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(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **UNIVERSITÉ PARIS DIDEROT - PARIS 7** [FR/FR]; 5, rue Thomas Mann, 75013 Paris (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, Rue Michel Ange, 75016 Paris (FR). **MEDECINE ET INNOVATION** [FR/FR]; 1 AVENUE FRÉDÉRIC LE PLAY, 75007 PARIS (FR).

(72) Inventors: **LE BUANEC, Hélène**; HOPITAL ST LOUIS - U976, 1 AVENUE CLAUDE VELLEFAUX, PAVILLON BAZIN, 75475 PARIS CEDEX 10 (FR). **ZAGURY, Daniel**; MEDECINE ET INNOVATION, 1 avenue Frédéric Le Play, 75007 PARIS (FR). **DUCHEZ, Sophie**; HOPITAL SAINT LOUIS - UMR7212, 1 Av Claude Vellefaux, 75010 PARIS (FR). **SCHIAVON, Valérie**; HOPITAL ST LOUIS - U976, 1 AVENUE CLAUDE VELLEFAUX, 75475 PARIS CEDEX 10 (FR). **BENSUSSAN, Armand**; Hôpital Saint Louis, 1 Avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris (FR).

(74) Agent: **COLLIN, Matthieu**; Inserm Transfert, 7 rue Watt, 75013 PARIS (FR).

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(54) Title: INVARIANT FOXP3+ REGULATORY T CELLS AND THERAPEUTIC USES THEREOF

(57) Abstract: The present invention relates to a novel population of invariant Foxp3+ regulatory T cells, an ex vivo method generating and expanding them and therapeutic uses thereof. The inventors aimed to determine the optimal conditions for inducing Foxp3 expression in invariant Tcells by assessing different nTreg polarizing medium for their capacity to induce the expression the differentiation of Foxp3+ cells with suppressive function. The inventors showed that tumor Ag-specific memory invTCR Vα24+ T cells ex vivo generated and expanded in the presence of the nTreg polarizing medium maintain their ability to perform suppressive function in pro-inflammatory conditions. In particular, the present invention relates to an isolated population of invariant Foxp3+ regulatory T cells having the following phenotype: CD3+ Vα24+ Foxp3+.

INVARIANT FOXP3$^+$ REGULATORY T CELLS AND THERAPEUTIC USES THEREOF

FIELD OF INVENTION

The present invention relates to a novel population of invariant Foxp3$^+$ regulatory T cells, an ex vivo method generating and expanding them and therapeutic uses thereof.

BACKGROUND OF INVENTION

The history of Tregs begins in 1970 (under the name of "suppressor T cells"). Unfortunately, due to lack of a molecular marker, research on suppressor T cells was stopped, until many years later, when Sakaguchi et al. (1995) identified CD25 as a phenotypic marker for suppressive CD4+ T cells in mice, which upon adoptive transfers could prevent development of autoimmune disease. These suppressive T cells were named nTregs and were later also found in humans within the CD4+CD25^{high} T cell population.

Two functionally distinct regulatory T-cell subsets are currently identified in peripheral tissues: T regulatory cells (Tregs) which control self-tolerance and anti-inflammatory IL-10–secreting type 1 regulatory T cells (Tr1) derived from Ag-stimulated T cells, which regulate inflammation-dependent adaptive immunity and minimize immunopathology. Tregs can develop both in the thymus (tTregs, thymic-derived Tregs formerly natural or nTreg) and in the periphery (pTregs, peripheral-induced Tregs, formerly induced, or iTreg) and silence autoreactive cells by cell–cell contact. Today, Tregs are routinely identified by the use of a combination of phenotypic markers: CD4, CD25, CD127, CD45RA and the canonical transcription factor FOXP3. Tregs may also exhibit others functional markers (CTLA-4, CD28, ICOS, GITR, CD39, TIGIT, FCLR3, TIM-3, Helios), maturation markers (HLA-DR, CD95) and homing molecules (CXCR3, CCR7, CXCR3, CCR5, CCR4, CLA) whose frequency and level depend on their maturation stages. Tr1 T cells originate from most immune-stimulated CD4+ helper T cell subtypes and down-modulate immune response by releasing IL-10.

The inventors isolated a new population of regulatory T cells, which are invariant $Foxp3^+$ regulatory T cells. The present invention thus provides the characteristics of this new population of invariant $Foxp3^+$ regulatory T cells, a method for ex vivo generating and expanding said invariant $Foxp3^+$ regulatory T cells and therapeutic uses of said invariant $Foxp3^+$ regulatory T cells.

SUMMARY

The present invention relates to an isolated population of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

In one embodiment, the isolated population of invariant $Foxp3^+$ regulatory T cells of the invention further expressing CD4 and CD25, thereby having the following phenotype: $CD3^+$ $CD4^+$ $V\alpha24^+$ $CD25^+$ $Foxp3^+$.

The present invention also relates to a method for generating ex vivo invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$, comprising

- culturing $CD3^+$ $V\alpha24^+$ T cells in the presence of an invariant T cell activator and the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator, ii) a TGFβ (Transforming growth factor beta) pathway activator, iii) a mTOR inhibitor, optionally iv) at least one cytokine selected in the group of IL-2, IL-7, IL-15 and TSLP, and optionally v) at least one TET enzymes activator (preferably selected from vitamin C and a NaHS hydrogen sulfide releasing agent) and/or at least one DNMT inhibitor (such as, for example, RG108, DAC or 5AC), for at least 5 days.

In one embodiment of the present invention, the invariant T cell activator is a polyclonal invariant T cell activator, preferably a $V\alpha24$ activator.

In another embodiment, the invariant T cell activator is an antigen-specific invariant T cell activator, preferably tolerogenic dendritic cells (DCs) expressing CD1 and pulsed with at least one non peptide lipid antigen.

In one embodiment, the cAMP activator is selected from the group comprising prostaglandin E2 (PGE2), an EP2 or EP4 agonist, a membrane adenine cyclase activator and metabotropic glutamate receptors agonists.

In one embodiment, the TGFβ pathway activator is selected from the group comprising TGFβ, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activin and nodal .

In one embodiment, the mTOR inhibitor is selected from the group comprising rapamycin, rapamycin analogs, wortmannin; theophylline; caffeine; epigallocatechin gallate (EGCG), curcumin, resveratrol; genistein, 3, 3-diindolylmethane (DIM), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PP242, PP30, Torin1, Ku-0063794, WAY-600, WYE-687, WYE-354, GNE477, NVP-BEZ235, PI-103, XL765 and WJD008.

In one embodiment of the present invention, the method of the invention further comprises an expansion step, wherein the invariant $Foxp3^+$ regulatory T cells obtained by the generation method as described above are cultured in the presence of an invariant T cell activator and the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator, ii) a TGFβ (Transforming growth factor beta) pathway activator, iii) a mTOR inhibitor, optionally iv) at least one cytokine selected in the group of IL-2, IL-7, IL-15 and TSLP, and optionally v) at least one TET enzymes activator (preferably selected from vitamin C and a NaHS hydrogen sulfide releasing agent) and/or at least one DNMT inhibitor (such as, for example, RG108, DAC or 5AC), for at least 5 days.

The present invention also relates to an ex vivo generated invariant $Foxp3^+$ regulatory T cell population obtainable by the method according to the invention, wherein said $Foxp3^+$ regulatory T cells have the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

Another object of the present invention is an ex vivo generated and expanded invariant $Foxp3^+$ regulatory T cell population obtainable by the method according to the invention, wherein said $Foxp3^+$ regulatory T cells have the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

A further object of the present invention is an immunogenic product comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

The present invention also relates to a pharmaceutical composition comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$and at least one pharmaceutically acceptable excipient.

The present invention further relates to a vaccine composition comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$and at least one adjuvant.

Another object of the present invention is the immunogenic product, pharmaceutical composition or vaccine composition according to the present invention for use in treating cancer.

A further object of the present invention is a pharmaceutical composition comprising invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ and at least one pharmaceutically acceptable excipient.

The present invention also relates to a pharmaceutical composition according to the invention for use in cell therapy.

The present invention further relates to a pharmaceutical composition according to the invention for use in treating inflammatory or autoimmune diseases or for preventing transplant rejection or graft versus host disease (GVHD).

DEFINITIONS

As used herein, "regulatory T cells" or "Treg" refers to cells functionally committed, i.e. capable of suppressive activity (i.e. inhibiting proliferation of conventional T cells), either by cell-cell contact or by MLR suppression (Mixed Lymphocytes Reaction). These cells include different subpopulations including but not limited to, MHCII restricted $CD4^+$ $Foxp3^+$ regulatory T cells, γδ $Foxp3^+$ regulatory T cells and invariant $Foxp3^+$ regulatory T cells.

As used herein, "invariant $Foxp3^+$ regulatory T cells" refers to cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$. These cells recognize non peptide lipid antigens under CD1 restriction.

As used herein, "γδ $Foxp3^+$ regulatory T cells" refers to cells having the following phenotype: $\gamma\delta TCR^+$ $Foxp3^+$. These cells recognize non peptide phospho antigens with no MHC (major histocompatibility complex) restriction.

As used herein, "MCHII restricted $CD4^+$ $Foxp3^+$ regulatory T cells" refers to cells having the following phenotype: $CD4^+CD25^+Foxp3^+$. These cells are thymic derived or peripherally induced. These cells can be identified by their αβTCR (T cell receptor) and recognize peptides (including foreign or self peptides) presented by restricted MHC class II (major histocompatibility complex class II) molecules.

As used herein, the term "treatment" refers to therapeutic treatment and prophylactic and preventive measures, wherein the object is to prevent or slow down (lessen, diminish) the targeted pathological disorder or condition. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. A subject or mammal is successfully "treated" for a disease if, after receiving a therapeutic amount of invariant $Foxp3^+$ regulatory T cells or a

therapeutically amount of inactivated invariant Foxp3$^+$ regulatory T cells according to the present invention, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of pathogenic cells; reduction in the percent of total cells that are pathogenic; and/or relief to some extent, of one or more of the symptoms associated with the specific disease or condition; reduced morbidity and mortality, and improvement in quality of life issues. The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician.

As used herein, "therapeutically effective amount" refers to the number of invariant Foxp3$^+$ regulatory T cells or of inactivated invariant Foxp3$^+$ regulatory T cells that is aimed at inducing a therapeutic response, without causing significant negative or adverse side effects to the target. A therapeutically effective amount may be administered prior to the onset of the disease to be treated, for a prophylactic or preventive action. Alternatively or additionally, the therapeutically effective amount may be administered after initiation of the disease to be treated, for a therapeutic action.

As used herein, "therapeutic response" refers to a therapeutic benefit induced by the invariant Foxp3$^+$ regulatory T cell therapy or the invariant Foxp3$^+$ regulatory T cell vaccination in a subject. A therapeutic response may include the fact of (1) delaying or preventing the onset of the disease to be treated; (2) slowing down or stopping the progression, aggravation, or deterioration of one or more symptoms of the disease to be treated; (3) bringing about ameliorations of the symptoms of the disease to be treated; (4) reducing the severity or incidence of the disease to be treated; or (5) curing the disease to be treated.

As used herein, "about" preceding a figure means more or less 10% of the value of said figure.

As used herein, "subject or patient" refers to a mammal, preferably a human. In the present invention, the terms subject and patient may be used with the same meaning. Examples of non-human mammal include a pet such as a dog, a cat, a domesticated pig, a rabbit, a ferret, a hamster, a mouse, a rat and the like; a primate such as a chimp, a

monkey, and the like; an economically important animal such as cattle, a pig, a rabbit, a horse, a sheep, a goat. In one embodiment, the subject is awaiting the receipt of, or is receiving medical care or was/is/will be the object of a medical procedure, or is monitored for the development of a disease. In one embodiment, the subject is an adult (for example a subject above the age of 18). In another embodiment, the subject is a child (for example a subject below the age of 18). In one embodiment, the subject is a male. In another embodiment, the subject is a female.

As used herein, "allogeneic cells" refers to cells isolated from one subject (the donor) and infused in another (the recipient or host).

As used herein, "autologous cells" refers to cells that are isolated and infused back into the same subject (recipient or host).

DETAILED DESCRIPTION

The present invention relates to an isolated population of $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

The present invention relates to an isolated population of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$. The term "invariant" as used herein includes the term "semi-invariant", where the semi-invariant T cells are T cells not expressing $V\beta11$.

In one embodiment, the isolated population of the invention is a population of semi-invariant $Foxp3^+$ T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+V\beta11^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$. In one embodiment, the invariant $Foxp3^+$ regulatory T cells $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ express the TCR $V\beta1$ chain. In one embodiment, the invariant $Foxp3^+$ regulatory T cells $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ express the TCR $V\beta2$ chain. In one embodiment, the invariant $Foxp3^+$ regulatory T cells $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ express the TCR $V\beta4$ chain. In one embodiment, the invariant $Foxp3^+$ regulatory T cells $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ express the TCR $V\beta8$ chain. In one embodiment, the invariant $Foxp3^+$ regulatory T cells $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ express the TCR $V\beta5.1$ chain.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CTLA4$^+$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD45RO$^+$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD127$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD161$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD45RO$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CTLA4$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD45RO$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD45RO$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are

$CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD45RO^+$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD45RO^+$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD127^-$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD127^-$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD161^-$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD25^+$ $CTLA4^+$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD25^+$ $CD45RO^+$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD25^+$ $CD127^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD25^+$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD25^+$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CTLA4^+$ $CD45RO^+$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CTLA4^+$ $CD127^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CTLA4^+$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CTLA4^+$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD45RO^+$ $CD127^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD45RO^+$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD45RO^+$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD127^-$ $CD161^-$. In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD127^-$ $CD56^-$.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are $CD3^+$ $V\alpha24^+$ $Foxp3^+$ $CD4^+$ $CD161^-$ $CD56^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CTLA4$^+$ CD45RO$^+$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CTLA4$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CTLA4$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CTLA4$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD45RO$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD45RO$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD45RO$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD127$^-$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD127$^-$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD25$^+$ CD161$^-$ CD56$^-$.

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In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CTLA4$^+$ CD45RO$^+$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CTLA4$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CTLA4$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CTLA4$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CD45RO$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CD45RO$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CD45RO$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CD127$^-$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CD127$^-$ CD56$^-$.

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In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$ CD45RO$^+$ CD127$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$ CD45RO$^+$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$ CD45RO$^+$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$ CD127$^-$ CD161$^-$. In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CTLA4$^+$ CD127$^-$ CD56$^-$.

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In another embodiment, the invariant Foxp3$^+$ regulatory T cells are CD3$^+$ Vα24$^+$ Foxp3$^+$ CD4$^+$ CD25$^+$ CTLA4$^+$ CD45RO$^+$ CD127$^-$ CD161$^-$ CD56$^-$.

In another embodiment, the invariant Foxp3$^+$ regulatory T cells are Vα24$^+$ Jα18$^+$.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells of the invention are human cells.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells express Foxp3 with a median fluorescence intensity (MFI) at least equivalent to the Foxp3 MFI measured in naïve regulatory T cells. As used herein, "naïve regulatory T cells" refer to T cells having for phenotype Foxp3$^+$CD45RA$^+$CD4$^+$CD25$^+$CD127$^-$.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells express Foxp3 with a median fluorescence intensity (MFI) of at least 2000.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells express Foxp3 with a median fluorescence intensity (MFI) of at least 2 or 3 fold the Foxp3 MFI measured in naïve regulatory T cells.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells express Foxp3 with a median fluorescence intensity (MFI) of at least 2000, 3000, 4000, 5000, 10000, 20000, 30000, 40000, 50000, 60000, 70000.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells population comprises at least about 65% of the CD3$^+$ Vα24$^+$ cells expressing Foxp3. The expression "at least about 65%" includes, without limitation, about 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 752%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 82%, 93%, 94%, 95%, 96%, 97%, 98%, 99% and 100%.

In one embodiment, the invariant Foxp3$^+$ regulatory T cell population is isolated. As used herein, the term "isolated population" refers to a cell population that is removed from its natural environment (such as the peripheral blood) and that is isolated, purified or separated, and is at least about 75% free, 80% free, 85% free and preferably about 90%, 95%, 96%, 97%, 98%, 99% free, from other cells with which it is naturally present, but which lack the cell surface markers based on which the cells were isolated.

In one embodiment, the Foxp3$^+$ regulatory T cell or the Foxp3$^+$ regulatory T cell population is frozen.

As used herein, the term "expression" may refer alternatively to the transcription of a molecule (i.e. expression of the mRNA) or to the translation (i.e. expression of the protein) of a molecule. In one embodiment, detecting the expression may correspond to an intracellular detection. In another embodiment, detecting the expression may correspond to a surface detection, i.e. to the detection of molecule expressed at the cell surface. In another embodiment, detecting the expression may correspond to an extracellular detection, i.e. to the detection of secretion. In another embodiment, detecting the expression may correspond to intracellular, surface and/or extracellular detections. Methods for determining the expression level are well-known from the skilled artisan, and include, without limitation, determining the transcriptome (in an embodiment wherein expression relates to transcription of a molecule) or proteome (in an embodiment wherein expression relates to translation of a cytotoxic molecule) of cells.

In one embodiment of the invention, the expression of the molecules is assessed at the mRNA level. Methods for assessing the transcription level of a molecule are well known in the prior art. Examples of such methods include, but are not limited to, RT-PCR, RT-qPCR, Northern Blot, hybridization techniques such as, for example, use of microarrays, and combination thereof including but not limited to, hybridization of amplicons obtained by RT-PCR, sequencing such as, for example, next-generation DNA sequencing (NGS) or RNA-seq (also known as "Whole Transcriptome Shotgun Sequencing") and the like. In another embodiment of the invention, the expression of the molecules is assessed at the protein level. Methods for determining a protein level in a sample are well-known in the art. Examples of such methods include, but are not limited to, immunohistochemistry, Multiplex methods (Luminex), western blot, enzyme-linked immunosorbent assay (ELISA), sandwich ELISA, fluorescent-linked immunosorbent assay (FLISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), flow cytometry (FACS) and the like.

In another embodiment, determining the expression level of at least one molecule corresponds to detecting and/or quantifying binding of a ligand to a molecule. In one embodiment, said ligand is an antibody specific of said molecule, and the method of the invention comprises detecting and/or quantifying a complex formed between said antibody and said molecule. The complex can be detected if the ligand has been for example, but not limited to, covalently coupled with a detectable molecule such as an

antibody constant fragment (Fc) or a fluorescent compound (e.g. Cyanine dye, Alexa dye, Quantum dye, etc). The complex can also be detected if the ligand has been tagged with different means well known to the person skilled in the art. For example, but without limitation, a tag used with the invention can be a tag selected from the group comprising or consisting of Hemaglutinin Tag, Poly Arginine Tag, Poly Histidine Tag, Myc Tag, Strep Tag, S-Tag, HAT Tag, 3x Flag Tag, Calmodulin-binding peptide Tag, SBP Tag, Chitin binding domain Tag, GST Tag, Maltose-Binding protein Tag, Fluorescent Protein Tag, T7 Tag, V5 Tag and Xpress Tag. The use of the ligand therefore allows on the one hand the identification and detection of the molecule depending on the ligand used, and on the other hand the quantification of the complex formed.

In one embodiment, determining the expression level of molecules is conducted by flow cytometry, immunofluorescence or image analysis, for example high content analysis. Preferably, the determination of the expression level of molecules is conducted by flow cytometry. In one embodiment, before conducting flow cytometry analysis, cells are fixed and permeabilized, thereby allowing detecting intracellular proteins.

In one embodiment, determining the expression level of a molecule in a cell population comprises determining the percentage of cells of the cell population expressing the molecule (i.e. cells "+" for the molecule). Preferably, said percentage of cells expressing the molecule is measured by FACS.

