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(54) Title: USE OF THE F359L MISSENSE IRF4 VARIANT FOR INCREASING THE STABILITY OF REGULATORY T CELLS

(57) **Abstract:** Uses of Tregs, in particular CAR-Tregs represent a very promising approach for inducing and maintaining tolerance. However, Tregs could lose Treg identity in an inflammatory environment. There is thus a great deal at stake to "lock" Tregs in their Treg lineage identity. Recently, the inventors identified a missense *IRF4* gene variant (p.F359L) in a family with a primary immune deficiency (PID), which combines hypogammaglobulinemia and skin manifestation. Unexpectedly, the transduction of CD4+ T cells with a lentiviral vector encoding either the wild-type or the mutated IRF4 gene, induced a strong and sustained CD25 expression in the latter compared to the former. The inventors extended this finding by showing a similar result in Tregs, along with enhanced expression of HELIOS, a Treg transcription factor, linked to IL-2-dependent Treg stability. In line with this, the inventors found that Tregs expressing the IRF4 variant demonstrate an enhanced IL-2 sensitivity. Accordingly, the results suggest that transgenic expression of this IRF4 mutant in Tregs might push them toward highly suppressive and stable effector cells. Thus, the present invention relates to the use of the F359L missense IRF4 variant for increasing the stability of regulatory T cells.

USE OF THE F359L MISSENSE IRF4 VARIANT FOR INCREASING THE STABILITY OF REGULATORY T CELLS

5 **FIELD OF THE INVENTION:**

The present invention is in the field of medicine, in particular immunology.

BACKGROUND OF THE INVENTION:

Transplantation is one of the most challenging and complex areas of medicine, and involves the
10 transfer of a tissue or organ from a donor to a recipient patient. It offers the possibility to replace the recipient's damaged or defective tissue or organ with a functional one and can significantly improve the health and well-being of the recipient. Organ transplantation has undergone substantial improvements in both the prevention and treatment of acute rejection, but subclinical episodes and chronic graft dysfunction still heavily impact medium- and long-term
15 graft survival.

Rejection arises due to sensitisation of the cell-mediated immune system of the recipient to the foreign (allogeneic) antigens of the donor. In particular, the recipient's immune system reacts to the major histocompatibility complex (MHC) molecules presented on the surface of the
20 donor tissues (the graft). The MHC molecules are expressed on the surface of cells in all jawed vertebrates, and are responsible for displaying antigens to cytotoxic T cells. MHC molecules also contribute to the risk of graft versus host disease (GVHD) after haematopoietic stem cell transplantation. Matching of MHC class I and II genes is essential for the success of transplantation. Graft-versus-host disease (GVHD) is associated with significant morbidity and
25 mortality. Mortality rates as a direct or indirect consequence of GVHD can reach 50% despite the prophylactic use of immunosuppressive drugs like cyclosporine, tacrolimus, ATG, methotrexate, and mycophenolate mofetil which are administered for prevention of GVHD. In humans, genes encoding for MHC molecules are referred to as human leukocyte antigen (HLA) genes. The most intensely studied HLA genes are the nine so-called classical MHC genes:
30 HLA-A, HLA-B, HLA-C, HLA-DPA 1 , HLA-DPB 1 , HLA-DQA 1 , HLA-DQB 1 , HLA-DRA, and HLA-DRB 1 . In humans, the MHC is divided into three regions: Class I, II, and III. The A, B, and C genes belong to MHC class I, whereas the six D genes belong to class II.

Immunological rejection is typically alleviated by administering immunosuppressant drugs to the recipient, both prior to and after the transplantation. Immunosuppressants decrease the activity of the recipient's immune system, thereby preventing it from attacking the donor tissue or organ and thus allowing better graft retention. However, the administration of immunosuppressants does not result in the patient developing long-term tolerance to the allograft, and, therefore, most patients must undergo immunosuppressive therapy for the lifetime of the graft (typically 5-10 years) or the remainder of their lives. Furthermore, immunosuppressants are known to cause a number of complications, largely owing to their non-specificity. Emerging therapeutic strategies, among them induction of tolerance to donor antigens, are moving to the clinical stage after years of experimental model work.

Among natural mechanisms and tolerance-inductive strategies, the uses of different types of regulatory cells (Tregs) are among the most promising ones. In particular, the uses of CD4+ and CD8+ Tregs have been highlighted in recent years. Preclinical studies have thus shown that donor-specific Tregs were far more potent than polyclonal Tregs at inducing and maintaining transplant tolerance. In this respect, the use of Chimeric Antigen Receptors was recently found very efficient at redirecting Treg (named CAR-Tregs) toward donor-specific antigens in transplant models.

However, clinical implementation of CAR-Tregs still faces a number of hurdles. One of the greatest concerns is that CAR-Tregs could lose Treg identity in an inflammatory environment, and become CAR-ex-Tregs. Such cells, engineered to recognized donor antigen, could potentially become harmful for the allograft. There is thus a great deal at stake to “lock” CAR-Tregs in their Treg lineage identity. In addition, there is a medical need for empowering CAR-Tregs through enhanced suppressive function, as well as improved *in vivo* IL-2-dependent fitness and stability.

The interferon regulatory factor 4 (IRF4 aliases: NF-EM5, Pip, LSIRF, ICSAT, MUM1) belongs to a family of nine human IRF transcription factors (*De Silva NS, Simonetti G, Heise N, Klein U. The diverse roles of IRF4 in late germinal center B-cell differentiation: IRF4 in late GC B-cell differentiation. Immunol Rev. 2012 May;247(1):73–92.*) and is in contrast to the other members of this family not regulated by interferons.

The IRF4 protein (as all members of the IRF family) consists of two conserved functional domains: a N-terminal helix-turn-helix DNA-binding domain (DBD) containing conserved tryptophan residues and a C-terminal interferon activation domain (IAD) shown to be critical in mediating protein-protein interaction *(Remesh SG, Santosh V, Escalante CR. Structural Studies of IRF4 Reveal a Flexible Autoinhibitory Region and a Compact Linker Domain. J Biol Chem. 2015 Nov;290(46):27779–90*). The IRF4 protein contains unlike other IRF members at its C-terminal end a flexible autoinhibitory region which directly binds the DBD and modulates interaction with target DNA *(Remesh SG, Santosh V, Escalante CR. Structural Studies of IRF4 Reveal a Flexible Autoinhibitory Region and a Compact Linker Domain. J Biol Chem. 2015 Nov;290(46):27779–90*).

Several reports have provided important insights about the role of IRF4 in Treg biology. In mouse CD4+ T cells, the co-transduction of *IRF4* and *FOXP3* transgenes induces dramatic changes in the transcriptomic profile and recapitulates most of the hallmark Treg transcriptomic signature (*Fu, W. et al. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. Nat. Immunol. 13, 972–980 (2012)*). In contrast, the transduction of *FOXP3* alone in CD4+ T cells is not enough to induce the Treg transcriptomic signature. This finding indicates that FOXP3 and IRF4 collaborates in shaping Treg-specific program. Furthermore, Treg-specific IRF4 expression was found to endow Tregs with the ability to suppress Th2-mediated immune responses (*Zheng, Y. et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature 458, 351–356 (2009)*). More recently, IRF4 emerged as a key instructor to commit human Tregs into an effector and highly suppressive differentiation pathway (*Alvisi, G. et al. IRF4 instructs effector Treg differentiation and immune suppression in human cancer. J. Clin. Invest. 130, 3137–3150 (2020)*).

The inventors recently identified a novel *IRF4* mutation in a family with a primary immune deficiency (PID), which combines hypogammaglobulinemia and skin manifestation. However, the role of said variant in the context of Tregs has never been investigated.

SUMMARY OF THE INVENTION:

The present invention is defined by the claims. In particular, the present invention relates to the use of the F359L missense IRF4 variant for increasing the stability of regulatory T cells.

DETAILED DESCRIPTION OF THE INVENTION:

Recently, the inventors identified a missense *IRF4* gene variant (Chr6: 401753; hg19 build *137*; NM_002460.3, exon 7, c.1075 T>C, p.F359L) in a family with a primary immune deficiency (PID), which combines hypogammaglobulinemia and skin manifestation. The variant is located in the interferon activation domain (IAD) of the IRF4 protein. Unexpectedly, the transduction of CD4+ T cells with a lentiviral vector encoding either the wild-type or the mutated IRF4 gene, induced a strong and sustained CD25 expression in the latter compared to the former. The inventors extended this finding by showing a similar result in Tregs, along with enhanced expression of HELIOS, a Treg transcription factor, linked to IL-2-dependent Treg stability (*Kim, H.-J. et al. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. Science 350, 334–339 (2015)*). In line with this, the inventors found that Tregs expressing the IRF4 variant demonstrate an enhanced IL-2 sensitivity. Accordingly, the results suggest that transgenic expression of this IRF4 mutant in Tregs might push them toward highly suppressive and stable effector cells.

Main definitions:

As used herein, the terms “**polypeptide**”, “**peptide**”, and “**protein**” are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component. Polypeptides when discussed in the context of gene therapy refer to the respective intact polypeptide, or any fragment or genetically engineered derivative thereof, which retains the desired biochemical function of the intact protein.

As used herein, the term “**polynucleotide**” refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two

complementary single-stranded forms known or predicted to make up the double-stranded form.

As used herein, the expression "**derived from**" refers to a process whereby a first component (e.g., a first polypeptide), or information from that first component, is used to isolate, derive or make a different second component (e.g., a second polypeptide that is different from the first one).

As used herein, the term "**mutation**" has its general meaning in the art and refers to a substitution, deletion or insertion. In particular, the term "**substitution**" means that a specific amino acid residue at a specific position is removed and another amino acid residue is inserted into the same position. Within the specification, the mutation are references according to the standard mutation nomenclature.

As used herein, the "**percent identity**" between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described below. The percent identity between two amino acid sequences can be determined using the Needleman and Wunsch algorithm (Needleman, Saul B. & Wunsch, Christian D. (1970). "A general method applicable to the search for similarities in the amino acid sequence of two proteins". Journal of Molecular Biology. 48 (3): 443–53.). The percent identity between two nucleotide or amino acid sequences may also be determined using for example algorithms such as EMBOSS Needle (pair wise alignment; available at www.ebi.ac.uk). For example, EMBOSS Needle may be used with a BLOSUM62 matrix, a "gap open penalty" of 10, a "gap extend penalty" of 0.5, a false "end gap penalty", an "end gap open penalty" of 10 and an "end gap extend penalty" of 0.5. In general, the "percent identity" is a function of the number of matching positions divided by the number of positions compared and multiplied by 100. For instance, if 6 out of 10 sequence positions are identical between the two compared sequences after alignment, then the identity is 60%. The % identity is typically determined over the whole length of the query sequence on which the analysis is performed. Two molecules having the same primary amino acid sequence or nucleic acid sequence are identical irrespective of any chemical and/or biological

modification. According to the invention, a first amino acid sequence having at least 90% of identity with a second amino acid sequence means that the first sequence has 90; 91; 92; 93; 94; 95; 96; 97; 98; 99 or 100% of identity with the second amino acid sequence.

5 As used herein, the term "**conservative sequence modifications**" refers to amino acid modifications that do not significantly affect or alter the biologic function of the protein containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into a protein by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated 10 mutagenesis. A “**conservative substitution**” is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, 15 hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. Amino acid substitutions may further be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or 20 the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent 25 conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Other families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, 30 serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

As used herein, the term “**engineered**” refers to an aspect of having been manipulated and altered by the hand of man. In particular, the term “**engineered cell**” refers to a cell that has been subjected to a manipulation, so that its genetic, epigenetic, and/or phenotypic identity is altered relative to an appropriate reference cell such as otherwise identical cell that has not been so manipulated. In some embodiments, the manipulation is or comprises a genetic manipulation. In some embodiments, a genetic manipulation is or comprises one or more of (i) introduction of a nucleic acid not present in the cell prior to the manipulation (i.e., of a heterologous nucleic acid); (ii) removal of a nucleic acid, or portion thereof, present in the cell prior to the manipulation; and/or (iii) alteration (e.g., by sequence substitution) of a nucleic acid, or portion thereof, present in the cell prior to the manipulation. In some embodiments, a an engineered cell is one that has been manipulated so that it contains and/or expresses a particular agent of interest (e.g., a protein, a nucleic acid, and/or a particular form thereof) in an altered amount and/or according to altered timing relative to such an appropriate reference cell. Those of ordinary skill in the art will appreciate that reference to an “**engineered cell**” herein may, in some embodiments, encompass both the particular cell to which the manipulation was applied and also any progeny of such cell.

As used herein, the term “**expression**” of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (e.g., by transcription); (2) processing of an RNA transcript (e.g., by splicing, editing, 5′ cap formation, and/or 3′ end formation); (3) translation of an RNA into a polypeptide or protein; and/or (4) post-translational modification of a polypeptide or protein.

As used herein, the term "**encoding**" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as, for example, a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*e.g.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified,

a "**polynucleotide sequence encoding an amino acid sequence**" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase "**polynucleotide sequence that encodes a protein or a RNA**" may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

As used herein, the term "**IRF4**" has its general meaning in the art and refers to the interferon regulatory factor 4 encoded by the *IRF4* gene. The term is also known as NF-EM5, Pip, LSIRF, ICSAT, or MUM1. IRF4 belongs to a family of nine human IRF transcription factors (*De Silva NS, Simonetti G, Heise N, Klein U. The diverse roles of IRF4 in late germinal center B-cell differentiation: IRF4 in late GC B-cell differentiation. Immunol Rev. 2012 May;247(1):73–92*) and is in contrast to the other members of this family not regulated by interferons. An exemplary amino acid sequence of IRF4 is represented by SEQ ID NO:1.

