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(54) Title: METHODS FOR TESTING THE PRESENCE OF PROTECTIVE ANTIGEN-INDUCED MEMORY CD8+ T CELLS

(57) Abstract: The present invention relates to methods for testing the presence of protective antigen- induced memory CD8+ T cells and uses thereof. In particular, the present invention relates to a method for testing the presence of at least one population of protective antigen-induced memory CD8+ T cells in a sample obtained from a subject comprising the steps of i) incubating the sample obtained from the subject with the antigen for which the presence of the antigen-induced memory CD8+ T cells is sought ii) determining the secretion level of at least one cytokine selected from the group consisting of GM-CSF, CCL1 and CCL9 iii) comparing the secretion level determined at step ii) with its corresponding predetermined reference value and iv) concluding that the population of protective antigen-induced memory CD8+ T cells is present in the sample when a difference between the secretion level determined at step ii) with the predetermined reference value is determined.

METHODS FOR TESTING THE PRESENCE OF PROTECTIVE ANTIGEN-INDUCED MEMORY CD8+ T CELLS

FIELD OF THE INVENTION:

The present invention relates to methods for testing the presence of protective antigen-induced memory CD8+ T cells and uses thereof.

BACKGROUND OF THE INVENTION:

One hallmark of the immune system is its capability to respond faster to a second exposure to the same antigen: a phenomenon called memory. This property is the basis for protective vaccination against infectious diseases or tumors. Immunological memory is based on antigen-specific memory B and T cells that are antigen specific cells that will persist long after infection has resolved.

The naive T cell compartment is composed of a huge diversity of naive $CD8^+$ T cells bearing antigen specific receptors (TCR, for T Cell Receptor). During the primary response (first encounter with an antigen), the rare naive $CD8^+$ T cells that are antigen specific are activated, expand and differentiate into effector cells within the first 7 to 10 days of the primary response. $CD8^+$ T cell activation is a complex process which depends on the recognition of peptide (derived from pathogen-antigens) loaded onto MHC class I molecules at the surface of professional Antigen Presenting Cells (APC) such as dendritic cells (DC). Three signals are required for optimal $CD8^+$ T cell activation: TCR recognition of peptide MHC class I complex (signal 1), T cell CD28 engagement by costimulatory molecules expressed at the surface of APC following the recognition of danger signals such as PAMPS (Pathogen Associated Molecular Pattern) (signal 2) and detection of inflammatory cytokines (signal 3). After their activation, naive cells differentiate into effector $CD8^+$ T cells. Effector cells kill pathogen-infected cells through cytotoxicity notably mediated by the release of cytotoxic granules containing perforin and granzymes. Effector $CD8^+$ T cells also produce cytokines such as IFNγ and TNFα, which are key anti-viral players. Following pathogen elimination, the majority of effector $CD8^+$ T cells undergo apoptosis (contraction phase) while some of them will differentiate into memory cells.

Memory cells can be distinguished from naive cells based on their expression of a number of surface markers such as CD44 and CXCR3. Functionally, memory $CD8^+$ T cells are resting cells but are hyper-responsive, allowing them to rapidly re-express effector

functions upon TCR re-stimulation. The $CD8^+$ T cell memory compartment is heterogeneous. The memory compartment is composed of antigen-induced memory cells, for example the ones that have been generated after pathogen encounter (pathogen-induced memory $CD8^+$ T cells) and that represent true memory cells. However the memory compartment is also composed of cytokine-induced memory phenotype cells (innate and Homeostatic Proliferation (HP) memory-phenotype $CD8^+$ T cells). Among the antigen-induced memory T cells, two subsets have been well characterized: T_{CM} (T Central Memory cells) and T_{EM} (T Effector Memory cells). These cells are generated following infection or tumor rejection. These subsets both express CD44 and CXCR3 but differ in their homing and effector capacities. T_{CM} home to secondary lymphoid organs, express IL-2 and vigorously proliferate upon antigen re-encounter whereas T_{EM} gain access to peripheral tissues, display potent cytotoxicity but proliferate poorly. Another antigen-induced memory subset is the T inflammatory memory subset (T_{IM}). These cells, described in the team, are generated by priming F5 TCR transgenic mice with antigen under sterile inflammatory conditions (i.e. in the absence of pathogen-derived signals). T_{IM} have been shown to be involved in the recall antigen-specific contact-hypersensitivity reactions. T_{IM} are arrested at a relatively early memory-stage and are able to further differentiate into T_{CM} or T_{EM} memory $CD8^+$ T cells upon antigenic re-stimulation by PAMP-matured DC or after viral infection.

It is critical to be able to evaluate the magnitude and the kinetic of memory $CD8^+$ T cell generation for understanding the regulation of the immune responses but also to be able to evaluate efficiency of vaccine candidates. One way to do so is to track and analyse antigen-specific $CD8^+$ T cells, but that requires the knowledge of the specific epitopes, i.e. antigen specificities, that will dominate the immune response. However, the full spectrum of antigenic epitopes harbored by a pathogen or a vaccine is often partially unknown. One other way would be to follow the whole antigen-induced population. Unfortunately, as described above, memory compartment is composed of antigen-induced and cytokine-induced memory cells and the phenotypic markers used to detect these memory cells (CD44, CXCR3, IFNγ) do not allow to discriminate between them. The identification of a biomarker that would be specific for protective antigen-induced memory cells would allow monitoring more closely the generation of memory $CD8^+$ T cells in response to an infection or vaccination.

SUMMARY OF THE INVENTION:

The present invention relates to methods for testing the presence of protective antigen-induced memory CD8+ T cells and uses thereof. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a method for testing the presence of at least one population of protective antigen-induced memory CD8+ T cells in a sample obtained from a subject comprising the steps of i) incubating the sample obtained from the subject with the antigen for which the presence of the antigen-induced memory CD8+ T cells is sought ii) determining the secretion level of at least one cytokine selected from the group consisting of GM-CSF, CCL1 and CCL9 iii) comparing the secretion level determined at step ii) with its corresponding predetermined reference value and iv) concluding that the population of protective antigen-induced memory CD8+ T cells is present in the sample when a difference between the secretion level determined at step ii) with the predetermined reference value is determined.

As used herein the term "CD8+ T cells" has its general meaning in the art and refers to a subset of T cells which express CD8 on their surface, are MHC class I-restricted, and function as cytotoxic T cells. "CD8" molecules are differentiation antigens found on dendritic cells, on thymocytes and on cytotoxic and suppressor T-lymphocytes. CD8 antigens are members of the immunoglobulin supergene family and are associative recognition elements in major histocompatibility complex class I-restricted interactions. As used herein the term “memory CD8+ T cells” has its general meaning in the art and refers to a subset of T CD8+ cells that have previously encountered and responded to their cognate antigen. Upon a second encounter with their cognate antigen, memory CD8+ T cells mount a faster and stronger immune response than the one associated with a first time antigen-encounter. As used herein the term “antigen-induced memory CD8+ T cell” refers to a subset of memory T CD8+ cells that is generated following stimulation by its cognate antigen. The term “antigen-induced memory CD8+ T cell” is defined in opposition with cytokine-induced memory CD8+ T cells and “protective antigen-induced memory CD8+ T cell” refers the antigen-induced memory CD8+ T cells that are able to confer protection in the subject when they encounter their cognate antigen.

In some embodiments, the subject is a mouse. In some embodiments, the subject is a human. Subjects may be male or female and may be of any age, including prenatal (i.e., in utero), neonatal, infant, juvenile, adolescent, adult, and geriatric subjects. For example, the subject can be a healthy subject, a subject suffering from a given disease or a subject who was vaccinated.