The terms "expressing (or +)" and "not expressing (or -)" are well known in the art and refer to the expression level of the cell marker of interest, in that the expression level of the cell marker corresponding to "+" is high or intermediate, also referred as "+/-". The cell marker corresponding to "-" is a null expression level of the cell marker or also refers to less than 10 % of a cell population expressing the said cell marker.

The expression level of the cell marker of interest is determined by comparing the Median Fluorescence Intensity (MFI) of the cells from the cell population stained with fluorescently labeled antibody specific for this marker to the fluorescence intensity (FI) of the cells from the same cell population stained with fluorescently labeled antibody with an irrelevant specificity but with the same isotype, the same fluorescent probe and originated from the same specie (referred as Isotype control). The cells from the population stained with fluorescently labeled antibody specific for this marker and that

show equivalent MFI or a lower MFI than the cells stained with the isotype controls are not expressing this marker and then are designated (-) or negative. The cells from the population stained with fluorescently labeled antibody specific for this marker and that show a MFI value superior to the cells stained with the isotype controls are expressing this marker and then are designated (+) or positive.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells are a different population than the MHCII restricted $CD4^+$ $Foxp3^+$ regulatory T cells as shown in Figure 1, wherein MHCII restricted $CD4^+$ $Foxp3^+$ regulatory T cells have the following phenotype $CD3^+$ $CD4^+$ $TCR\alpha\beta^+$ $CD25^+$ $Foxp3^+$ $CD127^-$ $CTLA\text{-}4^+$ whereas invariant $Foxp3^+$ regulatory T cells have the following phenotype $CD3^+$ $CD4^+$ $V\alpha24^+$ $Foxp3^+$ $CTLA4^+$ $CD25^+$ $CD127^-$. As shown in Figure 1, invariant $Foxp3^+$ regulatory T cells have a Foxp3 MFI superior to the Foxp3 MFI of MHCII restricted $CD4^+$ $Foxp3^+$ regulatory T cells.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells are a different population than the invariant NK T cells as shown in Figure 2, wherein the invariant NK T cells have the following phenotype $V\alpha24^+$ $CD56^{+/-}$ $CD161^+$ whereas the invariant $Foxp3^+$ regulatory T cells have the following phenotype $V\alpha24^+$ $CD56^-$ $CD161^-$. In addition, the invariant NK T cells have the following phenotype $CD127^+$ $CD25^-$ $CTLA4^-$ whereas the invariant $Foxp3^+$ regulatory T cells have the following phenotype $CD127^-$ $CD25^+$ $CTLA4^+$.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells of the invention do not present a regulatory T cells specific demethylated region (TSDR) of the gene Foxp3. In one embodiment, the invariant $Foxp3^+$ regulatory T cells of the invention present a regulatory T cells specific demethylated region (TSDR) of the gene Foxp3. In one embodiment, the invariant $Foxp3^+$ regulatory T cells present a percentage of demethylation of the TSDR of the gene FOXP3 superior to at least 30%, 40%, 50%, whereas the invariant NK T cells present a percentage of Foxp3 promoter demethylation less than 10%. A protocol for measuring promoter demethylation percentage is shown in the Material and Method part of the Examples.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells present a percentage of enrichment of acetylated histone in Foxp3 promoter region superior to at least 10%, 20%,

30%, 40% or 50%. A protocol for measuring enrichment of acetylated histones in percentage is shown in the Material and Method part of the Examples.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells of the invention present a percentage of enrichment of acetylated histone in Foxp3 promoter region superior to at least 10%, 20%, 30%, 40% or 50%. A protocol for measuring enrichment of acetylated histones in percentage is shown in the Material and Method part of the Examples.

An example of phenotypic characteristics of the population of the invariant Foxp3$^+$ regulatory T cells of the invention is shown in Figure 4.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells are capable of suppressive activity similar to the suppressive activity of naïve CD4$^+$ CD25$^+$ CD45RA$^+$ CD127$^-$ regulatory T cells. Determination of the suppressive activity of a cell population is well known in the art and can be performed by conventional assays such as the standard polyclonal cell-cell contact Treg suppression assay or the autologous MLR suppression assay as described in the Examples.

The present invention also relates to a method for generating ex vivo invariant Foxp3$^+$ regulatory T cells as defined here above.

In one embodiment, the method for generating ex vivo invariant Foxp3$^+$ regulatory T cells, comprises:

- culturing CD3$^+$Vα24$^+$ T cells, preferably CD3$^+$Vα24$^+$CD45RA+ T cells, in the presence of an invariant T cell activator and the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator, ii) a TGFβ (Transforming growth factor beta) pathway activator, iii) a mTOR inhibitor, optionally iv) at least one cytokine selected from the group of IL-2, IL-7, IL-15 and TSLP (Thymic stromal lymphopoietin), and optionally v) at least one TET enzymes activator and/or at least one DNMT inhibitor, for at least 5 days,
- thereby obtaining a population of invariant Foxp3$^+$ regulatory T cells ex vivo generated, preferably from invariant naïve (CD45RA$^+$) T cells.

In one embodiment, the $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA^+$ T cells, are obtained by any technic well known in the art from a blood sample. In one embodiment, the $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA^+$ T cells, are isolated from PBMCs (peripheral blood mononuclear cells) by flow cytometry. In one embodiment, the $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA^+$ T cells, may be isolated from frozen PBMCs.

In one embodiment, the obtainment of isolated $CD3^+$ $V\alpha24^+$ T cells, preferably $CD3^+$ $V\alpha24^+$ $CD45RA^+$ T cells, may be improved by an optional first to a purification step. The $CD3^+$ $V\alpha24^+$ T cells, preferably $CD3^+$ $V\alpha24^+$ $CD45RA^+$ T cells, are stimulated with antigen pulsed tolerogenic DCs (for example ovalbumin pulsed tolerogenic DCs) in the presence of soluble anti-CD28 and anti-CD40 antibodies. In one embodiment, the time of stimulation ranges between 1 hour and 24 hours, preferably between 10 hours and 20 hours, more preferably during about 16 hours. After stimulation, cells are washed, for example with PBS, and stained with anti-CD154 and anti-CD4 antibodies for sorting. The purified $CD3^+$ $V\alpha24^+$ $CD154^+$ T cells are enriched and may be used for the following activation step.

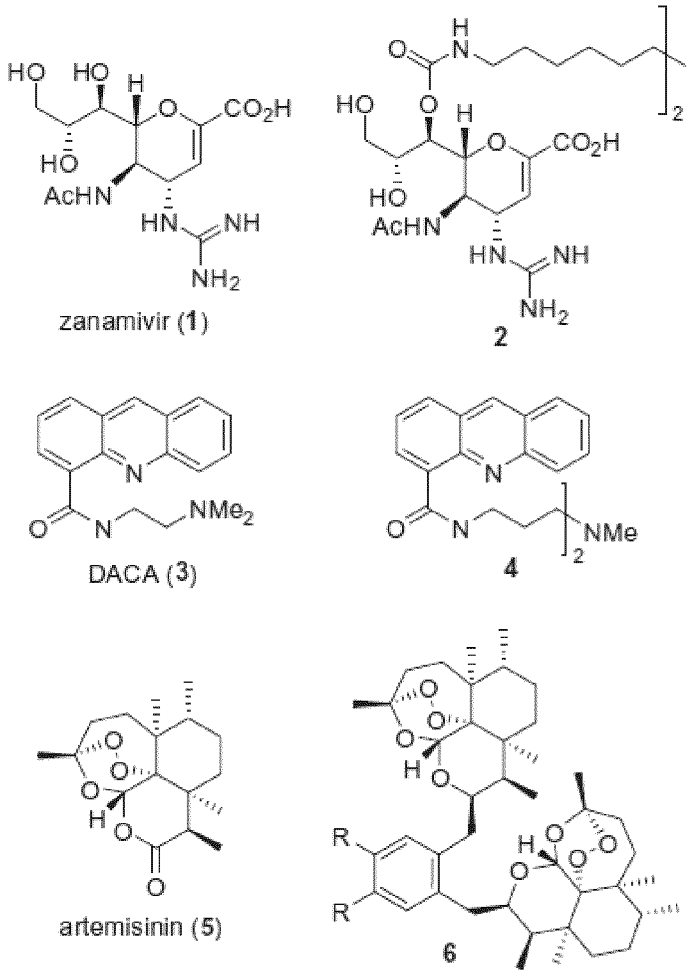
According to the invention, the $CD3^+V\alpha24^+$ T cells are activated in the presence of an invariant T cell activator. Said invariant T cell activator can be a polyclonal invariant T cell activator or an antigen-specific invariant T cell activator.

In the present invention, the polyclonal invariant T cell activator is a $V\alpha24$ activator. Examples of $V\alpha24$ activator include, but are not limited to, anti-$V\alpha24$ antibody such as 6B11 antibody (Montoya CJ et al. Immunology. 2007 Sep;122(1):1-14), or CD1 ligands including CD1a ligands, CD1b ligands, CD1c ligands and CD1d ligands, preferably CD1d ligands such as α-galactosylceramide (α-GalCer) and analogs thereof, such as for example HS44 (a synthetic amino cyclitolic ceramide analogue in which the sugar head group is a carba cyclitol ring that mimics glucose instead of galactose, and which has the O-glycosidic linkage replaced with an amide group), α-GalCer analogs of the table 1 herein below:

Table 1: Structure of α-GalCer analogs

Glycolipid	Structure
α-GalCer	R=$((CH_2)_{24}CH_3$
C10	R=$(CH_2)_5$Ph
6DW116C7	R=$(CH_2)_6$Ph
C11	R=$(CH_2)_7$Ph
6DW116C9	R=$(CH_2)_8$Ph
C15	R=$(CH_2)_9$Ph
C16	R=$(CH_2)_{10}$Ph
C27	R=$(CH_2)_{14}$Ph
C28	R=$(CH_2)_{20}$Ph
C29	R=$(CH_2)_{24}$Ph
C18	R=$(CH_2)_5$Ph(*p*-OMe)
C19	R=$(CH_2)_5$Ph(*p*-F)
C20	R=$(CH_2)_5$Ph(*p*-CF_3)
C21	R=$(CH_2)_5$Ph(*p*-Ph)
C22	R=$(CH_2)_7$Ph(*p*-OMe)
C23	R=$(CH_2)_7$Ph(*p*-F)
C24	R=$(CH_2)_7$Ph(*p*-CF_3)
C25	R=$(CH_2)_7$Ph(*p*-Ph)
7DW8-4	R=$(CH_2)_{10}$Ph(*p*-OMe)
7DW8-5	R=$(CH_2)_{10}$Ph(*p*-F)
7DW8-6	R=$(CH_2)_{10}$Ph(*p*-CF_3)
C26	R=$(CH_2)_{10}$Ph(*p*-Ph)
C13	R'= $(CH_2)_2$Ph
C12	R'= $(CH_2)_4$Ph
6DW138	R'= $(CH_2)_7$Ph
6DW135	R'= $(CH_2)_9$Ph

and homodimeric α-galactosylceramide analogs including the following:



wherein R is CO_2Me, CO_2H or CH_2OH;

α-glucuronyl- and α-galacturonyl-ceramides and analogs thereof; iGb3 (Isoglobotriosylceramide); N-glycolyl (NGc) gangliosides such as for example NGcGM3; glycosphingolipids or phosphoglycerolipids such as phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglyerol presented preferably by CD1d.

In one embodiment, the polyclonal invariant T cell activator is an anti-Vα24 antibody, preferably a m6B11 antibody.

In one embodiment, the polyclonal invariant T cell activator is soluble in the culture medium. In another embodiment, the polyclonal invariant T cell activator is coated to the culture plate.

In one embodiment, the polyclonal invariant T cell activator is used in the presence of feeder cells, preferably autologous feeder cells.

Feeder cells include, but are not limited to, ΔCD3 cells (T cell-depleted accessory cells), irradiated PBMCs, irradiated DCs, artificial APCs (antigen presenting cells), Sf9 cells, insect cells, a pool of PBMCs or a pool of B cells from different subjects, KCD40L cells EBV-transformed B cell lines and EBV-transformed lymphoblastoid cells (LCL).

Preferably, the feeder cells used in the invention are ΔCD3 cells that are isolated by negative selection from PBMCs by incubation with anti-CD3 coated beads and then irradiated at 3000 rad.

In one embodiment, the ratio T cells / feeder cells ranges from 1:100 to 1: 10 000, preferably from 1:1 000 to 1:5 000. Within the scope of the invention, the expression "from 1:100 to 1:10 000" includes, without limitation, 1:100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1 000, 1:1 250, 1:1 500, 1: 1 750, 1:2 000, 1:2 250, 1:2 500, 1: 2 750, 1:3 000, 1:3 250, 1:3 500, 1: 3 750, 1:4 000, 1:4 250, 1:4 500, 1: 4 750, 1:5 000, 1:5 250, 1:5 500, 1: 5 750, 1:6 000, 1:6 250, 1:6 500, 1: 6 750, 1:7 000, 1:7 250, 1:7 500, 1: 7 750, 1:8 000, 1:8 250, 1:8 500, 1: 8 750, 1:9 000, 1:9 250, 1:9 500, 1:9 750 and 1:10 000.

In the present invention, the antigen-specific invariant T cell activator is tolerogenic dendritic cells (DCs) expressing CD1, i.e. CD1a, CD1b, CD1c and/or CD1d, and pulsed with at least one non peptide lipid antigen. Preferably the tolerogenic DCs express CD1d.

In one embodiment, tolerogenic DCs express on their surface the major histocompatibility (MHC) class Ia and/or MHC class Ib. The MHC class Ia presentation refers to the "classical" presentation through HLA-A, HLA-B and/or HLA-C molecules whereas the MHC class Ib presentation refers to the "non-classical" antigen presentation through HLA-E, HLA-F, HLA-G and/or HLA-H molecules.

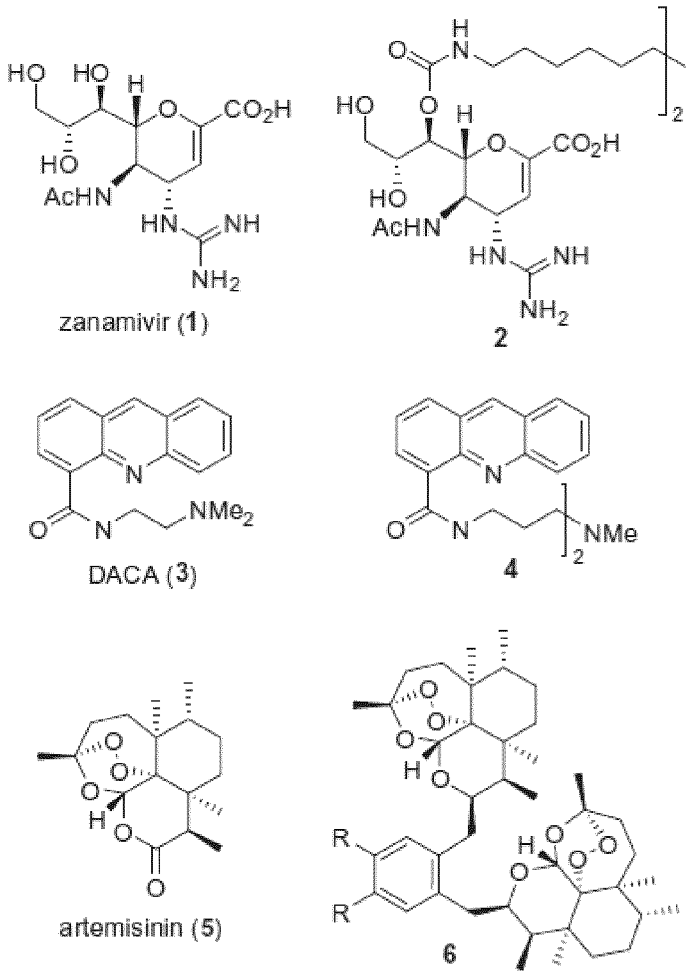
In one embodiment, tolerogenic DCs express 50% of MHC class Ia molecules and 50% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 45% of MHC class Ia molecules and 55% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 40% of MHC class Ia molecules and 60% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 35% of MHC class Ia molecules and 65% of MHC class Ib molecules on their surface. In

one embodiment, tolerogenic DCs express 30% of MHC class Ia molecules and 70% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 25% of MHC class Ia molecules and 75% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 20% of MHC class Ia molecules and 80% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 15% of MHC class Ia molecules and 85% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 10% of MHC class Ia molecules and 90% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express 5% of MHC class Ia molecules and 95% of MHC class Ib molecules on their surface. In one embodiment, tolerogenic DCs express only MHC class Ib molecules on their surface.

In one embodiment, tolerogenic DCs express 50% of HLA-A, HLA-B and/or HLA-C molecules and 50% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 45% of HLA-A, HLA-B and/or HLA-C molecules and 55% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 40% of HLA-A, HLA-B and/or HLA-C molecules and 60% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 35% of HLA-A, HLA-B and/or HLA-C molecules and 65% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 30% of HLA-A, HLA-B and/or HLA-C molecules and 70% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 25% of HLA-A, HLA-B and/or HLA-C molecules and 75% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 20% of HLA-A, HLA-B and/or HLA-C molecules and 80% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 15% of HLA-A, HLA-B and/or HLA-C molecules and 85% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 10% of HLA-A, HLA-B and/or HLA-C molecules and 90% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express 5% of HLA-A, HLA-B and/or HLA-C molecules and 95% of HLA-E molecules on their surface. In one embodiment, tolerogenic DCs express only HLA-E molecules on their surface.

In one embodiment, the non peptide lipid antigen is a recombinant antigen α-galactosylceramide and analogs such as for example HS44 (a synthetic amino cyclitolic

ceramide analogue in which the sugar head group is a carba cyclitol ring that mimics glucose instead of galactose, and which has the O-glycosidic linkage replaced with an amide group), α-GalCer analogs of the table 1 herein above, and homodimeric α-galactosylceramide analogs including the following:



wherein R is CO_2Me, CO_2H or CH_2OH;

α-glucuronyl- and α-galacturonyl-ceramides and analogs; iGb3 (Isoglobotriosylceramide); N-glycolyl (NGc) gangliosides such as for example NGcGM3; glycosphingolipids or phosphoglycerolipids such as phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglyerol presented preferably by CD1d.

In another embodiment, the non peptide lipid antigen is derived from immunogenic apoptotic bodies from cancer cells or immunogenic blebs from cancer cells or derived from tissue lysate.

Cancer cells may derive from tumor biopsy or from expansion of circulatory cancer cells.

Immunogenic apoptotic bodies from cancer cells may be obtained for example with anthracyclines including, without limitation, doxorubicin, daunorubicin, idarubicin and mitoxanthrone; oxaliplatin, UVC or γ-radiation treated cancer cells releasing apoptotic bodies or can be directly isolated from anthracyclines including doxorubicin, daunorubicin, idarubicin and mitoxanthrone; oxaliplatin; UVC or γ-radiation treated cancer.

Blebs constitute an important immunogenic particle. They are heterogenous vesicle formed at the surface of apoptotic cells. In one embodiment, the size of blebs ranges from 0.05 – 5 μm, preferably from 0.1 to 1 μm. Various Immunogenic cell death (ICD) inducers can induce the release of blebs from apoptotic or autophagic cells, such as, for example, irradiation at 5000 rad and several antineoplastic agents, including doxorubicin, oxaliplatin and cisplatin. Immunogenic cancer cell blebs may, in particular, be obtained from apoptotic cancer cells or from cancer cell autophagy following treatment by chemical or physical inducers.

Examples of tissue lysate include, but are not limited to, synovial liquid or inflammatory tissue lysate.

As used herein, "tolerogenic DCs" refers to DCs capable to induce tolerance. In one embodiment, tolerogenic DCs are capable of secreting more suppressive cytokines such as IL-10 and TGFβ than proinflammatory cytokines such as IL-12, IL-23 or TNFα. In one embodiment, DCs are defined as tolerogenic when they secrete IL-10 and IL-12 in a ratio IL-10: IL-12 > 1.