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SEQ ID NO:1 >sp|Q15306|IRF4_HUMAN Interferon regulatory factor 4
OS=Homo sapiens OX=9606 GN=IRF4 PE=1 SV=1
MNLEGGGRGGEFGMSAVSCGNGKLRQWLIDQIDSGKYPGLVWENEEKSIFRIPWKHAGKQ
DYNREEDAALFKAWALFKGKFREGIDKPDPPTWKTRLRCALNKSNDFEELVERSQLDISD
PYKVYRIVPEGAKKGAKQLTLEDPQMSMSHPYTMTTPYPSLPAQQVHNYMMPPLDRSWRD
YVPDQPHPEIPYQCPMTFGPRGHHWQGPACENGCQVTGTFYACAPPESQAPGVPTEPSIR
SAEALAFSDCRLHICLYYREILVKELTTSSPEGCRISHGHTYDASNLDQVLFPYPEDNGQ
RKNIEKLLSHLERGVVLWMAPDGLYAKRLCQSRIYWDGPLALCNDRPNKLERDQTCKLFD
TQQFLSELQAFAHHGRSLPRFQVTLCFGEEFPDPQRQRKLITAHVEPLLARQLYYFAQQN
SGHFLRGYDLPEHISNPEDYHRSIRHSSIQE
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As used herein, the term "**subject**", "**host**", "**individual**" or "**patient**" refers to a mammal, preferably a human being, male or female at any age that is in-need of a therapy.

As used herein, the term "**organ**" refers to a solid vascularized organ that performs a specific function or group of functions within an organism. The term organ includes, but is not limited to, heart, lung, kidney, liver, pancreas, skin, uterus, bone, cartilage, small or large bowel, bladder, brain, breast, blood vessels, esophagus, fallopian tube, gallbladder, ovaries, pancreas, prostate, placenta, spinal cord, limb including upper and lower, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, uterus.

As used herein, the term "**tissue**" refers to any type of tissue in human or animals, and includes, but is not limited to, vascular tissue, skin tissue, hepatic tissue, pancreatic tissue, neural tissue, urogenital tissue, gastrointestinal tissue, skeletal tissue including bone and cartilage, adipose

tissue, connective tissue including tendons and ligaments, amniotic tissue, chorionic tissue, dura, pericardia, muscle tissue, glandular tissue, facial tissue, ophthalmic tissue.

As used herein, the term "**cell**" refers to any eukaryotic cell. In some embodiments the cells are selected from the group consisting of multipotent hematopoietic stem cells derived from bone marrow, peripheral blood, or umbilical cord blood; or pluripotent (i.e. embryonic stem cells (ES) or induced pluripotent stem cells (iPS)) or multipotent stem cell-derived differentiated cells of different cell lineages such as cardiomyocytes, beta-pancreatic cells, hepatocytes, neurons, etc…

As used herein, the term "**population**" refers to a population of cells, wherein the majority (e.g., at least about 50%, preferably at least about 60%, more preferably at least about 70%, and even more preferably at least about 80%) of the total number of cells have the specified characteristics of the cells of interest and express the markers of interest (e.g. a population of human CAR-Tregs comprises at least about 50%, preferably at least about 60%, more preferably at least about 70%, and even more preferably at least about 80% of cells which have the highly suppressive functions and which express the particular markers of interest).

As used herein, the term "**Treg**" or "**T regulatory cell**" denotes a T lymphocyte endowed with a given antigen specificity imprinted by the TCR it expresses and with regulatory properties defined by the ability to suppress the response of conventional T lymphocytes or other immune cells. Such responses are known in the art and include, but are not limited to, cytotoxic activity against antigen-presenting target cells and secretion of different cytokines. Different types of Tregs exist and include, but are not limited to: inducible and thymic-derived Tregs, as characterized by different phenotypes such as CD4+CD25+/high, CD4+CD25+/highCD127-/low alone or in combination with additional markers that include, but are not limited to, FoxP3, neuropilin-1 (CD304), glucocorticoid-induced TNFR-related protein (GITR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152); T regulatory type 1 cells; T helper 3 cells. In some embodiments, the Treg is CD4+ Foxp3+ Treg or a CD8+ Foxp3+ Tregs or a CD4+CD45RC$^{low/-}$ Treg or a CD8+CD45RC$^{low/-}$ cells or CD4+Foxp3- Tr1 Tregs. All these Tregs can be modified with the IRF4 variant and / or transformed with the CAR of the present invention either upon direct ex vivo purification or upon in vitro expansion or differentiation from different precursor cells. Examples of in vitro amplification protocols can be found in

Battaglia et al., J. Immunol. 177:8338-8347 (2006), Putnam et al., Diabetes 58:652-662 (2009), Gregori et al., Blood 116:935-944 (2009).

As used herein, the term "**chimeric antigen receptor**" or "**CAR**" has its general meaning in
5 the art and refers to an artificially constructed hybrid protein or polypeptide containing the antigen binding domains of an antibody (e.g., scFv) linked to T- cell signalling domains. Characteristics of CARs include their ability to redirect T-cell specificity and reactivity toward a selected target in a non-MHC-restricted manner, exploiting the antigen-binding properties of monoclonal antibodies. Moreover, when expressed in T-cells, CARs advantageously do not
10 dimerize with endogenous T cell receptor (TCR) alpha and beta chains. The chimeric antigen receptor of the present invention typically comprises an extracellular hinge domain, a transmembrane domain, and an intracellular T cell signalling domain.

As used herein the term "**CAR-T cell**" refers to a T lymphocyte that has been genetically
15 engineered to express a CAR. The T lymphocytes that are genetically modified may be "derived" or "obtained" from the patient who will receive the treatment using the genetically modified T cells or they may be "derived" or "obtained" from a different patient. Thus the term "**CAR-Treg**" refers to a Treg that has been genetically engineered to express a CAR.

20 As used herein, the term "**antigen**" has its general meaning in the art and generally refers to a substance or fragment thereof that is recognized and selectively bound by an antibody or by a T cell antigen receptor, resulting in induction of an immune response. Antigens according to the invention are typically, although not exclusively, peptides and proteins. Antigens may be natural or synthetic and generally induce an immune response that is specific for that antigen.

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As used herein, the term "**auto-antigen**" means any self-antigen arising from the own body tissues which is mistakenly recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors.

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As used herein, the term "**HLA-A2**" has its general meaning in the art and refers to a HLA serotype within the HLA-A 'A' serotype group and is encoded by the HLA-A*02 allele group including the HLA-A*02:01, HLA-A*02:02, HLA-A*02:03, HLA-A*02:05, HLA-A*02:06, HLA-A*02:07 and HLA-A*02:11 gene products. HLA-A2 is very common in the Caucasian

population (40-50%) and provides an ideal cellular target for the first portion because it will be suitable for use in a high proportion of combinations of HLA-A2+ donors and HLA-A2- recipients.

As used herein the term "**antibody**" and "**immunoglobulin**" have the same meaning, and will be used equally in the present invention. The term "**antibody**" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. As such, the term antibody encompasses not only whole antibody molecules, but also antibody fragments as well as variants (including derivatives) of antibodies and antibody fragments. In natural antibodies, two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (l) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes three (α, δ, γ) to five (μ, ε) domains, a variable domain (VH) and three to four constant domains (CH1, CH2, CH3 and CH4 collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin and consists of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) can participate to the antibody binding site or influence the overall domain structure and hence the combining site. CDRs refer to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. The light and heavy chains of an immunoglobulin each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. An antigen-binding site, therefore, typically includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. Framework Regions (FRs) refer to amino acid sequences interposed between CDRs. The residues in

antibody variable domains are conventionally numbered according to a system devised by Kabat et al. This system is set forth in Kabat et al., 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA (hereafter "Kabat et al."). This numbering system is used in the present specification. The Kabat residue designations do not always correspond directly with the linear numbering of the amino acid residues in SEQ ID sequences. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or complementarity determining region (CDR), of the basic variable domain structure. The correct Kabat numbering of residues may be determined for a given antibody by alignment of residues of homology in the sequence of the antibody with a "standard" Kabat numbered sequence. The CDRs of the heavy chain variable domain are located at residues 31-35B (H-CDR1), residues 50-65 (H-CDR2) and residues 95-102 (H-CDR3) according to the Kabat numbering system. The CDRs of the light chain variable domain are located at residues 24-34 (L-CDR1), residues 50-56 (L-CDR2) and residues 89-97 (L-CDR3) according to the Kabat numbering system.

As used herein, the terms "**monoclonal antibody**", "**monoclonal Ab**", "**monoclonal antibody composition**", "**mAb**", or the like, as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody is obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprised in the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

As used herein the term "**human antibody**" as used herein, is intended to include antibodies having variable and constant regions derived from human immunoglobulin sequences. The human antibodies of the present invention may include amino acid residues not encoded by human immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "**human antibody**", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

As used herein, the term "**chimeric antibody**" refers to an antibody which comprises a VH domain and a VL domain of a non-human antibody, and a CH domain and a CL domain of a

human antibody. In some embodiments, a "**chimeric antibody**" is an antibody molecule in which (a) the constant region (*i.e.*, the heavy and/or light chain), or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity. Chimeric antibodies also include primatized and in particular humanized antibodies. Furthermore, chimeric antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. For further details, see Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). (see U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)).

As used hereon, the term "**humanized antibody**" refers to an antibody having variable region framework and constant regions from a human antibody but retains the CDRs of a previous non-human antibody. In some embodiments, a humanized antibody contains minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies and antibody fragments thereof may be human immunoglobulins (recipient antibody or antibody fragment) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, a humanized antibody/antibody fragment can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. Such antibodies are designed to maintain the binding specificity of the non-human antibody from which the binding regions are derived, but to avoid an immune reaction against the non-human antibody. These modifications can further refine and optimize antibody or antibody fragment performance. In general, the humanized antibody or antibody fragment thereof will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or a significant portion of the FR regions are those of a human immunoglobulin sequence. The humanized antibody or antibody fragment can also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human

immunoglobulin. For further details, see Jones et al., Nature, 321: 522-525, 1986; Reichmann et al., Nature, 332: 323-329, 1988; Presta, Curr. Op. Struct. Biol., 2: 593-596, 1992.

As used herein, the term "**antibody fragment**" refers to at least one portion of an intact antibody, preferably the antigen binding region or variable region of the intact antibody, that retains the ability to specifically interact with (*e.g.*, by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. "**Fragments**" comprise a portion of the intact antibody, generally the antigen binding site or variable region. Examples of antibody fragments include Fab, Fab', Fab'-SH, F(ab')2, and Fv fragments; diabodies; any antibody fragment that is a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "**single-chain antibody fragment**" or "**single chain polypeptide**"), including without limitation (1) single - chain Fv molecules (2) single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific antibodies formed from antibody fragments. Fragments of the present antibodies can be obtained using standard methods.

As used herein, the term "**scFv**" refers to a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked, e.g., via a synthetic linker, e.g., a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the specificity of the intact antibody from which it is derived. Unless specified, as used herein an scFv may have the VL and VH variable regions in either order, e.g., with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

As used herein, the term "**specificity**" refers to the ability of an antibody to detectably bind target molecule (e.g. an epitope presented on an antigen) while having relatively little detectable reactivity with other target molecules. Specificity can be relatively determined by binding or competitive binding assays, using, *e.g.*, Biacore instruments, as described elsewhere herein.

Specificity can be exhibited by, *e.g.*, an about 10:1, about 20:1, about 50:1, about 100:1, 10.000:1 or greater ratio of affinity/avidity in binding to the specific antigen versus nonspecific binding to other irrelevant molecules.

5 The term "**affinity**", as used herein, means the strength of the binding of an antibody to a target molecule (e.g. an epitope). The affinity of a binding protein is given by the dissociation constant Kd. For an antibody said Kd is defined as [Ab] x [Ag] / [Ab-Ag], where [Ab-Ag] is the molar concentration of the antibody-antigen complex, [Ab] is the molar concentration of the unbound antibody and [Ag] is the molar concentration of the unbound antigen. The affinity constant Ka 10 is defined by 1/Kd. Preferred methods for determining the affinity of a binding protein can be found in Harlow, et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Coligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, Meth. Enzymol. 92:589-601 (1983), which references are entirely incorporated herein by reference. 15 One preferred and standard method well known in the art for determining the affinity of binding protein is the use of Biacore instruments.

The term "**binding**" as used herein refers to a direct association between two molecules, due to, for example, covalent, electrostatic, hydrophobic, and ionic and/or hydrogen-bond 20 interactions, including interactions such as salt bridges and water bridges. In particular, as used herein, the term "**binding**" in the context of the binding of an antibody to a predetermined target molecule (e.g. an antigen or epitope) typically is a binding with an affinity corresponding to a K_D of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less.

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As used herein, the term "**tolerance**" refers to a failure to respond, or a reduced response, to an antigen (including auto-antigens, allergens and endogenously administered molecules). This may mean that a productive (immunogenic) response is not induced upon endogenous or exogenous exposure to said antigen. This response may be replaced, in part or completely, by 30 a tolerogenic response, i.e. an active process that further limits immunogenic responses. Examples of tolerogenic responses include, but are not limited to, generation of T regulatory cells, elimination of effector (conventional) T cells by apoptosis or their neutralization by anergy, and skewing of T cells and other immune cells towards phenotypes favouring a state of tolerance, e.g. production of regulatory cytokines such as interleukin-10 and TGF-β and of other

anti-inflammatory mediators and downregulated expression of co-stimulatory molecules. These immunological concepts are well known to the skilled in the art.

As used herein, the term "**transplantation**" and variations thereof refers to the insertion of a transplant (also called graft) into a recipient, whether the transplantation is syngeneic (where the donor and recipient are genetically identical), allogeneic (where the donor and recipient are of different genetic origins but of the same species), or xenogeneic (where the donor and recipient are from different species). Thus, in a typical scenario, the host is human and the graft is an isograft, derived from a human of the same or different genetic origins. In another scenario, the graft is derived from a species different from that into which it is transplanted, including animals from phylogenically widely separated species, for example, a baboon heart being transplanted into a human host. In some embodiments the donor of the transplant is a human. The donor of the transplant can be a living donor or a deceased donor, namely a cadaveric donor. In some embodiments, the transplant is an organ, a tissue or cells.

As used herein, the expression "**preventing or reducing transplant rejection**" is meant to encompass prevention or inhibition of immune transplant rejection, as well as delaying the onset or the progression of immune transplant rejection. The term is also meant to encompass prolonging survival of a transplant in a patient, or reversing failure of a transplant in a patient. Further, the term is meant to encompass ameliorating a symptom of an immune transplant rejection, including, for example, ameliorating an immunological complication associated with immune rejection, such as for example, interstitial fibrosis, chronic graft arteriosclerosis, or vasculitis.