The term "sample" as used herein refers to whole blood, saliva, urine, bronchoalveolar fluids, cerebrospinal fluids, or purified peripheral blood mononuclear cells (PBMC), or any of other biological fluids liable to contain CD8+ T cells. In some embodiments, the sample is 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. Such procedures are known to the expert in the art. In some embodiments, the sample is collected in tubes or other containers containing an appropriate anti-coagulant (e.g., lithium heparin or sodium citrate). For example, the crude whole blood specimen is unfractionated whole blood collected with appropriate anti-coagulant (e.g. EDTA). It contains plasma and blood cells (red blood cells, white blood cells). It may be a freshly isolated blood sample (<48h) or a blood sample which has been obtained previously and kept frozen until use.

In some embodiments, the incubation of the sample with the antigen is performed at the point of care locations such as physicians' offices, clinics, or outpatient facilities. Once incubation is complete, the requirement for fresh and active cells no longer exists. Cytokines are stable and, thus, the sample can be stored, frozen or shipped without special conditions.

Accordingly, in some embodiments, the sample is collected in suitable container (e.g. collection tube) containing the antigen.

In some embodiments, the population of CD8+ T cells is previously isolated. Standard methods for isolating CD8+ T cells are well known in the art. For example the methods may consist in collecting the population of CD8+ T cells present in the sample by using a binding partner directed against a specific surface marker of the CD8+ T cells (e.g. CD8). In some embodiments, the methods of the invention comprise bringing the sample into contact with a binding partner capable of selectively interacting with CD8+ T cells present in said sample. The binding partner may be an antibody that may be polyclonal or monoclonal, preferably monoclonal, directed against a specific surface marker of the CD8+ T cells. Typically said surface marker is CD8. In some embodiments, the binding partner may be an aptamer. In some embodiments, the population of memory CD8+ T cells are isolated from the sample by using a set of binding partners capable of selectively interacting with said cells. Typically, the set of binding partners may comprise a binding partner for CD8, and at least one binding partner specific for CD44, CD45RO/RA or CXCR3.

As used herein the term "antigen" is well understood in the art and refers to the portion of a macromolecule which is specifically recognized by a component of the immune system, e.g., an antibody or a T-cell antigen receptor. As used herein, the term "antigen" encompasses antigenic epitopes, e.g., fragments of an antigen which are antigenic epitopes. Epitopes are recognized by antibodies in solution, e.g. free from other molecules. Epitopes are recognized by T-cell antigen receptor when the epitope is associated with a class I or class II major histocompatibility complex molecule. Typically, the term “antigen” encompasses “antigen associated with a pathogenic organism”, “tumor associated antigen” and “allergen”.

In some embodiments, the antigen is a protein which can be obtained by recombinant DNA technology or by purification from different tissue or cell sources. Typically, said protein has a length higher than 10 amino acids, preferably higher than 15 amino acids, even more preferably higher than 20 amino acids with no theoretical upper limit. Such proteins are not limited to natural ones, but also include modified proteins or chimeric constructs, obtained for example by post-translational modifications, by changing selected amino acid sequences or by fusing portions of different proteins. In some embodiments of the invention, said antigen is a synthetic peptide. Typically, said synthetic peptide is 3-40 amino acid-long,

preferably 5-30 amino acid-long, even more preferably 8-20 amino acid-long. Synthetic peptides can be obtained by Fmoc biochemical procedures, large-scale multipin peptide synthesis, recombinant DNA technology or other suitable procedures. Such peptides are not limited to natural ones, but also include post-translationally modified amino acids, modified peptides or chimeric peptides, obtained for example by changing selected amino acid sequences or by fusing portions of different proteins.

In some embodiments, the antigen is a crude or partially purified tissue or cell preparation obtained by different biochemical procedures (e.g., fixation, lysis, subcellular fractionation, density gradient separation) known to the expert in the art.

In some embodiments, the antigen is a viral antigen. Examples of viral antigens include but are not limited to as 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 antigens (e.g. Dengue viral antigens, West Nile viral antigens), hepatitis viral antigens including antigens from HBV and HC, human immunodeficiency virus (HIV) antigens (e.g. gag, pol or nef), herpesvirus (such as cytomegalovirus and Epstein-Barr virus) antigens (e.g. pp65, IE1, EBNA-1, BZLF-1) and adenovirus.

In some embodiments, the antigen is a bacterial antigen. Examples of bacterial antigens 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. meningitidis* and *Haemophilus influenzae type b*.

In some embodiments, the antigen is a fungal antigen. Examples of fungal antigens 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 antigens (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 myeloma; HER-2/neu associated with breast cancer; 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, antigens of human T cell leukemia virus type 1 have been shown to induce specific cytotoxic T cell responses and antitumor immunity against the virus-induced human adult T cell leukemia (ATL).

In some embodiments, the antigen is selected from the group consisting of peptides, proteins, cells or tissues that constitute the molecular targets of an autoimmune response. Said molecular targets are expressed by the tissue(s) or cell(s) targeted by the autoimmune response. Expression of autoimmunity-associated self antigens can be limited to the target tissue or be extended to additional body compartments. Autoimmunity-associated antigens can be initially identified as being targets of autoantibody or T cell immune responses, or based on their selective expression by the target tissue. Some examples of autoimmunity-associated protein antigens are 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 type 1 diabetes; myeloperoxydase and proteinase 3 for granulomatosis with polyangiitis; myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) in multiple sclerosis. Examples of autoimmunity-associated peptide antigens are derived from the above said protein antigens following processing by APCs – including DC – and presentation in the context of different HLA Class I or Class II molecules. Therefore, said peptide antigens are different depending not only on their source antigens, but also on the HLA molecules by which they are presented. For example, a list of type 1 diabetes-associated peptide antigens for both mouse and human can be found in DiLorenzo et al., Clin.Exp.Immunol. 148:1, 2007.

Autoimmunity-associated peptide antigens also include post-translationally modified amino acid sequences and sequences derived from alternative splicing isoforms.

In some embodiments, the antigen is an allergen. Allergens of interest according to the present invention include antigens found in foods such as fruits (e.g., melons, strawberries, pineapple and other tropical fruits), peanuts, peanut oil, other nuts, milk proteins, egg whites, shellfish, tomatoes, etc.; airborne antigens such as grass pollens, animal danders, house mite feces, etc.; drug antigens such as penicillins and related antibiotics, sulfa drugs, barbiturates, anticonvulsants, insulin preparations (particularly from animal sources of insulin), local anesthetics (e.g., Novocain), and iodine (found in many X-ray contrast dyes); insect venoms and agents responsible for allergic dermatitis caused by blood sucking arthropods such as Diptera, including mosquitos (Anopheles sp., Aedes sp., Culiseta sp., Culex sp.), flies (Phlebotomus sp., Culicoides sp.) particularly black flies, deer flies and biting midges, ticks (Dermmacenter sp., Omithodoros sp., Otobius sp.), fleas (e.g., the order Siphonaptera, including the genera Xenopsylla, Pulex and Ctenocephalides felis felis); and latex. The specific allergen may be any type of chemical compound such as, for example, a polysaccharide, a fatty acid moiety, a protein, etc.

In some embodiments, the incubation time of step i) ranges from 5 to 48 hours, more preferably 5 to 36 hours and even more preferably 12 to 24 hours or a time period in between. Thus in some embodiments, the incubation time is 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 26 hours, 30 hours, 36 hours, 42 hours, or 48 hours.

As used herein, the term “GM-CSF” has its general meaning in the art ant refers to the Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) also known as colony stimulating factor 2 (CSF2).

As used herein, the term “CCL1” has its general meaning in the art and refers to Chemokine (C-C motif) ligand 1, also known as I-309, P500, SCYA1, SISe, or TCA3.

As used herein, the term "CCL9" has its general meaning in the art and refers to Chemokine (C-C motif) ligand 9, also known as CCF18; MRP-2; MIP-1 gamma, Scya9; or Scya10.