Methods for obtaining tolerogenic DCs are well-known in the art. An exemplary method is the generation of tolerogenic DCs from $CD14^+$ monocytes. For example, $CD14^+$ monocytes are cultured in the presence of GM-CSF and IL-4, or in the presence of GM-CSF and IFNα, for the generation of immature DCs.

Methods for inhibiting MHC class Ia molecules expression or inducing the expression of HLA-E molecules on the surface of tolerogenic DCs are well-known.

The inhibition of the TAP transporter (transporter associated with antigen processing) leads to a decreased expression of MHC class Ia molecules thereby promoting HLA-E molecules expression on the surface of tolerogenic DCs.

Exemplary methods to inhibit the TAP transporter in the endoplasmic reticulum include, but are not limited to, CRISPR-CAS-9 technology, silencing RNA, transfected DCs with the UL-10 viral protein from the CMV (cytomegalovirus) or the use of viral proteins.

Examples of viral proteins able to inhibit the TAP transporter include, but are not limited to, HSV-1 ICP47 protein, varicella-virus UL49.5 protein, cytomegalovirus US6 protein or gammaherpesvirus EBV BNLF2a protein.

Another method is the use of a chemical product to inhibit the expression of MHC class Ia molecules without changing HLA-E expression on the surface of tolerogenic DCs. Examples of chemical products include, but are not limited to, 5'- methyl-5'-thioadenosine or leptomycin B.

Methods for inducing the expression of CD1, i.e. CD1a, CD1b, CD1c and/or CD1d on the surface of the tolerogenic DCs are well-known in the art. For example, sulfatide can be used for the expression of CD1a; rifampin and a number of its derivative (e.g., rifabutin) for the expression of CD1b; cholesteryl esters and acylated steryl glycosides for the expression of CD1c and rosiglitazone; retinoic acid; RARα agonist such as AM580, CD437, AM80, BMS961, NRX195183, All-trans-retinoic acid, 9-cis-Retinoic acid, Ch55, TTNPB (4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), tamibarotene; PPARγ agonist such as ciglitazone, darglitazone, edaglitazone, genistein, indomethacin, GW 1929, LG100754, LT175, CAY10506, nTZDpa, pioglitazone, 15-deoxy-Δ-12,14-Prostaglandin J2, S26948, telmisartan, tesaglitazar, carnosic acid, troglitazone, bezafibrate, 13(S)-Hydroxyoctadeca-9Z,11E-dienoic acid, oxidized low-density lipoprotein (oxLDL) for the expression of CD1d. The tolerogenic DCs expressing CD1 thus obtained are then pulsed with the non peptide ligand antigen as described here above.

In one embodiment, rosiglitazone can be used at a concentration ranging from 0.1 μM to 10 μM.

In one embodiment, AM580 can be used at a concentration ranging from 1 nM to 10 μM.

In one embodiment, pioglitazone can be used at a concentration ranging from 1 nM to 10 μM.

In one embodiment, BMS961 can be used at a concentration ranging from 1 nM to 100 nM.

In one embodiment, TTNPB can be used at a concentration ranging from 1 nM to 1 mM.

In one embodiment, the cAMP activator added in the culture allows the activation of the cAMP pathway. Examples of cAMP activators include, but are not limited to PGE2 (prostaglandin E2), an EP2 or EP4 agonist, a membrane adenine cyclase activator such as forskolin, or metabotropic glutamate receptors agonists. Examples of PGE2 include, but are not limited to, PGE2 of ref P5640 or P0409 (Sigma-Aldrich), PGE2 of ref 2296 (R&D Systems), PGE2 of ref 2268 (BioVision), PGE2 of ref 72192 (Stemcell), PGE2 of ref ab144539 (Abcam), and PGE2 of ref 14010 (Cayman Chemical).

In one embodiment, the cAMP activator, preferably PGE2 is used at a concentration ranging from 0.01 μM to 10 μM. Within the scope of the invention, the expression "from 0.01 μM to 10 μM" includes, without limitation, 0.02 μM, 0.03 μM, 0.04 μM, 0.05 μM, 0.06 μM, 0.07 μM, 0.08 μM, 0.09 μM, 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM, 0.9 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM, 4 μM, 4.5 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM. In certain embodiments, PGE2 is at a concentration ranging from 0.03 μM to 1.5 μM.

In one embodiment, the TGFβ pathway activator added in the culture allows the activation of the TGFβ pathway. Examples of TGFβ pathway activators include, but are not limited to, TGFβ family (TGFβ1, TGFβ2, TGFβ3), bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activin, and nodal. Examples of TGFβ include, but are not limited to, TGFβ1 of ref T7039 (Sigma-Aldrich), TGFβ2 of ref T2815 (Sigma-Aldrich), TGFβ3 of ref T5425 (Sigma-Aldrich), human TGFβ1 of ref P01137 (R&D system), human TGFβ1 of ref 580702 (Biolegend), TGFβ1 of ref HZ-1011 (HumanZyme), human TGFβ1 of ref 14-8348-62 (Affymetrix eBioscience).

In one embodiment, the pathway activator is used at a concentration ranging from 1 ng/ml to 20 ng/ml. Within the scope of the invention, the expression "from 1 ng/ml to 20 ng/ml" includes, without limitation, 2 ng/ml, 2.5 ng/ml, 3 ng/ml, 3.5 ng/ml, 4 ng/ml, 4.5 ng/ml,

5 ng/ml, 5.5 ng/ml, 6 ng/ml, 6.5 ng/ml, 7 ng/ml, 7.5 ng/ml, 8 ng/ml, 8.5 ng/ml, 9 ng/ml, 9.5 ng/ml, 10 ng/ml, 11 ng/ml, 12 ng/ml, 13 ng/ml, 14 ng/ml, 15 ng/ml, 16 ng/ml, 17 ng/ml, 18 ng/ml, 19 ng/ml. In certain embodiments, TGFβ is at a concentration ranging from 2.5 ng/ml to 7.5 ng/ml.

In one embodiment, the mTOR inhibitor added in the culture allows the inhibition of the mTOR pathway. Examples of mTOR inhibitor include, but are not limited to, rapamycin (also named sirolimus) and its analogs (termed rapalogs); wortmannin; theophylline; caffeine; epigallocatechin gallate (EGCG); curcumin; resveratrol; genistein; 3, 3-diindolylmethane (DIM); LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one); PP242; PP30; Torin1; Ku-0063794; WAY-600; WYE-687; WYE-354; and mTOR and PI3K dual-specificity inhibitors such as GNE477, NVP-BEZ235, PI-103, XL765 and WJD008. Examples of rapamycin include, but are not limited to, rapamycin of ref R0395 (Sigma-Aldrich), rapamycin of ref S1039 (Selleckchem), rapamycin of ref 1292 (Tocris), rapamycin of ref R-5000 (LC Laboratories), rapamycin of ref tlrl-rap (InvivoGen), rapamycin of ref ab120224 (Abcam), rapamycin of ref R0395 (Sigma-Aldrich).

Examples of compounds of the same chemical class than rapamycin used clinically include, but are not limited to, Everolimus (code name RAD001), Temsirolimus (code name CCI-779, NSC 683864), Zotarolimus (code name ABT-578).

In one embodiment, the mTOR inhibitor, preferably rapamycin, is used at a concentration ranging from 0.1 nM to 50 nM. Within the scope of the invention, the expression "from 0.1 nM to 50 nM" includes, without limitation, 0.2 nM, 0.3 nM, 0.4 nM, 0.5 nM, 0.6 nM, 0.7 nM, 0.8 nM, 0.9 nM, 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 7 nM, 8 nM, 9 nM, 10 nM, 11 nM, 12 nM, 13 nM, 14 nM, 15 nM, 16 nM, 17 nM, 18 nM, 19 nM, 20 nM, 21 nM, 22 nM, 23 nM, 24 nM, 25 nM, 26 nM, 27 nM, 28 nM, 29 nM, 30 nM, 31 nM, 32 nM, 33 nM, 34 nM, 35 nM, 36 nM, 37 nM, 38 nM, 39 nM, 40 nM, 41 nM, 42 nM, 43 nM, 44 nM, 45 nM, 46 nM, 47 nM, 48 nM, 49 nM.

In one embodiment, at least one cytokine selected from IL-2, IL-7, IL-15 and TSLP can be added in the culture.

In one embodiment, IL-2 is used at a concentration ranging from 10 IU/ml to 1000 IU/ml. Within the scope of the invention, the expression "from 10 IU/ml to 1000 IU/ml" includes, without limitation, 15 IU/ml, 20 IU/ml, 25 IU/ml, 30 IU/ml, 35 IU/ml, 40 IU/ml, 45 IU/ml, 50 IU/ml, 55 IU/ml, 60 IU/ml, 65 IU/ml, 70 IU/ml, 75 IU/ml, 80 IU/ml, 85 IU/ml, 90 IU/ml, 95 IU/ml, 100 IU/ml, 150 IU/ml, 200 IU/ml, 250 IU/ml, 300 IU/ml, 350 IU/ml, 400 IU/ml, 450 IU/ml, 500 IU/ml, 550 IU/ml, 600 IU/ml, 650 IU/ml, 700 IU/ml, 750 IU/ml, 800 IU/ml, 850 IU/ml, 900 IU/ml, 950 IU/ml. In certain embodiments, IL-2 is used at a concentration ranging from 50 IU/ml to 250 IU/ml.

In one embodiment, IL-7 is used at a concentration ranging from 1 ng/ml to 100 ng/ml. Within the scope of the invention, the expression "from 1 ng/ml to 100 ng/ml" includes, without limitation, 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 45 ng/ml, 50 ng/ml, 55 ng/ml, 60 ng/ml, 65 ng/ml, 70 ng/ml, 75 ng/ml, 80 ng/ml, 85 ng/ml, 90 ng/ml, 95 ng/ml, 100 ng/ml.

In one embodiment, IL-15 is used at a concentration ranging from 1 ng/ml to 50 ng/ml. Within the scope of the invention, the expression "from 1 ng/ml to 50 ng/ml" includes, without limitation, 2 ng/ml, 3 ng/ml, 4 ng/ml, 5 ng/ml, 6 ng/ml, 7 ng/ml, 8 ng/ml, 9 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 45 ng/ml. In certain embodiments, IL-15 is used at a concentration ranging from 10 ng/ml to 30 ng/ml.

In one embodiment, TSLP is used at a concentration ranging from 1 ng/ml to 100 ng/ml. Within the scope of the invention, the expression "from 1 ng/ml to 100 ng/ml" includes, without limitation, 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 45 ng/ml, 50 ng/ml, 55 ng/ml, 60 ng/ml, 65 ng/ml, 70 ng/ml, 75 ng/ml, 80 ng/ml, 85 ng/ml, 90 ng/ml, 95 ng/ml, 100 ng/ml.

Examples of TET activators include but are not limited to vitamin C and NaHS hydrogen sulfide releasing agents (also known as H2S donors).

In one embodiment, vitamin C is used at a concentration ranging from 1 to 100 μg/ml.

In one embodiment, NaHS hydrogen sulfide releasing agent is used at a concentration ranging from 0.25 to 8 mM.

Examples of DNMT inhibitors include but are not limited to 2-(1,3-dioxo-1,2-dihydro-2H-isoindol-2-yl)-3-(1H-indol- 3-yl) propanoic acid (RG108), 5-aza-2`-deoxycytidine (decitabine or DAC) and 5-azacytidine (5AC).

In one embodiment, RG108 is used at a concentration ranging from 20 to 500 μM.

In one embodiment, DAC is used at a concentration ranging from 0.1 to 2 μM.

In one embodiment, 5AC is used at a concentration ranging from 0.1 to 10 μM.

In one embodiment neutralizing antibodies can be added to the culture to prevent the generation of other populations of regulatory T cells.

Examples of neutralizing antibodies include, but are not limited to, anti-IFNγ, anti-IL-4, and/or anti-IL12 antibodies.

Examples of anti-IFNγ antibodies include, but are not limited to, Affymetrix eBioscience (Ref 14-7318), R&D systems (Ref MAB285), Novus Biologicals (Ref AF-485-NA).

Examples of anti-IL-4 antibodies include, but are not limited to, R&D Systems (Ref MAB304, MAB204, or MAB204), Affymetrix eBioscience (Ref 14-7048), GeneTex (Ref GTX10755).

Examples of anti-IL-12 antibodies include, but are not limited to, Affymetrix eBioscience (Ref 16-7129 or 16-8126), Biolegend (Ref 508803), R&D systems (Ref MAB219, AF-219, or AB-219).

In one embodiment, the culture medium used in the culture of the invention comprises (i) one or more pH buffering system(s); (ii) inorganic salt(s); (iii) trace element(s); (vi) hormone(s); (vii) carbon/energy source(s).

Examples of inorganic salts include, but are not limited to, calcium bromide, calcium chloride, calcium phosphate, calcium nitrate, calcium nitrite, calcium sulphate, magnesium bromide, magnesium chloride, magnesium sulphate, potassium bicarbonate, potassium bromide, potassium chloride, potassium dihydrogen phosphate, potassium disulphate, di- potassium hydrogen phosphate, potassium nitrate, potassium nitrite, potassium sulphite, potassium sulphate, sodium bicarbonate, sodium bromide, sodium chloride, sodium disulphate, sodium hydrogen carbonate, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium sulphate and a mix thereof.

Examples of Trace elements include, but are not limited to cobalt (Co), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), zinc (Zn) and the salts thereof.

Examples of free amino acids include, but are not limited to, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, L-tryptophan, L-tyrosine, L-valine and a mix thereof.

Examples of vitamins include, but are not limited to, biotin (vitamin H); D-calcium-pantothenate; choline chloride; folic acid (vitamin B9); myo-inositol; nicotinamide; pyridoxal (vitamin B6); riboflavin (vitamin B2); thiamine (vitamin B1); cobalamin (vitamin B12); acid ascorbic; α-tocopherol (vitamin E) and a mix thereof.

Examples of carbon/energy sources include, but are not limited to D-glucose; pyruvate; lactate; ATP; creatine; creatine phosphate; and a mix thereof.

In one embodiment, the culture medium is a commercially available cell culture medium, in particular selected in a group comprising the IMDM (Iscove's Modified Dulbecco's Medium) from GIBCO® or the RPMI 1640 medium from GIBCO®.

In another embodiment, the culture medium is a serum-free culture medium such as the AIM-V medium from GIBCO®, the X-VIVO 10, 15 and 20 media from LONZA.

In another embodiment, the culture medium can be further supplemented with additional compound(s), in particular selected in a group comprising foetal bovine serum, pooled human AB serum, cytokines and growth factors; antibiotic(s), in particular selected in a group comprising penicillin, streptomycin and a mix thereof.

In one embodiment, the culture medium is IMDM.

In some particular embodiments, the culture medium comprises IMDM cell culture medium; from 1% (w/w) to 5% (w/w) of foetal bovine serum; from 10 IU/ml to 200 IU/ml of penicillin; from 10 IU/ml to 200 IU/ml of streptomycin; from 0.1 mM to 10 mM of a mixture of non-essential amino acids, in particular amino acids selected in a group comprising alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine; from 0.5 mM to 10 mM of glutamine from 10 mM to 25 mM of HEPES pH 7.6-7.8.

In one embodiment, the medium is a nTreg polarizing medium. The inventors define a "nTreg polarizing medium" as a medium such as RPMI medium comprising at least one cAMP activator as described hereabove, at least one TGFβ pathway activator as described here above and at least one mTor inhibitor as described hereabove. In a preferred embodiment, the "nTreg polarizing medium" refers to a RPMI medium comprising TGFβ, rapamycin and PGE2.

In another embodiment, the medium is an inflammatory medium. The inventors define an "inflammatory medium" as a medium such as IMDM comprising inflammatory cytokines such as for example IL-1β (10 ng/ml), IL-6 (30 ng/ml), IL-21 (50 ng/ml), IL-23 (30 ng/ml), IL-2 (100 UI/ml).

In one embodiment, the culture for generating the invariant $Foxp3^+$ regulatory T cells of the invention is performed during at least 5 days, at least 6 days, at least 7 days, at least 8 days. Within the scope of the invention, the expression "at least 5 days" includes, without limitation, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days.

In one embodiment, a portion of the culture medium is discarded once, twice, three times, four times or five times during the time course of the generation culture and replaced with the same volume of fresh culture medium. Within the scope of the invention the term "portion" is intended to mean at least 20% (v/v), at least 25% (v/v), at least 30% (v/v), at least 35% (v/v), at least 40% (v/v), at least 45% (v/v), at least 50% (v/v), at least 55% (v/v), at least 60% (v/v), at least 65% (v/v), at least 70% (v/v), at least 75% (v/v) of the volume of the culture medium. In certain embodiments, 40% (v/v) to 60% (v/v) of the volume of the culture medium of step a) is discarded. In certain embodiments, the volume that is discarded is replaced with an identical volume of fresh culture medium. Within the scope of the invention, the expression "fresh culture medium" refers to a culture medium that has not been in contact with any CD3+ T cells.

In one embodiment, the method for generating ex vivo invariant $Foxp3^+$ regulatory T cells comprises:

- culturing $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA+$ T cells, in the presence of ΔCD3 feeder cells and coated 6B11 mAb and the following agents: i) PGE2, ii) TGFβ, iii) rapamycin, optionally iv) IL-2 and/or IL-15, and optionally v) vitamin C for at least 5 days,
- thereby obtaining a population of invariant $Foxp3^+$ regulatory T cells ex vivo generated, preferably from invariant naïve ($CD45RA^+$) T cells.

In another embodiment, the method for generating ex vivo invariant $Foxp3^+$ regulatory T cells comprises:

- culturing $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA+$ T cells, in the presence of tolerogenic DCs expressing CD1d (incubation with AM580 during about 24h) and pulsed with aGalactosyleramide and in the presence of ΔCD3 feeder cells and the following agents: i) PGE2, ii) TGFβ, iii) rapamycin, optionally iv) IL-2 and/or IL-15, and optionally v) vitamin C, for at least 5 days,

thereby obtaining a population of invariant $Foxp3^+$ regulatory T cells ex vivo generated, preferably from invariant naïve ($CD45RA^+$) T cells.

The present invention also relates to an ex vivo method of generation and expansion of invariant $Foxp3^+$ regulatory T cells, comprising:

- generating the invariant $Foxp3^+$ regulatory T cells as described here above,
- expanding the invariant $Foxp3^+$ regulatory T cells generated by contacting them with an invariant T cell activator (preferably either ΔCD3 feeder cells and coated 6B11 mAb or tolerogenic DCs expressing CD1d (incubation with AM580 during about 24h) and pulsed with aGalactosyleramide in the presence of ΔCD3 feeder cells) in the presence of the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator (preferably PGE2), ii) a TGFβ (Transforming growth factor beta) pathway activator (preferably TGFβ), iii) a mTOR inhibitor (preferably rapamycin), optionally iv) at least one cytokine selected in the group of IL-2, IL-7, IL-15 and TSLP (preferably IL-2 and/or IL-15), and optionally v) at least one TET enzymes activator (preferably selected from vitamin C and a NaHS hydrogen sulfide releasing agent) and/or at least one DNMT inhibitor (such as, for example, RG108, DAC or 5AC), for at least 5 days,

- thereby obtaining an expanded population of invariant Foxp3$^+$ regulatory T cells.

In one embodiment, the invariant Foxp3$^+$ regulatory T cell population generated ex vivo is isolated by flow cytometry based on the following phenotype: CD3$^+$Vα24$^+$CD45RO$^+$Foxp3$^+$.

In one embodiment, the isolated invariant Foxp3$^+$ regulatory T cell population thus obtained is then expanded ex vivo by culturing these cells in the presence of a polyclonal T cell activator. Examples of polyclonal T cell activator are listed hereinabove. Alternatively, other examples of polyclonal T cell activators that may be used during expansion include, but are not limited to, mitogen such as PMA/ionomycin, super-antigen, anti-CD3 antibody… Preferably, the anti-CD3 monoclonal antibody is coated. In one embodiment, the polyclonal T cell activator can be used in the presence of feeder cells as described here above.

