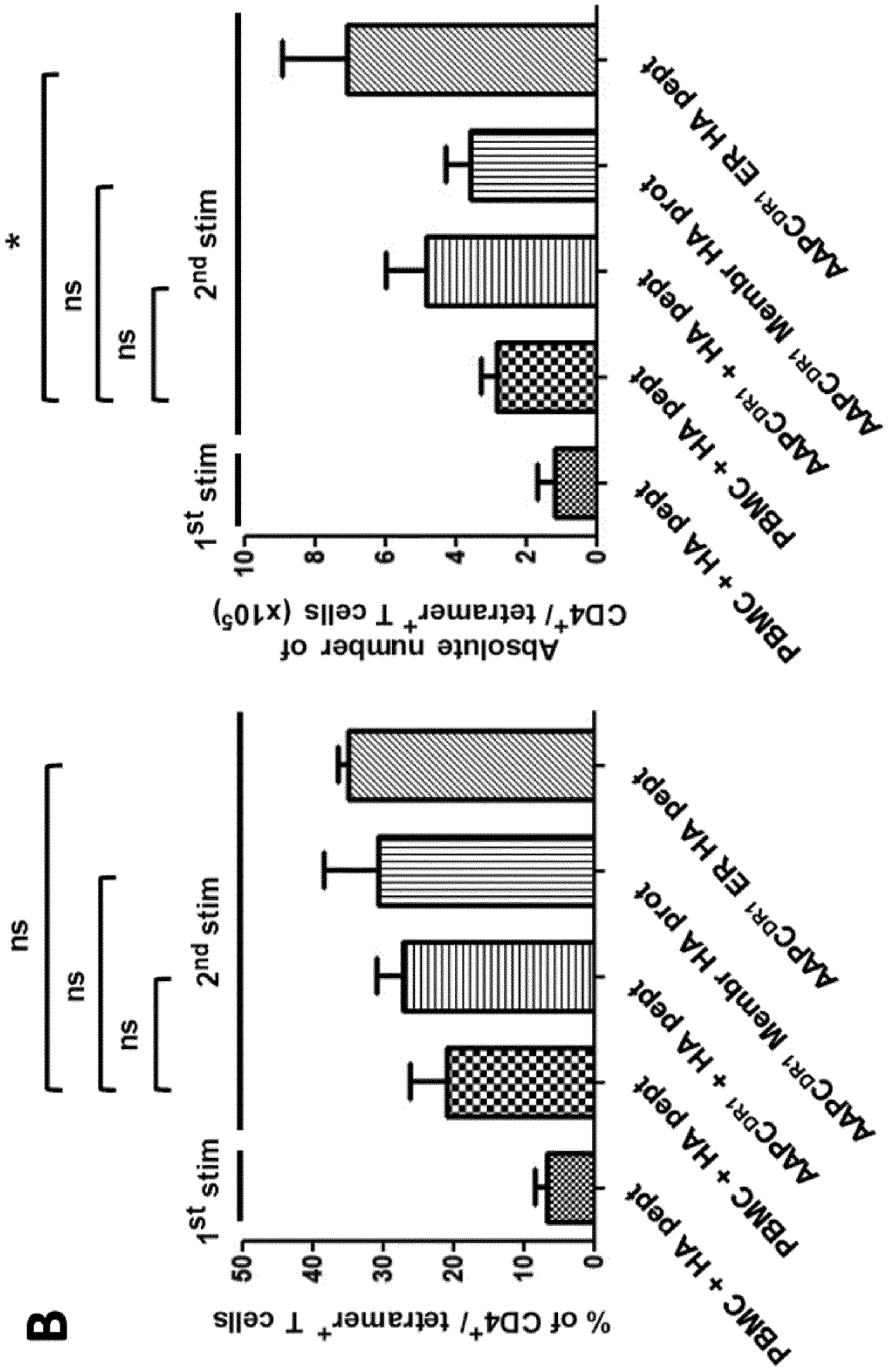

B
% of CD4+/ tetramer+ T cells
0
10
20
30
40
50
1st stim
2nd stim
ns
ns
ns
PBMC + HA pept
PBMC + HA pept
AAPCDR1 + HA pept
AAPCDR1 Member HA prot
AAPCDR1 ER HA pept
Absolute number of CD4+/ tetramer+ T cells (x10^5)
0
2
4
6
8
10
1st stim
2nd stim
ns
ns
*
PBMC + HA pept
PBMC + HA pept
AAPCDR1 + HA pept
AAPCDR1 Member HA prot
AAPCDR1 ER HA pept