Methods for quantifying secretion of a cytokine in a sample are well known in the art. For example, any immunological method such as but not limited to ELISA, multiplex strategies, ELISPOT, immunochromatography techniques, proteomic methods, Western blotting, FACS, or Radioimmunoassays may be applicable to the present invention.

Typically said methods comprise contacting the sample with a binding partner capable of selectively interacting with the cytokine present in the sample. The binding partner may be an antibody that may be polyclonal or monoclonal, preferably monoclonal. In some embodiments, the binding partner may be an aptamer.

In some embodiments, the binding partner is labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal. As used herein, the term "labelled", with regard to the antibody, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labelling of the probe or antibody by reactivity with a detectable substance. An antibody or aptamer of the invention may be labelled with a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I123, I124, In111, Re186, Re188.

The above mentioned assays generally involve the binding of the binding partner (ie. antibody or aptamer) to a solid support (e.g. tube). Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, plastic or glass (e.g. blood collection tubes).

In some embodiments, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize said cytokine(s). A sample containing or suspected of containing said cytokine(s) is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

In some embodiments, an Enzyme-linked immunospot (ELISpot) method may be used. Typically, the sample is transferred to a plate which has been coated with the desired anti-cytokine capture antibodies. Revelation is carried out with biotinylated secondary Abs and standard colorimetric or fluorimetric detection methods such as streptavidin-alkaline phosphatase and NBT-BCIP and the spots counted.

In some embodiments, when multi-cytokine secretion quantification is required, use of beads bearing binding partners of interest may be preferred. In some embodiments, the bead may be a cytometric bead for use in flow cytometry. Such beads may for example correspond to BD™ Cytometric Beads commercialized by BD Biosciences (San Jose, California). Typically cytometric beads may be suitable for preparing a multiplexed bead assay. A multiplexed bead assay, such as, for example, the BD(TM) Cytometric Bead Array, is a series of spectrally discrete beads that can be used to capture and quantify soluble antigens. Typically, beads are labelled with one or more spectrally distinct fluorescent dyes, and detection is carried out using a multiplicity of photodetectors, one for each distinct dye to be detected. A number of methods of making and using sets of distinguishable beads have been described in the literature. These include beads distinguishable by size, wherein each size bead is coated with a different target-specific antibody (see e.g. Fulwyler and McHugh, 1990, Methods in Cell Biology 33:613-629), beads with two or more fluorescent dyes at varying concentrations, wherein the beads are identified by the levels of fluorescence dyes (see e.g. European Patent No. 0 126,450), and beads distinguishably labelled with two different dyes, wherein the beads are identified by separately measuring the fluorescence intensity of each of the dyes (see e.g. U.S. patent Nos. 4,499,052 and 4,717,655). Both one-dimensional and two-dimensional arrays for the simultaneous analysis of multiple antigens by flow cytometry are available commercially. Examples of one-dimensional arrays of singly dyed beads

distinguishable by the level of fluorescence intensity include the BD(TM) Cytometric Bead Array (CBA) (BD Biosciences, San Jose, California) and Cyto-Plex(TM) Flow Cytometry microspheres (Duke Scientific, Palo Alto, California). An example of a two-dimensional array of beads distinguishable by a combination of fluorescence intensity (five levels) and size (two sizes) is the QuantumPlex(TM) microspheres (Bangs Laboratories, Fisher, Ind.). An example of a two-dimensional array of doubly-dyed beads distinguishable by the levels of fluorescence of each of the two dyes is described in Fulton et al. (1997, Clinical Chemistry 43(9):1749-1756). The beads may be labelled with any fluorescent compound known in the art such as e.g. FITC (FL1), PE (FL2), fluorophores for use in the blue laser (e.g. PerCP, PE-Cy7, PE-Cy5, FL3 and APC or Cy5, FL4), fluorophores for use in the red, violet or UV laser (e.g. Pacific blue, pacific orange). In another particular embodiment, bead is a magnetic bead for use in magnetic separation. Magnetic beads are known to those of skill in the art. Typically, the magnetic bead is preferably made of a magnetic material selected from the group consisting of metals (e.g. ferrum, cobalt and nickel), an alloy thereof and an oxide thereof. In another particular embodiment, bead is bead that is dyed and magnetized.

Typically, the predetermined reference value is a threshold value or a cut-off value. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement of the cytokine secretion levels in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the secretion levels of the cytokine(s) in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured concentrations of cytokines in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off

values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and 1-specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When AUC>0.5, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, the threshold value is derived from the cytokine secretion level determined in a control sample derived from one or more subjects for whom the presence of the population of protective antigen-induced memory CD8+ T cells is absent.

Typically, it is concluded that the population of protective antigen-induced memory CD8+ T cells is present in the sample when the secretion level determined at step i) is higher that its corresponding predetermined reference value.

In some embodiments, the secretion levels of GM-CSF and CCL1 are determined in the sample.

In some embodiments, the secretion levels of GM-CSF and CCL9 are determined in the sample.

In some embodiments, the secretion levels of CCL1 and CCL9 are determined in the sample.

In some embodiments, the secretion levels of GM-CSF, CCL1 and CCL9 are determined in the sample.

In some embodiments, a score which is a composite of the secretion levels of the different cytokines (i.e. GM-CSF, CCL1 and CCL9) is determined and compared to a corresponding predetermined reference value wherein a difference between said score and said reference value is indicative of the presence or absence of the population of protective antigen-induced memory CD8+ T cells.

The method of the invention may be useful in a wide variety of diagnostic assays, clinical studies, and monitoring assays.

In some embodiments, the method of the present invention is useful for determining whether a subject has developed a memory T cell response in a context of an infection with a pathogenic organism or a disease such as cancer, allergy, an autoimmune disorder, etc. Thus, e.g., the method is useful for detecting a population of protective antigen-induced memory CD8+ T cells that is specific for antigens associated with a pathogen; for a tumor-associated antigen; for an allergen; or for a self antigen.

In some embodiments, the method of the present invention is useful for identifying to which allergen(s) a subject will be sensitive, e.g., to which allergen(s) a subject exhibits an allergic reaction. Thus, e.g. a plurality of samples obtained from the subject are exposed to a panel of allergens, so that the allergen(s) to which the populations of protective antigen-induced memory CD8+ T cells which in the sample are reactive can be identified.

In some embodiments, the method of the present invention is useful for determining the efficacy of a treatment for a disorder. In some embodiments, the present invention provides methods of determining the response of a subject to treatment for an infection with a pathogen. In some embodiments, the present invention provides methods of determining the response of a subject to treatment for cancer. In some embodiments, the present invention provides methods of determining the response of a subject to treatment for allergy.

In some embodiments, the method of the present invention is useful for clinical studies, e.g., to compare the level of protective antigen-induced memory CD8+ T cells from one subject to another, e.g., in response to treatment, in the absence of treatment, etc.

In some embodiments, the method of the present invention is useful for testing efficacy of a vaccine (e.g. for the prophylactic treatment of an infection or for the treatment of cancer). Typically, when the presence of the population of protective antigen-induced memory CD8+ T cells is determined indicates that the vaccine preparation was efficient. The method may be thus particularly suitable for screening combinations of antigens and/or immunoadjuvants for the preparation of vaccine preparation.