As used herein, the term "**transplant rejection**" encompasses both acute and chronic transplant rejection. "**Acute rejection**" is the rejection by the immune system of a tissue transplant recipient when the transplanted tissue is immunologically foreign. Acute rejection is characterized by infiltration of the transplant tissue by immune cells of the recipient, which carry out their effector function and destroy the transplant tissue. The onset of acute rejection is rapid and generally occurs in humans within a few weeks after transplant surgery. Generally, acute rejection can be inhibited or suppressed with immunosuppressive drugs such as rapamycin, cyclosporin and the like. "**Chronic rejection**" generally occurs in humans within several months to years after engraftment, even in the presence of successful

immunosuppression of acute rejection. Fibrosis is a common factor in chronic rejection of all types of organ transplants.

As used herein, the term "**Graft- Versus-Host Disease**" or "**GVHD**" refers to a common and serious complication wherein there is a reaction of donated immunologically competent lymphocytes against a transplant recipient's own tissues. GVHD is a possible complication of any transplant that uses or contains hematopoietic stem cells from either a related or an unrelated donor.

As used herein, the term "**autoimmune disease**" refers to the presence of an autoimmune response (an immune response directed against an auto- or self-antigen) in a subject. Autoimmune diseases include diseases caused by a breakdown of self-tolerance such that the adaptive immune system, in concert with cells of the innate immune system, responds to self-antigens and mediates cell and tissue damage. In some embodiments, autoimmune diseases are characterized as being a result of, at least in part, a humoral and/or cellular immune response.

As used herein, the term "**allergy**" generally refers to an inappropriate immune response characterized by inflammation and includes, without limitation, food allergies, respiratory allergies and other allergies causing or with the potential to cause a systemic response such as, by way of example, Quincke's oedema and anaphylaxis.

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

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

As used herein, the term “**pharmaceutical composition**” refers to a composition described
15 herein, or pharmaceutically acceptable salts thereof, with other agents such as carriers and/or excipients. The pharmaceutical compositions as provided herewith typically include a pharmaceutically acceptable carrier.

As used herein, the term “**pharmaceutically acceptable carrier**” includes any and all solvents,
20 diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical-Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the
25 preparation thereof.

The Tregs of the present invention

The first object of the present invention related to a regulatory T cell (Treg) that is engineered
30 to express a polypeptide that comprises an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated.

In some embodiments, the amino acid residue (F) at position 359 is substituted. In some embodiments, the amino acid residue (F) at position 359 is substituted by a leucine (L) residue.

Therefore, in some embodiments, the regulatory T cell (Treg) is engineered to express a polypeptide that comprises an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 and that comprises the mutation F359L.

In some embodiments, the Treg of the present invention is also engineered to express a chimeric antigen receptor (CAR) having specificity for an antigen of interest.

In some embodiments, the CAR typically comprises an ectodomain (extracellular domain) and an endodomain (cytoplasmic domain), joined by a transmembrane domain. The ectodomain, expressed on the surface of the cell, comprises an antigen binding domain or receptor domain and optionally a spacer (or hinge) region linking the antigen binding domain to the transmembrane domain. The transmembrane domain is typically a hydrophobic alpha helix that spans across the lipid bilayer of the cell membrane. The endodomain of the CAR is composed of an intracellular signalling module that induces Treg activation upon antigen binding. The endodomain may include several signalling domains, as explained infra.

The extracellular domain of the CAR thus comprises an antigen binding domain that specifically binds or recognizes the antigen of interest.

In some embodiments, such antigen binding domain is an antibody, preferably a single chain antibody. Preferably, the antibody is a humanized antibody. Particularly, such antigen binding domain is an antibody fragment selected from fragment antigen binding (Fab) fragments, F(ab')2 fragments, Fab' fragments, Fv fragments, recombinant IgG (rlgG) fragments, single chain antibody fragments, single chain variable fragments (scFv), single domain antibodies (e.g., sdAb, sdFv, nanobody) fragments, diabodies, and multi-specific antibodies formed from antibody fragments. In some embodiments, the antibodies are single-chain antibody fragments comprising a variable heavy chain region and/or a variable light chain region, such as scFv. Particularly, such antigen binding domain is selected from a Fab and a scFv.

In some embodiments, when the antigen targeting domain is a scFv, the scFv can be derived from the variable heavy chain (VH) and variable light chain (VL) regions of an antigen-specific

mAb linked by a flexible linker. The scFv retains the same specificity and a similar affinity as the full antibody from which it is derived. The peptide linker connecting scFv VH and VL domains joins the carboxyl terminus of one variable region domain to the amino terminus of the other variable domain without compromising the fidelity of the VH–VL paring and antigen-binding sites. Peptide linkers can vary from 10 to 30 amino acids in length. In some embodiments, the scFv peptide linker is a Gly/Ser linker and comprises one or more repeats of the amino acid sequence Gly-Gly-Gly-Ser or Gly-Gly-Gly-Gly-Ser.

In some embodiments, the scFv is specific for HLA-A2. In some embodiments, the scFv comprises the amino acid sequence as set forth in SEQ ID NO:2.

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SEQ ID NO : 2>scFv_anti-HLA-A2
DVVMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGT
DFTFTISSLQPEDFATYYCQQYSSFPLTFGGGTKVDIKGGGGSGGGGSGGGGSQVQLVQSGGGVVQPGG
SLRVSCAASGVTLSDYGMHWVRQAPGKGLEWMAFIRNDGSDKYYADSVKGRFTISRDNSKKTVSLQMSS
LRAEDTAVYYCAKNGESGPLDYWYFDLWGRGTLVTV
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The CAR optionally comprises a spacer or hinge domain linking the antigen binding domain to the transmembrane domain.

In some embodiments, the CAR comprises a hinge sequence between the antigen binding domain and the transmembrane domain and/or between the transmembrane domain and the cytoplasmic domain. One ordinarily skilled in the art will appreciate that a hinge sequence is a short sequence of amino acids that facilitates flexibility. In particular, the spacer or hinge domain linking the antigen binding domain to the transmembrane domain is designed to be sufficiently flexible to allow the antigen binding domain to orient in a manner that allows antigen recognition. The hinge may be derived from or include at least a portion of an immunoglobulin Fc region, for example, an IgG1 Fc region, an IgG2 Fc region, an IgG3 Fc region, an IgG4 Fc region, an IgE Fc region, an IgM Fc region, or an IgA Fc region. In certain embodiments, the hinge domain includes at least a portion of an IgG1, an IgG2, an IgG3, an IgG4, an IgE, an IgM, or an IgA immunoglobulin Fc region that falls within its CH2 and CH3 domains. Exemplary hinges include, but are not limited to, a CD8a hinge, a CD28 hinge, IgG1/IgG4 (hinge-Fc part) sequences, IgG4 hinge alone, IgG4 hinge linked to CH2 and CH3 domains, or IgG4 hinge linked to the CH3 domain, those described in Hudecek et al. (2013) Clin. Cancer Res., 19:3153, international patent application publication number WO2014031687, U.S. Pat. No. 8,822,647 or published app. No. US2014/0271635. As hinge

domain, the invention relates to all or a part of residues 118 to 178 of CD8a (GenBank Accession No. NP_001759.3), residues 135 to 195 of CD8 (GenBank Accession No. AAA35664), residues 315 to 396 of CD4 (GenBank Accession No. NP_000607.1), or residues 137 to 152 of CD28 (GenBank Accession No. NP_006130.1) can be used. Also, as the spacer domain, a part of a constant region of an antibody H chain or L chain (CHI region or CL region) can be used. Further, the spacer domain may be an artificially synthesized sequence. In some embodiments, for example, the hinge sequence is derived from a CD8 alpha molecule or a CD28 molecule.

The transmembrane domain of the CAR functions to anchor the receptor on the cell surface. The choice of the transmembrane domain may depend on the neighbouring spacer and intracellular sequences.

In some embodiments, the transmembrane domain is derived either from a natural or from a synthetic source. Where the source is natural, the domain in some aspects is derived from any membrane -bound or transmembrane protein. Transmembrane regions include those derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T- cell receptor, CD28, CD3 zeta, CD3 epsilon, CD3 gamma, CD3 delta, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, ICOS/CD278, GITR/CD357, NKG2D, and DAP molecules. Alternatively the transmembrane domain in some embodiments is synthetic. In some aspects, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In some aspects, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. A transmembrane domain is thermodynamically stable in a membrane. It may be a single alpha helix, a transmembrane beta barrel, a beta-helix of gramicidin A, or any other structure. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the intracellular signalling domain(s) of the CAR. A glycine-serine doublet may provide a suitable linker.

The role of the intracellular domain of the CAR is to produce an activation signal to the Treg as soon as the extracellular domain has recognized the antigen. In particular, the intracellular domain of the CAR triggers or elicits activation of at least one of the normal effector functions of the Treg. Examples of intracellular domain sequences that are of particular use in the

invention include those derived from an intracellular signalling domain of a lymphocyte receptor chain, a TCR/CD3 complex protein, an Fc receptor subunit, an IL-2 receptor subunit, CD3ζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, CD66d, CD278(ICOS), FcsRI, DAP10, and DAP12. It is particularly preferred that the intracellular domain in the CAR comprises a cytoplasmic signalling sequence derived from CD3ζ. The intracellular domain of the CAR can be designed to comprise a signalling domain (such as the CD3ζ signalling domain) by itself or combined with costimulatory domain(s). A costimulatory molecule can be defined as a cell surface molecule that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, CD244 (2B4), ICOS, lymphocyte function-associated antigen- 1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, CD8, CD4, b2c, CD80, CD86, DAP10, DAP12, MyD88, BTNL3, and NKG2D. The intracellular signalling portion of the above recited co-stimulatory domains can be used alone or in combination with other co-stimulatory domains. In particular, the CAR can comprise any combination of two or more co-stimulatory domains from the group consisting of CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, CD244 (2B4), ICOS, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, CD8, CD4, b2c, CD80, CD86, DAP10, DAP12, MyD88, BTNL3, and NKG2D.

The CAR of the invention may be a first generation, a second generation, or a third generation CAR as described hereabove. Preferably, the CAR is a second or third generation CAR. Typically, "**first-generation CARs**" contain a single signalling domain. CARs containing a signalling domain together with one additional costimulatory domain are termed "**second generation**" while those containing a signalling domain together with two additional costimulatory domains are listed as "**third generation**". For example, first-generation CARs contain solely the CD3ζ chain as a single signalling domain. Second- and third-generation CARs consist of one or two additional costimulatory signalling domains, respectively, such as CD28, CD27, OX-40 (CD134) and 4-1BB (CD137). For example, second-generation CAR may contain CD3ζ and CD28 signalling domains, while third-generation CAR may contain CD3ζ, CD28 and either OX40 (CD134) or 4-1BB (CD137). "TRUCKs" represent the recently developed "**fourth-generation**" CARs. TRUCKs (T cells redirected for universal cytokine killing) are CAR-redirected T cells used as vehicles to produce and release a transgenic product that accumulates in the targeted tissue. The product, for example a pro-inflammatory cytokine, may be constitutively produced or induced once the T cell is activated by the CAR. Other

substances such as enzymes or immunomodulatory molecules may be produced in the same way and deposited by CAR-redirected T cells in the targeted lesion. This strategy involves two separate transgenes expressing for example (i) the CAR and (ii) a cell activation responsive promoter linked to a cytokine such as IL-12. Consequently, immune stimulatory cytokine such as IL-12 is secreted upon CAR engagement. In a particular embodiment, the CAR is a CAR of fourth generation as defined above.

Thus, for example, the CAR can be designed to comprise a signalling domain such as the CD3ζ signalling domain and a least one co-stimulatory signalling domain selected from CD28 and CD40, CD28 and 4-1BB (CD137), CD28 and OX40 (CD134), and CD28 and LFA-1. In some embodiments, the CAR of the present invention comprises the comprises the CD3ζ signalling domain and the 4-1BB co-stimulatory signalling domain as described in *Lamarthée B, Marchal A, Charbonnier S, Blein T, Leon J, Martin E, Rabaux L, Vogt K, Titeux M, Delville M, Vinçon H, Six E, Pallet N, Michonneau D, Anglicheau D, Legendre C, Taupin JL, Nemazanyy I, Sawitzki B, Latour S, Cavazzana M, André I, Zuber J. Transient mTOR inhibition rescues 4-1BB CAR-Tregs from tonic signal-induced dysfunction. Nat Commun. 2021 Nov 8;12(1):6446. doi: 10.1038/s41467-021-26844-1. PMID: 34750385; PMCID: PMC8575891.*

