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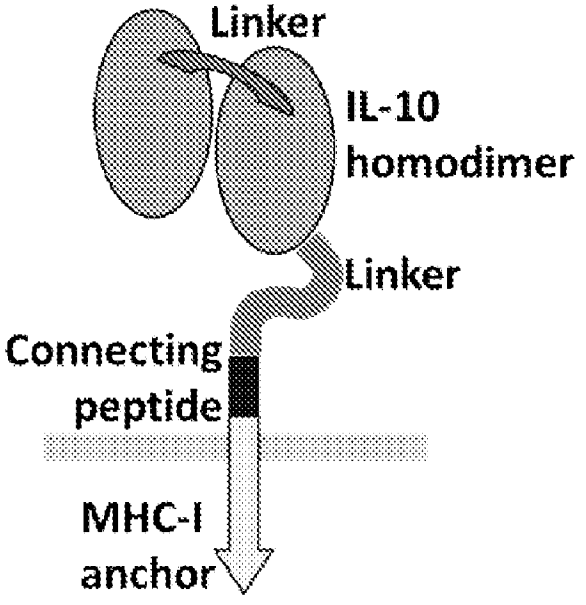
(54) Titre : TREGS GENETIQUEMENT REPROGRAMMES EXPRIMANT IL-10 LIE A UNE MEMBRANE
(54) Title: GENETICALLY REPROGRAMMED TREGS EXPRESSING MEMBRANE-BOUND IL-10

Fig. 1



(57) Abrégé/Abstract:
A nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch, optionally through a flexible hinge, is provided as well as a mammalian regulatory T cell (Treg) comprising and expressing the nucleic acid molecule and uses thereof.

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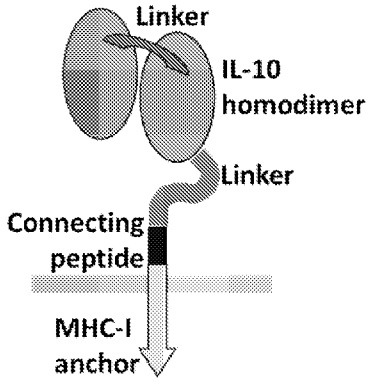
Fig. 1



(57) **Abstract:** A nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch, optionally through a flexible hinge, is provided as well as a mammalian regulatory T cell (Treg) comprising and expressing the nucleic acid molecule and uses thereof.

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Genetically Reprogrammed Tregs Expressing Membrane-Bound IL-10

FIELD OF THE INVENTION

The present invention relates in general to genetically reprogrammed regulatory T cells expressing membrane-bound IL10 and their use in increasing systemic immunosuppression and treating diseases manifested in excessive activity of the immune system.

BACKGROUND OF THE INVENTION

Harnessing CD4 regulatory T cells (Tregs) for suppressing local inflammation and restoring immunological balance holds great promise in the treatment of pathologies as diverse as autoimmune diseases, inflammatory bowel diseases, allergies, atherosclerosis, transplant rejection, graft-versus-host disease and more. However, Tregs, either natural (nTregs) or induced (iTregs) form only a minor fraction in the entire human CD4 T cell population. Consequently, there is an urgent need for the development of Treg-based therapies for recruiting, inducing, or engineering autologous or allogeneic Tregs at adequate numbers and stable phenotype which are critical for clinical efficacy and safety of treatment.

SUMMARY OF INVENTION

In one aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch, optionally through a flexible hinge, referred to herein as mem-IL10.

In a different aspect, the present invention provides a composition comprising the nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch as defined herein.

In a further aspect, the present invention provides a viral vector comprising any one of the nucleic acid molecules comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch as defined above.

In another aspect, the present invention provides a composition comprising the viral vector as defined above.

In still another aspect, the present invention provides a mammalian regulatory T cell (Treg) comprising any one of the nucleic acid molecules as defined above, or the viral vector as defined above.

In yet an additional aspect, the present invention provides a method of preparing allogeneic or autologous Tregs with a stable Tr1 phenotype, the method comprising contacting CD4 T cells with the nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 as defined above, or a viral vector comprising it, thereby endowing said CD4 T cells with a stable Tr1 phenotype, and thus preparing Tregs with a stable Tr1.

In still an additional aspect, the present invention provides a method for increasing immune suppression in a subject in need, comprising administering to said subject the mammalian Treg expressing on its surface a homodimeric membrane-bound IL-10 as defined above.

In certain embodiments, the present invention provides a method of treating or preventing a disease, disorder or condition in a subject, comprising administering to said subject the mammalian Treg expressing on its surface a homodimeric IL-10 as defined above, wherein said disease, disorder or condition is manifested in excessive or otherwise unwanted activity of the immune system, such as an autoimmune disease, allergy, asthma, and organ and bone marrow transplantation.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 depicts a schematic presentation of membrane-anchored homodimeric IL-10.

Figs. 2A-2D show analysis of memIL-10 expression in T cells and its effect on IL-10 receptor (IL-10R) and CD49b. Human Jurkat or primary, peripheral blood lymphocyte-derived CD4 T cells (**A, B**) and mouse B3Z or NOD splenic CD4 T cells (**C, D**) were electroporated with 10 μg of *in-vitro* transcribed mRNA encoding human or mouse memIL-10, respectively. Cells were analyzed by flow cytometry 24 hours (**A-C**) or 48 hours (**D**, left and right) post-transfection. Human or mouse memIL-10 and IL-10R and human CD49b were analyzed by monoclonal antibodies specific to the respective human or mouse proteins, respectively.

Figs. 3A-D depict schematic presentations of native IL-10 homodimer bound to its cell surface receptor (**A**) and of the three membrane-anchored derivatives of IL-10 (mem-IL10): (**B**) mem-IL10 with short linker; (**C**) mem-IL10 with long linker; and (**D**) mem-IL10 linked to IL-10Rβ (IL-10Rβ fusion).