Figure 4B

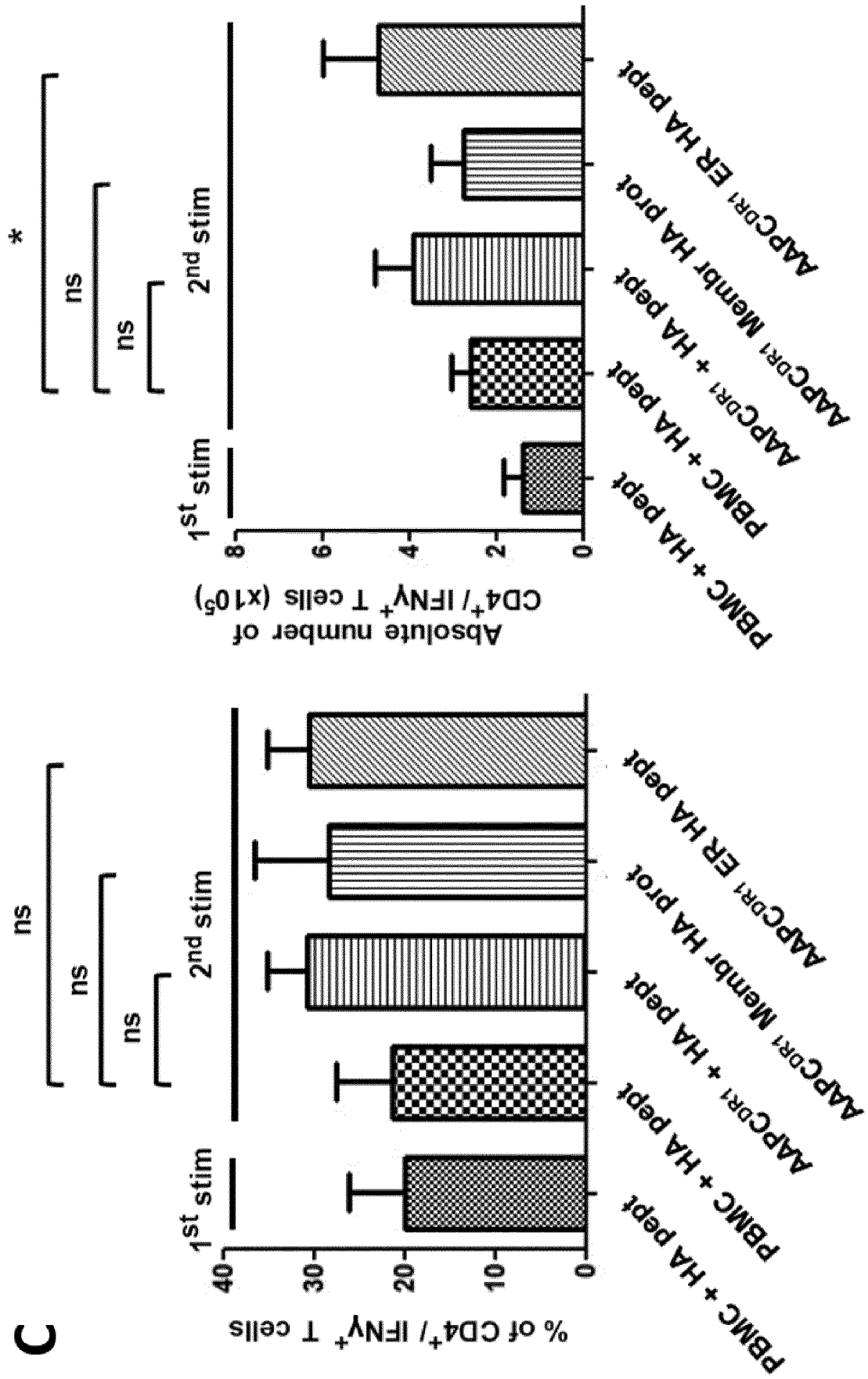

C
% of CD4+/ IFNγ+ T cells
0
10
20
30
40
1st stim
2nd stim
ns
ns
ns
PBMC + HA pept
PBMC + HA pept
AAPCDR1 + HA pept
AAPCDR1 Member HA prot
AAPCDR1 ER HA pept
Absolute number of CD4+/ IFNγ+ T cells (x10^5)
0
2
4
6
8
1st stim
2nd stim
ns
ns
*
PBMC + HA pept
PBMC + HA pept
AAPCDR1 + HA pept
AAPCDR1 Member HA prot
AAPCDR1 ER HA pept