In some embodiments, the CAR of the present invention is specific for HLA-A2. In some embodiments, the CAR comprises the amino acid sequence as set forth in SEQ ID NO:3.

```
SEQ ID NO :3 >CAR (scFv anti-HLA2_signal-CD3z)
DVVMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGT
DFTFTISSLQPEDFATYYCQQYSSFPLTFGGGTKVDIKGGGGSGGGGSGGGGSQVQLVQSGGGVVQPGG
SLRVSCAASGVTLSDYGMHWVRQAPGKGLEWMAFIRNDGSDKYYADSVKGRFTISRDNSKKTVSLQMSS
LRAEDTAVYYCAKNGESGPLDYWYFDLWGRGTLVTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
AVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPY
APPRDFAAYRSRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL
YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
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In a particular embodiment, multiple CARs such as CARs binding to different antigens, may be expressed by a single Treg-cell.

Methods for preparing the Tregs of the present invention

The Treg of the present is preparing by any conventional method well known in the art. Typically, the Tregs are transduced in order to express a polynucleotide that encodes for the IRF4 variant of the present invention.

5 Thus, a further object of the present invention relates to a method of preparing a Treg of the present invention, comprising the steps consisting of introducing into a Treg a polynucleotide that encodes for the polypeptide comprising an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated.

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It is contemplated that a polynucleotide construct encoding the IRF4 variant can be introduced into the Tregs as naked DNA or in a suitable vector. Naked DNA generally refers to the DNA encoding the IRF4 variant contained in a plasmid expression vector in proper orientation for expression. Physical methods for introducing a polynucleotide construct into a host cell include 15 calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Other means can be used including colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. In some embodiments, the polynucleotide construct encoding the IRF4 variant is introduced into the 20 Treg by a viral vector that is an adeno-associated virus (AAV), a retrovirus, lentivirus, bovine papilloma virus, an adenovirus vector, a vaccinia virus, a polyoma virus, or an infective virus. In some embodiments, the vector is a retroviral. Retroviruses may be chosen as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and for 25 being packaged in special cell- lines. In order to construct a retroviral vector, a polynucleotide encoding for the IRF4 variant is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line is constructed containing the gag, pol, and/or env genes but without the LTR and/or packaging components. When a recombinant plasmid containing a cDNA, together with 30 the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media. The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of

cell types. Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes gag, pol, and env, contain other genes with regulatory or structural function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses (HIV 1, HIV 2) and the Simian Immunodeficiency Virus (SIV). Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes env, vif, vpr, vpu and nef are deleted making the vector biologically safe. Lentiviral vectors are known in the art, see, e.g. U.S. Pat. Nos. 6,013,516 and 5,994,136, both of which are incorporated herein by reference. In general, the vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection and for transfer of the nucleic acid into a host cell. The gag, pol and env genes of the vectors of interest also are known in the art. Thus, the relevant genes are cloned into the selected vector and then used to transform the target cell of interest. Recombinant lentivirus capable of infecting a non-dividing cell wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat is described in U.S. Pat. No. 5,994,136, incorporated herein by reference. This describes a first vector that can provide a nucleic acid encoding a viral gag and a pol gene and another vector that can provide a nucleic acid encoding a viral env to produce a packaging cell. Introducing a vector providing a heterologous gene into that packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest. The env preferably is an amphotropic envelope protein which allows transduction of cells of human and other species. Typically, the vector of the present invention include "**control sequences'**", which refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell. Another nucleic acid sequence, is a "**promoter**" sequence, which is used herein in its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence. Transcription promoters can include "**inducible promoters**" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), "**repressible promoters**" (where expression of a polynucleotide sequence operably linked to

the promoter is induced by an analyte, cofactor, regulatory protein, etc.), and "**constitutive promoters**". In some embodiments, the polynucleotide that encodes for the IRF4 variant is encoded by a nucleic acid molecule whose sequence has been codon optimized for expression in a mammalian cell. Codon optimization refers to the discovery that the frequency of 5 occurrence of synonymous codons (i.e., codons that code for the same amino acid) in coding DNA is biased in different species. Such codon degeneracy allows an identical polypeptide to be encoded by a variety of nucleotide sequences. A variety of codon optimization methods is known in the art, and include, e.g., methods disclosed in at least U.S. Pat. Nos. 5,786,464 and 6,114,148. In order to confirm the presence of the polynucleotide that encodes for the IRF4 10 variant in the Treg, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known such as Southern and Northern blotting, RT-PCR and quantitative PCR; or "biochemical" assays, such as detecting the presence or absence of a particular peptide.

15 The same approach as above described are used for engineering the Tregs for expressing a CAR of interest.

Thus, a further object of the present invention relates to a method of preparing a CAR-Treg of the present invention, comprising the steps consisting of introducing into a Treg i) a 20 polynucleotide that encodes for the polypeptide comprising an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated and ii) a polynucleotide that encodes for the CAR.

25 Use of bicistronic polynucleotides or vectors encoding for both the IRF4 variant and the CAR are particularly suitable for preparing the CAR-Tregs of the present invention. As used herein the term "**bicistronic**" refers to a polynucleotide or vector that comprises two cistrons, i.e. comprising two genes.

30 Once the population of Tregs is obtained, functionality of the cells may be evaluated according to any standard method which typically include a suppressive assay. Cell surface phenotype of the cells with the appropriate binding partners can also be confirmed. Quantifying the secretion of various cytokines may also be performed. Methods for quantifying secretion of a cytokine in a sample are well known in the art. For example, any immunological method such as but not

limited to ELISA, multiplex strategies, ELISPOT, immunochromatography techniques, proteomic methods, Western blotting, FACS, or Radioimmunoassays may be applicable to the present invention.

5 In particular, the Treg of the present invention is characterized by the expression of CD4, CD25, FOXP3, HELIOS and HLA-DR.

Methods of therapy:

10 A further object of the present invention relates to a method of inducing tolerance to one antigen of interest in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the population of Tregs of the present invention that are specific for said antigen.

15 The population of Tregs prepared as described above can be thus utilized in methods and compositions for adoptive immunotherapy in accordance with known techniques, or variations thereof that will be apparent to those skilled in the art based on the instant disclosure. See, e.g., US Patent Application Publication No. 2003/0170238 to Gruenberg et al; see also US Patent No. 4,690,915 to Rosenberg.

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Currently, most adoptive immunotherapies are autolymphocyte therapies (ALT) directed to treatments using the patient's own immune cells. These therapies involve processing the patient's own lymphocytes to enhance the tolerance response towards specific antigens. Typically, the treatments are accomplished by removing the patient's lymphocytes and exposing 25 these cells in vitro to biologics and drugs to convey them to a Treg profile. Once the Tregs are engineered to express the IRF4 variant, these ex vivo cells are reinfused into the patient to enhance the immune system to induce tolerance.

Pharmaceutical compositions:

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In some embodiments, the Tregs are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "**pharmaceutically acceptable**" carrier) in a treatment-effective amount.

Thus a further object of the present invention relates to a pharmaceutical composition comprising a population of Tregs of the present invention and a pharmaceutically acceptable carrier.

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Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin. A treatment-effective amount of cells in the composition is dependent on the relative
10 representation of the T cells with the desired specificity, on the age and weight of the recipient, on the severity of the targeted condition and on the immunogenicity of the targeted Ags. These amount of cells can be as low as approximately 10^3/kg, preferably $5x10^3$/kg; and as high as 10^7/kg, preferably 10^8/kg. The number of cells will depend upon the ultimate use for which the composition is intended, as will the type of cells included therein. For example, if cells that are