In some embodiments, the method of the present invention is useful for determining whether a subject shall be vaccinated (or revaccinated). Typically, when the presence of the population of protective antigen-induced memory CD8+ T cells cannot be determined, then the subject is vaccinated (or revaccinated) for said antigen, pathogen…

Yet another object of the invention relates to a kit for performing a method of the invention, said kit comprising means for quantifying the secretion of the cytokines in the sample. Typically the kit includes means (e.g. antibodies, aptamers such as above described) for determining the secretion levels in a sample of at least one cytokine selected from the group consisting of GM-CSF, CCL1 and CCL9 that are indicative of the presence of at least one population of protective antigen-induced memory CD8+ T cells in the sample. In some embodiments, the kit comprises means for determining the secretion levels of GM-CSF and CCL1. In some embodiments, the kit comprises means for determining the secretion levels of GM-CSF and CCL9. In some embodiments, the kit comprises means for determining the secretion levels of CCL1 and CCL9. In some embodiments, the kit comprises means for determining the secretion levels of GM-CSF, CCL1 and CCL9. The kit may also include the antigen, an antibody, or a set of antibodies as above described. In some embodiments, the antigen, the antibody or set of antigens and antibodies are labelled as above described. In some embodiments, the kit comprises a suitable container (e.g. collection tube) containing the antigen, an antibody, or a set of antibodies as above described. The kit may also contain other suitably packaged reagents and materials needed for the particular detection protocol, including solid-phase matrices, if applicable, and standards.

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. Comparison of protective capacity between two memory CD8 T cell populations. Survival curve of infected mice. Mice were transferred with $5x10^4$ naive (CD8+CD44 low) or TIM (CD8+CD44 int) or Flu-TM (CD8+CD44 high) CD8 T cells and infected intranasally 48h later with a lethal dose of Flu-NP68 ($1x10^7$ $TCID_{50}$). Data are mean of 2 independent experiments with at least 5 mice per group. Total numbers of mice per condition are indicated in brackets. Wilcoxon log rank test.

Figure 2. Validation of the gene-expression signature associated with protective memory CD8 response. (A) Cytokines profile of stimulated naive, T_{IM} or Flu-memory CD8 T cells. Supernatants were collected after 48h of NP68 peptide stimulation. Cytokines production was measured by bead-based multiplexing technology for GMCSF and IL10 or by ELISA for CCL1, CCL9 and IL21. Data are mean ± SD of 3 independent experiments with a pool of four or five mice per group. (B-C) Cytokines profile of human sorted naive or memory CD8 T cells after stimulation. Supernatants were collected after 48h of anti-CD3+anti-CD28 stimulation or anti-CD3+anti-CD28 stimulation plus IL12+IL18 stimulation. Cytokines production was measured by bead-based multiplexing technology for IFNγ and GMCSF (Bioplex Human Cytokine Group I 6-plex Assay, Bio-Rad) or by ELISA for CCL1. Data are mean ± SD of 5 healthy donors. *,$p < 0.05$ unpaired Student's t test.

Figure 3. IL12 and IL-18 induce the production of GM-CSF by Flu-TM memory cells. Cytokines expression profile of naive, TIM and Flu-memory CD8 T cells. Supernatants were collected 48h after IL12+IL18 stimulation. Cytokines production was measured by bead-based multiplexing technology for GMCSF and IL10 or by ELISA for CCL1, CCL9. Data are mean ± SD of 3 independent experiments with a pool of 4 or 5 mice per group. *,$p < 0.05$ unpaired Student's t test.

EXAMPLE 1:

CD8 memory cells induced by an acute viral infection are endowed with new properties that contribute to the timely control of the same pathogen when reencountered. The acquisition of such properties is sought when vaccinating individuals. The development of CD8 based vaccine is in part hampered by the lack of hallmarks that would clearly distinguish

efficient memory cells from memory cells that are not fully differentiated and have not developed the full pattern of protective memory traits. Memory CD8 cells induced in response to acute viral infection are known to generate protective CD8 memory cells. This is in contrast to memory CD8 T cell generated in response to recombinant vaccines or peptide immunisation. In order to define the quality uniquely associated with protective memory cells, we devised a system to compare the gene expression signature of two qualities of memory CD8 T cells: memory CD8 T cells generated following an infection with life influenza virus or peptide immunisation. Selection of high affinity TCR's has been associated with memory response efficiency, hence to avoid selection of CD8 T cells clones with different affinities in the different experimental models, both memory cells types were generated using naïve F5 TCR transgenic cells that are specific for NP68, an influenza nucleoprotein epitope. Peptide induced memory cells (TIM) have been previously described: they correspond to an early differentiation step in the naïve to memory CD8 differentiation pathway and are able to further differentiate in TCM/TEM when recruited in a secondary anti-viral response. Flu induced memory cells where generated by intranasal infection of C57BL/6J mice that where grafted with F5 CD8 T cells. Naïve, TIM and influenza induced memory cells (Flu-TM) were compared for their surface phenotype and capacity to produce cytokines upon stimulation with their cognate peptide. TIM memory cells, similarly to Flu-TM F5 cells, express increased levels of CD44, CXCR3 and CD122 that distinguish them from naïve cells. TIM memory cells all belong to the TCM subset in contrast to Flu-induced memory that contains both TEM and TCM memory cells. An increased protection against infection has been associated with the presence of poly-functional CD8 or CD4 memory T cells able to produce multiple cytokine. We measured the frequency of poly-functional CD8 T cells among TIM or Flu-TM F5 cells following peptide re-stimulation, for one combination of 4 cytokines IFNγ, IL2, CCL3 and CCL5. A poly-functionality index that numerically evaluates the degree and variation of poly-functionality was calculated for each subset. As expected naïve CD8 T naïve cells were not poly-functional in contrast to Flu-TM F5 cells that showed a high poly-functionality index. The poly-functionality index of TIM was also high compared to naïve cells but was half that of Flu-TM F5. We next measured the capacity of the different subsets of CD8 T cells to protect against a lethal dose of influenza virus. Naïve C57BL/6J mice were grafted with an identical numbers of F5 CD8 T cells obtained from the spleens of naïve, TIM or Influenza primed mice, before being infected with a lethal dose of influenza virus intra-nasally. Results in Figure 1 show that Flu-TM F5 CD8 T cells are able to protect naïve mice, against a lethal dose of virus. This is in contrast to what is observed with naïve F5 cells or

TIM F5 cells. Hence, Flu-TM F5 CD8 T cells display intrinsic functional capacities, acquired following their priming by the virus that allow them to curtail a lethal infection by the influenza virus. This is contrast to peptide-induced memory F5-TIM or Naive F5 CD8 T cells.

In order to identify at the gene expression level the differences associated with Flu-TM and TIM memory cells we performed a global transcriptional analysis of naïve F5 CD8 T cells, TIM and Flu-TM F5 cells. To capture the poised genes that are more rapidly expressed by memory cells, arrays were performed with RNA from quiescent (Homeostatic condition – H) or NP68 peptide stimulated (restimulated condition-R) CD8 T cells. We first performed a principal-component analysis (PCA), a method that identifies linear combinations of gene-expression profiles (principal components) that best explain variance across a data set. The first two components account for more than 70% of the variability; samples are distributed on the first component according to the homeostatic/restimulated factor. The second component correlates with the CD8 differentiation stage, with TIM memory holding an intermediated position between naive cells and Flu-TM F5. We next compared the list of genes that are differentially expressed by TIM or Flu-TM F5 compared to naïve CD8 T cells in homeostatic and re-stimulation conditions. The large majority of genes differentially expressed in TIM compared to naïve cells are also expressed in Flu-TM in both homeostatic and re-stimulated conditions (respectively 88% and 93% shared genes). Similarly, a signalling pathway impact analysis reveals that most signalling pathways that are impacted by differential gene-expression in TIM memory compared to naïve cells are also affected in Flu-TM. These results confirm at the gene expression level that TIM are partially differentiated memory cells compared to Flu-induced memory cells. Importantly, Flu-TM have more differentially expressed genes per perturbed pathway, which is seen also on the overall amount of differentially expressed genes in Flu-TM compared to TIM.