Fig. 4 shows cell surface expression of the three memIL-10 derivatives in Jurkat cells 24 hours post-mRNA electroporation. Human Jurkat CD4 T cells were electroporated with 10 μg of each of the indicated mRNAs (sL and lL stand for short and long linker, respectively). Twenty four hours cells were analyzed by flow cytometry for surface expression of IL-10.

Figs. 5A-C show that memIL-10 expression in CD4 T cells induces spontaneous

phosphorylation of STAT3. Mouse CD4 T cells were either electroporated with irrelevant mRNA (Irr. mRNA), mRNA encoding short linker memIL-10 (sLmemIL-10), long linker memIL-10 (lLmemIL-10) or IL-10 linked to the IL-10Rβ chain (memIL-10Rβ) or treated with soluble recombinant IL-10 (sIL-10) at 20 ng/ml. Twenty four hours later cells were subjected to flow cytometry analysis for surface IL-10 (**A**), surface IL-10Rα chain (**B**) or intracellularly for phosphorylated STAT3 (pSTAT3) (**C**).

Figs. 6A-B show analysis of retrovirally transduced mouse CD4 T cells expressing memIL-10. Phenotypic analysis of short-linker memIL-10-ransduced mouse CD4 T cells (v-memIL-10), 48 hours (**A**) and 6 days (**B**) post-transduction. Analysis was performed in parallel on memIL-10(+) and memIL-10(-) cells growing in the same cell culture, staining for LAG-3, CD49b and PD-1. As a positive control non-transduced cells were treated with soluble IL-10 (sIL-10). Mock, cells treated with identical protocol as retrovirally transduced cells but without exposure to viral particles.

Fig. 7 shows secretion of IL-10 by activated, memIL-10 transduced mouse CD4 T cells. Cells from the same experiment as in **Fig. 6** were stimulated by an anti-TCR-CD3 mAb (2C11) and their growth medium was subjected to an IL-10 ELISA. Mock- and GFP-transduced T cells serves as negative controls.

Figs. 8A-C show phenotypic characterization of memIL-10 transduced human CD4 T cells. CD4 T cells were isolated by magnetic beads from peripheral blood mononuclear cells prepared from a blood sample of a healthy donor. Cells were grown in the presence of the anti-CD3 and anti-CD28 antibodies and IL-2 to the desired number and transduced with recombinant retrovirus encoding memIL-10 or an irrelevant gene (Irr.), or treated with soluble IL-10 (sIL-10). Cells were grown in the presence of IL-2 and samples were taken for flow cytometry analysis for the indicated cell surface markers at day 1 (**A**), day 5 (**B**) and day 18 (**C**). At day 18 non-transduced Tregs were added to the analysis for comparison of cell surface markers. At each time point cells expressing memIL-10 (Pos, solid frame)) were analyzed side by side with cells from the same culture which do not express IL-10 (Neg, dotted frame).

Fig. 9 shows a second experiment phenotyping memIL-10-transduced human CD4 T cells. Cells were prepared and transduced with memIL-10 and analyzed 4 days later for the indicated markers as described in the legend to **Fig. 8**. Non-transduced (Naïve) and mock-transduced (Mock) CD4 cells served as negative controls. MemIL-10 positive cells were compared to memIL-10 negative cells from the same culture as well as to naïve CD4 T cells grown in the presence of 50, 100 or 300 ng/ml sIL-10. Shown are % of positively stained cell in each sample. Double pos, % of cells stained positive for LAG-3 and CD49b.

DETAILED DESCRIPTION OF THE INVENTION

It has been found in accordance with the present invention that genetically reprogramming T cells to constitutively express membrane-bound IL-10 confers a stable Tr1 phenotype to the T cells.

The type of Treg cell selected is of critical importance for successful clinical implementation. Tr1 cells are a subset of CD4(+) FoxP3(+/-) Tregs which are induced in the periphery in a TCR- and antigen-specific manner upon chronic exposure to antigen on dendritic cells in the presence of IL-10 (1, 2). These cells are characterized by a non-proliferative (anergic) state, high production of IL-10 and TGF-β but only minimally of IL-2 and none of IL-4 or IL-17 and the ability to suppress effector T cells (Teffs) in a cell-to-cell contact-independent manner. Andolfi et al. demonstrated that the enforced expression of IL-10 in human CD4 T cells, accomplished by lentiviral transduction, was sufficient for endowing these cells with a stable Tr1 phenotype in an autocrine fashion (3). This study also showed that exposure of these cells to IL-2 could temporarily reverse the anergic state of these IL-10-induced Tr1 cells. Importantly, two cell surface markers, CD49b and LAG-3, have been identified, which are stably and selectively co-expressed on human (and mouse) Tr1 cells and allow their isolation and flow cytometry analysis for purity of the cell population (4).

The present invention provides a gene encoding a membrane-anchored derivative of IL-10 (mem-IL10). Native IL-10 is a homodimer (5, 6) and it was found herein that imparting a functional homodimeric configuration on its membrane-anchored form provides an IL-10-driven safe lock guaranteeing permanent preservation of the Tr1 phenotype, while avoiding IL-10 secretion in the absence of antigenic stimulation. Safety wise, as IL-10 does not signal T cell proliferation, the autonomous activation of the IL-10 signaling pathway is not associated with risk of uncontrolled cell growth.

In this invention we achieve an anti-inflammatory effect for imposing immune suppression, for the first time, by modifying Tregs to express membrane IL-10. Furthermore, since IL-10 does not induce T cell proliferation it can be expressed constitutively through stable viral transduction with no risk of inducing autonomous cell proliferation and cellular transformation.

In one aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch, optionally through a flexible hinge, referred to herein as mem-IL10.

In certain embodiments, the isolated nucleic acid molecule does not comprise a nucleotide sequence encoding for additional different proteins except for mem-IL-10, but may comprise additional control elements such as promoters and terminators.

In certain embodiments, the homodimeric IL-10 comprises a first and a second IL-10 monomer connected in a single-chain configuration such that the C-terminus of the first IL-10 monomer is linked to the N-terminus of the second IL-10 monomer via a first flexible linker.