15 specific for a particular Ag are desired, then the population will contain greater than 70%, generally greater than 80%, 85% and 90-95% of such cells. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 ml or less, even 250 ml or 100 ml or less. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed the desired total amount of cells.

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Diseases:

In some embodiments, the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing, at least one symptom of a disease or condition caused
25 by inappropriate or unwanted immune system activity against an antigen. The subject may be identified or diagnosed as having done so or as likely to do so based on a variety of factors, for example, family history and/or genetic testing of e.g. the mother and/or father, siblings, other relatives (grandparents, aunts, uncles, cousins, etc.), presence of other disease biomarkers such as (auto)antibodies directed against different (self-)antigens. Generally, the subject is known to
30 have a genetic predisposition to development of an autoimmune disease, an allergy or other unwanted immune response. By "**is known to have a genetic predisposition**", we mean that one or both parents or siblings may have the disease or condition, and/or are known to be carriers of gene(s) that is/are associated with the disease or condition, so that the statistically probability of the subject having or developing the disease is at least 1, 5, 10, 20, 30, 40, 50,

60, 70, 80, 90%, or is 100%. The determination may be based on observation of the health of the parents, siblings or of the subject, or on genetic testing of the same and identification of a gene or genes in a form known to be associated with or to cause the disease or condition, e.g. to have a particular sequence such as an allele, mutation, insertion, deletion, etc. The risk of disease may or may not also be confirmed by genotypying subject’s cells and/or by assessing them by suitable biomarkers, including non-genetic biomarkers such as, by way of example, antibodies and other immune phenotypes or epigenetic modifications. Those of skill in the art will also recognize that such genetic traits may not be “all or nothing”, in that gene dosage may apply. Nevertheless, if a subject is deemed to be at risk, and if the life of a subject can be lengthened or improved by the practice of the present methods, then the subject is a viable candidate for treatment.

In some embodiments, the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an autoimmune disease. Examples of autoimmune disease include, without limitation, acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/Anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticaria, axonal and neuronal neuropathies, Behcet's disease, bullous pemphigoid, autoimmune cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal ostomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogans syndrome, cold agglutinin disease, congenital heart block, coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), discoid lupus, Dressler's syndrome, endometriosis, eosinophilic fasciitis, erythema nodosum, experimental allergic encephalomyelitis, Evans syndrome, fibromyalgia, fibrosing alveolitis, giant cell arteritis (temporal arteritis), glomerulonephritis, Goodpasture's syndrome, granulomatosis with polyangiitis (GPA), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis,

hypogammaglobulinemia, hypergammaglobulinemia, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, inflammatory bowel disease, insulin-dependent diabetes (type 1), interstitial cystitis, juvenile arthritis, Kawasaki syndrome, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease (LAD), lupus (SLE), Lyme disease, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease (MCTD), monoclonal gammopathy of undetermined significance (MGUS), Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's), autoimmune neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS syndrome, polyarteritis nodosa, type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatica, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis, psoriasis, psoriatic arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red cell aplasia, Raynaud's phenomenon, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, temporal arteritis/Giant cell arteritis, thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, transverse myelitis, ulcerative colitis, undifferentiated connective tissue disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, Waldenstrom's macroglobulinemia (WM), and Wegener's granulomatosis [Granulomatosis with Polyangiitis (GPA)].

In some embodiments, the subject is predisposed or believed to be predisposed to developing, or who has already developed or is developing an allergy. The term encompasses allergy, allergic disease, hypersensitive associated disease or respiratory disease associated with airway inflammation, such as asthma or allergic rhinitis. In some embodiments, the method of the present invention is effective in preventing, treating or alleviating one or more symptoms related to anaphylaxis, drug hypersensitivity, skin allergy, eczema, allergic rhinitis, urticaria,

atopic dermatitis, dry eye disease, allergic contact allergy, food hypersensitivity, allergic conjunctivitis, insect venom allergy, bronchial asthma, allergic asthma, intrinsic asthma, occupational asthma, atopic asthma, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). Hypersensitivity associated diseases or disorders that may be treated by the method of the present invention include, but are not limited to, anaphylaxis, drug reactions, skin allergy, eczema, allergic rhinitis, urticaria, atopic dermatitis, dry eye disease [or otherwise referred to as Keratoconjunctivitis sicca (KCS), also called keratitis sicca, xerophthalmia], allergic contact allergy, food allergy, allergic conjunctivitis, insect venom allergy and respiratory diseases associated with airway inflammation, for example, IgE mediated asthma and non-IgE mediated asthma. The respiratory diseases associated with airway inflammation may include, but are not limited to, rhinitis, allergic rhinitis, bronchial asthma, allergic (extrinsic) asthma, non-allergic (intrinsic) asthma, occupational asthma, atopic asthma, exercise induced asthma, cough-induced asthma, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD).

In some embodiments, the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an immune reaction against molecules that are exogenously administered for therapeutic or other purposes and may trigger an unwanted immune response. Non-limiting examples of this kind include immune reactions against replacement therapeutics in the context of genetic deficiencies, which include, but are not limited to, haemophilia A, haemophilia B, congenital deficiency of other clotting factors such as factor II, prothrombin and fibrinogen, primary immunodeficiencies (e.g. severe combined immunodeficiency, X-linked agammaglobulinemia, IgA deficiency), primary hormone deficiencies such as growth hormone deficiency and leptin deficiency, congenital enzymopathies and metabolic disorders such as disorders of carbohydrate metabolism (e.g. sucrose-isomaltase deficiency, glycogen storage diseases), disorders of amino acid metabolism (e.g. phenylketonuria, maple syrup urine disease, glutaric acidemia type 1), urea cycle disorders (e.g. carbamoyl phosphate synthetase I deficiency), disorders of organic acid metabolism (e.g. alcaptonuria, 2-hydroxyglutaric acidurias), disorders of fatty acid oxidation and mitochondrial metabolism (e.g. medium-chain acyl-coenzyme A dehydrogenase deficiency), disorders of porphyrin metabolism (e.g. porphyrias), disorders of purine or pyrimidine metabolism (e.g. Lesch-Nyhan syndrome), disorders of steroid metabolism (e.g. lipoid congenital adrenal hyperplasia, congenital adrenal hyperplasia), disorders of mitochondrial function (e.g. Kearns-Sayre syndrome), disorders of peroxisomal function (e.g. Zellweger syndrome), lysosomal

storage disorders (e.g. Gaucher's disease, Niemann Pick disease). In the case of genetic deficiencies, the proposed method may not only allow to reinstate immune tolerance against the replacement therapeutics that are used to treat the disease, but also reinstate the biological activity for which said therapeutics are administered. Other therapeutics for which said method may be suitable to limit undesired immune responses include other biological agents such as, by way of example, cytokines, monoclonal antibodies, receptor antagonists, soluble receptors, hormones or hormone analogues, coagulation factors, enzymes, bacterial or viral proteins. For example, hemophilic children can be treated prophylactically with periodic coagulation factor (e.g. factor VIII) replacement therapy, which decreases the chance of a fatal bleed due to injury. In addition to the expense and inconvenience of such treatment, repeated administration results in inhibitor antibody formation in some patients against the coagulation factor. If the antibodies in these patients are low titer antibodies, patients are treated with larger doses of blood coagulation factors. If the antibodies are high titer antibodies, treatment regimens for these patients become much more complex and expensive. In some embodiments, the therapeutic protein is produced in the subject following gene therapy suitable e.g. for the treatment of congenital deficiencies. Gene therapy typically involves the genetic manipulation of genes responsible for disease. One possible approach for patients, like those with hemophilia deficient for a single functional protein, is the transmission of genetic material encoding the protein of therapeutic interest. However, the repeated administration of gene therapy vectors, such as viral vectors, may also trigger unwanted immune responses against the therapeutic protein introduced in the vector and/or against other components of the vector. Thus, the method of the present invention can be suitable for overcoming the body's immune response to gene therapy vectors such as viral vectors. Viral vectors are indeed the most likely to induce an immune response, especially those, like adenovirus and adeno-associated virus (AAV), which express immunogenic epitopes within the organism. Various viral vectors are used for gene therapy, including, but not limited to, retroviruses for X-linked severe combined immunodeficiency (X-SCID); adenoviruses for various cancers; adeno-associated viruses (AAVs) to treat muscle and eye diseases; lentivirus, herpes simplex virus and other suitable means known in the art.