To identify genes which expression levels could uniquely be associated with spleen Flu-TM CD8 and could represent a signature for protection against a lethal intranasal infection, we searched for gene that showed a significant differential expression between the different differentiation-stages naïve, Tim or Flu-TM in homeostatic or re-stimulated conditions. To identify the significantly differentially expressed probe-sets we performed a three way discretized comparison. Briefly, a discretized fold change profile was defined for each probe-set in the following comparison (Tim – Na; Flu – Na; Flu – Tim), a value of either 1 (positive FC) or -1 (negative FC) was attributed to the comparison if the adjusted p-value was below 0.05, or 0 if the adjusted p-value was superior to 0.05. Six distinct classes of discretized FC profiles were found and annotated from P1 to P6 with a large majority of genes

contained in the first three clusters (P1 to P3). A principal component analysis performed on the different array-samples, using the P1 to P3 gene lists, shows that P3 and P2 genes discriminate Naïve from memory TIM and Flu-TM in contrast to P1 genes that segregate TIM and Naïve apart from Flu-TM. This being true for gene lists obtained when comparing gene expression by CD8 T cells in homeostatic conditions (HP) or following restimulation (RP).

In homeostatic conditions the HP2 and HP3 gene-lists that differentiate memory cells, TIM or Flu-TM, from naïve contain genes that are classically associated with memory such as Klrc1, Ccl5, CD44, Cxcr3 or IFNγ, Asns, which have a HP3 and HP2 profile respectively. The pattern of expression of some of these genes was validated at RNA or protein levels. To assess the conserved expression of these memory gene signatures, we performed a meta-analysis of 8 expression datasets comparing naïve and memory CD8 T cells gene-expression-profiles in homeostatic conditions. Memory CD8 T cells, in these models, were generated using 4 different pathogens (Vaccinia, Listeria, VSV and LCMV) and 3 different TCR transgenic mice (P14, F5 and OT1). The majority of genes in the P2 and P3 clusters are present in the meta-analysis i.e. are differentially expressed by memory cells compared to naïve cells in all these different models. Next we concentrated our analysis on genes that have a P1 profile in homeostatic conditions i.e. a significant increased expression in Flu-TM, but not TIM, compare to quiescent naïve cells. These P1 genes are also up-regulated in VV-TM and LM-TM F5 memory cells generated in response to two other pathogens vaccinia-NP68 (VV-NP68) and *Listeria Monocytogenes*-NP68 (LM-NP68), indicating that this HP1 signature was not restricted to influenza generated memory CD8 T cells. Genes in this signature code for proteins, that are viral infection restricting factors (e.g. IFITM1, IFITM3, GBP7), that protect against cellular or environmental stress (e.g. ABCB1a, Car2, ERN1, serpin3G), that regulate immune activation processes (e.g. FGL2, ANXA1, Havcr2, NRARP) or that are involved in the regulation of cell migration (e.g. CCR2, CCR5, ITGA1, ITGB1, GPR183, GPR15, RGS16), This last group includes genes that code for proteins that have been shown to play an essential role in the recruitment of CD8 T cells in the lung following infection by a pathogen. The chemokine receptor CCR5 has been shown to be necessary for the recruitment of peripheral memory CD8 T cells to the lung and the ITGA1/ITGB1 genes code for VLA1 an integrin involved in the retention and survival of CD8 memory cells in the lung. To validate if this differential expression resulted in a different migration pattern following a virus induced stimulation, we transferred equivalent numbers of naïve, TIM or Flu-TM F5 cells in naïve C57BL/6J host that were infected intra-nasally with an infectious dose of NP68-influenza. The number of F5 cells in different organs was monitored at

different times following infection. Similar numbers of F5 cells were found in all groups on day 6 post-infection, indicating that at the peak of the response the different subsets of transferred F5 cells are activated and have a similar distribution. An increased number of F5 CD8 T cells was found in the lung of mice having been transferred with Flu-TM CD8 T cells compared to naïve or TIM recipient mice, at early time points, day 3 and day 4 following infection. The number of TIM in the lung although lower than Flu-TM was however also significantly increased compared to the number of naïve F5 cells. To discriminate between intravascular and tissue location, we performed *in vivo* anti-CD45, intravascular staining on day 4 following infection. The majority of Flu-TM are localized within the tissue in contrast to TIM and naïve F5 cells that were mainly found in the vasculature. These results indicated that the HP1 gene expression signature is associated with the capacity of Flu-TM to be more rapidly recruited to the lung parenchyma. This early recruitment was associated with increased levels of IFNγ in the broncho-alveolar lavage of Flu-TM recipient mice. This property of Flu-TM CD8 T cells could contribute to the protection they can confer against a lethal dose of influenza virus.

We next analysed genes that were differentially expressed following a brief NP68 peptide re-stimulation *in vitro*. Genes were classified in three groups, RP2 and RP3 that segregate naïve from memory cells and RP1 that segregates Flu-TM from naïve and TIM cells. These RP gene lists contain a number of HP genes that maintain their differential expression following peptide stimulation and poised genes that were not differentially expressed in homeostatic conditions. We focused our analysis on these poised genes as they might reveal new functions associated with memory CD8 T cells efficiency. Hence we analysed genes that were not significantly up regulated by memory cells compared to naïve cells in homeostatic conditions (HP0) but showed a significant differential expression in memory CD8 T cells, following peptide stimulation (RP). The expression profiles of 10 genes showing the highest FC when comparing Naïve and Flu-TM was validated on VV-TM cells. The HP0RP2 genes, that show a similar expression profile in Flu-TM and TIM, contain two transcription factors IRF8 and TRAF5 that control the optimal generation of effector CD8 T cells and two cytokines IL-3 and XCL1 that have been previously shown to be poised in memory CD4 or CD8 T cells. The HP0RP3 highlights new poised genes such as IL1RL1 that codes for the receptor for the IL33 alarmin that is produced by non hematopoietic cells during the course of viral infections and that plays an essential role in the development of anti-viral effector CD8 T cells, transcription factors (ZBTB1, ZBTB25, ZBTB32) involved in regulating T cells activation or differentiation or proteins involved in cellular response to

stress (Nfe2l2) or infection (IFIH1). Hence the majority of the RP2 and RP3 poised genes seem to be involved in the generation, regulation or function of memory CD8T cells. The poised-gene-signature HP0RP1 associated with protective Flu-TM is strongly enriched in cytokines (IL10, IL21 and CSF2) and CC-chemokines (CCL1 and CCL9). Among these only CSF2/GM-CSF is known to be expressed by human memory CD8 cells (Newell *et al.*, Immunity 2012). Expression of the other cytokines by memory CD8 T cells has not been previously described, their expression by memory CD8 T cells at the mRNA level was validated by PCR. The production of the poised RP1 cytokine GM-CSF and chemokines CCL1 and CCL9 at the protein level was measured following peptide stimulation. Results in Figure 2A indicate that all three proteins are secreted in significantly larger quantities by Flu-TM compared to TIM or naïve F5 cells. Memory CD8 T-cells also increase their functional abilities and secrete certain cytokines such as IFNγ in respond to IL-12 and IL-18 two cytokines associated with inflammatory milieu. Figure 3 shows that the production of GM-CSF, but not CCL1 or CCL9, by Flu-TM was strongly induced by IL-12 and IL-18 indicating that its production by memory CD8 T cells follows the same pattern as IFNγ.