Flexible peptide linkers are well-known in the art. Empirical linkers designed by researchers are generally classified into three categories according to their structures: flexible linkers, rigid linkers, and *in vivo* cleavable linkers as defined e.g. in (7–9), each one of which is incorporated by reference as if fully disclosed herein.

As stated above, the first linker is a flexible linker and its structure is selected from any one of the linkers disclosed in (7–9). In principle, to provide flexibility, the linkers are generally composed of small, non-polar (e.g. Gly) or polar (e.g. Ser or Thr) amino acids, such an underlying sequence of alternating Gly and Ser residues. Solubility of the linker and associated homodimeric IL-10 may be enhanced by including charged residues; e.g. two positively charged residues (Lys) and one negatively charged residue (Glu). The linker may vary from 2 to 31 amino acids, optimized for each condition so that the linker does not impose any constraints on the conformation or interactions of the linked partners in lengths, such as between 12 and 18 residues.

In certain embodiments, the first flexible linker has the amino acid sequence GSTSGSGKPGSGEGSTKG (SEQ ID NO: 1). In certain embodiments, the first flexible linker is encoded by a nucleotide sequence e.g. as set forth in SEQ ID NO: 2.

In certain embodiments, the flexible hinge comprises a polypeptide selected from the following polypeptides or variants thereof:

- The hinge region of CD8α, (for example as set forth in SEQ ID NO: 3; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 4)
- The hinge region of the heavy chain of IgG (for example as set forth in SEQ ID NO: 5; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 6)
- The hinge region of the heavy chain of IgD (for example as set forth in SEQ ID NO: 7; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 8).
- The extracellular stretch of the IL-10R β chain (as set forth in SEQ ID NO: 9; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 10); and
- A second flexible linker comprising an amino acid sequence of up to 28 amino acids comprising at least one Gly4Ser(Gly3Ser)$_2$ sequence, e.g. comprising one Gly$_4$Ser(Gly$_3$Ser)

sequence (SEQ ID NO: 11; for example encoded by a nucleotide sequence as set forth in SEQ ID NO: 12), or two $Gly_4Ser(Gly_3Ser)$ sequences with one or two Ser residues inserted between them.

In certain embodiments, the second flexible linker comprises a 21 amino acid sequence comprising the amino acid sequence $Gly_4Ser(Gly_3Ser)_2$ (referred to herein as "short linker"; SEQ ID NO: 13; for example encoded by a nucleotide sequence as set forth in SEQ ID NO: 14).

In certain embodiments, the second flexible linker consists of a 28 amino acid spacer comprising the amino acid sequence $Gly_4Ser(Gly_3Ser)_2Ser_2(Gly_3Ser)_3$ (referred to herein as "long linker"; SEQ ID NO:15; for example encoded by a nucleotide sequence as set forth in SEQ ID NO: 22) and the connecting peptide of SEQ ID NO: 16.

In certain embodiments, the second flexible linker of any one of the above embodiments further comprises an 8 amino acid bridge of the sequence SSQPTIPI (referred to herein as "connecting peptide"; SEQ ID NO: 17; for example encoded by a nucleotide sequence as set forth in SEQ ID NO: 18) derived from the membrane-proximal part of the connecting peptide of HLA-A2.

In certain embodiments, the transmembrane-intracellular stretch of the mem-IL10 is derived from the heavy chain of a human MHC class I molecule selected from an HLA-A, HLA-B or HLA-C molecule, preferably HLA-A2 (as set forth in SEQ ID NO: 19; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 20); human CD28 (as set forth in SEQ ID NO: 21; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 22); or human IL-10R β chain (as set forth in SEQ ID NO: 23; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 24).

In certain embodiments, the amino acid sequence of the complete mem-IL10 comprises or essentially consists of the homodimeric IL-10 linked via the short second flexible linker and the connecting peptide to the transmembrane-intracellular stretch of HLA-A2 as set forth in SEQ ID NO: 25; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 26.

In certain embodiments, the amino acid sequence of the complete mem-IL10 comprises or essentially consists of the homodimeric IL-10 linked via the long second flexible linker and the connecting peptide to the transmembrane-intracellular stretch of HLA-A2 as set forth in SEQ ID NO: 27; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 28).

In certain embodiments, the mem-IL-10 is fused to the IL-10Rβ extracellular domain (for example as set forth in SEQ ID NO: 9) via a second flexible linker, and optionally further to the IL-10Rβ transmembrane & cytosolic domains (for example as set forth in SEQ ID NO: 23).

In certain embodiments, the mem-IL-10 is fused to the N-terminus of an essentially complete IL-10R β chain via the short linker (as set forth in SEQ ID NO: 29; e.g. encoded by a nucleotide sequence as set forth in SEQ ID NO: 23).

The polypeptides making up the mem-IL10 of the present invention that are encoded by the nucleic acid molecules of the invention are not limited to those defined herein by specific amino acid sequences but may also be variants of these oligopeptides or have amino acid sequences that are substantially identical to those disclosed above. A "substantially identical" amino acid sequence as used herein refers to a sequence that differs from a reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site of the molecule, and provided that the polypeptide essentially retains its functional properties. A conservative amino acid substitution, for example, substitutes one amino acid with another of the same class, *e.g.*, substitution of one hydrophobic amino acid with another hydrophobic amino acid, a polar amino acid with another polar amino acid, a basic amino acid with another basic amino acid and an acidic amino acid with another acidic amino acid. One or more amino acids can be deleted from the peptide, thus obtaining a fragment thereof without significantly altering its biological activity.

In certain embodiments, the amino acid sequence of the complete membrane-bound IL-10 or each one of the various sub-regions of the membrane-bound IL-10 as disclosed above i.e. the homodimeric IL-10 in which the first and second IL-10 monomers are connected in a single-chain configuration via a first flexible linker; the first flexible linker *per se*, the flexible hinge; and the transmembrane-intracellular stretch, is at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, or at least 98% identical to SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29.