In another embodiment, the isolated invariant Foxp3$^+$ regulatory T cell population thus obtained is then expanded ex vivo by culturing these cells in the presence of antigen-specific invariant T cell activator as described here above. In one embodiment, the antigen-specific T cell activator can be used in the presence of feeder cells as described here above.

In one embodiment, the culture for expanding the ex vivo generated invariant Foxp3$^+$ regulatory T cells of the invention is performed during at least 5 days, at least 6 days, at least 7 days, at least 8 days. Within the scope of the invention, the expression “at least 5 days” includes, without limitation, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days.

In one embodiment, a portion of the culture medium is discarded once, twice, three times, four times or five times during the time course of the generation culture and replaced with the same volume of fresh culture medium. Within the scope of the invention the term “portion” is intended to mean at least 20% (v/v), at least 25% (v/v), at least 30% (v/v), at least 35% (v/v), at least 40% (v/v), at least 45% (v/v), at least 50% (v/v), at least 55% (v/v), at least 60% (v/v), at least 65% (v/v), at least 70% (v/v), at least 75% (v/v) of the volume of the culture medium. In certain embodiments, 40% (v/v) to 60% (v/v) of the

volume of the culture medium of step a) is discarded. In certain embodiments, the volume that is discarded is replaced with an identical volume of fresh culture medium. Within the scope of the invention, the expression "fresh culture medium" refers to a culture medium that has not been in contact with any CD3+ T cells.

In one embodiment, invariant $Foxp3^+$ regulatory T cells are generated ex vivo by culturing $CD3^+V\alpha24^+CD45RA^+$ T cells obtained from PBMCs by flow cytometry (5.10^3 cells/ml) in the presence of autologous ΔCD3 feeder cells (125 10^5 cells/ml) and coated 6B11 mAb (2 μg/ml) in the presence of PGE2 (1 μM), TGFβ (5 ng/ml), Rapamycin (10 nM) and IL-2 (100 UI/ml) in IMDM-5. On day 1, IL-2 (100 UI/ml) and IL-15 (10 ng/ml) are added to the culture. Every 3 days, half of the medium volume is discarded and replaced by fresh medium comprising PGE2 (50 nM), TGFβ (5 ng/ml), Rapamycin (1 nM), IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Once cells begin to expand, they can be split every 2 or 3 days and cultured in the presence of ΔCD3 feeder cells and coated 6B11 mAb every 9 days in a medium comprising PGE2 (1 μM), TGFβ (5 ng/ml), Rapamycin (10 nM) and IL-2 (100 UI/ml).

In another embodiment, invariant $Foxp3^+$ regulatory T cells are generated ex vivo by culturing $CD3^+V\alpha24^+CD45RA^+$ T cells (5.10^3 cells/ml) obtained from PBMCs by flow cytometry (5.10^3 cells/ml) in the presence of tolerogenic DCs expressing CD1d (incubation with AM580 during about 24h) and pulsed with αGalactosyleramide and in the presence of ΔCD3 feeder cells (125 10^5 cells/ml), PGE2 (1 μM), TGFβ (5 ng/ml), Rapamycin (10 nM) and IL-2 (100 UI/ml) in IMDM-5. On day 1, IL-2 (100 UI/ml), IL-15 (10 ng/ml) and TGFβ (5 ng/ml), are added to the culture. Every 3 days, half of the medium volume is discarded and replaced by fresh medium comprising PGE2 (50 nM), TGFβ (5 ng/ml), Rapamycin (1 nM), IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Once cells begin to expand, they can be split every 2 or 3 days and restimulated every 9 days with tolerogenic DCs pulsed with αGalactosylceramide in the presence of ΔCD3 feeder cells and PGE2 (1 μM), TGFβ (5 ng/ml), Rapamycin (10 nM) and IL-2 (100 UI/ml).

In this embodiment, tolerogenic DCs were obtained by culturing $CD14^+$ monocytes isolated from PBMCs in the presence of AIMV supplemented with GMCSF (100 ng/ml),

IL-4 (10 ng/ml) and AM580 (100 nM). At day 3 and 6, the medium is discarded and replaced by fresh medium comprising GM-CSF and IL-4. On day 6, the tolerogenic DCs are pulsed for 24 hours in the presence of αGalactosylceramide (100 ng/ml) and AM580 (100 nM).

The present invention also relates to invariant $Foxp3^+$ regulatory T cells obtainable by the ex vivo generation method as described here above.

The present invention also relates to invariant $Foxp3^+$ regulatory T cells obtainable by the ex vivo generation and expansion method as described here above.

In one embodiment, the population of invariant $Foxp3^+$ regulatory T cells obtained by the generation and expansion method of the invention comprises at least 10^6, 10^7, 10^8, 10^9, 10^{10} cells.

In one embodiment, the population of invariant $Foxp3^+$ regulatory T cells obtained by the generation and expansion method of the invention has a phenotype similar to the phenotype of isolated invariant $Foxp3^+$ regulatory T cells as described here above.

In another embodiment, the population of invariant $Foxp3^+$ regulatory T cells obtained by the generation and expansion method of the invention has the property to remain functionally stable when placed in inflammatory condition.

As used herein, "functionally stable" refers to no secretion or a low secretion of IL-17, i.e. inferior to 200 ng/ml, 100 ng/ml, 50 ng/ml and still capable of suppressive capacity, i.e. inhibiting proliferation of conventional T cells as shown in the Examples.

As used herein, "inflammatory condition" refers to a medium enriched in aromatic acid, preferably in tryptophan, such as for example IMDM, comprising inflammatory cytokines such as for example IL-1β (10 ng/ml), IL-6 (30 ng/ml), IL-21 (50 ng/ml), IL-23 (30 ng/ml), IL-2 (100 UI/ml). A method for determining if a population of regulatory T cells remains functionally stable in inflammatory condition comprises culturing the regulatory T cells in the inflammatory condition medium as described here above in the presence of anti-CD3 (4 μg/ml), preferably coated, and anti-CD28 (4 μg/ml), preferably in a soluble form. After 36h to 72h of culture, the presence of IL-17 in the culture supernatant is measured. The recognition of IL-17 in the culture supernatant may be carried out by conventional methods known in the art such as, for example, a sandwich ELISA anti-IL-

17. Briefly, after coated the plate with a capture anti-IL-17 antibody, the culture supernatant is added to each well with a dilution series. After incubation, a detection anti-IL-17 antibody is added to each well. The ELISA is developed by any colorimetric means known in the art such as, for example, using detection antibody labelled with biotin, a poly-streptavidin HRP amplification system and an o-phenylenediamine dihydrochloride substrate solution. An IL-17 level inferior to 200 ng/ml, 100 ng/ml, 50 ng/ml corresponds to no secretion or low secretion of IL-17.

Without wishing to be bound to a theory, the inventors state that the stroma of malignant tumor cells comprises TILs (Tumor-infiltrating lymphocytes) that are highly enriched in regulatory T cells and that exert an immune suppressive activity, in particular on NK cells, which likely accounts for the local cancer immune escape. The inactivated invariant $Foxp3^+$ regulatory T cells may represent an antigenic target to induce an immune response directed against the invariant $Foxp3^+$ regulatory T cells present in the TILs, thereby preventing their immune suppressive activity and allowing the cytotoxic activity of effector cells such as NK cells against the tumor cells. The inventors thus suggest using a vaccine composition comprising inactivated invariant $Foxp3^+$ regulatory T cells as active principle.

One object of the invention is an immunogenic product comprising inactivated invariant $Foxp3^+$ regulatory T cells as described here above.

In one embodiment, the immunogenic product comprises, consists essentially of or consists of inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$, as described hereinabove.

As used herein, the term “consisting essentially of”, with reference to a pharmaceutical composition, vaccine, immunogenic product or medicament, means that the at least one invariant $Foxp3^+$ regulatory T cell population or antibody of the invention is the only one therapeutic agent or agent with a biologic activity within said pharmaceutical composition, vaccine, immunogenic product or medicament.

In one embodiment, the immunogenic product comprises, consists essentially of or consists of inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype

$CD3^+$ $V\alpha24^+$ $Foxp3^+$ generated and optionally expanded ex vivo by the method as described here above.

One object of the invention is an immunogenic product comprising blebs from invariant $Foxp3^+$ regulatory T cells as described here above.

In one embodiment, the immunogenic product comprises, consists essentially of or consists of blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$, as described hereinabove.

One object of the invention is an immunogenic product comprising immunogenic dendritic cells (immunogenic DC) loaded with blebs from invariant $Foxp3^+$ regulatory T cells as described here above.

In one embodiment, the immunogenic product comprises, consists essentially of or consists of immunogenic dendritic cells (immunogenic DC) loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$, as described hereinabove.

As used herein, "immunogenic DCs" refers to DCs capable to induce an immunogenic response. In one embodiment, immunogenic DCs have the following phenotype: $CD80^{high}$ $CD83^{high}$ $CD86^{high}$ HLA class I^{high} and HLA class II^{high} and secrete IL-10 and IL-12 with a ratio IL-12/IL-10 >1.

Methods for obtaining immunogenic DCs are well-known in the art. An exemplary method the generation of immunogenic DCs from $CD14^+$ monocytes. For example, $CD14^+$ monocytes are cultured in the presence of GM-CSF (about 20 ng/mL) and IFNα (about 10 ng/mL) for obtaining MoDC. Maturation of MoDC may be induced by a cytokine cocktail consisting of IL-1β (about 10 ng/ml), IL-6 (about 10 ng/ml) TNFα (about 200 U/ml), and PGE2 (about 10 ng/ml).

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of the immunogenic product as described here above and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of inactivated invariant $Foxp3^+$ regulatory T cells having the

following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ generated and expanded ex vivo by the method as described here above and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ as described here above and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of immunogenic DC loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ as described here above and at least one pharmaceutically acceptable excipient.

As used herein, the term “excipient” refers to any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by regulatory offices, such as, for example, FDA Office or EMA.

By "pharmaceutically acceptable" is meant that the ingredients of a pharmaceutical composition are compatible with each other and not deleterious to the subject to which it is administered. Examples of pharmaceutically acceptable excipient include, but are not limited to, water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like or combinations thereof.

Another object of the invention is a vaccine composition comprising, consisting essentially of or consisting of the immunogenic product as described here above.

Another object of the invention is a vaccine composition comprising, consisting essentially of or consisting of inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

Another object of the invention is a vaccine composition comprising, consisting essentially of or consisting of inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ generated and expanded ex vivo by the method as described here above.

Another object of the invention is a vaccine composition comprising, consisting essentially of or consisting of blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ as described here above.

Another object of the invention is a vaccine composition comprising, consisting essentially of or consisting of immunogenic DC loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ as described here above.

As used herein, "inactivated" T cells refers to T cells that are viable but has reduced or no effector function, i.e. have lost any pathogenic potential. Examples of cell surface markers of inactivated T cells include, but are not limited to, 7-Aminoactinomycin D (7-AAD), calreticulin and heat shock protein 90 (HSP-90). Therefore, inactivated T cells express 7-AAD and/or calreticulin and/or HSP-90. The inactivated invariant $Foxp3^+$ regulatory T cells of the invention have lost their suppressive activity but are still immunogenic. An example of T cell effector function assay is, but not limited to, T-cell proliferation assay. T-cell proliferation may be assessed on fixed T cells *versus* non-fixed T cells. Briefly, the T-cell proliferation assay aims at determining the percentage of living proliferating cells in fixed *versus* non-fixed T cells by flow cytometry. After staining the T cells with CFSE, anti-CD3 antibody and 7-AAD, the living proliferating cells are defined as the CFSE ^{low} fraction in gated $CD3^+$ $7\text{-}AAD^-$ cells.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells are inactivated by any method well-known in the art. Examples of method for inactivating cells include, but are not limited to, irradiation, preferably with about 2500 to 3000 rads and/or chemical inactivation such as exposure to cisplatin, carboplatin, oxaliplatin, mitomycine C or antracycline.

In one embodiment, the vaccine composition of the invention further comprises at least one adjuvant. Examples of adjuvant that can be used in the vaccine composition include,

but are not limited to, ISA51; emulsions such as CFA, MF59, montanide, AS03 and AF03; mineral salts such as alum, calcium phosphate, iron salt, zirconium salt, and AS04; TLR ligands such as TLR2 ligands (such as outer-surface protein A or OspA), TLR3 ligands (such as poly I:C), TLR4 ligands (such as MPL and GLA), TLR5 ligands, TLR7/8 ligands (such as imiquimod), TLR9 ligands (such as CpG ODN); polysacharrides such as chitin, chitosan, α-glucans, β-glucans, fructans, mannans, dextrans, lentinans, inulin-based adjuvants (such as gamma inulin); TLR9 and STING ligands such as K3 CpG and cGAMP. As used herein, "adjuvant" refers to an agent that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses.

In one embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells are specific of at least one non-peptide lipid antigens presented by CD1 molecules as described here above.

In another embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells are specific of at least one non-peptide lipid antigens presented by CD1 molecules that were present on apoptotic bodies of cancer cells.

In another embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells are specific of at least one non-peptide lipid antigens presented by CD1 molecules that were present on blebs of cancer cells.

In one embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells present in the immunogenic product, pharmaceutical composition or vaccine composition of the invention are human invariant Foxp3$^+$ regulatory T cells.

In one embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells present in the immunogenic product, pharmaceutical composition or vaccine composition of the invention are autologous invariant Foxp3$^+$ regulatory T cells.

In one embodiment, the inactivated invariant Foxp3$^+$ regulatory T cells present in the immunogenic product, pharmaceutical composition or vaccine composition of the invention are allogenic invariant Foxp3$^+$ regulatory T cells.

In another embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention may be personalized for a patient. As used herein, a "personalized" immunogenic product or vaccine composition refers to the use of invariant Foxp3$^+$ regulatory T cells generated and expanded ex vivo with at least one

patient specific epitope. In this embodiment, the invariant Foxp3$^+$ regulatory T cells to be used as immunogenic product or in the vaccine composition are generated and expanded ex vivo in the presence of apoptotic bodies of cancer cells or of blebs obtained from the patient, thereby providing at least one patient specific epitope.

In one embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention comprises, consists essentially of or consists of inactivated invariant Foxp3$^+$ regulatory T cells as active principle.

In one embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention comprises, consists essentially of or consists of at least 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} inactivated invariant Foxp3$^+$ regulatory T cells as active principle.

In one embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention comprises, consists essentially of or consists of about 10^4, $5x10^4$, 10^5, $5x10^5$, 10^6, $5x10^6$, 10^7, $5x10^7$, 10^8, $5x10^8$, 10^9, $5x10^9$, 10^{10}, inactivated invariant Foxp3$^+$ regulatory T cells as active principle.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells, the inactivated invariant Foxp3$^+$ regulatory T cells, the immunogenic product, the pharmaceutical composition or the vaccine composition of the invention are/is frozen.

In one embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention may be administrated to the subject by subcutaneaous, intramuscular, intraperitoneal or intravenous injection, or directly into the tumor.

In one embodiment, the immunogenic product, pharmaceutical composition or vaccine composition of the invention may be administrated to the subject at least once, twice, 3 times, 4 times, 5 times in a year. Example of regime of administration includes, but is not limited to, administration of the immunogenic product or vaccine composition at day 0, 4 weeks after day 0, 8 weeks after day 0, 12 weeks after day 0 and 24 weeks after day 0.

Another object of the invention is a method for treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of inactivated

invariant Foxp3$^+$ regulatory T cells or of the immunogenic product, pharmaceutical composition or vaccine composition of the invention as described here above.

Another object of the invention is a method for eliciting an immune response against invariant Foxp3$^+$ regulatory T cells present in the TILs of a subject affected with a cancer, comprising administering to the subject a therapeutically effective amount of inactivated invariant Foxp3$^+$ regulatory T cells or of the immunogenic product, pharmaceutical composition or vaccine composition of the invention as described here above.

Examples of cancer that can be treated with the immunogenic product, pharmaceutical composition or vaccine composition of the invention include, but are not limited to, adrenocortical carcinoma, anal cancer, bladder cancer, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal, pineal tumors, hypothalamic glioma, breast cancer, carcinoid tumor, carcinoma, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, extrahepatic bile duct cancer, ewings family of tumors (pnet), extracranial germ cell tumor, eye cancer, intraocular melanoma, gallbladder cancer, gastric cancer, germ cell tumor, extragonadal, gestational trophoblastic tumor, head and neck cancer, hypopharyngeal cancer, islet cell carcinoma, laryngeal cancer, leukemia, acute lymphoblastic leukemia, oral cavity cancer, liver cancer, lung cancer, small cell lymphoma, AIDS-related, lymphoma, central nervous system (primary) lymphoma, cutaneous T-cell lymphoma, Hodgkin's disease, non-Hodgkin's disease, malignant mesothelioma, melanoma, merkel cell carcinoma, metastatic squamous carcinoma, multiple myeloma, plasma cell neoplasms, mycosis fungoides, myelodysplastic syndrome, myeloproliferative disorders, nasopharyngeal cancer, neuroblastoma, oropharyngeal cancer, osteosarcoma, ovarian epithelial cancer, ovarian germ cell tumor, ovarian low malignant potential tumor, pancreatic cancer, exocrine, pancreatic cancer, paranasal sinus and nasal cavity cancer, parathyroid cancer, pheochromocytoma cancer, pituitary cancer, plasma cell neoplasm, rhabdomyosarcoma, rectal cancer, renal cell cancer, salivary gland cancer, Sezary syndrome, Kaposi's sarcoma, small intestine cancer, soft tissue sarcoma, thymoma, malignant thyroid cancer, urethral cancer, uterine cancer, sarcoma, unusual cancer of childhood, vaginal cancer, vulvar cancer or Wilms' tumor, benign conditions associated with chemotherapy treatments, such as, lupus, rheumatoid arthritis and skin diseases.

In one embodiment, the cancer that can be treated with the immunogenic product, pharmaceutical composition or vaccine composition of the invention include, but is not limited to, breast cancer, prostate cancer, ovarian cancer and glioblastoma.

Another object of the invention is a method for preparing the immunogenic product of the invention, comprising:

- providing a biological sample, preferably a blood sample, from the subject to be treated and optionally a tumor sample, from the subject to be treated,
- generating and expanding ex vivo as described here above invariant $Foxp3^+$ regulatory T cells from the $CD3^+V\alpha24^+$ T cells, preferably $CD3^+V\alpha24^+CD45RA+$ T cells, isolated from the biological sample,
- inactivating the invariant $Foxp3^+$ regulatory T cells obtained in the previous step,
- thereby obtaining the immunogenic product of the invention.

In a preferred embodiment, the generation and expansion steps are carried out in the presence of tolerogenic dendritic cells (DCs) expressing CD1, i.e. CD1a, CD1b, CD1c and/or CD1d, and pulsed with apoptotic tumor bodies or blebs obtained from the tumor sample of the subject.

Another object of the invention is a method for treating cancer in a subject in need thereof, comprising administrating to the subject the immunogenic product, pharmaceutical composition or vaccine composition of the invention.

Another object of the invention is a method for treating cancer in a subject in need thereof, comprising:

- preparing an immunogenic product as described here above,
- optionally preparing a pharmaceutical composition or a vaccine composition comprising the immunogenic product,
- optionally submitting the subject to plasmapheresis,
- administrating to the subject the immunogenic product, pharmaceutical composition or vaccine composition of the invention.

Without wishing to be bound by a theory, the inventors suggest that the invariant $Foxp3^+$ regulatory T cells of the invention, which are committed to exert immune suppressive

function, may be capable of inhibiting autoreactive pathogenic immune effector cells including CD4+, CD8+, B cells or innate NK cells, which, in turn, are no longer able to exert their cytotoxic properties towards the self-cells.