In some embodiments, the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an immune reaction against a grafted organ, a grafted tissue, or a grafted population of cells. Typically, the subject may have been transplanted with a graft selected from the group consisting of heart, kidney, lung, liver, pancreas, pancreatic islets, brain tissue, stomach, large intestine, small intestine, cornea, skin, trachea, bone, bone

marrow, muscle, or bladder. The method of the invention is indeed particularly suitable for preventing or suppressing an immune response associated with rejection of a donor tissue, cell, graft, or organ transplant by a recipient subject. Graft-related diseases or disorders include graft versus host disease (GVDH), such as associated with bone marrow transplantation, and immune disorders resulting from or associated with rejection of organ, tissue, or cell graft transplantation (e.g., tissue or cell allografts or xenografts), including, e.g., grafts of skin, muscle, neurons, islets, organs, parenchymal cells of the liver, etc. With regard to a donor tissue, cell, graft or solid organ transplant in a recipient subject, it is believed that the adoptive immunotherapy according to the invention may be effective in preventing acute rejection of such transplant in the recipient and/or for long-term maintenance therapy to prevent rejection of such transplant in the recipient (e.g., inhibiting rejection of insulin-producing islet cell transplant from a donor in the subject recipient suffering from diabetes). Thus, the adoptive immunotherapy of the invention is useful for preventing Host-Versus-Graft-Disease (HVGD) and Graft- Versus-Host-Disease (GVHD). Typically, Tregs of the present invention may be administered to the subject before and/or after transplantation. In some embodiments, Tregs of the present invention may be administered to the subject on a periodic basis before and/or after transplantation.

Antigens:

In some embodiments, the antigen is an auto-antigen. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors. It may be, by way of example, an auto-antigen of the following non-limiting list: acethylcholine receptor, actin, adenin nucleotide translocator, β-adrenoreceptor, aromatic L-amino acid decarboxylase, asioaloglycoprotein receptor, bactericidal/permeability increasing protein (BPi), calcium sensing receptor, cholesterol side chain cleavage enzyme, collagen type IV αγ-chain, cytochrome P450 2D6, desmin, desmoglein-1, desmoglein-3, F-actin, GM-gangliosides, glutamate decarboxylase, glutamate receptor, H/K ATPase, 17-α-hydroxylase, 21-hydroxylase, IA-2 (ICAS12), insulin, insulin receptor, intrinsic factor type 1, leucocyte function antigen 1, myelin associated glycoprotein, myelin basic protein, myelin oligodendrocyte protein, myosin, P80-coilin, pyruvate deshydrogenase complex E2 (PDC-E2), sodium iodide symporter, SOX-10, thyroid and eye muscle shared protein, thyroglobulin, thyroid peroxydase, thyrotropin receptor, tissue transglutaminase, transcription coactivator p75, tryptophan hydroxylase, tyrosinase, tyrosine hydroxylase, ACTH, aminoacyl-tRNA-hystidyl synthetase, cardiolipin, carbonic anhydrase II,

cebtromere associated proteins, DNA-dependant nucleosome-stimulated ATPase, fibrillarin, fibronectin, glucose 6 phosphate isomerase, beta 2-glycoprotein I, golgin (95, 97, 160, 180), heat shock proteins, hemidesmosomal protein 180, histone H2A, H2B, keratin, IgE receptor, Ku-DNA protein kinase, Ku-nucleoprotein, La phosphoprotein, myeloperoxydase, proteinase 3, RNA polymerase I-III, signal recognition protein, topoisomerase I, tubulin, vimenscin, myelin associated oligodendrocyte basic protein (MOBP), proteolipid protein, oligodendrocyte specific protein (OSP/Claudin 11), cyclic nucleotide 3′ phosphodiesterase (CNPase), BP antigen 1 (BPAGl-e), transaldolase (TAL), human mitochondrial autoantigens PDC-E2 (Novo 1 and 2), OGDC-E2 (Novo 3), and BCOADC-E2 (Novo 4), bullous pemphigoid (BP)180, laminin 5 (LN5), DEAD-box protein 48 (DDX48) or insulinoma-associated antigen-2.

In some embodiments, when the subject suffers from multiple sclerosis, the autoantigen is selected from the group consisting of myelin-related antigens (e.g. myelin basic protein (MBP) (e.g. MBP83-102 peptide), myelin oligodendrocyte glycoprotein (MOG) (e.g. MOG35-55 peptide) and proteolipid protein (PLP) (e.g. PLP139-151 peptide). When the subject suffers from Type I diabetes (T1D), the autoantigen is selected from the group consisting of insulin, insulin precursor proinsulin (Prolns), glutamic acid decarboxylase 65 (GAD65), glial fibrillary acidic protein (GFAP), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulinoma-associated antigen-2 (IA-2) and zinc transporter 8 (ZnT8). When the autoimmune disease is rheumatoid arthritis, the autoantigen is type II collagen (CTII).

In some embodiments, the antigen is a food antigen. In some embodiments, the food antigen is selected from non-limiting list: bovine antigens such as lipocalin, Ca-binding S100, alpha-lactalbumin, lactoglobulins such as beta-lactoglobulin, bovine serum albumin, caseins. Food-antigens may also be atlantic salmon antigens such as parvalbumin, chicken antigens such as ovomucoid, ovalbumin, Ag22, conalbumin, lysozyme or chicken serum albumin, peanuts, shrimp antigens such as tropomyosin, wheat antigens such as agglutinin or gliadin, celery antigens such as celery profilin, carrot antigens such as carrot profilin, apple antigens such as thaumatin, apple lipid transfer protein, apple profilin, pear antigens such as pear profilin, isoflavone reductase, avocado antigens such as endochitinase, apricot antigens such as apricot lipid transfer protein, peach antigens such as peach lipid transfer protein or peach profilin, soybean antigens such as HPS, soybean profilin or (SAM22) PR- 10 prot.

In some embodiments, the antigen is an inflammatory antigen selected from the group consisting of to myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), oligodendrocyte myelin oligoprotein (OMGP), myelin associated oligodendrocyte basic protein (MOBP), oligodendrocyte specific protein (OSP/Claudin 1), heat shock proteins, oligodendrocyte specific proteins (OSP), NOGO A, glycoprotein Po, peripheral myelin protein 22 (PMP22), 2',3'-cyclic nucleotide 3 -phosphodiesterase (CNPase), citrulline- substituted cyclic and linear filaggrin peptides, type II collagen peptides, human cartilage glycoprotein 39 (HCgp39) peptides, HSP, heterogeneous nuclear ribonucleoprotein (hnRNP) A2 peptides, hnRNP Bl, hnRNP D, Ro60/52, HSP60, 65, 70 and 90, BiP, keratin, vimentin, fibrinogen, collagen type I, III, IV and V peptides, annexin V, Glucose 6 phosphate isomerase (GPI), acetyl-calpastatin, pyruvate deshydrogenase (PDH), aldolase, topoisomerase I, snRNP, PARP, Scl-70, Scl-100, phospholipid antigen including anionic cardiolipin and phosphatidylserine, neutrally charged phosphatidylethanolamine and phosphatidylcholine, matrix metalloproteinase, fibrillin, and aggrecan.

In some embodiments, the antigen is an allergen in particular an inhaled allergen, an ingested allergen or a contact allergen. Examples of allergens include, but are not limited to, inhaled allergens derived from pollens (Cup, Jun), house dust mites (Der, Gly, Tyr, Lep), dog, cat and rodents (Can, Fel, Mus, Rat). Examples of contact allergens include, but are not limited to, heavy metals (such as nickel, chrome, gold), latex, haptens such as halothane, hydralazine.

In some embodiments, the antigen is as an allo-antigen. Allo-antigens include, but are not limited to, antigens expressed by the allograft, proteins expressed in the course of gene therapy (and also viral antigens issued from the viral vector used) as well as therapeutic proteins. The alloantigen is selected from the group consisting of HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, HLA-DM, HLA-DO, HLA-DP, HLA-DQ, HLA-DR, minor antigens such as HA-1, HA-2, HA-3, HA-8, HB-1, UGT2B 17, BCL2A1, HY B7, HY A2, HY Al, HY B60, HY B8, HY DQ5, HY DRB3, and blood group antigens A and B. In some embodiments, the alloantigen is HLA-A2.

In some embodiments, the antigen is a molecule that is exogenously administered for therapeutic or other purposes and may trigger an unwanted immune response. While frequently neutralising the biological activity that said molecules are meant to induce, such immune

responses may have additional deleterious effects unrelated to the purpose for which the molecules were originally administered. Examples of this kind include immune reactions against therapeutic clotting factor VIII in haemophilia A or factor IX in haemophilia B, against different enzymes in congenital enzymopathies and, more in general, during any kind of 5 replacement therapies in the context of genetic deficiencies. Allo-immunization responses against antigens expressed by tissues or hematopoietic and/or blood cells transplanted into an individual are equally considered.

The invention will be further illustrated by the following figures and examples. However, these 10 examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

15 **Figure 1.** (**A**) Experimental design. CD25+ CD127- CD45RA+ CD45RO- CD4+ naïve Tregs were sorted, activated and transduced with a lentiviral vector encoding either the wild-type *IRF4* gene and GFP, or the *IRF4* variant and GFP. Efficiently transduced cells were sorted as GFP+ cells at day 8, before phenotypic studies. **(B)** Representative dot plot showing a higher CD25 and HLA-DR expression (upper panel) and higher HELIOS expression (lower panel) in 20 Tregs transduced with the IRF4 variant compared to those transduced with the wild-type IRF4 or mock-transduced (GFP-negative cells). **(C)** Graphs depicting two (HLA-DR) or three (CD25 / HELIOS) independent experiments, where CD25 and HLA-DR expression in Tregs with enforced IRF4 expression is normalized to those of mock-transduced Tregs. MT: mock-transduced; WT: Wild-Type *IRF4*; Mut: mutated *IRF4*. (**D**) HLA-DR relative expression (when 25 normalized with untransduced Tregs)