In certain embodiments, the amino acid sequence of the complete membrane-bound IL-10 or each one of the various sub-regions of the membrane-bound IL-10 as disclosed above i.e. the homodimeric IL-10 in which the first and second IL-10 monomers are connected in a single-chain configuration via a first flexible linker; the first flexible linker *per se*, the flexible hinge; and the transmembrane-intracellular stretch, as well as the whole construct, is 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%,

89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98, or 99% identical to SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29.

In certain embodiments, the isolated nucleic acid molecule comprises a polynucleotide sequence encoding the complete membrane-bound IL-10 or each one of the various sub-regions of the membrane-bound IL-10 as disclosed above i.e. the homodimeric IL-10 in which the first and second IL-10 monomers are connected in a single-chain configuration via a first flexible linker; the first flexible linker *per se*, the flexible hinge; and the transmembrane-intracellular stretch, as well as the whole construct, that is at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, or at least 98% identical to one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30.

In certain embodiments, the isolated nucleic acid molecule comprises a polynucleotide sequence encoding the complete membrane-bound IL-10 or each one of the various sub-regions of the membrane-bound IL-10 as disclosed above i.e. the homodimeric IL-10 in which the first and second IL-10 monomers are connected in a single-chain configuration via a first flexible linker; the first flexible linker *per se*, the flexible hinge; and the transmembrane-intracellular stretch, as well as the whole construct is 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98, or 99% identical to one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30.

In certain embodiments, the isolated nucleic acid molecule comprises a polynucleotide sequence encoding the complete membrane-bound IL-10 or each one of the various sub-regions of the membrane-bound IL-10 as disclosed above i.e. the homodimeric IL-10 in which the first and second IL-10 monomers are connected in a single-chain configuration via a first flexible linker; the flexible linker *per se*, the flexible hinge; and the transmembrane-intracellular stretch, as well as the whole construct as set forth in one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30.

In a different aspect, the present invention provides a composition comprising the nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch as defined in any of the above embodiments.

In certain embodiments the nucleic acid molecule is the sole nucleic acid molecule in the composition, i.e. the composition does not comprise additional nucleic acid molecules comprising nucleotide sequences encoding for additional different proteins.

The nucleic acid molecules of the present invention are delivered into T cells for the purpose of enforcing a stable Tr1 phenotype using any well-known method in the field: For example, Matuskova and Durinikova (10) teach that there are two systems for the delivery of transgenes into a cell – viral and non-viral. The non-viral approaches are represented by polymer nanoparticles, lipids, calcium phosphate, electroporation/nucleofection or biolistic delivery of DNA-coated microparticles.

There are two main types of vectors that can be used in accordance with the present invention depending on whether the DNA is integrated into chromatin of the host cell or not. Retroviral vectors such as those derived from gammaretroviruses or lentiviruses persist in the nucleus as integrated provirus and reproduce with cell division. Other types of vectors (e.g. those derived from herpesviruses or adenoviruses) remain in the cell in the episomal form.

Thus, in a further aspect, the present invention provides a viral vector comprising anyone of the nucleic acid molecules comprising a nucleotide sequence encoding a homodimeric IL-10 linked to a transmembrane-intracellular stretch as defined above.

In certain embodiments, the viral vector is selected from a modified virus derived from a virus selected from the group consisting of a retrovirus, lentivirus, gammavirus, adenovirus, adeno-associated virus, pox virus, alphavirus, and herpes virus.

In particular embodiments, the vector is a retrovirus, such as a modified gammavirus, lentivirus, murine stem cell virus, moloney murine leukemia virus, bovine leukaemia virus, Rous sarcoma virus, and spumavirus. In fact, of the 52 clinical trials evaluating CAR-T cell in solid tumors which are listed in (11), 24 use retroviral vectors and 9 use lentiviral vectors. It is also noted that the two FDA-approved CAR products for the treatment of B cell malignancies are Kymriah™ (lentiviral vector) and Yescarta™ (gamma-retroviral vector). Thus, good candidates for the viral vector of the present invention may be retroviral vectors, lentiviral vectors and gamma-retroviral vectors. For example, the retrovirus may be derived from moloney murine leukemia virus or murine stem cell virus sequences (gamma-retroviral vectors).

In certain embodiments, the nucleic acid molecule is the sole polypeptide encoded by the nucleotide sequence, i.e. the nucleic acid molecule of the viral vector does not encode for additional different proteins, but may comprise additional control elements such as promoters and terminators.

In another aspect, the present invention provides a composition comprising the viral vector as defined above.

In still another aspect, the present invention provides a mammalian regulatory T cell (Treg) comprising any one of the nucleic acid molecules as defined above, or the viral vector as defined above.

In certain embodiments, the mammalian Treg expresses on its surface a homodimeric IL-10 that is linked to a transmembrane-intracellular stretch, optionally through a flexible hinge.

In a certain embodiment, the mammalian Treg is a human Treg.

In certain embodiments, the mammalian Treg has a stable Tr1 phenotype (that is, not losing their regulatory activity (12) exhibiting the cell-surface markers CD49b and LAG-3.

In yet an additional aspect, the present invention provides a method of preparing allogeneic or autologous Tregs with a stable Tr1 phenotype, the method comprising contacting CD4 T cells with the nucleic acid molecule comprising a nucleotide sequence encoding a homodimeric IL-10 as defined above, or a viral vector comprising it, thereby endowing said CD4 T cells with a stable Tr1 phenotype, and thus preparing Tregs with a stable Tr1.

Methods for preparing CD4 T cells are well known in the art and may be performed e.g. by the method disclosed below in the Examples section.

Methods for creating recombinant retroviral and lentiviral vectors and using them for transducing T cells are also well-known in the art and are usually performed using commercial kits including packaging cells, plasmids and transfection reagents, which are offered by many companies, including Invitrogen®, Sigma®, Clontech®, Cell Biolabs®, SBI®, Genecopoeia® and many others. The methods are thus performed along with the guidelines supplied with the commercial kits.