CCL1 in contrast to CCL9 is conserved in humans, we thus determined if it was also behaving as a poised gene in human memory CD8 T cells. We purified human memory CD8 T cells based on their phenotype and measured their capacity to produce CCL1 and GM-CSF or IFNγ as a control following stimulation with anti-CD3/CD28 in the presence or absence of IL12/IL18. As shown in Figure 2B and 2C, similarly to GM-CSF or IFNγ, the CCL1 chemokine was produced by memory phenotype but not naïve CD8 T cells. This indicates that the CCL1 gene is also poised in human memory CD8 T cells. The strong enrichment of the HP0RP1 signature in soluble mediators, which can act on innate and adaptive immune response, suggests that Flu-TM memory CD8 T cells could impact the host immune response when re-stimulated *in vivo*. We thus measured the consequences of Flu-TM activation on a naïve CD8 T cell response. Naïve mice transferred with naïve or memory CD8 T cells TIM or Flu-TM were infected with the recombinant VV-NP68 and the response of naïve CD8 T cells to the dominant vaccinia virus epitope B8R was monitored by tetramer staining. Infection by the VV-NP68 lead to a strong stimulation of transferred naïve and memory F5 CD8 T cells as illustrated by the large number of effector cells recovered from the spleen at the peak of the response. Naïve cells generating significantly more effector cells in the spleen than memory cells as also observed when F5 CD8 were re-stimulated with influenza virus. The response of host-derived naïve CD8 to the B8R epitope was also measured in the spleen. Naïve mice

having received Flu-TM memory F5 T cells showed a strong increase in the frequency of B8R CD8 T cells compared to mice having received naïve or TIM memory F5 cells.

REFERENCES:

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.

Cytometry by Time-of-Flight Shows Combinatorial Cytokine Expression and Virus-Specific Cell Niches within a Continuum of CD8+ T Cell Phenotypes

Evan W. Newell, Natalia Sigal, Sean C. Bendall, Garry P. Nolan, and Mark M. Davis. Immunity 36, 142–152, January 27, 2012

CLAIMS:

1. A method for testing the presence of at least one population of protective antigen-induced memory CD8+ T cells in a sample obtained from a subject comprising the steps of i) incubating the sample obtained from the subject with the antigen for which the presence of the antigen-induced memory CD8+ T cells is sought ii) determining the secretion level of at least one cytokine selected from the group consisting of GM-CSF, CCL1 and CCL9 iii) comparing the secretion level determined at step ii) with its corresponding predetermined reference value and iv) concluding that the population of protective antigen-induced memory CD8+ T cells is present in the sample when a difference between the secretion level determined at step ii) with the predetermined reference value is determined.

2. The method of claim 1 wherein the subject is a mouse.

3. The method of claim 1 wherein the subject is a human.

4. The method of claim 1 wherein the sample is a sample of purified peripheral blood mononuclear cells.

5. The method of claim 1 wherein the antigen is an antigen associated with a pathogenic organism, a tumor associated antigen or an allergen.

6. The method of claim 5 wherein the antigen is a viral, bacterial or fungal antigen.

7. The method of claim 1 wherein the secretion levels of GM-CSF and CCL1 are determined in the sample.

8. The method of claim1 wherein the secretion levels of GM-CSF and CCL9 are determined in the sample.

9. The method of claim 1 wherein the secretion levels of CCL1 and CCL9 are determined in the sample.

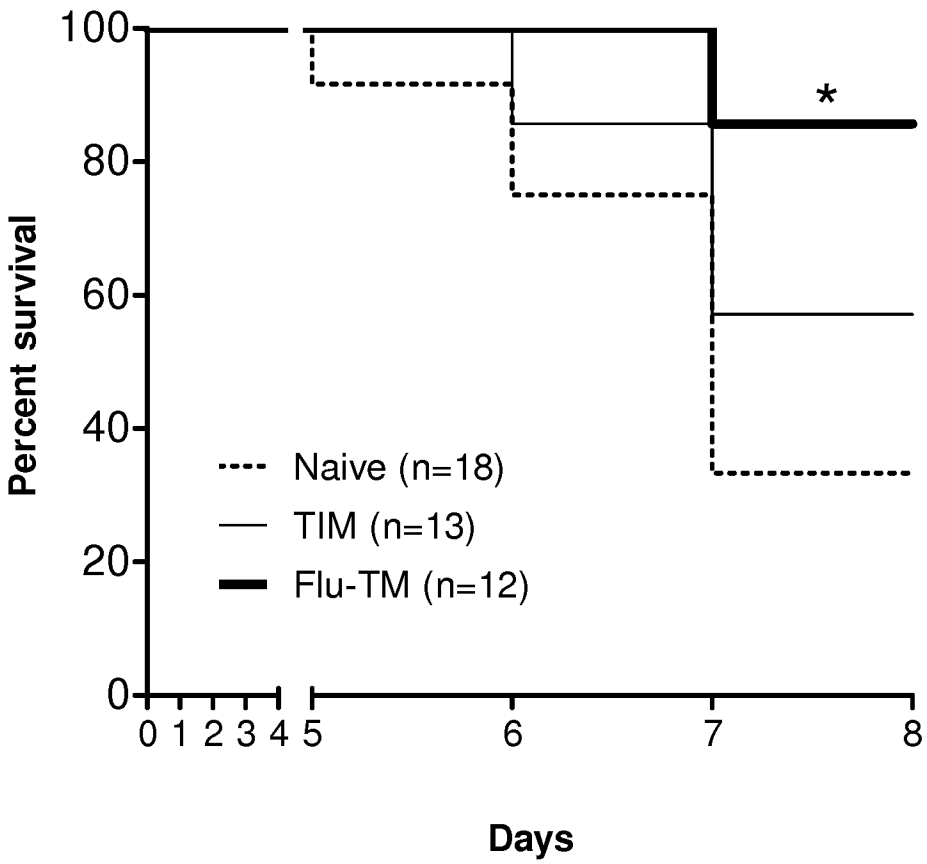
10. The method of claim 1 wherein the secretion levels of GM-CSF, CCL1 and CCL9 are determined in the sample.

11. Use of the method of claim 1 for determining whether a subject has developed a memory T cell response in a context of an infection with a pathogenic organism, a disease such as cancer, an allergy, or an autoimmune disorder.

12. Use of the method of claim 1 for testing efficacy of a vaccine wherein when the presence of the population of protective antigen-induced memory CD8+ T cells indicates that the vaccine preparation was efficient.

13. Use of the method of claim 1 for screening combinations of antigens and/or immunoadjuvants for the preparation of vaccine preparation.

14. A kit for performing the method according to any one of claims 1 to 13 wherein said kit comprises means for determining the secretion level of at least one cytokine selected from the group consisting of GM-CSF, CCL1 and CCL9 that are indicative of the presence of at least one population of protective antigen-induced memory CD8+ T cells in the sample.


100
80
60
40
20
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Percent survival
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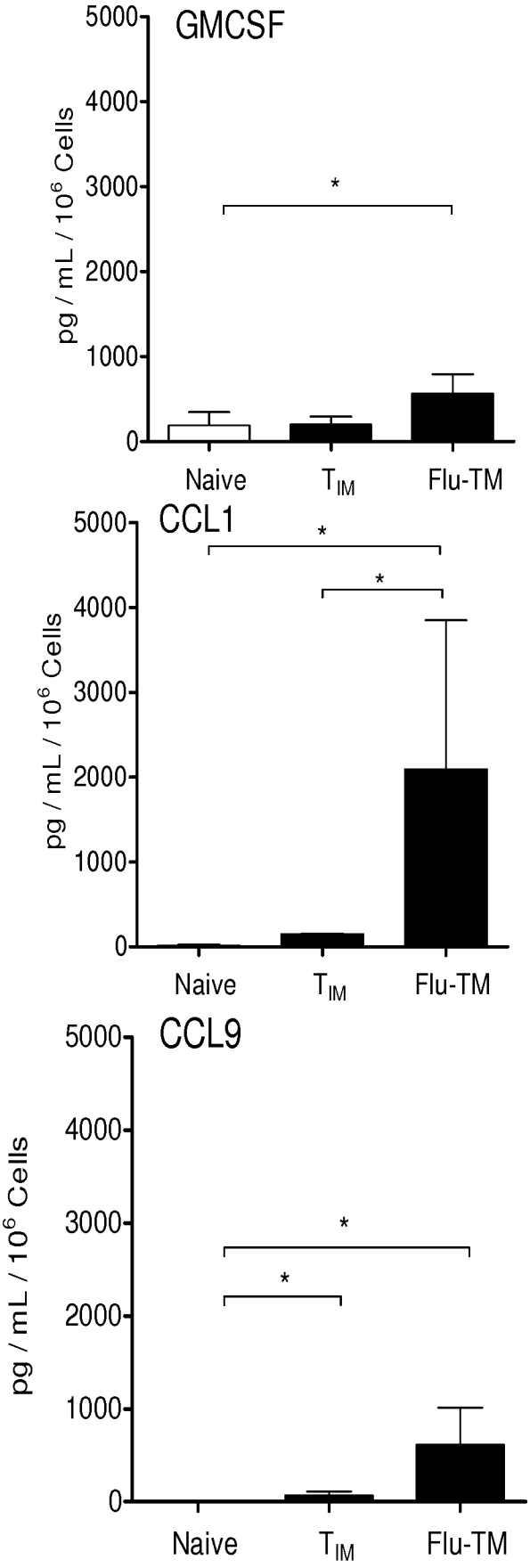
TIM (n=13)
Flu-TM (n=12)
0 1 2 3 4 5
6
7
8
Days