One object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of the invariant $Foxp3^+$ regulatory T cells or invariant $Foxp3^+$ regulatory T cell population as described here above and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ and at least one pharmaceutically acceptable excipient.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of invariant $Foxp3^+$ regulatory T cells having the following phenotype $CD3^+$ $V\alpha24^+$ $Foxp3^+$ generated and expanded ex vivo by the method as described here above and at least one pharmaceutically acceptable excipient.

One object of the invention is the invariant $Foxp3^+$ regulatory T cells or the invariant $Foxp3^+$ regulatory T cell population or the pharmaceutical composition as described here above for use in adoptive therapy.

Another object of the invention is the invariant $Foxp3^+$ regulatory T cells or the invariant $Foxp3^+$ regulatory T cell population or the pharmaceutical composition as described here above for use in treating inflammatory or autoimmune diseases.

Examples of inflammatory or autoimmune diseases include, but are not limited to, acute disseminated encephalomyelitis, acute necrotizing haemorrhagic leukoencephalitis, Addison's disease, agammaglobulinaemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome, autoimmune angioedema, autoimmune aplastic anaemia, autoimmune dysautonomia, autoimmune haemolytic anaemia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease, autoimmune myocarditis, autoimmune oophoritis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura, autoimmune thyroid disease, autoimmune urticaria, axonal

and neuronal neuropathies, Balo disease, Behcet's disease, bullous pemphigoid, cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy, chronic recurrent multifocal osteomyelitis, 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, discoid lupus, Dressler's syndrome, endometriosis, eosinophilic esophagitis, eosinophilic fasciitis, erythema nodosum, experimental allergic encephalomyelitis, Evans syndrome, fibromyalgia, fibrosing alveolitis, giant cell arteritis, giant cell myocarditis, glomerulonephritis, Goodpasture's syndrome, granulomatosis with Polyangiitis (Wegener's syndrome), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, haemolytic anaemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, idiopathic pulmonary fibrosis, idiopathic thrombocytopenic purpura, IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, interstitial cystitis, juvenile arthritis, juvenile diabetes (Type 1 diabetes), juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease, lupus, Lyme chronic disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease, Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica, neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, paediatric autoimmune neuropsychiatric disorders associated with Streptococcus, paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria, Parry Romberg syndrome, Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anaemia, POEMS syndrome, polyarteritis nodosa, type I, II, and III autoimmune polyglandular syndromes, polymyalgia rheumatic, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, psoriatic arthritis, pyoderma gangrenosum, pure red cell aplasia, Raynauds phenomenon, reactive arthritis, 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 and testicular autoimmunity, Stiff person syndrome, subacute bacterial endocarditis, Susac's syndrome, sympathetic ophthalmia, systemic lupus erythematosus, Takayasu's arteritis, temporal arteritis/Giant cell arteritis, thrombocytopenic purpura, Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, ulcerative colitis, undifferentiated connective tissue disease, uveitis, vasculitis, vesiculobullous dermatosis and vitiligo.

Examples of inflammatory or autoimmune diseases include, but are not limited to, rheumatoid arthritis, type 1 diabetes, and multiple sclerosis.

Another object of the invention is the invariant $Foxp3^+$ regulatory T cells or the invariant $Foxp3^+$ regulatory T cell population or the pharmaceutical composition as described here above for use in preventing transplant rejection or graft versus host disease (GVHD).

In one embodiment, the invariant $Foxp3^+$ regulatory T cells are specific of at least one non-peptide lipid antigens presented by CD1 molecules as described here above.

In another embodiment, the invariant $Foxp3^+$ regulatory T cells are specific of at least one non-peptide lipid antigens presented by CD1 molecules that were present in tissue lysates.

Examples of tissue lysate include, but are not limited to, synovial liquid, Langerhans islets lysate, beta islets lysate…

In one embodiment, the pharmaceutical composition of the invention comprises, consists essentially of or consists of at least 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} invariant $Foxp3^+$ regulatory T cells as active principle.

In one embodiment, the pharmaceutical composition of the invention comprises, consists essentially of or consists of about 10^4, $5x10^4$, 10^5, $5x10^5$, 10^6, $5x10^6$, 10^7, $5x10^7$, 10^8, $5x10^8$, 10^9, $5x10^9$, 10^{10} invariant $Foxp3^+$ regulatory T cells as active principle.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells, the invariant $Foxp3^+$ regulatory T cell population or the pharmaceutical the invention are/is frozen.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells present in the pharmaceutical composition of the invention are human invariant $Foxp3^+$ regulatory T cells.

In one embodiment, the invariant $Foxp3^+$ regulatory T cells present in the pharmaceutical composition of the invention are autologous invariant $Foxp3^+$ regulatory T cells.

In one embodiment, the invariant Foxp3$^+$ regulatory T cells present in the pharmaceutical composition of the invention are allogenic invariant Foxp3$^+$ regulatory T cells.

In one embodiment, the pharmaceutical composition of the invention may be administrated to the subject by subcutaneaous, intramuscular, intraperitoneal or intravenous injection.

In one embodiment, the pharmaceutical composition of the invention may be administrated to the subject at least once, twice, 3 times, 4 times, 5 times per week.

In another embodiment, the pharmaceutical composition of the invention may be administrated to the subject at least once, twice, 3 times, 4 times, 5 times per month.

In another embodiment, the pharmaceutical composition of the invention may be administrated to the subject at least once, twice, 3 times, 4 times, 5 times per 3 months.

Another object of the invention is a method for treating inflammatory or autoimmune diseases in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the invariant Foxp3$^+$ regulatory T cells or the invariant Foxp3$^+$ regulatory T cell population or the pharmaceutical composition as described here above.

It has been shown in the art that T cell vaccination induces regulatory networks that specifically suppress the immunogenic T cells by activating T cells specific for a clonotype-specific determinant (anti-idiotypic response). In addition, anti-ergotypic responses directed at activation markers (corresponding to the ergotope) may also partially account for the suppression of the regulatory T cell population targeted.

Another object of the invention is an antibody recognizing the TCR (T cell receptor) of the invariant Foxp3$^+$ regulatory T cells of the invention.

In one embodiment, the antibody recognizing the TCR of the invariant Foxp3$^+$ regulatory T cells of the invention recognizes at least one of the CDR1, CDR2 and CDR3 (complementary determining region 1, 2 and 3) of the TCR.

In another embodiment, the antibody recognizing the TCR of the invariant Foxp3$^+$ regulatory T cells of the invention recognizes the CDR3 of the TCR.

Another object of the invention is a pharmaceutical composition comprising, consisting essentially of or consisting of said antibody and at least one pharmaceutically acceptable excipient.

Another object of the invention is the use of said antibody for treating cancer in a subject in need thereof.

In one embodiment, the antibodies directed against the invariant $Foxp3^+$ regulatory T cells of the invention consist of antibodies produced following immunization of a mammal, including a human, with the immunogenic composition as described here above.

In another embodiment, the antibodies may also be obtained by cloning the relevant DNA material encoding them, starting for example from B cells obtained from the said mammal, including from the said human.

In another embodiment, the antibodies may also be obtained by sequencing the amino acid sequences of the antibodies collected from the said mammal, including from the said human, and then synthesize a DNA molecule encoding the antibody or a portion thereof comprising the CDR thereof, for producing relevant recombinant antibodies directed against the invariant $Foxp3^+$ regulatory T cells of the invention.

Preparing antibodies directed against the invariant $Foxp3^+$ regulatory T cells of the invention by immunization with the immunogenic composition of the invention may be easily performed by a skilled in the art, using the common technical knowledge from the state in the art.

Alternatively the antibodies directed against the invariant $Foxp3^+$ regulatory T cells of the invention may be obtained after immortalization of the human B lymphocytes producing them; their cDNA can also be cloned and used further for producing them or their derivatives through recombinant DAN technology.

The term "antibody" herein is used to refer to a molecule having a useful antigen binding specificity. Those skilled in the art will readily appreciate that this term may also cover polypeptides which are fragments of or derivatives of antibodies yet which can show the same or a closely similar functionality. Such antibody fragments or derivatives are intended to be encompassed by the term antibody as used herein. By "antibody" or "antibody molecule" for the purpose of passive immunotherapy, it is intended herein not only whole immunoglobulin molecules but also fragments thereof, such as Fab, F(ab')2,

Fv and other fragments thereof that retain the capacity to bind and inactivate the invariant $Foxp3^+$ regulatory T cells. Similarly, the term antibody includes genetically engineered derivatives of antibodies such as single chain Fv molecules (scFv) and domain antibodies (dAbs).

In some embodiments, an antibody directed against the invariant $Foxp3^+$ regulatory T cells of the invention consists of a polyclonal antibody.

In some embodiments, an antibody directed against the invariant $Foxp3^+$ regulatory T cells of the invention consists of a monoclonal antibody.

The term "monoclonal antibody" is used herein to encompass any isolated Ab's such as conventional monoclonal antibody hybridomas, but also to encompass isolated monospecific antibodies produced by any cell, such as for example a sample of identical human immunoglobulins expressed in a mammalian cell line.

The variable heavy (VH) and variable light (VL) domains of the antibody are involved in antigen recognition, a fact first recognized by early protease digestion experiments. Further confirmation was found by "humanization" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison et al. (1984) Proc. Natl. Acad. Sci. USA 81, 6851-6855). That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better et al (1988) Science 240, 1041); Fv molecules (Skerra et al (1988) Science 240, 1038); single-chain Fv (ScFv) molecules where the V.sub.H and V.sub.L partner domains are linked via a flexible oligopeptide (Bird et al (1988) Science 242, 423; Huston et al (1988) Proc. Natl. Acad. Sci. USA 85, 5879) and single domain antibodies (dabs) comprising isolated V domains (Ward et al (1989) Nature 341, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991, Nature 349, 293-299).

The term "ScFv molecules" encompasses molecules wherein the VH and VL partner domains are linked via a flexible oligopeptide. Engineered antibodies, such as ScFv antibodies, can be made using the techniques and approaches described in J. Huston et al,

(1988) "Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single chain Fv analogue produced in E. coli", Proc. Natl. Acad. Sci. USA, 85, pp. 5879-5883, and in A. Pluckthun, (1991) "Antibody engineering; Advances from use of E. coli expression systems", Bio/technology 9 (6): 545-51, incorporated herein by reference.

Suitable monoclonal antibodies which are reactive as described herein may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies; A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Application", S G R Hurrell (CRC Press, 1982).

A further embodiment encompasses humanized antibodies where the regions of the murine antibody that contacted the antigen, the Complementarity Determining Regions (CDRs) were transferred to a human antibody framework. Such antibodies are almost completely human and seldom cause any harmful antibody responses when administered to patients. Several chimeric or humanized antibodies have been registered as therapeutic drugs and are now widely used within various indications (Borrebaeck & Carlsson, 2001, Curr. Opin. Pharmacol. 1: 404-408).

It is preferred if the antibody is a humanized antibody. Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies. Humanized antibodies can be made using the techniques and approaches described in Verhoeyen et al (1988) Science, 239, 1534-1536, and in Kettleborough et al, (1991) Protein Engineering, 14 (7), 773-783.

In another embodiment, antibodies also encompass completely human antibodies, which may be produced using recombinant technologies. Typically, large libraries comprising billions of different antibodies are used. In contrast to the previous technologies employing chimerization or humanization of e.g. murine antibodies this technology does not rely on immunization of animals to generate the specific antibody. Instead the recombinant libraries comprise a huge number of pre-made antibody variants wherein it is likely that the library will have at least one antibody specific for any antigen.

The frequency of administration may be determined clinically by following the decline of antibody titers in the serum of patients over time, but in any event may be at a frequency

of 1 to 52 times per year, and most preferably between 1 and 12 times per year. Quantities of antibody may vary according to the severity of the disease, or half-life of the antibody in the serum, but preferably will be in the range of 1 to 10 mg/kg of patient body weight, and preferably within the range of 1 to 5 mg/kg of patient, and most preferably 1 to 2 mg/kg of patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Detection of FOXP3 expression in a $CD3^+$ invTCR $V\alpha24^+$ CD1-restricted T cell subset within peripheral blood. (A-B) Representative histograms showing Foxp3 expression (%) and fluorescence intensity (MFI) in (A) $CD3^+$ TCR αβ MHCII-restricted T cell subset and in (B) $CD3^+$ invTCR $V\alpha24^+$ CD1-restricted T cell subset. (C) Phenotypic and functional regulatory capacity of both $Foxp3^+$ $CD3^+$ T cell subsets: frequency (%) and expression levels (MFI) of Treg-associated markers including, CD127, CD25 and CTLA-4 and cell-cell contact suppressive capacity as evaluated by the standard polyclonal cell-cell contact suppression assay.

Figure 2: $FOXP3^+$ $CD3^+$ TCR $V\alpha24^+$ CD1-restricted T cell subset do not display invariant NKT cell membrane phenotypic markers, including CD161 and CD56, while expressing Treg cell phenotypic hallmarks.

Figure 3: TCR Vβ diversity analysis in circulating $CD3^+$ TCR$V\alpha24^+$ $CD4^+$ expressing $FOXP3^+$. TCR Vβ repertoire was determined by seven-color flow cytometry antibodies directed against CD3, CD4, CD8, Vα24 and $Foxp3^+$ and 24 Vβ families. Distribution of the 24 Vβ segments in $CD3^+$ $V\alpha24^+$ $CD4^+$ $FOXP3^+$ cells is shown.

Figure 4: Different frequencies and phenotypic characteristics between $FOXP3^+$ and $FOXP3^-$ $CD3^+$ T cell populations, as defined by their variable TCR recognition in human peripheral blood (PBMCs) and in TIL isolated from breast tumor.

Figure 5: In vitro induction of tumor-Ag specific $CD3^+$ TCR $V\alpha24^+$ CD1-restricted T cells (invTreg) from stimulated naive $CD3^+$ TCR $V\alpha24^+$ T cells with different nTreg polarizing medium. Naive $CD3^+$ TCR $V\alpha24^+$ T cells were stimulated for 21 days with tumor - apoptotic breast tumor cell line pulsed autologous tDC as described in Fig 4 in

presence of IL-2 (100 IU/ml) and IL-15 (10 ng/ml). Where indicated, TGFβ, RAPA and PGE2 were added. (A) Overlay histogram displaying Foxp3 expression profiles of each of the generated invTreg. (B) Frequency and (C) expression level (evaluated by MFI) of Foxp3 in $CD3^+$ T cell culture. Dashed black line represents in (B) and (C) the frequency and the expression level of FOXP3 in naive Treg phenotypically defined by the expression a high level of CD45RA and CD25 and a low level of CD127.

Figure 6: Combination of TGFβ, RAPA and PGE2 induce the establishment and the expansion of tumor Antigen specific $FOXP3^+$ $CD3^+$ TCR $V\alpha24^+$ CD1-restricted T cell cells committed to exclusively exert regulatory activity, with an autologous MLR assay. $CD3^+$ TCR $V\alpha24^+$ $CD45RA^+$ T cells were stimulated with autologous tolerogenic DC pulsed with apoptotic breast tumor cell lines in presence of IL-2, IL-15 and nTreg polarizing medium. After 21 days of in vitro expansion in nTreg polarizing medium, suppressive capacity of ex vivo generated Tumor Ag-specific invariant $Foxp3^+$ Treg was evaluated in the presence of (A) a low or (B) high inflammatory medium. Fresh naïve Treg were used as control.

EXAMPLES

The present invention is further illustrated by the following examples.

Materials and Methods

Human Blood Sample. Blood samples from healthy individuals originated from Etablissement Français du Sang (EFS, Paris). Blood cells are collected using standard procedures.

Human tumor sample. Tumor tissue sample originated from patient with Luminal A and Luminal B Breast cancer (Institut Jean Godinot, Reims).

Cell Purification and Culture.

Peripheral blood mononuclear cells (PBMCs) are isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia). PBMCs are used either as fresh cells or

stored frozen in liquid nitrogen. T-cell subsets and T cell-depleted accessory cells (ΔCD3 cells) are isolated from either fresh or frozen PBMCs. T cell–depleted accessory cells (ΔCD3 cells) are isolated by negative selection from PBMCs by incubation with anti-CD3–coated Dynabeads (Dynal Biotech) and are irradiated at 3000 rad (referred to as ΔCD3-feeder).

$CD4^+$ T cells are negatively selected with a $CD4^+$ T-cell isolation kit (Miltenyi Biotec, yielding $CD4^+$ T-cell populations at a purity of 96–99%. Sub- sequently, selected $CD4^+$ T cells are labeled with anti-CD4 (13B8.2)-FITC (Beckman Coulter), anti-CD25(4E3)-APC (Miltenyi Biotec), and anti-CD127(R34.34)-PE (Beckman Coulter) before being sorted into $CD4^+CD127^{-/lo}CD25^{high}$ (pTregs) and $CD4^+CD127^+CD25^{neg/dim}$ [conventional helper CD4 T cells (Tconv)] subpopulations using a FACSAria III Cell Sorter (Becton Dickinson).

$CD14^+$ monocytes are isolated from PBMCs by positive selection using a MACS system.

$CD3^+$ $CD4^+$ $CD127^+$ $CD45RA^+$ $CD25^-$ $TCR\alpha\beta^+$ MHCII restricted (naive conventional $CD4^+$ T cells) are isolated from PBMCs after magnetic enrichment (MACS system: CD4 microbeads) and FACs sorting. Before the sorting step, enriched $CD3^+$ $CD4^+$ T cells are stained with anti-CD4 (13B8.2)-FITC (Beckman Coulter), anti-CD25(4E3)-APC (Miltenyi Biotec), and anti-CD127(R34.34)-PE (Beckman Coulter), anti-TCR αβ-BV421 (IP26) (Biolegend).

$CD3^+$ $CD45RA^+$ invTCR $V\alpha24^+$ CD1-restricted T cells are isolated from PBMCs after magnetic enrichment (MACS system: anti-iNKT microbeads and FACS sorting. Before the sorting step, enriched $CD3^+$ invTCR $V\alpha24^+$T cells are stained with anti-CD3 (UCHT-1) V450 anti-invariant TCR Vα24-JαQ (6B11)-PE (inv TCR Vα24-JαQ (Becton Dickinson) and anti-CD45RA (T6D11)-FITC (Miltenyi Biotec).

$CD3^+$ $CD45RA^+$ $CD27^+$ $TCR\gamma\delta^+$ unrestricted T cells are isolated from PBMCs after magnetic enrichment (MACS system: $TCR\gamma\delta^+$ T cell isolation kit) and FACS sorting. Before the sorting step, enriched $CD3^+$ $TCR\gamma\delta^+$ T cells are stained with anti-CD3 (UCHT-1) V450, anti- TCR pan$\gamma\delta^+$ PE (IMMU510) (Beckman Coulter), anti-CD27-

APC efluor 780 (O323) (ebioscience) and anti-CD45RA (T6D11)-FITC (Miltenyi Biotec).

T cell subsets are cultured either in IMDM supplemented with 5% SVF, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES (IMDM-5 media) in hypoxia 2%.

Breast cancer cell line and culture. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (USA). Cells are maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS). MCF-7 cells are treated with 5 μg/ml Doxorubicin for 24 h or by γ irradiation (20 Gy). Extent of apoptosis is monitored by flow cytometric analysis (FACS). Cells are extensively washed prior to feeding DCs.

TIL isolation. Tumor tissue was minced with scalpels and enzymatically digested by overnight incubation in collagenase Type IV (2 mg/ mL, Roche Diagnostic GmbH) in DMEM High Glucose medium supplemented with 2 mM glutamine (Gibco), 50 mg/mL gentamycin and 0.25 % Human Serum Albumin, at 37 °C on a rotary shaker.

Ex vivo generation of polyclonal functionally committed FOXP3 expressing regulatory T cells.