In short, according to a non-limiting example taught by the γ-Retrovirus Guide on the website of Addgene, the following components are needed: (a) γ-Retroviral transfer plasmid encoding a transgene of interest: The transgene sequence is flanked by long terminal repeat (LTR) sequences, which facilitate integration of the transfer plasmid sequences into the host genome. Typically it is the sequences between and including the LTRs that is integrated into the host genome upon viral transduction; (b) Packaging genes (viral Gag-Pol): Gag is a structural precursor protein, and Pol is a polymerase; and (c) Envelope gene (may be pseudotyped to alter infectivity).

As a non-limiting example, the three components described above (envelope, packaging, and transfer) are supplied by three types of plasmids, which are cotransfected into a 293T packaging cell line. This system provides the greatest flexibility to pseudotype γ-retrovirus using

different envelopes to modify tropism. Briefly, different envelope plasmids can direct the production of virus with various tropisms. A detailed non-limiting example of methods for preparation of recombinant retroviral stock and retroviral transduction of human CD4 T cells is found below in the Examples section.

In still an additional aspect, the present invention provides a method for increasing immune suppression in a subject in need, comprising administering to said subject the mammalian Treg expressing on its surface a homodimeric membrane-bound IL-10 as defined above.

In certain embodiments, the subject is in need of increasing immune suppression because of symptoms caused by a disease, disorder or condition, manifested in excessive or otherwise unwanted activity of the immune system.

Thus, in certain embodiments, the present invention provides a method of treating or preventing a disease, disorder or condition in a subject, comprising administering to said subject the mammalian Treg expressing on its surface a homodimeric IL-10 as defined above, wherein said disease, disorder or condition is manifested in excessive or otherwise unwanted activity of the immune system, such as an autoimmune disease, allergy, asthma, and organ and bone marrow transplantation.

In yet another aspect, the present invention is directed to the mammalian Treg expressing on its surface a homodimeric IL-10 as defined above, for use in increasing immune suppression in a subject in need.

In certain embodiments, the mammalian Treg expressing on its surface a homodimeric IL-10 as defined above, are for use in treating or preventing a disease, disorder or condition, manifested in excessive or otherwise unwanted activity of the immune system.

In certain embodiments, the mammalian Treg is for treating a human subject and the mammalian Treg is a human Treg.

The specific diseases defined as autoimmune diseases are well known in the art; for example, as disclosed in The Encyclopedia of Autoimmune Diseases, Dana K. Cassell, Noel R. Rose, Infobase Publishing, 14 May 2014, incorporated by reference in its entirety as if fully disclosed herein.

In certain embodiments, the autoimmune disease is selected from type 1 diabetes; rheumatoid arthritis; psoriasis; psoriatic arthritis; multiple sclerosis; systemic lupus erythematosus; inflammatory bowel disease, such as Crohn's disease and ulcerative colitis; Addison's disease; Graves' disease; Sjögren's syndrome; Hashimoto's thyroiditis; myasthenia gravis; vasculitis; pernicious anemia; celiac disease; and atherosclerosis.

In some embodiments, the subject is human and said mammalian Treg is human.

In some embodiments, Treg is an allogeneic Treg.

The stable Tr1 cells of the present invention may be used to increase immune suppression and treat diseases, disorders or conditions manifested in excessive or otherwise unwanted activity of the immune system without further genetic manipulation as evident from pre-clinical studies demonstrating that adoptive transfer of purified $CD4^+$ $CD25^+$ Tregs can inhibit or prevent disease in a range of models of autoimmune illness. These include, but are not restricted to systemic lupus erythematosus, inflammatory bowel disease, autoimmune encephalomyelitis, type 1 diabetes, autoimmune hepatitis and collagen-induced arthritis. Furthermore, adoptive transfer of these cells can protect against allograft rejection and graft versus host disease induced by allogeneic hematopoietic stem cell transplantation (13). In addition, a growing number of clinical trials evaluating the safety and efficacy of the adoptive transfer of *ex-vivo*-expanded, non-antigen-specific Tregs in the immunotherapy of a number of conditions and diseases, including graft-versus-host disease (GvHD), allograft rejection and type 1 diabetes (see (13) for review) show promise for this approach.

The beneficial clinical response observed in these studies may be improved in light of the cumulative evidence arguing that engagement of Tregs with antigen through their endogenous TCR enhances immune suppression (14–16).

The inventors of the present invention envision an approach in which the Tr1 cells are manipulated to express tissue-targeting proteins. For example, retinoic acid (RA) induces the expression of the gut-homing receptors integrin $\alpha4\beta7$ and chemokine receptor CCR9 in T cells and can exert this function *in vivo* following pre-incubation *ex-vivo* (17, 18). RA is also a key regulator of TGF-β-mediated suppression by Tregs and promotes Treg differentiation (19). RA has also been shown to enhance the conversion of naïve CD4 Teff cells into induced Tregs (20, 21) and to sustain Treg stability and function in the presence of IL-6 in an inflammatory environment (18). Preincubation with all-trans RA emerges as a feasible and simple procedure for equipping the reprogrammed Tr1 cells with gut homing capacity. The Tregs used in the methods for treating diseases as defined above may thus be contacted with retinoic acid prior to administration to the subject in order to equip the reprogrammed Tr1 cells with gut homing capacity and to sustain Treg stability and function in the presence of IL-6 in an inflammatory environment.

An attractive alternative solution capitalizes on the well-established ability to genetically redirect large numbers of T cells against cell surface antigens of choice using chimeric antigen receptors, or CARs (22).

In principle, CARs can also be used for reprogramming Tregs. Indeed, several laboratories have recently described the generation of functional mouse and human CAR-Tregs in different experimental settings ((23–29) and see (13, 16, 30, 31) for review). Redirecting Tregs through the transfer of exogenous TCR genes has also been reported (32–34).