Figure 1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pg / mL / 10^6 Cells
5000
4000
3000
2000
1000
0
*
Naive
T_IM
Flu-TM
CCL1
pg / mL / 10^6 Cells
5000
4000
3000
2000
1000
0
*
*
Naive
T_IM
Flu-TM
CCL9
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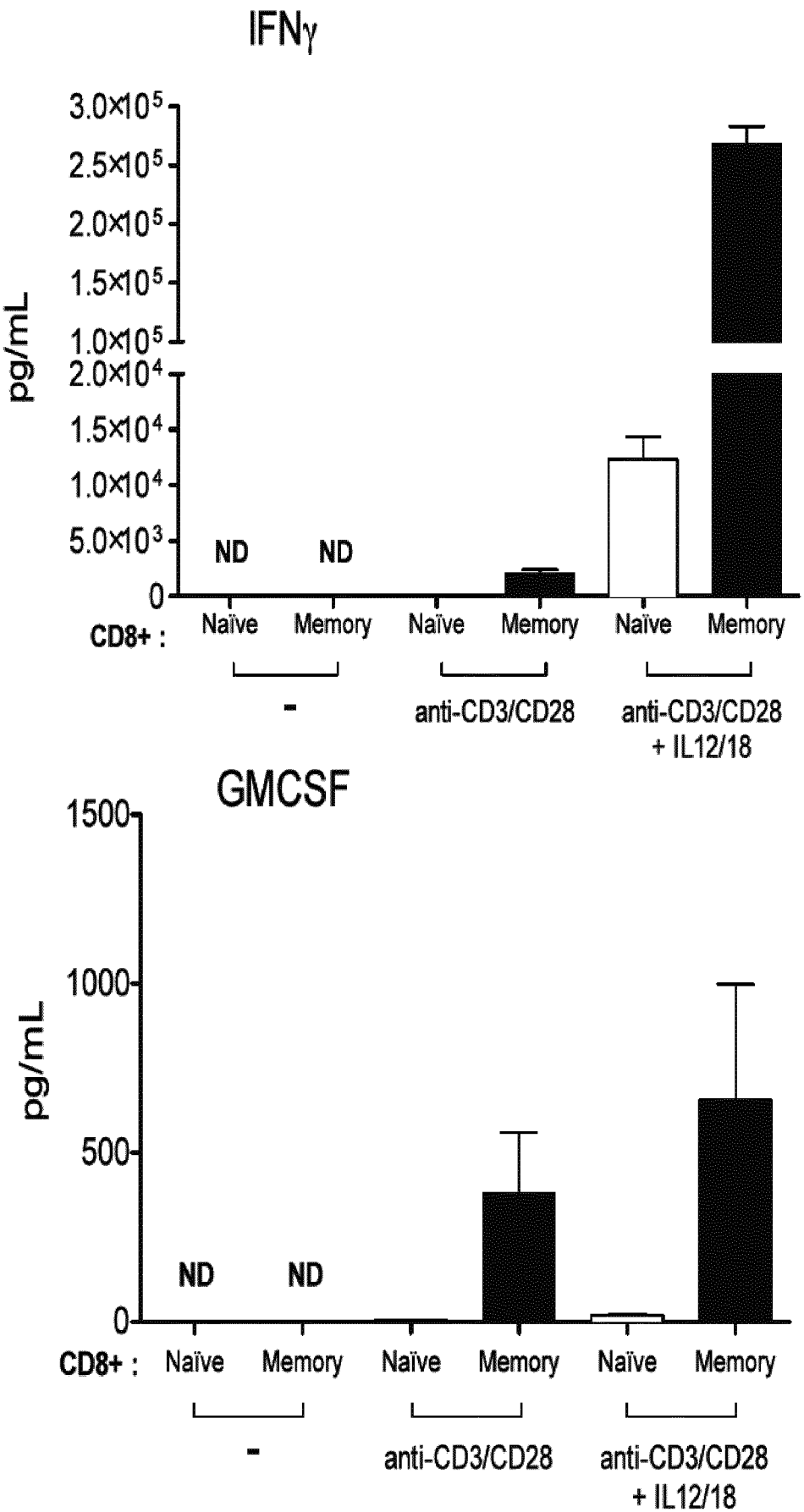
*
*
Naive
T_IM
Flu-TM

Figure 2A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1.0x10^4
5.0x10^3
0
pg/mL
ND
ND
CD8+ :
Naïve
Memory
Naïve
Memory
Naïve
Memory
-
anti-CD3/CD28
anti-CD3/CD28
+ IL12/18
GMCSF
1500
1000
500
0
pg/mL
ND
ND
CD8+ :
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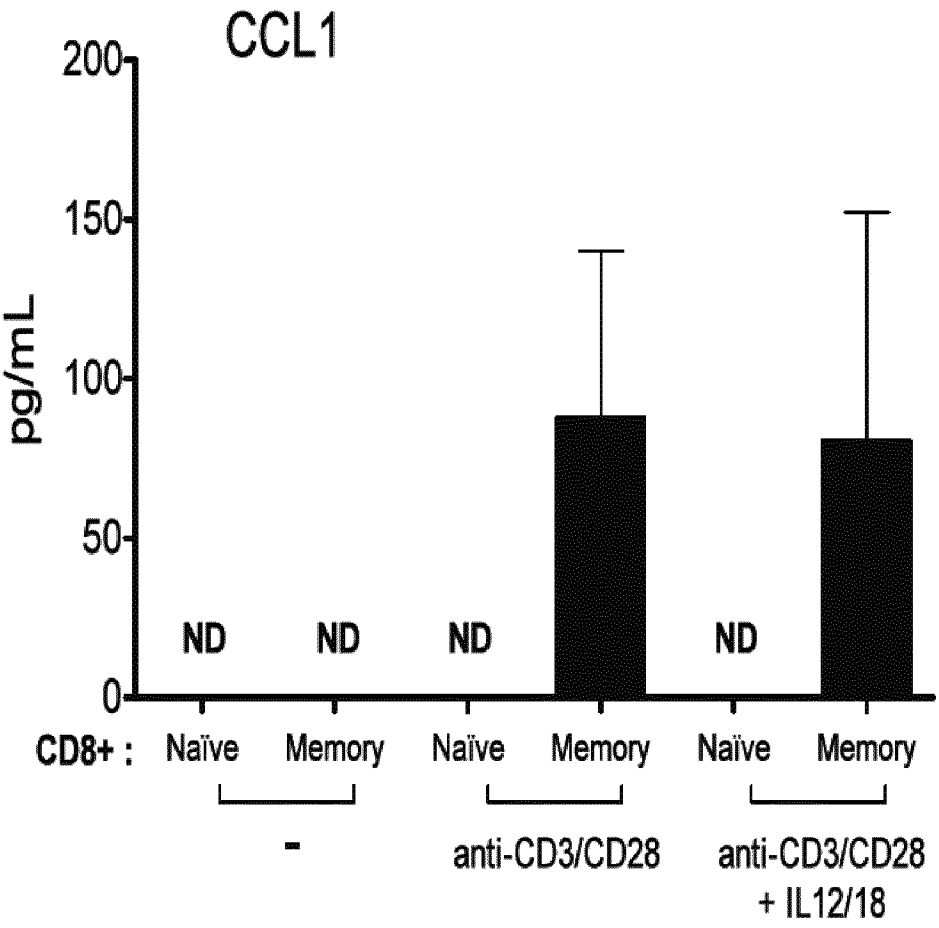
anti-CD3/CD28
anti-CD3/CD28
+ IL12/18