Figure 2. Naïve or memory Tregs were sorted, activated and transduced with a lentiviral vector encoding either the reporter gene alone (empty), or the wild-type *IRF4* gene (WT), or the *IRF4* variant (359). Transduced cells were sorted and phenotyped at day 9 **(A)** and 15 (**B)** with a 30 specific focus on FOXP3, HELIOS and CD25 expression. CD25 expression is depicted as Mean Fluorescence Intensity (MFI) while the percentage of double-positive FOXP3+ HELIOS+ is shown at the two time points.

Figure 3. Response to growing amounts of IL-2 in naïve (nTregs) or in memory (mTregs) regulatory T cells, transduced with either a mock vector (empty) or a vector encoding the wild-type (WT) or mutated (359) *IRF4* gene. IL-2 signaling was assessed by the measure of phosphorylated-STAT5 (p-STAT5) using flow cytometry following an overnight IL-2 starvation at day 16 of culture.

Figure 4. Representative dot plot showing a higher HELIOS expression (upper panel) and higher CD25 and HLA-DR expression (lower panel) in CAR-Tregs transduced with the IRF4 variant compared to those transduced with the wild-type IRF4 or not transduced cells.

Figure 5: Competitive fitness assay. $IRF4^{F359L}$-expressing GFP+ Tregs were plated with untransduced Tregs at a 1:1 ratio and cultured at growing IL-2 concentrations. The percentage of GFP+ cells was then assessed at day 5 and 7 of culture, indicating the proportion of $IRF4^{F359L}$-expressing cells according to IL-2 concentration. This experiment shows an enhanced fitness of $IRF4^{F359L}$-expressing Tregs at high IL-2 concentrations, when compared with unmodified Tregs.

Figure 6: Suppression test. IRF4 ^{F359L} –expressing Tregs exhibit higher and more sustained HLA-DR expression than wild-type IRF4-expressing and untransduced Tregs after activation. Notably, HLA-DR expression in Tregs was correlated with enhanced suppressive capacities (Baecher-Allan et . J Immunol 2006

EXAMPLE:

Methods

Human Treg purification:

Healthy donor peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were enriched using EasySep™ CD4+ Enrichment kit (StemCell®). Naïve Tregs $CD4+CD25^{high}$, $CD45RA^{high}$ $CD45RO^{low}$ $CD127^{low}$ were sorted using FACS ARIA II (BD Bioscience®). As control, effector T cells expressing $CD4+CD25^{low}CD127^{high}$ were sorted from the same donor. Sorted Tregs were stimulated with Dynabeads Human T-Activator CD3/CD28 (Gibco®) in Xvivo20® + 20% human AB serum + 1 000 U/mL of IL2 for Tregs and 100 U/mL of IL2 for Teff.

Flow Cytometry:

Surface staining was performed with the following antibodies and dyes, CD4 (Biolegend®), CD127 (BD Pharmingen®), CD25 (BD Pharmingen®), eF780 FVD (eBioscience®), CD45RA (Biolegend®), CD45RO (BD Pharmingen®). Cells were then fixed and permeabilized with the eBioscience FOXP3 / Transcription factor staining buffer set (Invitrogen®) following manufacturer's instructions for intracellular marker assessment. Intracellular staining was performed for FOXP3 (BD Biosciences ®), HELIOS (ThermoFisher®), TNFa (BD Biosciences®), IFNg (BD Pharmingen®), IL-17a (Biolegend®), P-STAT5 (BD Biosciences®). Cells were analysed using Fortessa X-20 analyzer (BD®) and post-acquisition analysis was performed using the software FlowJo®. For the P-STAT5 staining, the BD Phosflow Kit® was used. Cells were fixed for 15 minutes at 37 °C, then permeabilized 30 minutes on ice before being washed and stained.

IL-2 titration:

Sorted Tregs were washed in pre-warmed 1X PBS before being exposed for 15 minutes at 37°C to increasing concentrations of IL-2. Cells were immediately fixed for 15 minutes at 37°C using the BD Phosflow Kit® and then permeabilized 30 minutes on ice before being washed and incubated with P-STAT5 antibody (BD Biosciences®).

Leucocyte activation:

Cytokine secretion was stimulated with a Leucocyte Activation Cocktail (BD Biosciences®). This cocktail contains PMA (Phorbol 12-Myristate 13-Acetate) which activates protein Kinase C, Ionomycin (an antibiotic that induces cytokine secretion by the cells) and Brefeldin A an intra organelle transport inhibitor (BD GolgiPlug®). 2 µL of Leukocyte Activation Cocktail / mL of cell suspension was added and cells were incubated for 4 to 6 hours at 37°C. Cells were then washed in 1X PBS to stop the activation. The presence of Brefeldin A (BD GolgiPlug®) in the cocktail blocks protein transport, therefore an intracellular staining of those cytokines was required.

Lentiviral transduction:

Sorted Tregs were plated in flat-bottom 96-well plate. Prostaglandin E2 (10µM) and Lentiboost (250 µg/mL) were added before adding Lentiviral vectors coding for either wild type IRF4, IRF4 mutant or an empty vector with GFP as reporter gene. Transduction was performed at a

Multiplicity of Infection (MOI) of 30 during 6 hours at 37°C, 5% CO_2. GFP-positive cells were sorted 5 days post transduction using FACS Aria II (BD Biosciences®).

Results

5

Tregs transduced with the IRF4 variant showed higher CD25, HELIOS, and HLA-DR expression

Naïve Tregs were transduced and cultured as depicted (**Figure 1A**). Strikingly, transgenic
10 expression of the *IRF4* variant led not only to a greater proportion of CD25+ cells (**Figure 1B**), but also to a higher CD25 expression level, as assessed by the Mean Fluorescence Intensity (**Figure 1C**). Furthermore, the induction of HLA-DR, a marker of highly-suppressive Tregs (*Rosenblum, M. D., Way, S. S. & Abbas, A. K. Regulatory T cell memory. Nat. Rev. Immunol. 16, 90–101 (2016)*), indicates a state of activated effector Tregs (**Figure 1B-C**). Given the key
15 role of IL-2 signaling in Treg stability, expression of FOXP3 and HELIOS, two hallmark transcription factors of Treg lineage were analyzed. Notably, Tregs expressing the *IRF4* variant exhibited a significantly greater expression of HELIOS compared to the controls (**Figures 1B-C**). Moreover, IRF4 ^{F359L} –expressing Tregs exhibit higher and more sustained HLA-DR expression than wild-type IRF4-expressing and untransduced Tregs after activation (**Figure**
20 **1D**). Notably, HLA-DR expression in Tregs was correlated with enhanced suppressive capacities (Baecher-Allan et . J Immunol 2006).

CD25-positive Tregs show higher phosphorylation of STAT-5, even at low dose IL-2 exposure, than CD25-negative effector T cells:

25

In order to investigate whether high CD25 expression, resulting from IRF4 mutant expression, improves IL-2 signaling in Tregs, we studied STAT5 phosphorylation, a key player downstream the IL-2 receptor.

30 First, we addressed the question of whether constitutive high CD25 expression by Tregs is associated with enhanced phosphorylation of STAT5 upon low dose IL-2 exposure, in comparison with Teff. FACS-sorted Treg and Teff populations were exposed to increasing IL-2 concentrations (0, 1, 5 U/mL) for 15 minutes. The analysis of STAT5 phosphorylation was performed after snap fixation and subsequent permeabilization. No phosphorylated STAT5 was

observed in both resting *ex-vivo* populations, in absence of IL-2. Tregs exhibited a sizeable STAT5 phosphorylation at 1 IU/mL of IL-2, and high phosphorylated STAT5 at 5 IU/mL, in line with constitutive expression of high-affinity IL-2 receptor. In contrast, Teffs only displayed a low phosphorylated STAT5 level at the highest IL-2 concentration. (**data not shown**).

5

In addition, we compared IL-2 signalling between co-cultured Treg (CD25+ FOXP3+) and Teff (CD25low or – FOXP3-negative) cells. Cultured cells were separated from the beads and IL-2 at day 2 and 3 of culture, respectively. At day 4, cells were exposed to a titration of IL-2 (0, 1, 5, 10, 20 U/mL). The baseline phosphorylation of STAT5 was low in both CD25+ and CD25-
10 cells, yet was higher in CD25+ than in CD25- cells. Similarly, upon IL-2 stimulation, STAT5 phosphorylation increased with a dose-effect to greater levels in CD25+ FOXP3+ cells than in CD25- FOXP3- cells (**data not shown**).

Taken together, these results indicate that increased CD25 expression correlates with enhanced
15 IL-2 signaling, as assessed by STAT5 phosphorylation. We next analyzed whether the transgenic expression of the IRF4 variant, associated with increased expression of CD25 and HELIOS, increases IL-2 signaling in Tregs, and as such could promote a better fitness in low IL2 environment, and a more stable Treg lineage identity.

20 ***naïveTreg (nTreg) and memoryTreg (memTreg) showed increased CD25 expression alongside increased STAT5 phosphorylation when transduced with the IRF4 variant:***

To further investigate the impact of IRF4 variant expression in Tregs, naïve (CD45RA+) and memory (CD45RO+) Tregs were transduced with the *IRF4* variant, in comparison with the
25 wild-type *IRF4* and an empty vector expressing only the reporter gen GFP, and then expanded over a two-week culture.

Phenotypic characterization confirmed and extended our previous results, showing a far greater CD25 expression, by one order of magnitude at day 15, in IRF4 variant-expressing Tregs
30 compared to controls (**Figure 2A**). Increased CD25 expression was similarly observed in naïve and memory Tregs, which express the IRF4 variant. Notably, this finding correlates with a higher frequency of the most stable subset of human Tregs, co-expressing FOXP3 and HELIOS (**Figure 2B**).

Next, we investigated whether IRF4 variant expression would lead to enhanced IL-2 signaling. To this end, cultured Tregs were separated from the aCD3/CD28 beads at day 14 and starved from IL-2 at day 15, before a stimulation with titrated low amounts of IL-2 the following day. Memory Tregs (memTreg), transduced with the IRF4 variant, had a baseline phosphorylation of STAT5 far above those of the controls (**Figure 3**), yet failed to demonstrate a dose-effect response to growing amounts of IL-2. This finding suggests that pSTAT5 expression had already reached a plateau, and did not return to baseline levels during overnight IL-2 starvation (unlike untransduced naïve Tregs in the former experiments).