A recent work in this field (28) has employed lentiviral transduction for generating HLA-A2–specific human CAR-Tregs as a means for preventing xenogeneic GvHD in immunodeficient mice caused by HLA-$A2^+$ effector T cells. Indeed, *in-vivo* these CAR-Tregs were markedly superior to the same number CAR-Tregs of an irrelevant specificity in suppressing GvHD. The number of the HLA-A2 CAR-Tregs that were detectable in the blood of recipient mice peaked one week post-administration, remained stable for another week and then declined to near zero at the end of the third week.

Another example for the intended clinical use of CAR-Tregs has been reported recently, where retrovirally transduced human Tregs have been redirected at coagulation factor VIII (FVIII) in attempt to suppress the antibody response in replacement therapy for hemophilia A (29). Using a xenogeneic immunocompetent mouse model, strong suppression of the antibody response was evident 8 weeks post-immunization, although the introduced CAR-Tregs were already undetectable 2 weeks post-transfer.

Thus, the mammalian Tregs expressing on their surface a membrane-bound homodimeric IL-10 as defined herein and having a stable Tr1 phenotype are efficient agents for increasing immune suppression and treating diseases, disorders or conditions manifested in excessive or otherwise unwanted activity of the immune system; and agents that can be readily manipulated using techniques well-known in the art for increased efficacy. Furthermore, methods employing adoptive transfer of *ex-vivo*-expanded, non-antigen-specific as well as redirected antigen-specific Tregs are well known in the field of immunotherapy.

Definitions

The term "Tr1 cells" is used interchangeably herein with the terms "iTregs" or "type 1 cells" and refers to CD4 T cells that are characterized by the expression of two cell surface markers, CD49b and LAG-3, low, or no expression of FoxP3, a non-proliferative (anergic) state, high production of IL-10 and TGF-β, but only minimally of IL-2 and none of IL-4 or IL-17, and the ability to suppress effector T cells (Teffs) in a cell-to-cell contact-independent manner.

The term “treating” as used herein refers to means of obtaining a desired physiological effect. The effect may be therapeutic in terms of partially or completely curing a disease and/or symptoms attributed to the disease. The term refers to inhibiting the disease, i.e. arresting its development; or ameliorating the disease, i.e. causing regression of the disease.

As used herein, the terms "subject" or "individual" or "animal" or "patient" or "mammal," refers to any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired, for example, a human.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

The following exemplification of carriers, modes of administration, dosage forms, etc., are listed as known possibilities from which the carriers, modes of administration, dosage forms, etc., may be selected for use with the present invention. Those of ordinary skill in the art will understand, however, that any given formulation and mode of administration selected should first be tested to determine that it achieves the desired results.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local. In certain embodiments, the pharmaceutical composition is adapted for oral administration.

The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the active agent is administered.

The term “variant" as used herein refers to polynucleotides or polypeptides modified at one or more base pairs, codons, introns, exons, or amino acid residues, respectively, yet still retain the biological activity of a polypeptide of the naturally occurring sequence

Unless otherwise indicated, all numbers expressing identity or similarity or any other parameter are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in this description and attached claims are approximations that may vary by up to plus or minus 10% depending upon the desired properties sought to be obtained by the present invention.

The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Materials and Methods

Separation of human CD4 T cells

Peripheral blood monocytes (PBMCs) have been prepared from whole blood samples or pheresis products using a standard Ficoll-Paque (Sigma) separation procedure. Twenty four

hours post-separation (or after cell thawing) PBMCs were activated for 72 hours by plate-bound anti-CD3 Ab (OKT3) in the presence of soluble anti-CD28 and recombinant human IL-2. CD4 T cells were then separated using positive selection with magnetic beads (BD IMag™) and then placed in complete medium for a 24 hour rest before experimental use.

Preparation of recombinant retroviral stock

The memIL-10 gene was cloned into the commonly used MSGV1 retroviral vector via the BamHI-EcoRI restriction sites. The resulting plasmid, together with a plasmid carrying gag/pol and a plasmid carrying env were co-transfected to $3x10^6$ HEK293T cells placed in a 10 cm poly-D-lysine-coated plate in OptiMEM™ medium (a modification of Eagle's Minimum Essential Media, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factor) with no antibiotics, using a transfection reagent such as either Lipofectamine (Thermo Fisher Scientific®) or Fugene HD® (Promega®) according to the manufacturers' instructions. Next day cells were moved to complete medium with antibiotics and in the following day supernatant was collected and either frozen in aliquots or used directly for retroviral transduction.

Retroviral transduction of human CD4 T cells

Transduction was performed in non-coated 6-well tissue culture plates. Wells were coated with a gene transduction enhancer (RetroNectin®; Takara®) overnight. RetroNectin® is a 63 kD fragment of recombinant human fibronectin fragment (also referred to as rFN-CH-296) that enhances the efficiency of lentiviral- and retroviral-mediated gene transduction. RetroNectin® was removed and wells were washed, blocked with 2.5% sterile bovine serum albumin (BSA) in phosphate buffered saline (PBS) and washed again. Viral supernatant was diluted in Dulbecco's Modified Eagle's medium (DMEM) containing a transfection reagent such as Polybrene (Merck®) and moved to the RetroNectin®-coated wells at 4 ml/well. Plates were centrifuged at 2000xg for 2 hours at 32°C, supernatant was aspirated and 4 ml of CD4 T cells at 5x105 cells/ml in 50/50 AIM-V/RPMI medium + 300 IU/ml recombinant IL-2 were added to each well. Plates were centrifuged for 15 minutes at 1000xg and incubated at 37°C overnight. CD4 T cells were then moved to new coated 6-well tissue culture plates and 1 ml of fresh 50/50 medium + 300 IU/ml rIL-2 was added to each well. In the following days medium was replaced and cells were split as needed.

Example 1. Two IL-10 monomers linked together in tandem by a flexible linker and linked to a transmembrane-intracellular stretch via a short hinge region.