Figure 4C

A

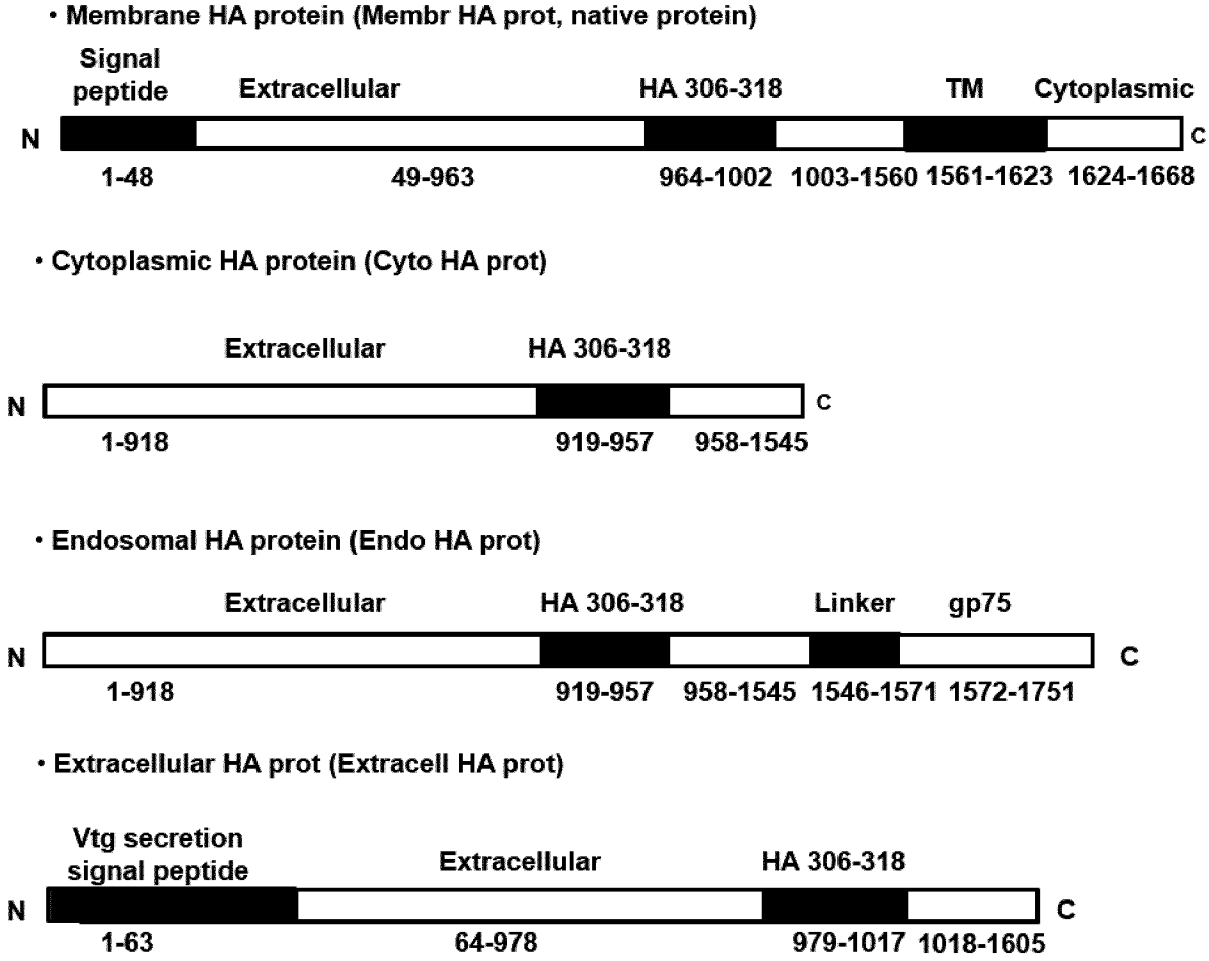
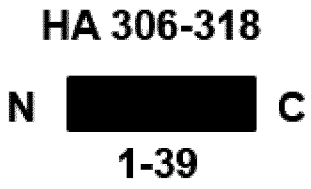

• Membrane HA protein (Membr HA prot, native protein)
Signal peptide
Extracellular
HA 306-318
TM
Cytoplasmic
N
C
1-48
49-963
964-1002
1003-1560
1561-1623
1624-1668
• Cytoplasmic HA protein (Cyto HA prot)
Extracellular
HA 306-318
N
C
1-918
919-957
958-1545
• Endosomal HA protein (Endo HA prot)
Extracellular
HA 306-318
Linker
gp75
N
C
1-918
919-957
958-1545
1546-1571
1572-1751
• Extracellular HA prot (Extracell HA prot)
Vtg secretion signal peptide
Extracellular
HA 306-318
N
C
1-63
64-978
979-1017
1018-1605