Ex vivo generation of polyclonal functionally committed FOXP3 expressing CD3+ TCRαβ+ MHCII restricted T cells: On day 0, T cells are seeded at 2,5 × 10^5/well in 48-well plates and stimulated with plate-bound anti-CD3 mAb (4 μg/ml) in the presence of ΔCD3-feeder (1 M). Cells are cultured in IMDM-5 media (IMDM supplemented with 5% SVF, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES) with PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM. On day 2, IL-2 (100IU/ml) are added to the culture. Every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml). On day 11, these $CD4^+$ T-cell lines were further expanded by restimulation with plate-bound anti-CD3 Abs (4μg/ml). The restimulations were performed in the presence of ΔCD3-feeder, PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM and IL-2 (100 UI/ml). Then every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2

(100 UI/ml). On day 20, the phenotype of the expanded $CD4^+$ T cells was assessed by flow cytometry. 75% of the stimulated naive conventional T cells that became $CD45RO^+$ express $FOXP3^+$.

Ex vivo generation of polyclonal functionally committed FOXP3 expressing invariant T cells: On day 0, T cells are seeded at 1×10^3/well in 96-well plates and stimulated with plate-bound anti-inv TCR Vα24-JαQ (6B11) mAb (2 μg/ml) in the presence of ΔCD3-feeder ($2.5x10^5$). Cells are cultured in IMDM-5 media with PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM, IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Every three days, IL-2 (100 UI/ml) and IL-15 (10 ng/ml) are added to the culture. On day 12, T cells are further expanded by restimulation with plate-bound anti- anti-inv TCR Vα24-JαQ (6B11) mAb (2 μg/ml) in the presence of ΔCD3-feeder, PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Then every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml) and IL-15 (10 ng/ml). On day 21, cells are analyzed by flow cytometry. 70% of the stimulated $CD3^+$ invTCR $Vα24^+$ RA^+ T cells that became $CD45RO^+$ express $Foxp3^+$.

Ex vivo generation of polyclonal functionally committed FOXP3 expressing TCRγδ⁺ T cells: On day 0, T cells are seeded at 1×10^3/well in 96-well plates and stimulated with plate-bound anti-TCRγδ mAb (2 μg/ml) in the presence of ΔCD3-feeder ($2.5X10^5$). Cells are cultured in IMDM-5 media (IMDM supplemented with 5% SVF, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES) with PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM, IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml) and IL-15 (10 ng/ml). On day 11, T cells were further expanded by restimulation with plate-bound anti-pan TCR γδ Abs (2μg/ml). The restimulations were performed in the presence of ΔCD3-feeder, PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM and IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Then every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml) and IL-15 (10 ng/ml). On day 21, cells are analyzed by flow cytometry. 65% of the stimulated $CD3^+$ $CD45RA^+$ $CD27^+$ $TCRγδ^+$ T cells that became $CD45RO^+$ express $Foxp3^+$.

Ex vivo generation of antigen specific functionally committed FOXP3 expressing T cells:

Ex vivo generation of antigen (Ovalbumun) specific functionally committed Foxp3 expressing CD3$^+$ TCRαβ$^+$ MHCII restricted T cells:

a) In vitro generation of ovalbumin- loaded Tolerogenic DC from CD14$^+$ monocytes (termed tolerogenic monocyte-derived DC (Tol-Mo-DC) : monocytes are cultured in 48-well flat-bottom plates containing 0,5 ml of AIMV per well supplemented with 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml human recombinant IL-4 for the generation of immature DC. At day 3, 500 µl of the medium containing cytokines was added. On day 6, Tol-Mo-DC are 1) removed from the wells, washed twice with IMDM-5 (IMDM supplemented with 5% SVF, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES, 2) added to wells of a 48-well plate at a concentration of 3 X 10^5 /ml in IMDM-5 and 3) pulsed in IMDM-5 with specific Ag (OVA).

b) Ex vivo generation and expansion of specific functionally committed FOXP3 expressing CD3$^+$ TCRαβ$^+$ MHCII restricted T cells: On day 0, ovalbumin pulsed tDC are 1) washed twice with IMDM-5 and 2) added to wells of a 48-well plate at a concentration of 3 X 10^5 /ml in IMDM-5 in the presence of 2 X 10^5 irradiated autologous feeders, PGE2 1 µM, and Rapa 10 nM. Purified naive conventional CD4$^+$ T cells (isolated from the previously frozen PBMC by FACS) are added to the pulsed tDC. On day 1, IL-2 (100IU/ml) and TGFβ (5ng/ml) are added to the coculture. Every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml (T cell cloning medium). On day 12, these T-cells are further expanded by restimulation with ova-pulsed tDC in the presence of ΔCD3-feeder, PGE2 1 µM, TGFβ 5 ng/ml, Rapa 10 nM, IL-2 (100 UI/ml). Once T cells begin to expand, they can be split every 2 to 3 days with T cell cloning medium and irradiated feeder. On day 21, cells are analyzed by flow cytometry. 85 % of the stimulated naive conventional CD4$^+$ T cells that

became CD45RO$^+$ express Foxp3$^+$. To confirm that the Ova-specific memory CD3$^+$ TCR$\alpha\beta^+$ MHCII restricted T cells are committed to exclusively exert regulatory activity, whatever culture condition of stimulation, after 21 days of expansion in nTreg polarizing medium, the ova-specific-pTreg are further cultured for 3 weeks either in nTreg polarizing medium (comprising the combination of IL-2, TGFβ, PGE2 and rapamycin) or TH-17 polarizing medium (IMDM medium containing IL-2 IL-1 IL-6, IL-21 IL-23 cytokines). The 21-day-expanded-Foxp3 expressing CD3$^+$ CD4$^+$ TCR$\alpha\beta^+$ MHCII restricted T cells are stimulated with plate-bound anti-CD3 mAb (4 µg/ml) in the presence of ΔCD3-feeder (1 M) in 48-well plates and every three days, half of the supernatant volume is discarded and replaced with fresh T cell cloning medium or TH-17 polarizing medium for 21 days.

Ex vivo generation of tumor-antigen specific functionally committed FOXP3 expressing CD3$^+$ invTCR Vα24$^+$ CD1d- restricted T cells:

a) In vitro generation of tumor-loaded Tolerogenic DC from CD14$^+$ monocytes (termed tolerogenic monocyte-derived DC (tDC): monocytes are cultured in 48-well flat-bottom plates containing 0,5 ml of AIMV per well supplemented with 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml human recombinant IL-4 and AM580 (100 nM) for the generation of immature DC expressing CD1d. At day 3, 500 µl of the medium containing cytokines are added. At day 5, a portion of tDCs are co-cultured with apoptotic MCF-7 cells at a DC/tumor cell ratio of 1:2 for 24h in AIMV with GM-CSF (100 ng/mL), IL-4 (10 ng/mL). Another portion of tDC are freezed at 2 x 10^6/per vial vial- in 90% FBS - 10% DMSO.

b) Ex vivo generation and expansion of tumor-antigen specific functionally committed Foxp3 expressing CD3$^+$ invTCR Vα24$^+$ CD1d- restricted T cells: On day 0, tumor-antigen pulsed tDC are 1) washed twice with IMDM-5 and 2) added to wells of a 48-well plate at a concentration of 3 X 10^5 /ml in IMDM-5 in the presence of 2 X 10^5 irradiated autologous feeders, PGE2 1 µM, and

Rapa 10 nM. Purified $CD3^+$ $CD45RA^+$ invTCR $V\alpha24^+$ CD1-restricted T cells (isolated from the previously frozen PBMC by FACS) are added to the pulsed tDC. On day 1, IL-2 (100IU/ml), IL-15 (10 ng/ml) and TGFβ (5ng/ml) are added to the coculture. Every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml) and IL-15 (10 ng/ml) (T cell cloning medium). On day 12, these T-cells are further expanded by restimulation with tumor Ag-pulsed tDC in the presence of ΔCD3-feeder, PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM, IL-2 (100 UI/ml) and IL-15 (10 ng/ml). Once T cells begin to expand, they can be split every 2 to 3 days with T cell cloning medium and irradiated feeder. On day 21, cells are analyzed by flow cytometry. 75 % of the stimulated $CD3^+$ $CD45RA^+$ invTCR $V\alpha24^+$ cells that became $CD45RO^+$ express $Foxp3^+$.

Ex vivo generation of phospho-antigen specific functionally committed FOXP3 expressing $CD3^+$ $TCR\gamma\delta^+$ unrestricted T cells:

a) In vitro generation of Tolerogenic DC from $CD14^+$ monocytes (termed tolerogenic monocyte-derived DC (Tol-Mo-DC): monocytes are cultured in 48-well flat-bottom plates containing 0,5 ml of AIMV per well supplemented with 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml human recombinant IL-4 for the generation of immature DC. At day 3, 500 μl of the medium containing cytokines was added. On day 6, generated Tol-Mo-DC are removed from the wells, washed twice with IMDM-5 (IMDM supplemented with 5% SVF, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES, freezed or used for the generation and expansion of phospho-antigen specific functionally committed FOXP3 expressing $CD3^+$ $TCR\gamma\delta^+$ unrestricted T cells.

b) Ex vivo generation and expansion of phospho-antigen specific functionally committed FOXP3 expressing $CD3^+$ $TCR\gamma\delta^+$ unrestricted T cells: On day 0, tDC are added to wells of a 48-well plate at a concentration of 3×10^5 /ml in IMDM-5 in the presence of 2×10^5 irradiated autologous feeders, PGE2

1 μM, and Rapa 10 nM and zoledronic acid (100 nM). Purified $CD3^+$ $CD45RA^+$ $TCR\gamma\delta^+$ unrestricted T cells (isolated from the previously frozen PBMC by FACS) are added to the pulsed tDC. On day 1, IL-2 (100IU/ml), IL-15 (10 ng/ml) and TGFβ (5ng/ml) are added to the coculture. Every three days, half of the supernatant volume is discarded and replaced with fresh IMDM-5 with IL-2 (100 UI/ml) and IL-15 (10 ng/ml) (T cell cloning medium). On day 12, these T-cells are further expanded by restimulation with tDC in the presence of ΔCD3-feeder, PGE2 1 μM, TGFβ 5 ng/ml, Rapa 10 nM, IL-2 (100 UI/ml), IL-15 (10 ng/ml) and zoledronic acid (100 nM). Once T cells begin to expand, they can be split every 2 to 3 days with T cell cloning medium and irradiated feeder. On day 21, cells are analyzed by flow cytometry. 75 % of the stimulated $CD3^+$ $CD45RA^+$ $TCR\gamma\delta^+$ T cells that became $CD45RO^+$ express $Foxp3^+$.

In vitro generation of stimulator cells for MLR assay: monocytes are cultured in 48-well flat-bottom plates containing 0,5 ml of RPMI-5 per well supplemented with 20 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml human recombinant IL-4 for the generation of immature DC (iDC). At day 3, 500 μl of the medium containing cytokines are added. At day 5, a portion of iDC are co-cultured with apoptotic MCF-7 cells at a DC/tumor cell ratio of 1:2 for 24h in RPMI 1640 supplemented with GM-CSF (20 ng/mL), IL-4 (20 ng/mL) and 5 % FBS. Another portion of iDC are freezed at 2 x 10^6 / per vial - in 90% FBS -10% DMSO. When indicated, pulsed DCs are matured with tumor necrosis factor α (TNF-α; 20 ng/mL final) and PGE2 (1 μM) for 2 days (mDC). In some experiments, TNF and PGE2 (at the same concentrations), or lipopolysaccharide (LPS; 10-1000 ng/mL; Sigma) are added directly to MLRs. Antigen-loaded DC stimulators are irradiated at 30 Gy.

Flow Cytometry Analysis.

mAb labeling. The following conjugated mAbs are used. a) for $CD3^+$ T cells : anti-CD4(SK3)-PerCP-eFluor 710, anti-TCRαβ(IP26)-APC (ebioscience), anti-CD25 (B1.49.9)-PeCy55, anti-CD127(R34.34)-APC-AF700 (Beckman Coulter), anti-CD3(UCHT1)- BB515 anti-invariant TCR Vα24-JaQ (6B11)-PE, anti-Foxp3

(259D/C7)-PE-CF594 and anti-CD152 (BNI3)-BV421, anti-CD161 (DX12) BV605 and anti-CD56(NCAM 16.2) BU395 (Becton Dickinson), anti-TCR αβ-BV421 (IP26) (Biolegend), anti- TCR pan $\gamma\delta^+$ PE (IMMU510) (Beckman Coulter) and anti-CD27- APC efluor 780 (O323) (ebioscience). Cells are stained for surface markers (at 4°C in the dark for 30 min) using mixtures of Ab diluted in PBS containing BSA/NaN_3 (0.5% BSA, 0.01% NaN_3) (FACS buffer). Foxp3 and CTLA-4 intracellular staining are performed with FOXP3 staining kit obtained from ebioscience according to the manufacturer's instructions. Appropriate isotype control Abs are used for each staining combination. Samples are acquired on a BD LSR FORTESSA flow cytometer using BD FACSDIVA 8.0.1 software (Becton Dickinson). Results are expressed in percentage (%) or in mean fluorescence intensity (MFI).

CFSE staining. Tconv are stained with 1 μM carboxy- fluorescein succinimidyl ester (CFSE) (CellTrace cell proliferation kit; Molecular Probes/Invitrogen) in PBS for 8 min at 37 °C at a concentration of 1×10^7 cells/mL. The labeling is stopped by washing the cell twice with RPMI 1640 culture medium containing 10% FBS. Cells are then resuspended at the desired concentration and subsequently used for proliferation assays.

7-AAD (7-amino-actinomycin D) staining. Apoptosis of stimulated CFSE- labeled or unlabeled nTregs and Tconv was determined using the 7-AAD assay. Briefly, cultured cells are stained with 20 μg/mL nuclear dye 7-AAD (Sigma-Aldrich) for 30 min at 4 °C. FSC/7-AAD dot plots distinguish living (FSC^{high}/7-AAD^-) from apoptotic (FSC^{high}/7-AAD^+) cells and apoptotic bodies (FSC^{low}/7- AAD^+) and debris (FSC^{low}/7- AAD^-). Living cells are identified as $CD3^+$ 7-AAD^- FSC^- cells.

Flow Cytometric Analysis of the TCR-Vβ Repertoire. The IOTest Beta Mark TCR-Vβ Repertoire kit (Beckman Coulter, Miami, FL) was used for the assessment of the following TCR-Vβ regions: Vβ 1, Vβ 2, Vβ 3, Vβ 4, Vβ 5.1, Vβ 5.2, Vβ 5.3, Vβ 7.1, Vβ 7.2, Vβ 8, Vβ 9, Vβ 11, Vβ 12, Vβ 13.1, Vβ 13.2, Vβ 13.6, Vβ 14, Vβ 16, Vβ 17, Vβ 18, Vβ 20, Vβ 21.3, Vβ 22, and Vβ 23. This kit includes 8 cocktails, each containing antibodies against 3 different TCR-Vβ regions covering 24 TCR-Vβ antigens and approximately 70% of the normal human TCR-Vβ repertoire. Analysis was performed on

fresh PBMCs. The panel included antibody against CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), iNKT (clone 6B11) and $Foxp3^+$ (clone 259D) to allow evaluation of $V\alpha24^+$ $CD4^+$ $FOXP3^+$, $V\alpha24^+$ $CD4^+$ $FOXP3^-$, $V\alpha24^+$ $CD4^-$ $CD8^-$ and $V\alpha24^+$ $CD8^+$ subpopulations.

Functional Assays.

T-cell proliferation. T-cell proliferation is assessed CFSE dilution assay in RPMI supplemented with 5% FBS, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, glutamax and 10 mM HEPES (RPMI-5 media) in normoxia. At coculture completion, stimulated CFSE-labeled Tconv are harvested, costained with anti-CD3 mAb and 7-AAD, and the percentage of living proliferating cells (defined as CFSE low fraction) in gated $CD3^+$ 7-AAD^- cells is determined by flow cytometry.

Standard polyclonal cell-cell contact Treg suppression assay: CFSE-labeled T_{conv} (4×10^4 per well), used as responder cells, are cultured with ΔCD3- feeder (4×10^4 per well) in the presence or absence of defined amounts of Foxp3 T cells (blood Treg or ex vivo generated T cells) for 4 to 5 d. Cultures are performed in round-bottom plates coated with 0.2 μg/mL anti-CD3 mAb in 200 μL of complete RPMI medium. Results are expressed as the percentage of proliferating CFSE low T cells or as a percentage of suppression calculated as follows: (100 × [(percentage of T_{conv} CFSE low cells − percentage of T_{conv} CFSE low in coculture with nTregs)/percentage of T_{conv} CSFE low cells,

Autologous MLR suppression assay: CFSE-labeled T_{conv} $CD4^+CD25^-$ T cells (5×10^4) are stimulated either with 1×10^4 pulsed iDC in RPMI-5 media or with 5×10^3 pulsed – mDC in IMDM-5 media supplemented with IL-2 (20 IU/ml) IL-1b (10 ng/ml), IL-6 (30 ng/ml), IL-21 (50 ng/ml) and IL-23 (30 ng/ml) in the presence or absence of defined amounts of Foxp3 T cells (blood Treg or ex vivo generated T cells) for 5 to 6 d. When indicated, culture are performed in IMDM-5 media supplemented with IL-2 (20 IU/ml) IL-1β (10 ng/ml), IL-6 (30 ng/ml), IL-21 (50 ng/ml) and IL-23 (30 ng/ml). Results are expressed as the percentage of proliferating CFSE low T cells or as a percentage of suppression calculated as follows: (100 × [(percentage of T_{conv} CFSE low cells −

percentage of T_{conv} CFSE low in coculture with nTregs)/percentage of T_{conv} CSFE low cells.

Measurement of DNA methylation: Classically, a stable Treg genetic signature consisted of highly demethylated CpG islands within the conserved non-coding sequence 2 (CNS2) of the Treg specific demethylation region (TSDR). DNA methylation analysis of the TSDR region of the gene FOXP3 was evaluated by quantitative PCR after bisulfite treatment of genomic DNA as previously described by Christopher Fuhrman (Fuhrman et al, Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226, 2015, Journal of immunology). Briefly Nucleotides were isolated with AllPrep DNA/RNA Mini Kit (Qiagen) or DNeasy tissue kit (Qiagen), as appropriate. Bisulfite treatment of genomic DNA was performed on 500 ng DNA with the EZ DNA Methylation Kit (Zymo Research). DNA standards originated from unmethylated bisulfite-converted human EpiTect control DNA (Qiagen) or universally methylated bisulfite-converted human control DNA (Zymo Research). To obtain a large quantity of standard, the TSDR was PCR-amplified using the following reaction: 50 µl reaction volume containing 25 µl of ZymoTaq PreMix buffer (Zymo Research) and 0.5 µM each of the primers FOXP3_TSDRfwd (5′-ATATTTTTAGATAGGGATATGGAGATGATTTGTTTGG-3′ SEQ ID NO: 1) and FOXP3_TSDRrev (5′-AATAAACATCACCTACCACATCCACCAACAC-3′ - SEQ ID NO: 2). After incubation at 95°C for 10 min, amplification was performed as follows: 50 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Amplified PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen). The concentration of purified control TSDR DNA was determined with a GE NanoVue spectrophotometer (GE Healthcare Life Sciences). TSDR real-time PCR was performed with probes that targeted methylated or demethylated target sequences. The reaction was performed in 96-well white trays with a Roche LightCycler 480 system (Roche Diagnostics). Each reaction contained 10 µl LightCycler 480 Probes Master Mix (Roche), 10 ng of bisulfite converted DNA sample or standards, 1 µM of each primer, and 150 nM of each probe with a final reaction value of 20 µl. The probes used for amplification were TSDR-Forward 5′-GGTTTGTATTTGGGTTTTGTTGTTATAGT-3′ (SEQ ID NO: 3) and TSDR-Reverse 5′-CTATAAAATAAAATATCTACCCTCTTCTCTTCCT-3′ (SEQ ID NO: 4). The

probes for target sequence detection were FAM-labeled methylated probe, FAM-CGGTCGGATGCGTC-MGB-NFQ (SEQ ID NO: 5), or VIC-labeled unmethylated probe, VIC-TGGTGGTTGGATGTGTTG-MGB-NFQ (SEQ ID NO: 6). All samples were tested in triplicate. The protocol for real-time amplification is as follows: after initial denaturation at 95°C for 10 min, the samples were subjected to 50 cycles at 95°C for 15 s and at 61°C for 1 min. Fourteen different ratios of fully methylated and demethylated template were used as real-time standards. A six-order polynomial equation was used to extrapolate the percentage of cells demethylated at the TSDR for each sample.