In the specific construct used here, two IL-10 monomers were linked together in tandem by a flexible linker of the sequence GSTSGSGKPGSGEGSTKG to create a homodimer, which was then linked to the transmembrane-intracellular stretch derived from the HLA-A2 heavy chain by a flexible hinge regions having a 21 amino acid spacer comprising the flexible linker $Gly_4Ser(Gly_3Ser)_2$ and an additional 8 amino acid bridge of the sequence SSQPTIPI derived from the membrane-proximal part of the connecting peptide of HLA-A2 (**Fig. 1**). Surface expression of memIL-10 and IL-10R on human and mouse CD4 T cells was then confirmed (**Fig. 2**).

Elevation of the CD49b integrin could be observed in (**A**) and upregulation of IL-10 receptor (IL-10R) was similar to that induced by recombinant IL-10 (rIL-10, (**B**)). Mouse memIL-10 was clearly expressed 48 hours post-transfection (**D, left**) and, as expected, memIL-10 blocked the binding of the anti-mouse IL-10R mAb we used, suggesting binding in-cis (35).

Example 2. Two IL-10 monomers linked together in tandem by a flexible linker and linked to a transmembrane-intracellular stretch via a long hinge region or the IL-10R β chain.

Our original memIL-10 constructs, both human and mouse, incorporated a hinge comprising a flexible linker of 21 amino acids (in addition to an 8 amino acid-long rigid spacer, now referred to herein as SmemIL-10 (S for short linker, see below).

In attempt to optimize our memIL-10 we have engineered and cloned two new versions of this membrane cytokine: In one, cloned first, we provided memIL-10 with a longer linker peptide (of 30 amino acids, termed LmemIL-10 for long) to facilitate optimal engagement with IL-10R (**Fig. 3, lower left**). To create another derivative we fused our dimeric IL-10 to the N-terminus of the IL-10R β chain as a new scaffold designed to endow it with direct access to the IL-10 binding site located on the IL-10R α chain, designated memIL-10RB (**Fig. 3, lower right**). Indeed, **Fig. 4** confirms surface expression of the three products in human Jurkat cells. Of note, it is expected that the level of surface expression of the memIL-10RB fusion protein depends on the availability of IL-10Rα chain. To evaluate expression and function of the three different memIL-10 configurations mouse CD4 T cells were transfected with mRNA encoding the three constructs and assayed for surface expression (Fig. **5A**), downregulation of surface IL-10R (**Fig. 5B**) and spontaneous phosphorylation of STAT3 (**Fig. 5C**). Indeed, in agreement with the results obtained in Jurkat cells, the constructs harboring the short and long linkers are

expressed at much higher levels than memILL-10Rβ and exhibit superior function, as evident from the greater reduction in surface IL-10R and the stronger induction of pSTAT3. As the short linker construct (sLmemIL-10) was superior to the long linker one (lLmemIL-10) in its ability to induce pSTAT3 also in repeated experiments (not shown) it was selected for further experiments.

Example 4. Expression and characterization of memIL-10 in retrovirally transduced mouse CD4 T cells.

To test expression and function of memIL-10 in retrovirally transduced T cells we first used splenic CD4 T cells purified with magnetic beads from C57BL/6 (B6) mice. As a negative control for memIL-10 transduced cells we used mock-transduced cells (Mock). Soluble IL-10 (sIL-10) was used in these experiments as a positive control. **Fig. 6** shows the results of a flow cytometry analysis of transduced cells vs. non-transduced ones which grew in the same culture and mock-transduced cells for the expression of the three Tr1-associated markers LAG-3, CD49b and PD-1 48 hours and 6 days post-transfection. Clear elevation of the 3 markers could indeed be observed already at day 2 which also persisted at day 6, pointing the expected phenotype. The ability of the transduced T cells to secrete IL-10 upon TCR-mediated activation confirmed the acquisition of Tr1-like functional properties **(Fig. 7)**.

Example 5. Assessing inhibitory effect of transduced cells on T effector cells.

To examine the ability of transduced cells to exert their inhibitory effect on neighboring Teff cells a coculture setting is designed which will allow us to selectively activate at will only one T cell population and not the other (obviously, anti-TCR/CD3 antibodies would activate all T cells in the coculture). To this end we will exploit two genes we have created, encoding the chimeric H-2K^b-CD3ζ (K^b-CD3ζ) and H-2K^d-CD3ζ (K^d-CD3ζ) MHC-I heavy chains. We have already shown that both genes selectively activate T cells following Ab-mediated cross-linking in magnitude that is comparable to TCR cross-linking. In the following series of functional experiments these tools are employed to mix mRNA-transfected Tr1 and Teff cells at different ratios for 3-4 days and use CFSE dilution and intracellular IFN-γ staining to assess the ability of activated Tr1 cells (vs. non-activated or RFP+ non-Tr1 cells) to suppress both proliferation and effector function of the activated Teffs.

Example 6. Assessing in-vivo persistence of IL-10-transduced cells and suppressive function in mouse models for human diseases.

To evaluate in-vivo persistence of the IL-10-transduced NOD or B6 CD4 T cells in syngeneic wild-type mice and maintenance of their phenotype a protocol we recently established in our T1D experimental system (36) is used. Briefly, 10x106 cells are injected into the tail vain. Spleen and peripheral lymph nodes are harvested 1, 7 and 14 days post-injection and CD4+IL-10+LAG-3+CD49b+ T cells are identified by flow cytometry (compared to background level of staining in non-injected mice).

The actual suppressive function of memIL-10-tarsduced T cells under physiological conditions in-vivo is then tested, employing mouse models for human diseases such as T1D or IBD.

Example 7. Expression and characterization of memIL-10 in retrovirally transduced human CD4 T cells.

For assessing the phenotypic and functional outcome of retroviral transduction of human CD4 T cells we isolated CD4 T cells from blood samples obtained from healthy donors through the Blood Services Center of Magen David Adom, Israel. The first of two independent *ex-vivo* experiments is presented in **Fig. 8**. In this experiment cells have been kept in culture eighteen days post-transduction and phenotypic analyses for the markers LAG-3, CD49b, PD-1, 4-1BB, CD25 and IL-10Rα were performed by flow cytometry at days 1, 5 and 18 post-transduction. Our results confirm that all these cell surface markers that are associated with the expected Tr1 phenotype were significantly increased in memIL-10-expressing cells compared to memIL-10-negative cells that grew in the same culture dish for the entire period of the experiment.