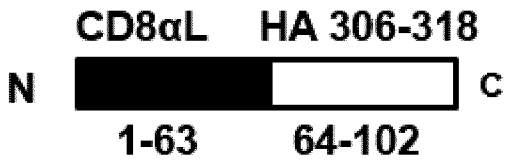
Figure 5A

B

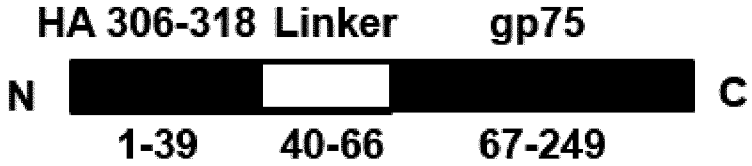
• Cytoplasmic HA peptide (Cyto HA pept)



• Endoplasmic Reticulum HA peptide (ER HA pept)



• Endosomal HA peptide (Endo HA pept)



• Extracellular HA peptide (Endo HA pept)

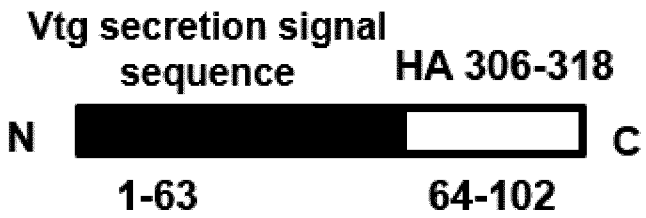


Figure 5B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/084788

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/10 C12N5/0783 A61K35/17 A61P37/02
ADD.

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)
C12N G01N 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 practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GARNIER A ET AL: "Artificial antigen-presenting cells expressing HLA class II molecules as an effective tool for amplifying human specific memory CD4 + T cells", IMMUNOLOGY AND CELL BIOLOGY, vol. 94, no. 7, August 2016 (2016-08), pages 662-672, XP055594821, ISSN: 0818-9641, DOI: 10.1038/icb.2016.25 cited in the application the whole document ----- -/--	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2020	10/03/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Teyssier, Bertrand

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHATILLON J-F ET AL: "Direct Toll-Like Receptor 8 signaling increases the functional avidity of human CD8+ T lymphocytes generated for adoptive T cell therapy strategies : TLR8 engagement increases CTL function", IMMUNITY, INFLAMMATION AND DISEASE, vol. 3, no. 1, March 2015 (2015-03), pages 1-13, XP055594829, ISSN: 2050-4527, DOI: 10.1002/iid3.43 -----	1-18,23, 24
A	DUPONT J ET AL: "Artificial antigen-presenting cells transduced with telomerase efficiently expand epitope-specific, human leukocyte antigen-restricted cytotoxic T cells", CANCER RESEARCH, vol. 65, no. 12, 15 June 2005 (2005-06-15) , pages 5417-5427, XP002355479, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-04-2991 -----	1-18,23, 24
A	LATOUCHE J-B ET AL: "Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells", NATURE BIOTECHNOLOGY, vol. 18, no. 4, April 2000 (2000-04), pages 405-409, XP002191046, ISSN: 1087-0156, DOI: 10.1038/74455 cited in the application the whole document -----	1-18,23, 24
X	BUTLER M O ET AL: "A panel of human cell-based artificial APC enables the expansion of long-lived antigen-specific CD4+ T cells restricted by prevalent HLA-DR alleles", INTERNATIONAL IMMUNOLOGY, vol. 22, no. 11, November 2010 (2010-11), pages 863-873, XP055386975, ISSN: 0953-8178, DOI: 10.1093/intimm/dxq440	19-22
A	the whole document -----	23,24
X	BORST J ET AL: "CD4+T cell help in cancer immunology and immunotherapy", NATURE REVIEWS IMMUNOLOGY, vol. 18, no. 10, 29 July 2018 (2018-07-29) , pages 635-647, XP036602043, ISSN: 1474-1733, DOI: 10.1038/S41577-018-0044-0 cited in the application the whole document -----	19-22

-/--

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/084788

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	COUTURE A ET AL: "HLA-Class II Artificial Antigen Presenting Cells in CD4+ T Cell-Based Immunotherapy", FRONTIERS IN IMMUNOLOGY, vol. 10, 17 May 2019 (2019-05-17), XP055594828, ISSN: 1664-3224, DOI: 10.3389/fimmu.2019.01081 the whole document -----	1-24

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