Figure 2B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100
50
0
pg/mL
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ND
ND
ND
CD8+ :
Naïve
Memory
Naïve
Memory
Naïve
Memory
-
anti-CD3/CD28
anti-CD3/CD28
+ IL12/18

Figure 2C

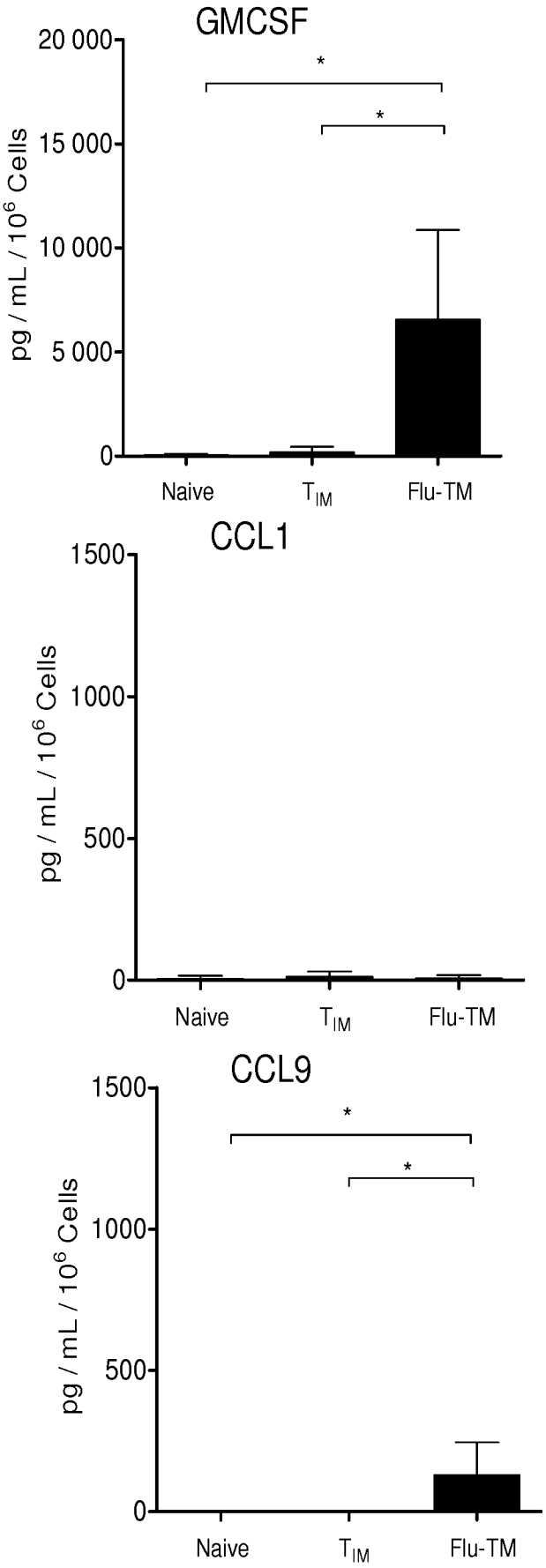
GMCSF
20 000
15 000
10 000
5 000
0
pg / mL / 10^6 Cells
*
*
Naive
T_IM
Flu-TM
CCL1
1500
1000
500
0
pg / mL / 10^6 Cells
Naive
T_IM
Flu-TM
CCL9
1500
1000
500
0
pg / mL / 10^6 Cells
*
*
Naive
T_IM
Flu-TM

Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/079883

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 G01N33/68
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)
G01N

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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/096397 A1 (INSERM INST NAT DE LA SANTÉ ET DE LA RECH MÉDICALE [FR]; UNIVERSITÉ CL) 26 June 2014 (2014-06-26) claims -----	1-13
X	K. TSUBOI ET AL: "Granulocyte-Macrophage Colony-Stimulating Factor Expressed in T Cells Mediates Immunity against Herpes Simplex Virus Type 1 Encephalitis", THE JOURNAL OF INFECTIOUS DISEASES, vol. 178, no. 1, 1 July 1998 (1998-07-01), pages 16-26, XP055191001, ISSN: 0022-1899, DOI: 10.1086/515588	14
Y	abstract, page 21, right-hand column, last paragraph, figure 4, page 24, right-hand column,, 1st paragraph through page 25, right-hand column, 2nd paragraph; -----	1-13

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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

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"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
7 March 2016	17/03/2016

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	Pilch, Bartosz

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/079883

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/015705 A2 (ROGER WILLIAMS HOSPITAL [US]; LUM LAWRENCE G [US]; ELFENBEIN GERLAD [U) 27 February 2003 (2003-02-27) Claims 48, 49, 53 -55;	1-13
X	US 2014/274925 A1 (JIN CHENGGANG [US] ET AL) 18 September 2014 (2014-09-18)	14
Y	Paragraph 102 , 120 , claims 1-5 ; 89-90;	1-8
Y	US 2013/288229 A1 (DEML LUDWIG [DE] ET AL) 31 October 2013 (2013-10-31) Paragraph 136 , claims 6 and 15 ;	1-13
X	EHLERS S ET AL: "DIFFERTIATION OF T CELL LYMPHOKINE GENE EXPRESSION: THE IN VITRO ACQUISITION OF T CELL MEMORY", THE JOURNAL OF EXPERIMENTAL MEDICINE, ROCKEFELLER UNIVERSITY PRESS, US, vol. 173, 1 January 1991 (1991-01-01), pages 25-36, XP002914397, ISSN: 0022-1007, DOI: 10.1084/JEM.173.1.25 summary, page 26, right-hand column, last paragraph through page 27, left-hand column, paragraph 2; figure 4;	14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/079883

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014096397 A1	26-06-2014	EP 2936161 A1	28-10-2015
		US 2015309048 A1	29-10-2015
		WO 2014096397 A1	26-06-2014
WO 03015705 A2	27-02-2003	AU 2002323236 A1	03-03-2003
		CA 2457143 A1	27-02-2003
		EP 1450828 A2	01-09-2004
		JP 2004538331 A	24-12-2004
		US 2003185823 A1	02-10-2003
		US 2011064689 A1	17-03-2011
		WO 03015705 A2	27-02-2003
US 2014274925 A1	18-09-2014	NONE	
US 2013288229 A1	31-10-2013	CN 103109191 A	15-05-2013
		DE 102010037622 A1	22-03-2012
		EA 201370070 A1	30-07-2013
		EP 2616816 A2	24-07-2013
		JP 2013539652 A	28-10-2013
		SG 188420 A1	31-05-2013
		US 2013288229 A1	31-10-2013
		WO 2012037937 A2	29-03-2012