Measurement of histone acetylation: Histone acetylation analysis of the four different sites of FOXP3 gene was evaluated by ChIP assay, as previously described by Ling Lu (Ling Lu et al, PNAS 2014). Briefly, 50,000 cells of each treated nTreg cell sample were harvested and cross-linked with 1% formaldehyde, and then lysed with 120μL of lysis buffer [50 mM Tris·HCl, pH 8.0, 10 mM EDTA, 1% (wt/vol) SDS, protease inhibitor mix (1:100 dilution; Sigma), 1 mM PMSF, 20 mM Na-butyrate]. The chromatin in the lysate was sonicated to 500–800-bp fragments and then diluted with 800μL of RIPA ChIP buffer [10 mM Tris·HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.1% (wt/vol) Na-deoxycholate, protease inhibitor mix (1:100 dilution; Sigma), 1 mM PMSF, and 20 mM Na- butyrate]. Dynabeads protein G (10μL; Invitrogen) was incubated with 1μg ofH3K4me3 (Abcam) or H3K9ac (Cell Signaling) or normal rabbit IgG negative control ChIP-grade antibodies for 2 h separately. Then, 100μL of the sheared chromatin was immunoprecipitated with pretreated antibody–bead complexes and another 100μL of the sheared chromatin for total input DNA extraction separately. Immunoprecipitated DNA was quantified by real-time PCR with following primers: promoter, 5′-ACC GTA CAG CGT GGT TTT TC-3′ (SEQ ID NO: 7) and 5′-CTA CCT CCC TGC CAT CTC CT-3′ (SEQ ID NO: 8); CNS1, 5′-CCC AAG CCC TAT GTG TGATT-3′ (SEQ ID NO: 9) and 5′-GTG TGT CAG GCC TTG TGC TA-3′ (SEQ ID NO: 10) ; CNS2, 5′-GTC CTC TCC ACAACC CAA GA-3′ (SEQ ID NO: 11) and 5′-GAC ACC ACG GAG GAA GAG AA -3′ (SEQ ID NO: 12); and CNS3, 5′-AGG TGC CGA CCT TTA CTG TG-3′ (SEQ ID NO: 13) and 5′- ACA ATA CGG CCT CCT CCT CT-3′ (SEQ ID NO: 14).

Results

a) Proportion of Foxp3$^+$ expressing T cells in circulating blood

In circulating blood, both CD3$^+$ TCRαβ$^+$ MHCII restricted T cells and CD3$^+$ invTCR Vα24$^+$ CD1-restricted T cells express Foxp3.

FOXP3 expressing CD3$^+$ TCRαβ$^+$ MHCII restricted T cells (PBMC) represents approximately 9% (1-12 %) of these CD3$^+$ TCRαβ T cells (Figure 1; upper right panel). Figure 1 (lower right panel) shows that these Foxp3$^+$ T cells express high levels of CD4, CD25 and CTLA4 and low level of CD127 while FOXP3- T cells are mainly CD127$^+$ T cells and express low levels of CD25 and CTLA4.

Foxp3 expressing CD3$^+$ invTCR Vα24$^+$ CD1-restricted T cells represent almost 9% (3-15 %) of the CD3$^+$ invTCR Vα24$^+$ CD1-restricted T cells population (see Figure 1, upper right panel). These Foxp3$^+$ T cells present a same phenotypic profile as Foxp3$^+$ CD3$^+$ TCRαβ, with respect to the CD4, CD25 and CTLA4 markers, with, however, an increased intensity of expression (as measured by the MFI value) for CD25 and CTLA markers (see Figure 1, lower panel) while FOXP3$^-$ CD3$^+$ invTCR Vα24$^+$ exhibit a similar phenotypic profile as FOXP3$^-$ CD3$^+$ TCRαβ.

Both Foxp3$^+$ T cells populations exhibit suppressive activity.

As further shown in Figure 2, FOXP3$^+$ CD3$^+$ invTCR Vα24$^+$ CD1-restricted T cell subset display low CD161 and CD56 levels, cell membrane phenotypic markers of invariant NKT, while expressing Treg cell phenotypic markers (CD25, CTLA4).

To test the TCR-Vβ repertoire on the CD3$^+$ CD4$^+$ Vα24$^+$ T subset cells expressing Foxp3$^+$, flow cytometry staining using TCR-Vβ repertoire kit (Beckman) is performed. TCR-Vβ families examined by the panel of available antibodies covered a mean of 70.18% (6 standard deviation of 8.268) of all analyzed cells, representing the majority of cells within each subset investigated.
The expression of 24 TCR-Vβ families is investigated in CD3$^+$ Vα24$^+$ T-cell subsets: CD3$^+$ Vα24$^+$ CD4$^+$ FOXP3$^+$and CD3$^+$ Vα24$^+$ CD4$^+$ FOXP3$^-$.

The repertoire analysis shows that $CD3^+$ $V\alpha24^+$ $CD4^+$ subset cells expressing $FOXP3^+$ exhibit a wide variety of TCR-V chains. This TCR-Vβ repertoire is relatively comparable to the TCR-Vβ repertoire of $CD3^+$ $V\alpha24^+$ $CD4^+$ $FOXP3^-$ subset with an increased Vβ usage involving Vβ1, Vβ2, Vβ4, Vβ8, or Vβ5.1 (Figure 3).

b) Optimal conditions for inducing Foxp3 expression in invariant Tcells

Starting from naive $CD3^+$ invTCR $V\alpha24^+$ T cells isolated from human PBMCs, different nTreg polarizing medium were assessed for their capacity to induce the expression the differentiation of $Foxp3^+$ cells with suppressive function.

Figure 5 shows that cultured naive $CD3^+$ invTCR $V\alpha24^+$ T cells exhibit a variable level of Foxp3 dependent on their culture condition of stimulation. Polarizing medium comprising the combination of IL-2, TGFβ, PGE2 and rapamycin results in a higher Foxp3 expression over combinations of IL-2, TGFβ and rapamycin, IL-2 and PGE2, or IL-2 alone. Moreover, the combination of IL-2, TGFβ, PGE2 and rapamycin results in an optimal intensity of Foxp3 expression in the invTCR $V\alpha24^+$ T cells, as compared to the other combinations.

Furthermore, it is interesting to note that only naive $CD3^+$ invTCR $V\alpha24^+$ T cells stimulated with the polarizing medium comprising the combination of IL-2, TGFβ, PGE2 and rapamycin express level and intensity of Foxp3 similar or higher to those of blood naïve regulatory T cells ($CD3^+$ TCRαβ $CD4^+$ $CD127^{-/low}$ $CD45RA^+$ $CD25^+$), corresponding to our positive control.

c) Tumor Ag-specific $CD3^+$ TCR $V\alpha24^+$ T cells maintain their ability to perform suppressive function in pro-inflammatory conditions

Figure 6A shows that tumor Ag-specific memory invTCR $V\alpha24^+$ T cells ex vivo generated and expanded in the presence of the nTreg polarizing medium above described are endowed of a higher suppressive activity than fresh Foxp3 expressing $CD3^+$ $CD4^+$ $TCR\alpha\beta^+$ MHCII restricted T cells when using an autologous MLR coculture assay.

Furthermore, Figure 6B shows that these tumor Ag-specific invTCR $V\alpha24^+$ T cells still

maintain their suppressive activity, when the autologous MLR coculture assay are performed in presence of a high inflammatory medium containing IL-2 IL-1 IL-6, IL-21 IL-23 cytokines, while fresh Foxp3 expressing $CD3^+$ $CD4^+$ $TCR\alpha\beta^+$ MHCII restricted T cells lose their suppressive activity.

5

CLAIMS

1. An isolated population of invariant Foxp3$^+$ regulatory T cells having the following phenotype: CD3$^+$ Vα24$^+$ Foxp3$^+$.

2. The isolated population of invariant Foxp3$^+$ regulatory T cells of claim **1** further expressing CD4 and CD25, thereby having the following phenotype: CD3$^+$ CD4$^+$ Vα24$^+$ CD25$^+$ Foxp3$^+$.

3. A method for generating ex vivo invariant Foxp3$^+$ regulatory T cells having the following phenotype: CD3$^+$ Vα24$^+$ Foxp3$^+$, comprising:
   - culturing CD3$^+$ Vα24$^+$ T cells in the presence of an invariant T cell activator and the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator, ii) a TGFβ (Transforming growth factor beta) pathway activator, iii) a mTOR inhibitor, optionally iv) at least one cytokine selected in the group of IL-2, IL-7, IL-15 and TSLP, and optionally v) at least one TET enzymes activator and/or at least one DNMT inhibitor, for at least 5 days.

4. The method according to claim **3**, wherein the invariant T cell activator is a polyclonal invariant T cell activator, preferably a Vα24 activator.

5. The method according to claim **3**, wherein the invariant T cell activator is an antigen-specific invariant T cell activator, preferably tolerogenic dendritic cells (DCs) expressing CD1 and pulsed with at least one non peptide lipid antigen.

6. The method according to anyone of claims **3** to **5**, wherein the cAMP activator is selected from the group comprising prostaglandin E2 (PGE2), an EP2 or EP4 agonist, a membrane adenine cyclase activator and a metabotropic glutamate receptors agonist.

7. The method according to anyone of claims **3** to **6**, wherein the TGFβ pathway activator is selected from the group comprising TGFβ, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activin and nodal .

8. The method according to anyone of claims **3** to **7**, wherein the mTOR inhibitor is selected from the group comprising rapamycin, rapamycin analogs, wortmannin; theophylline; caffeine; epigallocatechin gallate (EGCG), curcumin, resveratrol; genistein, 3, 3-diindolylmethane (DIM), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PP242, PP30, Torin1, Ku-0063794, WAY-600, WYE-687, WYE-354, GNE477, NVP-BEZ235, PI-103, XL765 and WJD008.

9. The method according to anyone of claims **3** to **8**, further comprising an expansion step, wherein the invariant $Foxp3^+$ regulatory T cells obtained by the generation method of claims **3** to **8** are cultured in the presence of an invariant T cell activator and the following agents: i) an cAMP (Cyclic adenosine monophosphate) activator, ii) a TGFβ (Transforming growth factor beta) pathway activator, iii) a mTOR inhibitor, optionally iv) at least one cytokine selected in the group of IL-2, IL-7, IL-15 and TSLP, and optionally v) at least one TET enzymes activator and/or at least one DNMT inhibitor, for at least 5 days.

10. An ex vivo generated invariant $Foxp3^+$ regulatory T cell population obtainable by the method according to anyone of claims **3** to **8**, wherein said $Foxp3^+$ regulatory T cells have the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

11. An ex vivo generated and expanded invariant $Foxp3^+$ regulatory T cell population obtainable by the method according to claim **9**, wherein said $Foxp3^+$ regulatory T cells have the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

12. An immunogenic product comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$, or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$.

13. A pharmaceutical composition comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or

immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$and at least one pharmaceutically acceptable excipient.

14. A vaccine composition comprising inactivated invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ or immunogenic dendritic cells loaded with blebs of invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$and at least one adjuvant.

15. The immunogenic product, pharmaceutical composition or vaccine composition according to anyone of claims **12** to **14** for use in treating cancer.

16. A pharmaceutical composition comprising invariant $Foxp3^+$ regulatory T cells having the following phenotype: $CD3^+$ $V\alpha24^+$ $Foxp3^+$ and at least one pharmaceutically acceptable excipient.

17. The pharmaceutical composition according to claim **16** for use in cell therapy.

18. The pharmaceutical composition according to claim **16** for use in treating inflammatory or autoimmune diseases or for preventing transplant rejection or graft versus host disease (GVHD).

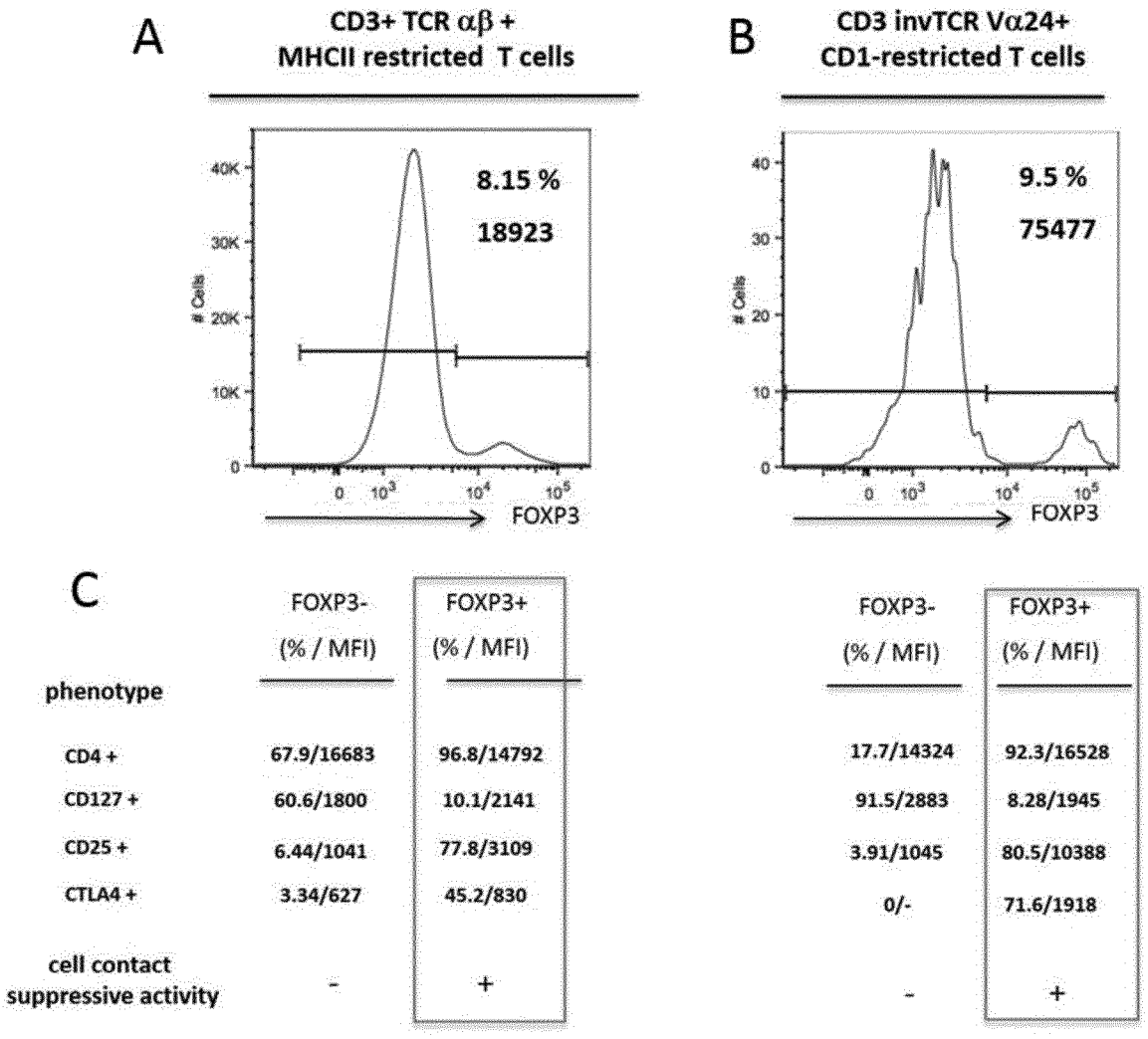

A
CD3+ TCR αβ +
MHCII restricted T cells
8.15 %
18923
Cells
FOXP3
B
CD3 invTCR Vα24+
CD1-restricted T cells
9.5 %
75477
Cells
FOXP3
C
phenotype
FOXP3-
(% / MFI)
FOXP3+
(% / MFI)
CD4 +
67.9/16683
96.8/14792
CD127 +
60.6/1800
10.1/2141
CD25 +
6.44/1041
77.8/3109
CTLA4 +
3.34/627
45.2/830
cell contact
suppressive activity
-
+
FOXP3-
(% / MFI)
FOXP3+
(% / MFI)
17.7/14324
92.3/16528
91.5/2883
8.28/1945
3.91/1045
80.5/10388
0/-
71.6/1918
-
+

FIG. 1

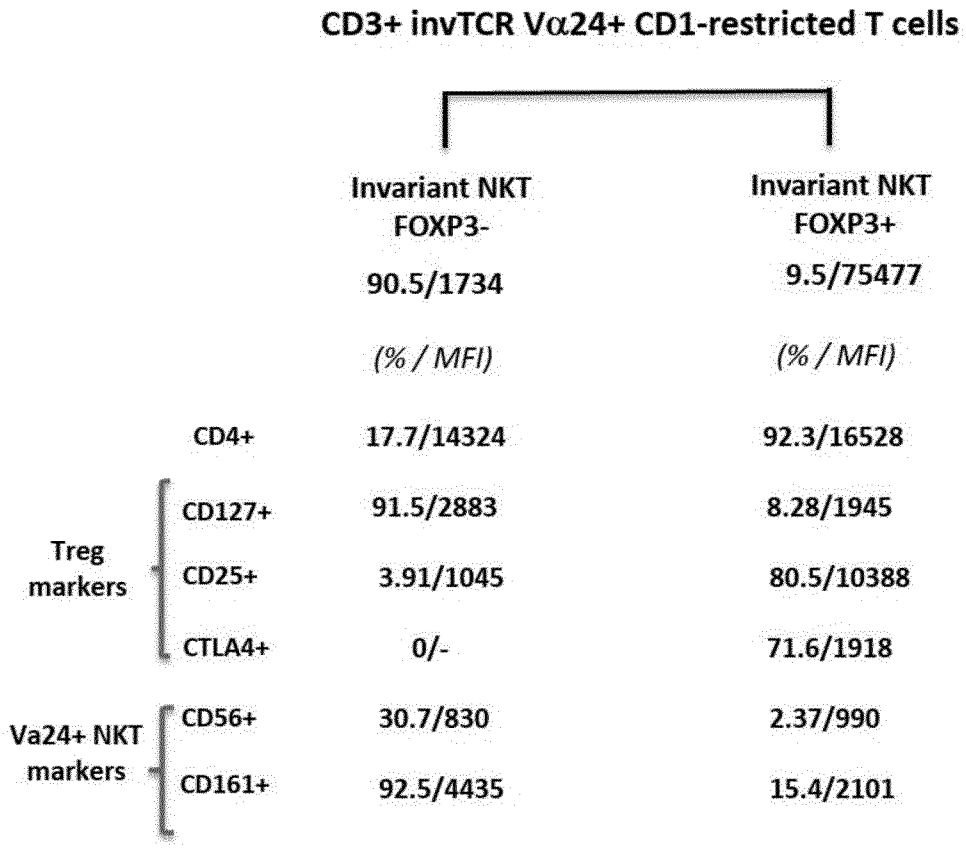

CD3+ invTCR Vα24+ CD1-restricted T cells
Invariant NKT FOXP3-
Invariant NKT FOXP3+
90.5/1734
9.5/75477
(% / MFI)
(% / MFI)
CD4+
17.7/14324
92.3/16528
Treg markers
CD127+
91.5/2883
8.28/1945
CD25+
3.91/1045
80.5/10388
CTLA4+
0/-
71.6/1918
Va24+ NKT markers
CD56+
30.7/830
2.37/990
CD161+
92.5/4435
15.4/2101