Naïve Tregs (nTreg), transduced with the IRF4 variant, showed a lower baseline expression of pSTAT5 compared to their memory counterparts (**Figure 3**). More importantly, they demonstrated a dose response to low amounts of IL-2, whereas the controls did not. These preliminary data suggest that naïve Tregs expressing the IRF4 variant had a higher IL-2 sensitivity than those expressing the wild-type IRF4.

Transgenic expression of the IRF4 variant in CAR-Tregs was associated with increased CD25 expression and with a greater maintenance of FOXP3 and HELIOS expression upon long-term culture:

As previously reported in *Lamarthée B, Marchal A, Charbonnier S, Blein T, Leon J, Martin E, Rabaux L, Vogt K, Titeux M, Delville M, Vinçon H, Six E, Pallet N, Michonneau D, Anglicheau D, Legendre C, Taupin JL, Nemazanyy I, Sawitzki B, Latour S, Cavazzana M, André I, Zuber J. Transient mTOR inhibition rescues 4-1BB CAR-Tregs from tonic signal-induced dysfunction. Nat Commun. 2021 Nov 8;12(1):6446. doi: 10.1038/s41467-021-26844-1. PMID: 34750385; PMCID: PMC8575891*, extensive *in vitro* expansion of CAR-Tregs, along with CAR tonic signal, might precipitate the loss of FOXP3 and HELIOS expression. In this respect, robust and sustained IL-2 signaling is critical for locking Treg lineage stability. We thus investigated whether forced expression of the IRF4 variant would protect CAR-Tregs from unstable phenotype. As a fact, CAR-Tregs expressing IRF4 variant demonstrated not only higher CD25 expression than controls, but also a better maintenance of FOXP3 and HELIOS expression (**Figure 4**).

Competitive fitness test and suppression analysis

IRF4^{F359L}-expressing GFP+ Tregs were plated with untransduced Tregs at a 1:1 ratio and cultured at growing IL-2 concentrations. The percentage of GFP+ cells was then assessed at

day 5 and 7 of culture, indicating the proportion of IRF4^{F359L}-expressing cells according to IL-2 concentration. This experiment shows an enhanced fitness of IRF4^{F359L}-expressing Tregs at high IL-2 concentrations, when compared with unmodified Tregs (**Figure 5**).

5 We also showed that IRF4 ^{F359L} –expressing Tregs elicit a greater suppression than the controls when co-cultured with conventional T cells stimulated with Treg suppression Inspector (Miltenyi Biotech) beads (**Figure 6**).

Discussion:

10

This work explored a way to further enforce (CAR)-Treg stability, fitness, and suppressive capacities through a gene addition-based strategy. We exploited the finding that transgenic expression of a PID-related IRF4 variant in CD4+ T cells was associated with an increased expression of CD25, the alpha chain of the IL-2 receptor. We extended this finding by showing 15 a similar result in Tregs, along with enhanced expression of HELIOS, a Treg transcription factor, linked to IL-2-dependent Treg stability (*Kim, H.-J. et al. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. Science 350, 334–339 (2015)*). In line with this, we found that Tregs expressing the IRF4 variant demonstrate an enhanced IL-2 sensitivity. Taken together, this set of data suggests that transgenic expression of this IRF4 20 mutant in Tregs might push them toward highly suppressive effector cells.

Molecular mechanisms underpinning the effect of IRF4 mutant on IL-2 signaling pathway have yet to be explored. In this respect, a recent study provided an important clue to link IRF4 and enhanced IL-2 signaling (*Diener, C. et al. Quantitative and time-resolved miRNA pattern of 25 early human T cell activation. Nucleic Acids Res. 48, 10164–10183 (2020)*). This study highlighted IRF4 and its complexes with SPI1 and BATF as instrumental for the transcriptional regulation of miR-155 in activated CD4+ T cells. In Tregs, miR155 was found to target and inhibit a major cytokine signaling regulator, named SOCS1 (*Lu, L.-F. et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting* SOCS1 protein. 30 *Immunity 30(1):80-91 (2009)*). Through this mechanism, Tregs exhibit an enhanced STAT5-dependent IL-2 signaling. Hence, miR155 expression is thought to provide a competitive fitness to Tregs, especially in IL-2-limited environments. We hypothesize that this IRF4 mutant can further increase miR155 expression in Tregs, accounting for a greater IL-2 sensitivity.

REFERENCES:

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

CLAIMS:

1. A regulatory T cell (Treg) that is engineered to express a polypeptide that comprises an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated.

2. The Treg of claim 1 wherein the amino acid residue (F) at position 359 is substituted. In some embodiments, the amino acid residue (F) at position 359 is substituted by a leucine (L) residue.

3. The Treg of claim 1 that is engineered to express a polypeptide that comprises an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 and that comprises the mutation F359L.

4. The Treg of claim 1 that is further engineered to express a chimeric antigen receptor (CAR) having specificity for an antigen of interest.

5. The Treg of claim 4 wherein the CAR is specific for HLA-A2.

6. The Treg of claim 5 wherein the CAR comprises the scFV that consists of the amino acid sequence as set forth in SEQ ID NO:2.

7. The Treg of claim 4 wherein the CAR comprises the comprises the CD3ζ signalling domain and the CD28 co-stimulatory signalling domain.

8. The Treg of claim 7 wherein the CAR consists of the amino acid sequence as set forth in SEQ ID NO:3.

9. A method of preparing the Treg of claim 1, comprising the steps consisting of introducing into a Treg a polynucleotide that encodes for the polypeptide comprising an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated.

10. The method of claim 9 comprising the steps consisting of introducing into a Treg i) a polynucleotide that encodes for the polypeptide comprising an amino acid sequence having at least 90% of identity with the amino acid sequence as set forth in SEQ ID NO: 1 wherein the amino acid residue (F) at position 359 is mutated and ii) a polynucleotide that encodes for the CAR.

11. A method of inducing tolerance to one antigen of interest in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the population of Tregs according to any one of claim 1 to 6 that are specific for said antigen.

5 12. The method of claim 11 wherein the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an autoimmune disease.

13. The method of claim 11 wherein the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an allergy.

14. The method of claim 11 wherein the subject is predisposed or believed to be predisposed
10 to developing, or has already developed or is developing an immune reaction against molecules that are exogenously administered for therapeutic or other purposes.

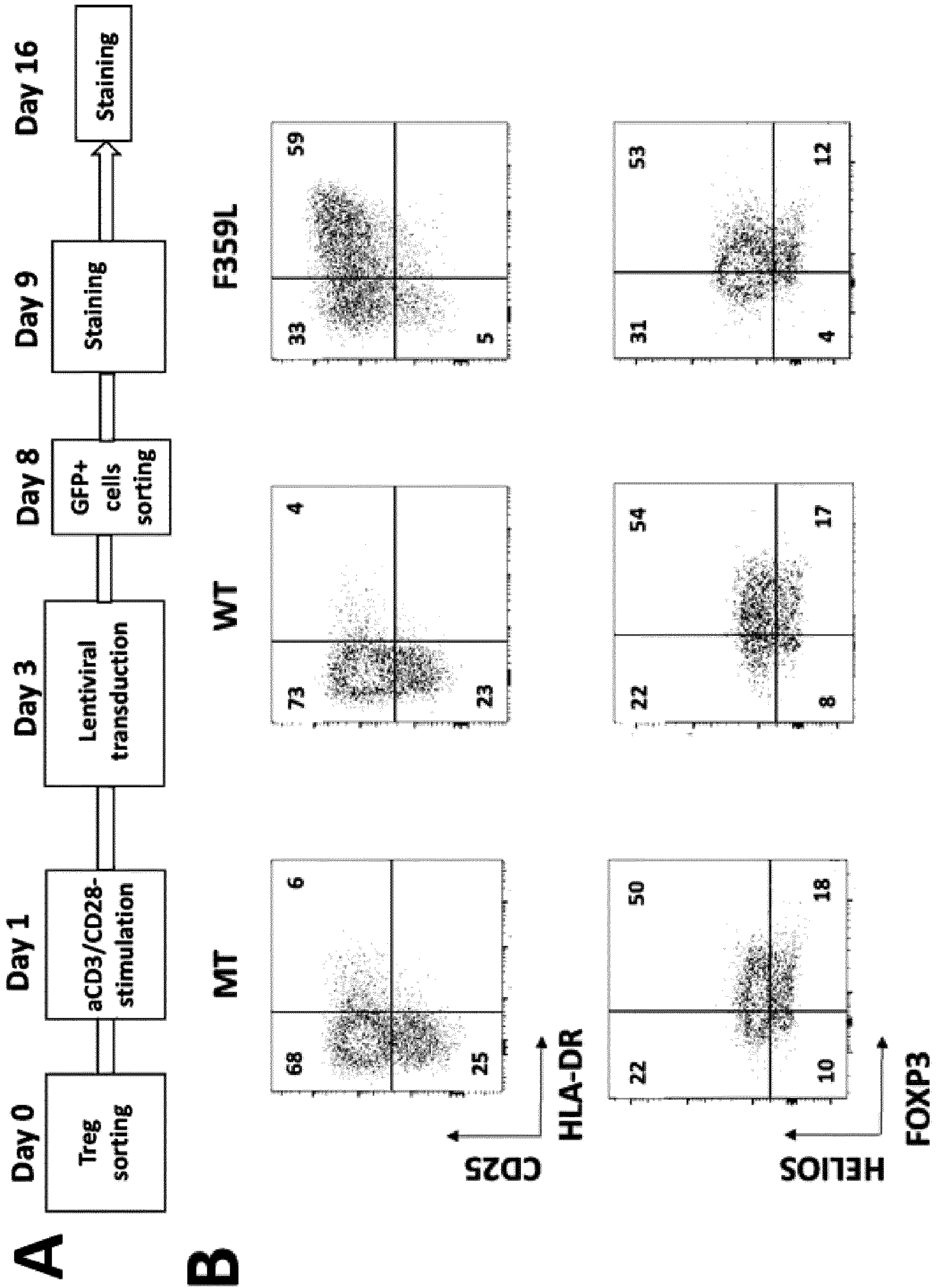
15. The method of claim 11 wherein the subject is predisposed or believed to be predisposed to developing, or has already developed or is developing an immune reaction against a grafted population of cells, a grafted tissue or a grafted organ.

15 16. The method of claim 11 wherein the subject suffers from Host-Versus-Graft-Disease (HVGD) and Graft- Versus-Host-Disease (GVHD).

17. The method of claim 11 wherein the antigen is an auto-antigen, an allergen, an allo-antigen or a molecule that is exogenously administered for therapeutic purposes.

18. The method of claim 17 wherein the allo-antigen is HLA-A2.

20 19. A pharmaceutical composition comprising the population of Tregs according to any one of claims 1 to 6 and a pharmaceutically acceptable carrier.



Figures 1A and 1B

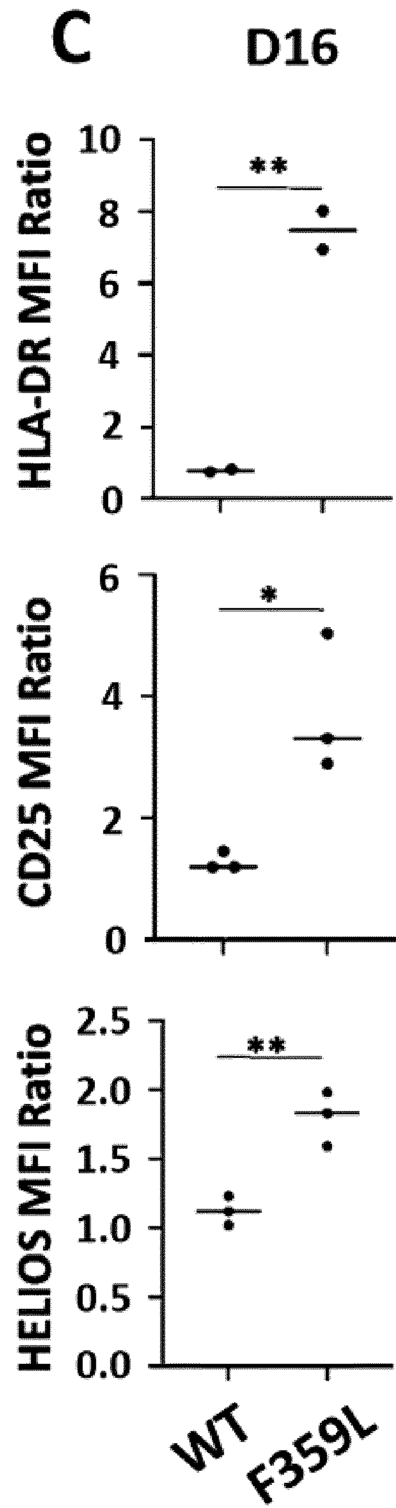


Figure 1C

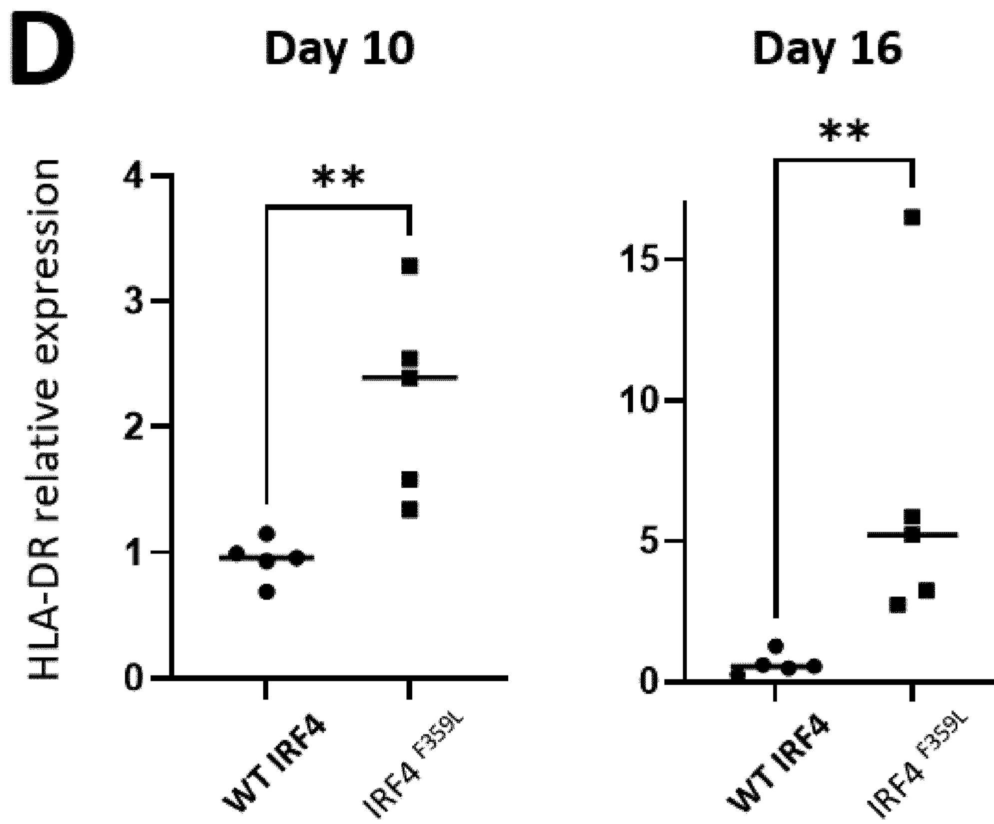


Figure 1D

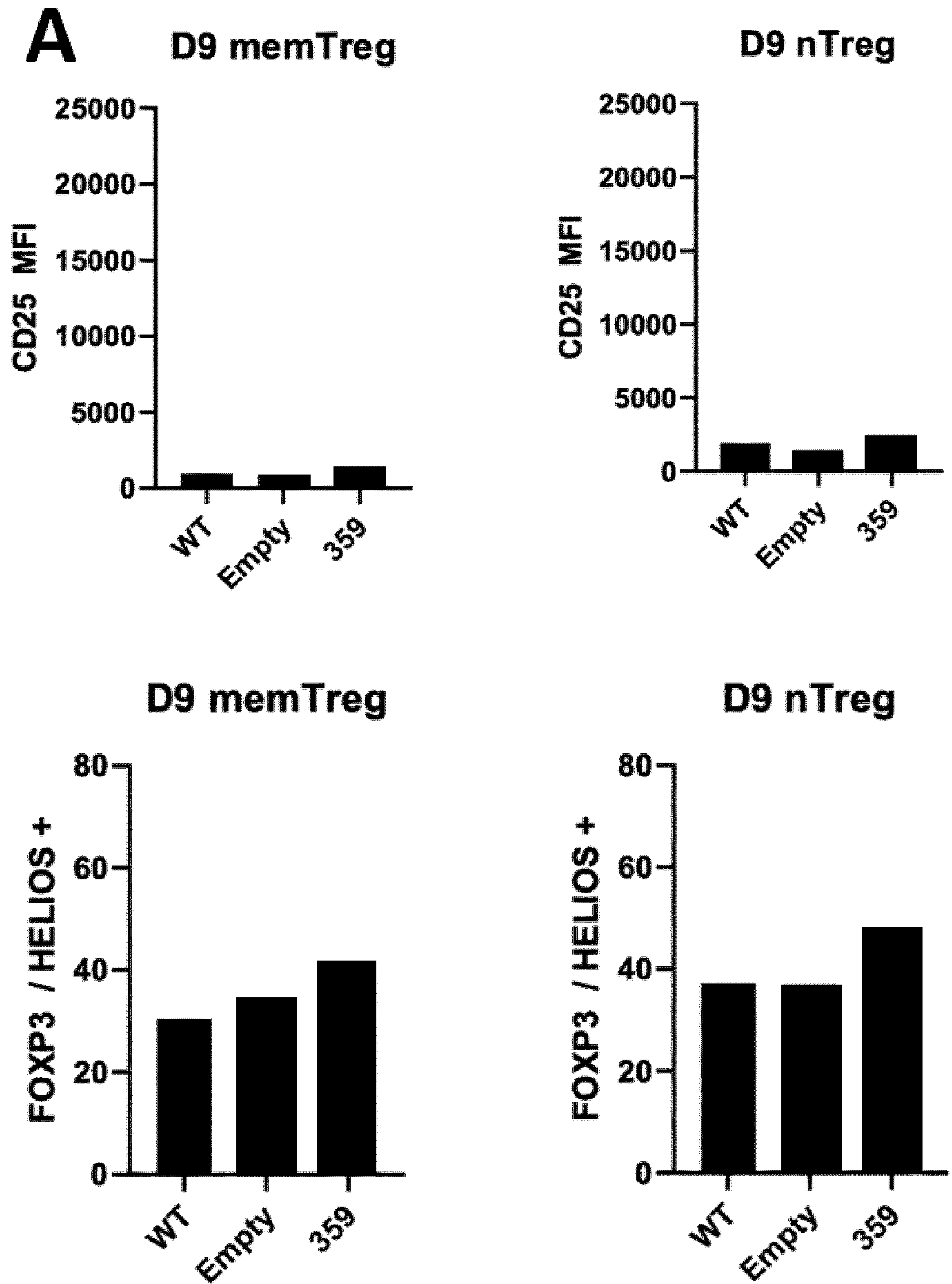


Figure 2A

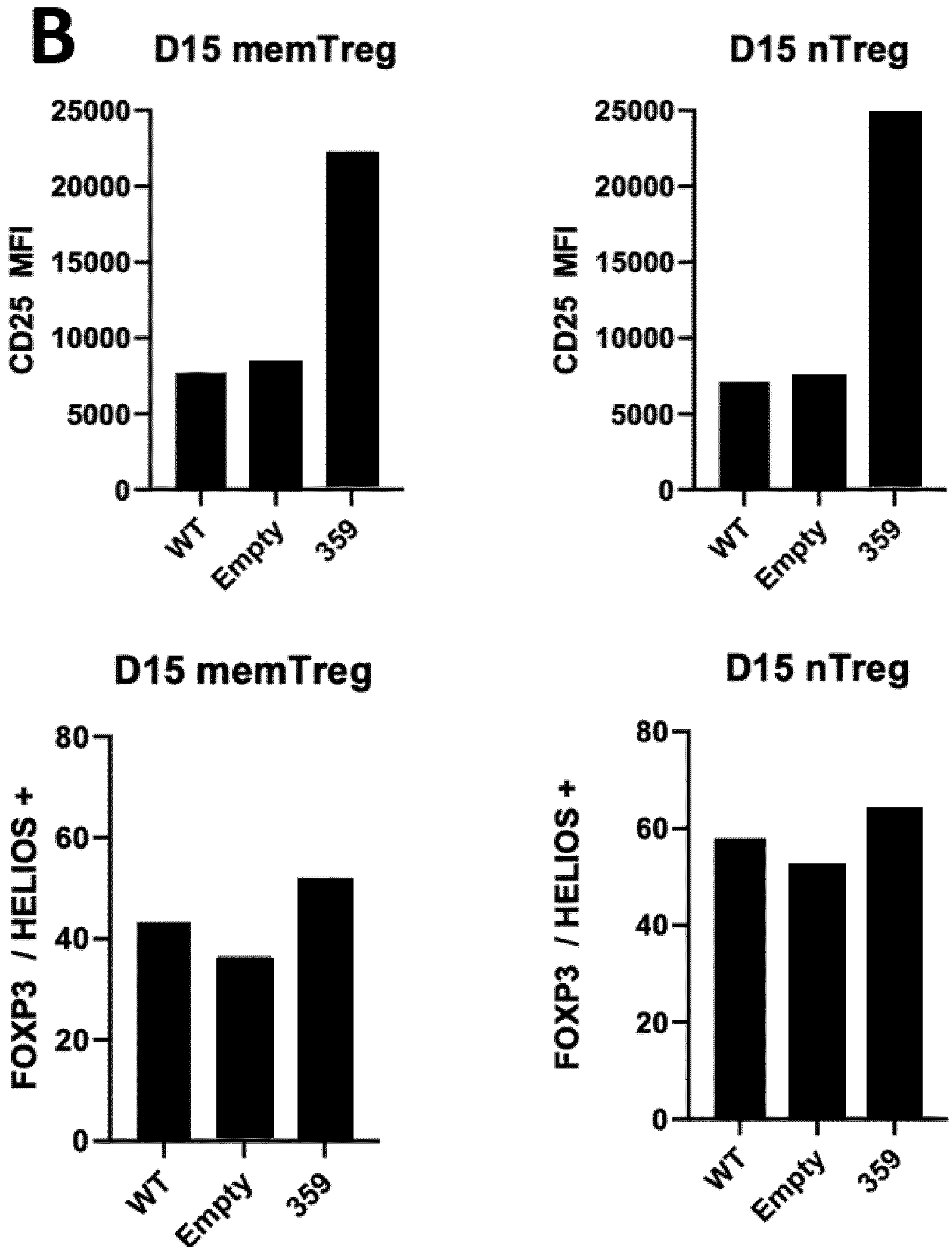


Figure 2B

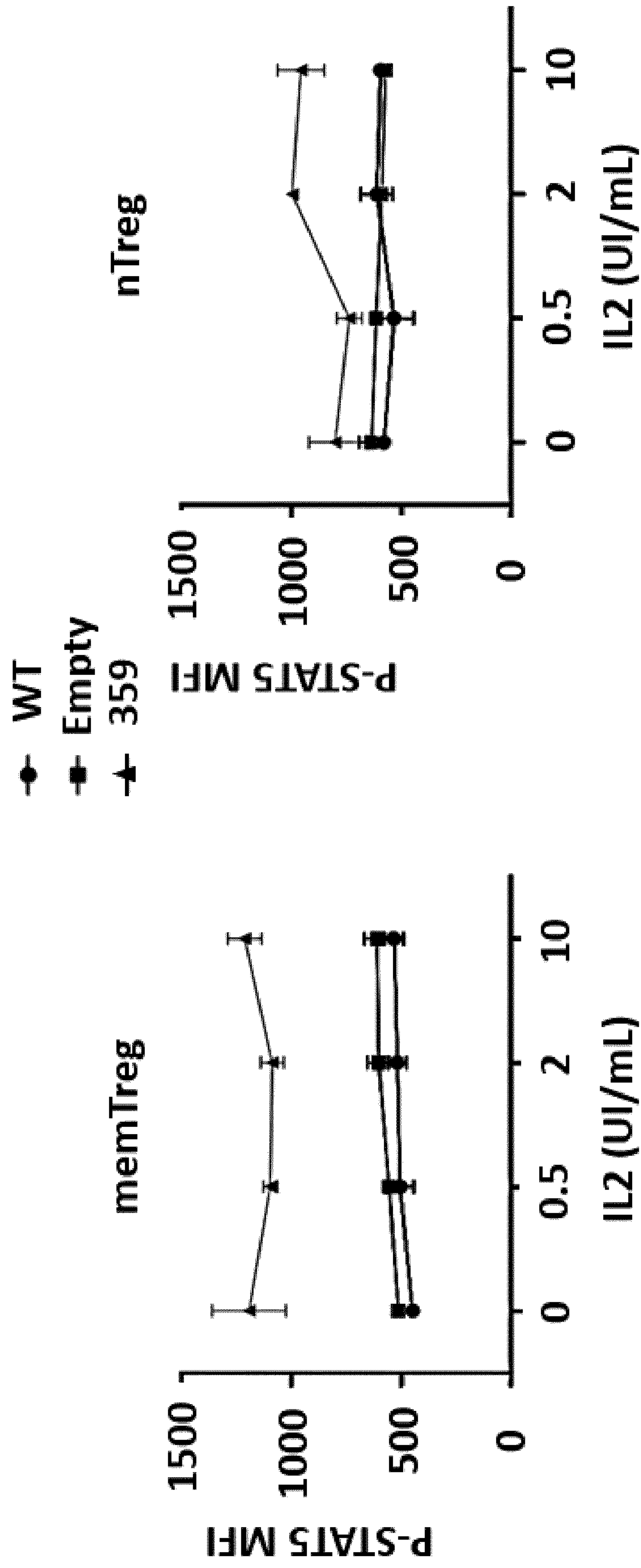


Figure 3

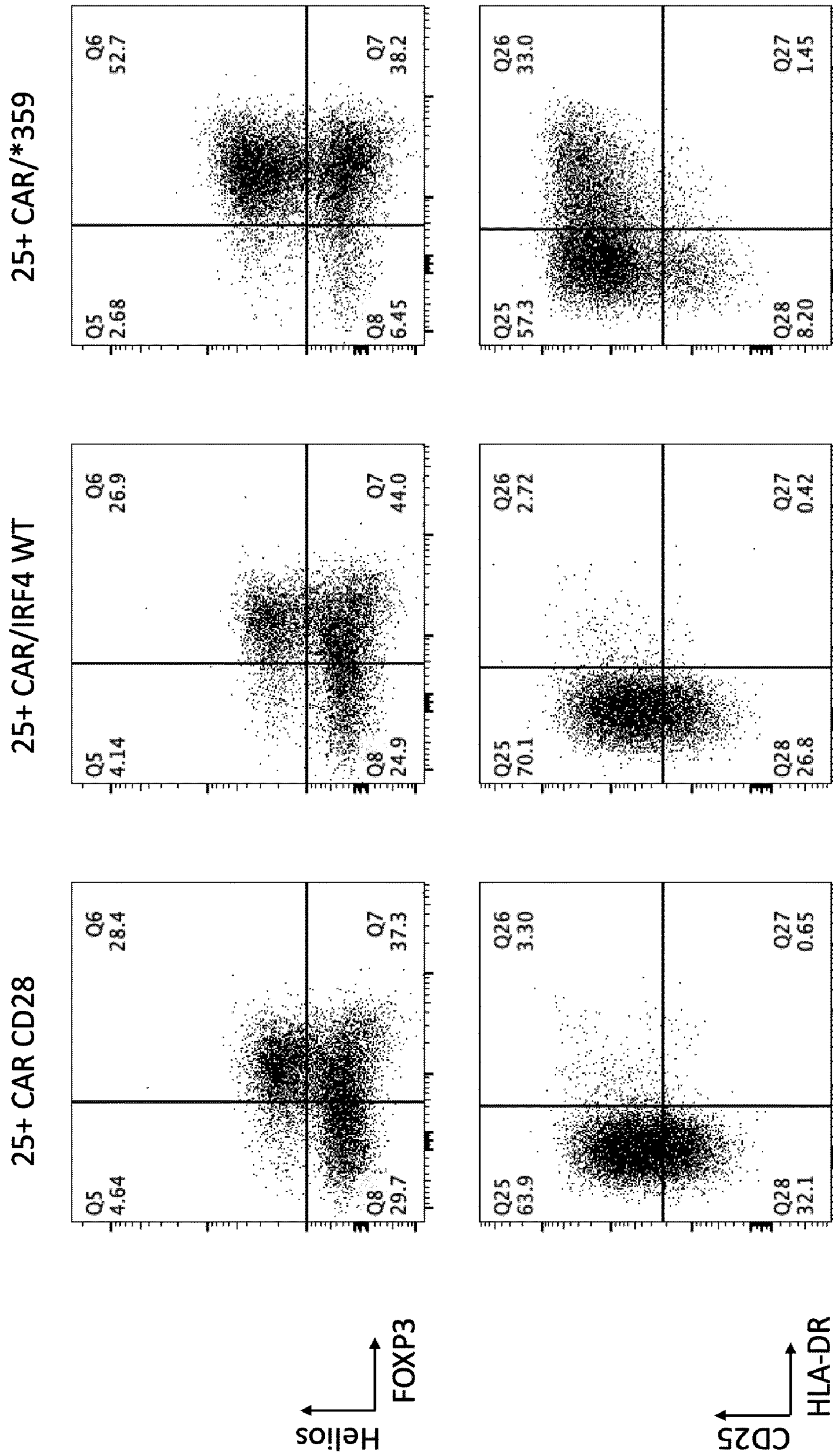


Figure 4

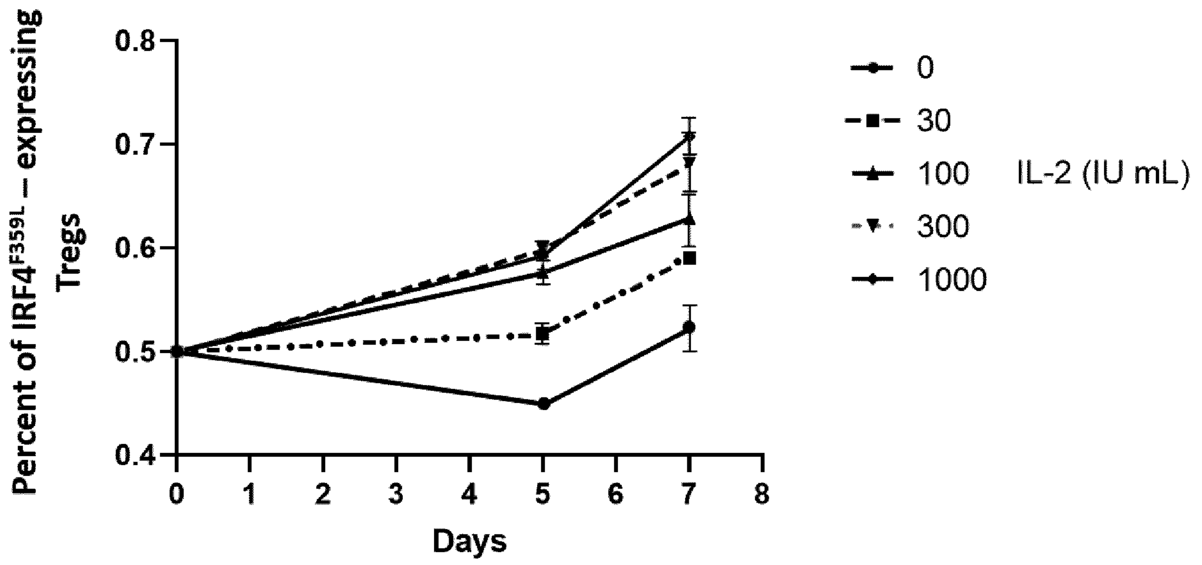


Figure 5

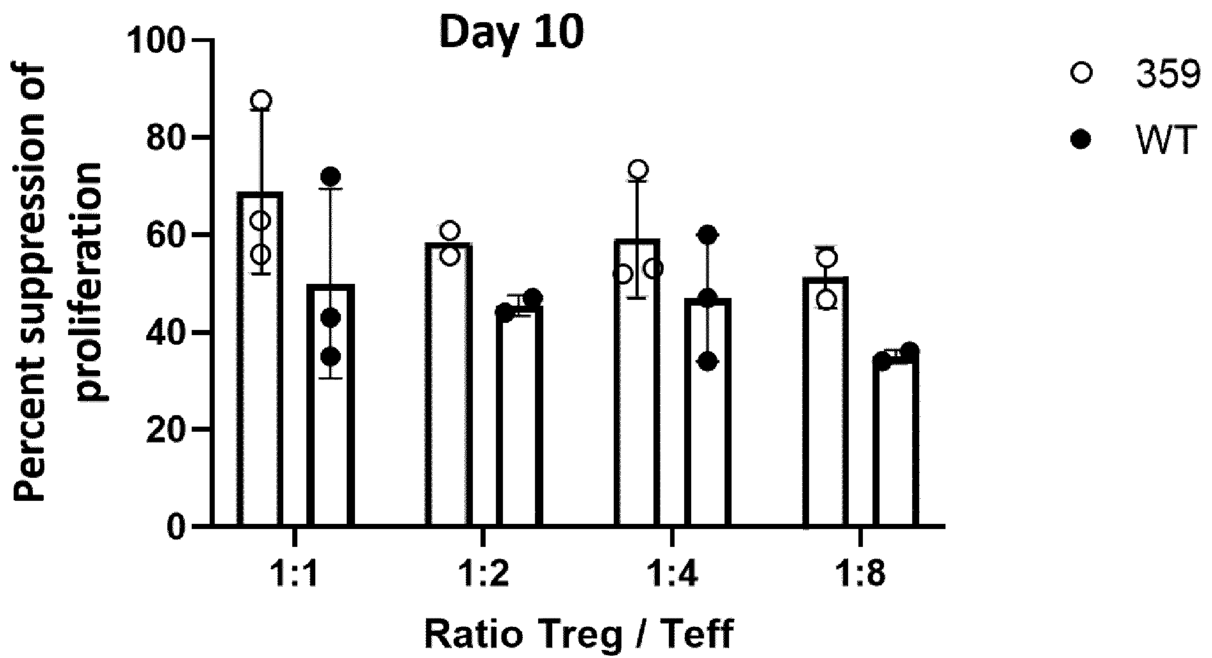


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/065361

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/065361

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/47 C12N5/0783 C07K14/725 C07K14/705
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2022/169687 A1 (MAHNE ASHLEY [US] ET AL) 2 June 2022 (2022-06-02) claims 144-146 ----- -/--	1-19

[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
13 September 2023	21/09/2023

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	Wiesner, Martina

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHERIAN MATHEW A. ET AL: "An activating mutation of interferon regulatory factor 4 (IRF4) in adult T-cell leukemia", JOURNAL OF BIOLOGICAL CHEMISTRY , vol. 293, no. 18 5 March 2018 (2018-03-05), pages 6844-6858, XP093000431, US ISSN: 0021-9258, DOI: 10.1074/jbc.RA117.000164 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5936815/pdf/zbc6844.pdf figure 1 -----	1-19
A	GUÉRIN ANTOINE ET AL: "IRF4 haploinsufficiency in a family with Whipple's disease", ELIFE , vol. 7 1 January 2018 (2018-01-01), page 32340, XP093000448, DOI: 10.7554/eLife.32340 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5915175/pdf/elife-32340.pdf figure 2 -----	1-19
A	CHOI JAEHYUK ET AL: "Genomic landscape of cutaneous T cell lymphoma", NATURE GENETICS, NATURE PUBLISHING GROUP US, NEW YORK, vol. 47, no. 9, 20 July 2015 (2015-07-20), pages 1011-1019, XP037923082, ISSN: 1061-4036, DOI: 10.1038/NG.3356 [retrieved on 2015-07-20] figure 1	1-19
A	-& CHOI JAEHYUK ET AL: "Supplementary Text and Figures - Genomic landscape of cutaneous T cell lymphoma", NATURE GENETICS, vol. 47, no. 9, 20 July 2015 (2015-07-20), XP093000456, New York ISSN: 1061-4036, DOI: 10.1038/ng.3356 Retrieved from the Internet: URL:https://www.nature.com/articles/ng.3356> table 5 -----	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/065361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2022169687 A1	02-06-2022	US 2022143134 A1	12-05-2022
		US 2022169687 A1	02-06-2022
		US 2023104151 A1	06-04-2023
		WO 2022103789 A1	19-05-2022