FIG. 2

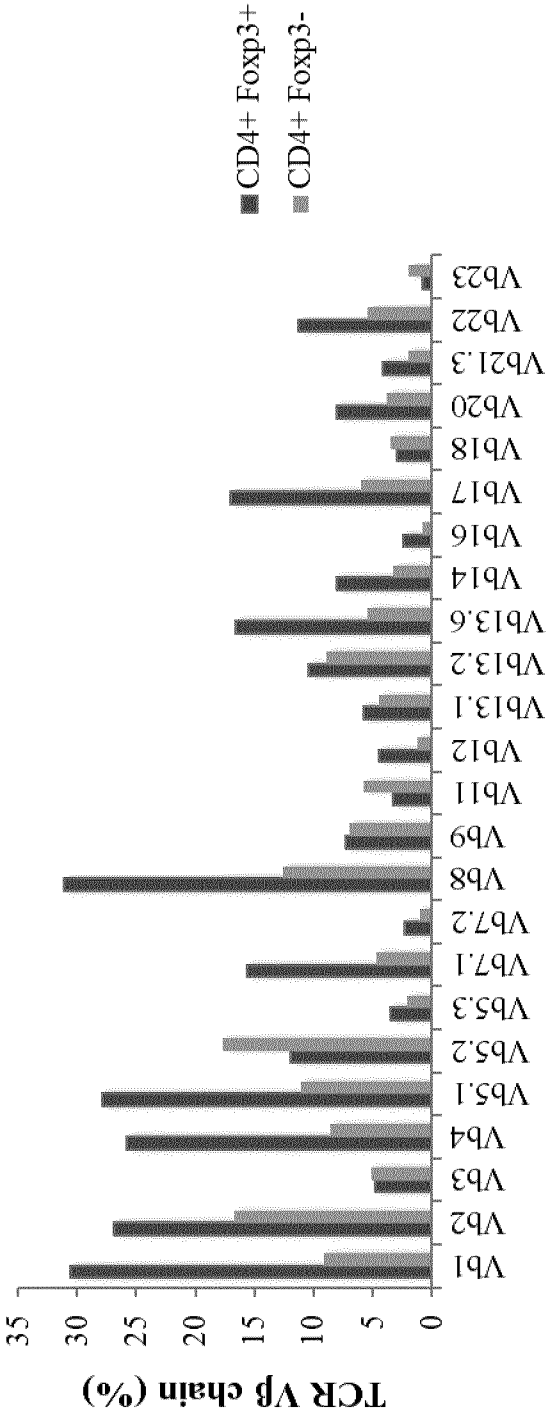

CD4+ Foxp3+
CD4+ Foxp3-
TCR Vβ chain (%)
0
5
10
15
20
25
30
35
Vb1
Vb2
Vb3
Vb4
Vb5.1
Vb5.2
Vb5.3
Vb7.1
Vb7.2
Vb8
Vb9
Vb11
Vb12
Vb13.1
Vb13.2
Vb13.6
Vb14
Vb16
Vb17
Vb18
Vb20
Vb21.3
Vb22
Vb23

FIG. 3

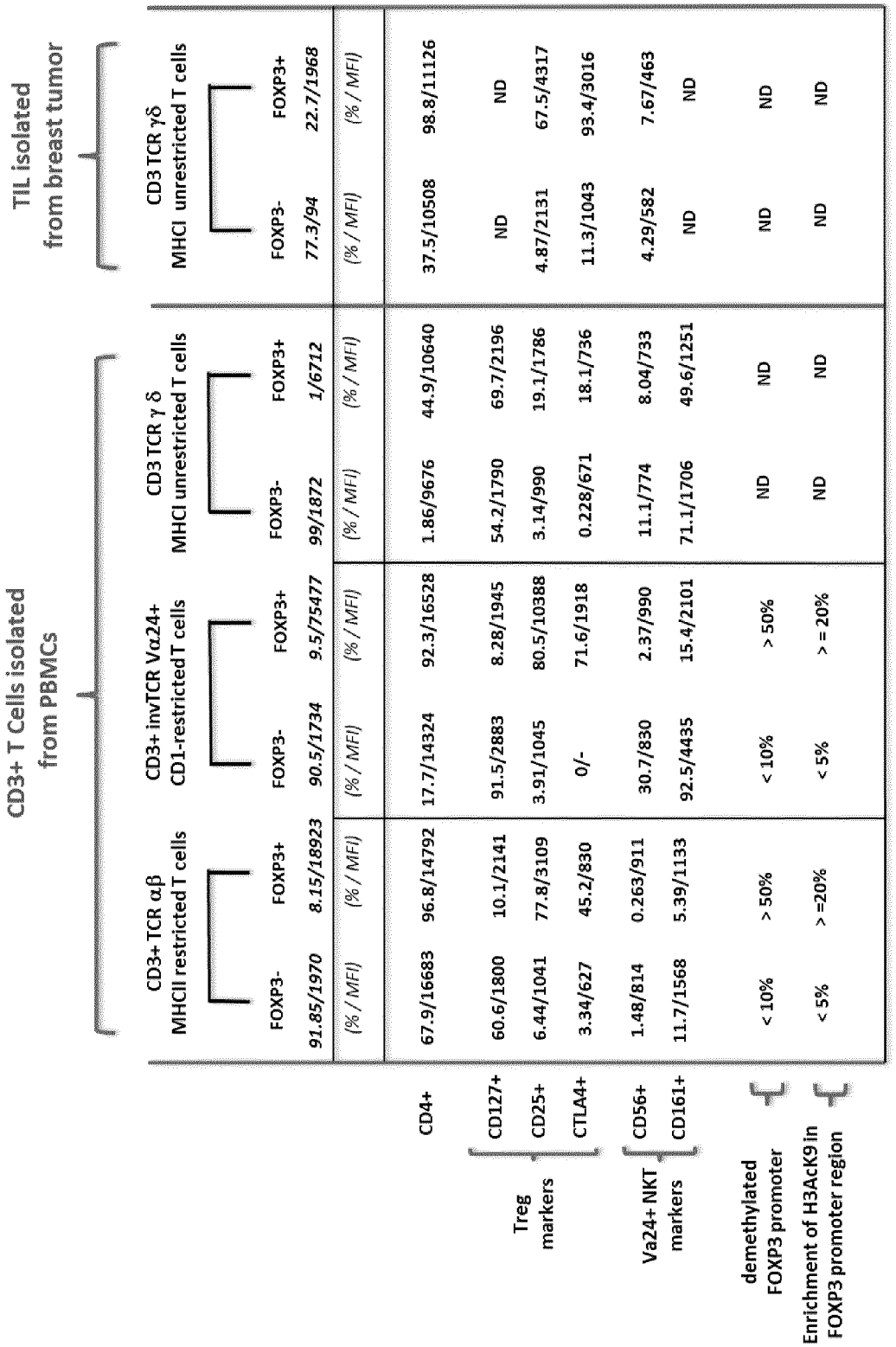

CD3+ T Cells isolated from PBMCs
TIL isolated from breast tumor
CD3+ TCR αβ MHCII restricted T cells
CD3+ invTCR Vα24+ CD1-restricted T cells
CD3 TCR γ δ MHCI unrestricted T cells
CD3 TCR γδ MHCI unrestricted T cells
FOXP3- FOXP3+ FOXP3- FOXP3+ FOXP3- FOXP3+ FOXP3- FOXP3+
91.85/1970 8.15/18923 90.5/1734 9.5/75477 99/1872 1/6712 77.3/94 22.7/1968
(% / MFI) (% / MFI) (% / MFI) (% / MFI) (% / MFI) (% / MFI) (% / MFI) (% / MFI)
CD4+ 67.9/16683 96.8/14792 17.7/14324 92.3/16528 1.86/9676 44.9/10640 37.5/10508 98.8/11126
Treg markers
CD127+ 60.6/1800 10.1/2141 91.5/2883 8.28/1945 54.2/1790 69.7/2196 ND ND
CD25+ 6.44/1041 77.8/3109 3.91/1045 80.5/10388 3.14/990 19.1/1786 4.87/2131 67.5/4317
CTLA4+ 3.34/627 45.2/830 0/- 71.6/1918 0.228/671 18.1/736 11.3/1043 93.4/3016
Va24+ NKT markers
CD56+ 1.48/814 0.263/911 30.7/830 2.37/990 11.1/774 8.04/733 4.29/582 7.67/463
CD161+ 11.7/1568 5.39/1133 92.5/4435 15.4/2101 71.1/1706 49.6/1251 ND ND
demethylated FOXP3 promoter
< 10% > 50% < 10% > 50% ND ND ND ND
Enrichment of H3AcK9 in FOXP3 promoter region
< 5% > =20% < 5% > = 20% ND ND ND ND

FIG. 4

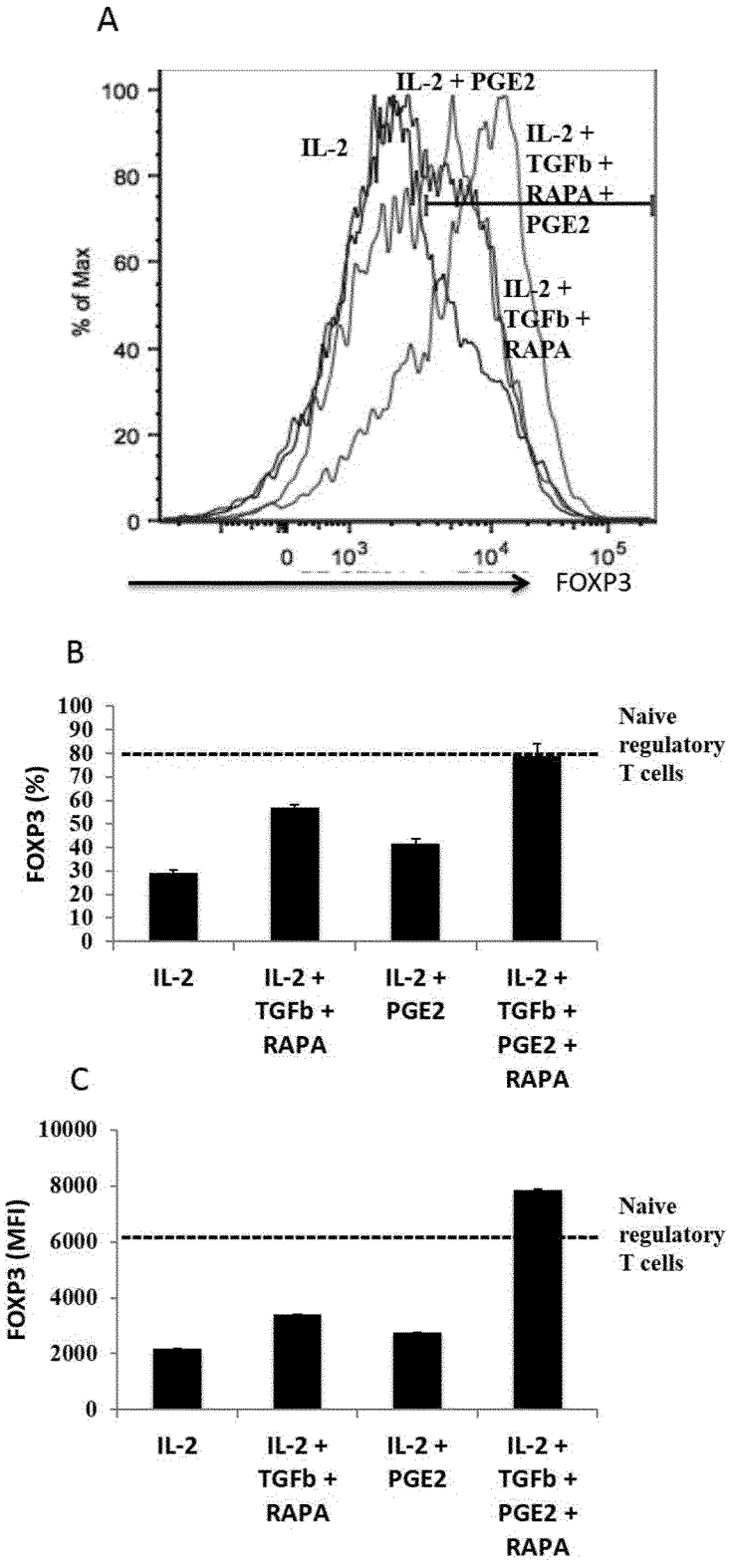

A
IL-2 + PGE2
IL-2
IL-2 + TGFb + RAPA + PGE2
IL-2 + TGFb + RAPA
% of Max
100
80
60
40
20
0
0
10^3
10^4
10^5
FOXP3
B
FOXP3 (%)
100
90
80
70
60
50
40
30
20
10
0
Naive regulatory T cells
IL-2
IL-2 + TGFb + RAPA
IL-2 + PGE2
IL-2 + TGFb + PGE2 + RAPA
C
FOXP3 (MFI)
10000
8000
6000
4000
2000
0
Naive regulatory T cells
IL-2
IL-2 + TGFb + RAPA
IL-2 + PGE2
IL-2 + TGFb + PGE2 + RAPA

FIG. 5

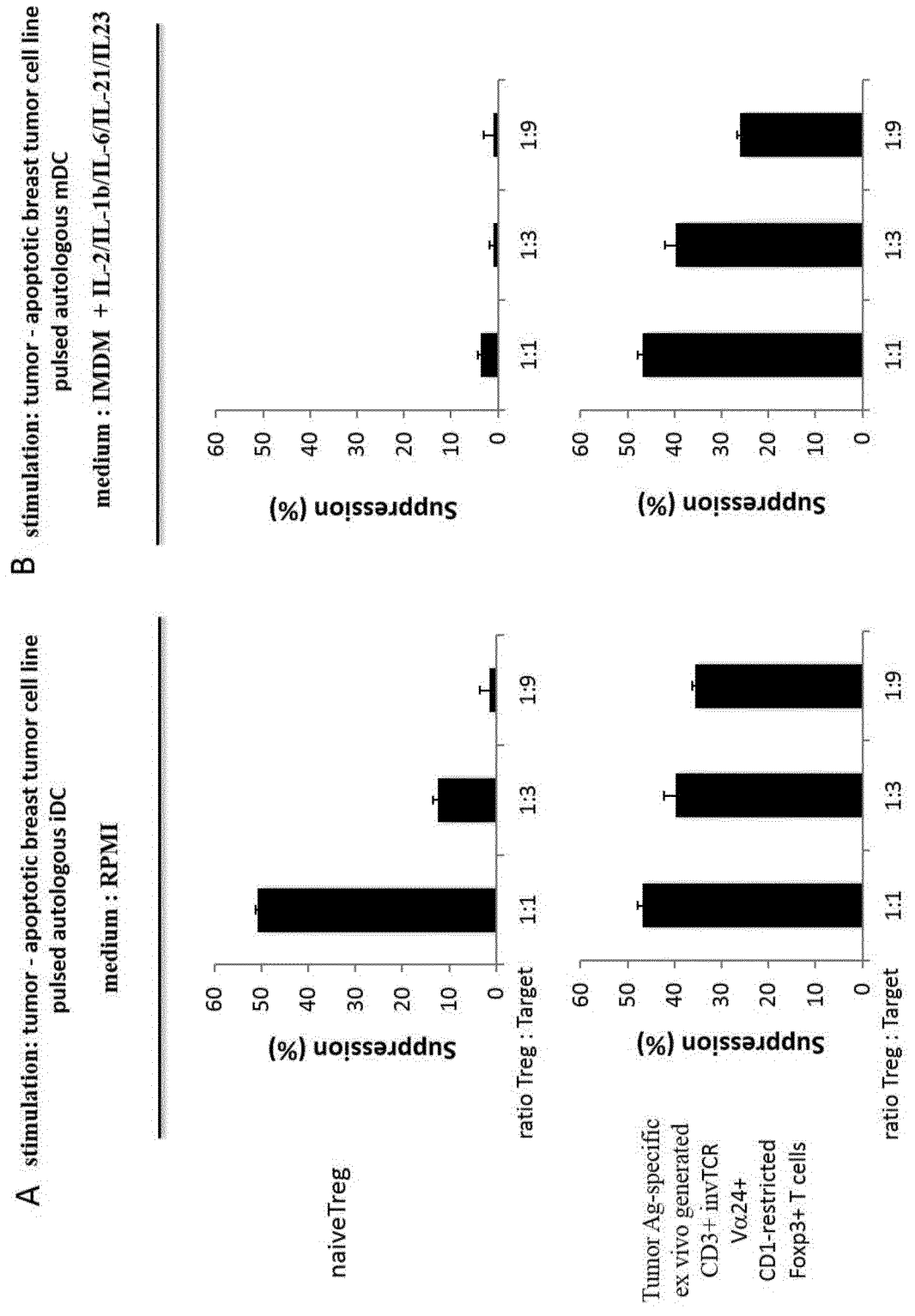

A
stimulation: tumor - apoptotic breast tumor cell line pulsed autologous iDC
medium : RPMI
naiveTreg
Tumor Ag-specific ex vivo generated CD3+ invTCR Vα24+ CD1-restricted Foxp3+ T cells
Suppression (%)
ratio Treg : Target
1:1
1:3
1:9
B
stimulation: tumor - apoptotic breast tumor cell line pulsed autologous mDC
medium : IMDM + IL-2/IL-1b/IL-6/IL-21/IL23

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/069822

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0783
ADD. A61K35/17 A61K39/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAGANARI OHKURA ET AL: "FOXP3regulatory T cells: control of FOXP3 expression by pharmacological agents", TRENDS IN PHARMACOLOGICAL SCIENCES, vol. 32, no. 3, 1 March 2011 (2011-03-01), pages 158-166, XP028170472, ISSN: 0165-6147, DOI: 10.1016/J.TIPS.2010.12.004 [retrieved on 2010-12-17] page 158 - page 160 page 161, left-hand column, paragraph 3 page 163; table 1	1-18

A	US 2013/195919 A1 (VON ANDRIAN ULRICH [US] ET AL) 1 August 2013 (2013-08-01) page 30 - page 36; examples 1-9	1-18

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[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

"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
18 October 2017	25/01/2018

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	Heiduschat, Carola

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	BERNARDO ET AL: "Decreased circulating iNKT cell numbers in refractory coeliac disease", CLINICAL IMMUNOLOGY, ACADEMIC PRESS, US, vol. 126, no. 2, 31 October 2007 (2007-10-31), pages 172-179, XP022415739, ISSN: 1521-6616, DOI: 10.1016/J.CLIM.2007.09.005 page 173, left-hand column, paragraph 2 - right-hand column, paragraph 3 page 176, right-hand column, paragraph 3 - page 177, right-hand column, paragraph 2	1-18
A	H. TUOVINEN ET AL: "Most human thymic and peripheral-blood CD4+CD25+ regulatory T cells express 2 T-cell receptors", BLOOD, vol. 108, no. 13, 15 December 2006 (2006-12-15), pages 4063-4070, XP055348655, US ISSN: 0006-4971, DOI: 10.1182/blood-2006-04-016105 page 4065, right-hand column page 4068, right-hand column	1-18
A	MONTOYA CARLOS J ET AL: "Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11", IMMUNOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 122, no. 1, 1 September 2007 (2007-09-01), pages 1-14, XP009175292, ISSN: 0019-2805, DOI: 10.1111/J.1365-2567.2007.02647.X cited in the application page 1 - page 6 page 9	1-18
A	Peter T Lee ET AL: "Brief Definitive Report Distinct Functional Lineages of Human V $\bar{}$ 24 Natural Killer T Cells", J. Exp. M̄ed, 4 March 2002 (2002-03-04), pages 637-641, XP055347737, Retrieved from the Internet: URL:http://jem.rupress.org/content/195/5/637/tab-pdf [retrieved on 2017-02-20] the whole document	1-18

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/116908 A1 (UNIV FLORIDA [US]) 31 July 2014 (2014-07-31) page 14	1-18
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