The second experiment was performed on a different blood sample and flow cytometry performed for LAG-3, CD49b and PD-1 (**Fig. 9**) are in line with the results obtained in the first experiment. From these two experiments it can be concluded that long-term expression of memIL-10 in human CD4 T cells via retroviral transduction endows these cells with a TR-1-like phenotype.

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CLAIMS

1. A nucleic acid molecule comprising a nucleotide sequence encoding a membrane-bound homodimeric IL-10 (mem-IL10) comprising a homodimeric IL-10 linked to a transmembrane-intracellular stretch.

2. The nucleic acid molecule of claim 1, wherein said homodimeric IL-10 is linked to said transmembrane-intracellular stretch through a flexible hinge.

3. The nucleic acid molecule of claim 1 or 2, wherein said homodimeric IL-10 comprises a first and a second IL-10 monomer connected in a single-chain configuration such that the C-terminus of the first IL-10 monomer is linked to the N-terminus of the second IL-10 monomer via a first flexible linker.

4. The nucleic acid molecule of claim 3, wherein said first flexible linker has the amino acid sequence GSTSGSGKPGSGEGSTKG (SEQ ID NO: 1).

5. The nucleic acid molecule of any one of claims 2 to 4, wherein said flexible hinge comprises a polypeptide selected from a hinge region of CD8α, a hinge region of a heavy chain of IgG, a hinge region of a heavy chain of IgD; an extracellular stretch of an IL-10R β chain; and a second flexible linker comprising an amino acid sequence of up to 28 amino acids comprising at least one Gly4Ser(Gly3Ser)$_2$ sequence.

6. The nucleic acid molecule of claim 5, wherein said second flexible hinge comprises a 21 amino acid sequence comprising the amino acid sequence $Gly_4Ser(Gly_3Ser)_2$ (referred to herein as "short linker"; SEQ ID NO: 13).

7. The nucleic acid molecule of claim 5, wherein said second flexible linker comprises a 28 amino acid sequence comprising the amino acid sequence $Gly_4Ser(Gly_3Ser)_2Ser_2(Gly_3Ser)_3$ (referred to herein as "long linker"; SEQ ID NO: 15).

8. The nucleic acid molecule of any one of claims 5 to 7, wherein said polypeptide further comprises an amino acid bridge of the sequence SSQPTIPI (SEQ ID NO: 17;).

9. The nucleic acid molecule of any one of claims 1 to 8, wherein said transmembrane-intracellular stretch is derived from a heavy chain of a human MHC class I molecule selected from an HLA-A, HLA-B or HLA-C molecule, preferably HLA-A2; human CD28; or human IL-10R β chain.

10. The nucleic acid molecule of any one of claims 1 to 9, wherein the amino acid sequence of the complete mem-IL10 comprises or essentially consists of the homodimeric IL-10 linked via the short second flexible linker and the connecting peptide to the transmembrane-intracellular stretch of HLA-A2 as set forth in SEQ ID NO: 25.

11. The nucleic acid molecule of any one of claims 1 to 9, wherein the amino acid sequence of the complete mem-IL10 comprises or essentially consists of the homodimeric IL-10 linked via the long second flexible linker and the connecting peptide to the transmembrane-intracellular stretch of HLA-A2 as set forth in SEQ ID NO: 27.

12. The nucleic acid molecule of any one of claims 1 to 9, wherein the homodimeric IL-10 is linked to the N-terminus of an essentially complete human IL-10R β chain via the short linker as set forth in SEQ ID NO: 29.

13. A composition comprising the nucleic acid molecule of any one of claims 1 to 12.

14. A viral vector comprising the nucleic acid molecule of any one of claims 1 to 12.

15. The viral vector of claim 14, which is a modified virus derived from a virus selected from a retrovirus, lentivirus, gammavirus, adenovirus, adeno-associated virus, pox virus, alphavirus, and herpes virus.

16. A composition comprising the viral vector of claim 14 or 15.

17. A mammalian regulatory T cell (Treg) comprising the nucleic acid molecule of any one of claims 1 to 12 or the viral vector of claim 14 or 15.

18. The mammalian Treg of claim 17, expressing on its surface a homodimeric IL-10 that is linked to a transmembrane-intracellular stretch.

19. The mammalian Treg of claim 17 or 18, which is a human Treg.

20. The mammalian Treg of any one of claims 17 to 19 having a stable Tr1 phenotype exhibiting the cell-surface markers CD49b and LAG-3.

21. The mammalian Treg of claim 20, further exhibiting PD-1, 4-1BB, CD25 and IL-10Rα.

22. A method of preparing allogeneic or autologous Tregs with a stable Tr1 phenotype, the method comprising contacting CD4 T cells with the nucleic acid molecule of any one of claims 1

to 12 or a viral vector comprising it, thereby endowing said CD4 T cells with a stable Tr1 phenotype, and thus preparing Tregs with a stable Tr1 phenotype.

23. The mammalian Treg of any one of claims 17 to 21, for use in increasing immune suppression in a subject in need thereof.

24. The mammalian Treg of 23, for use in treating or preventing a disease, disorder or condition, manifested in excessive or unwanted activity of the immune system.

25. The mammalian Treg for the use of claim 24, wherein said disease, disorder or condition is selected from an autoimmune disease, allergy, asthma, and organ and bone marrow transplantation.

26. The mammalian Treg for the use of claim 25, wherein the autoimmune disease is selected from type 1 diabetes; rheumatoid arthritis; psoriasis; psoriatic arthritis; multiple sclerosis; systemic lupus erythematosus; inflammatory bowel disease, such as Crohn's disease and ulcerative colitis; Addison's disease; Graves' disease; Sjögren's syndrome; Hashimoto's thyroiditis; myasthenia gravis; vasculitis; pernicious anemia; celiac disease; and atherosclerosis.

27. The mammalian Treg for the use of any one of claims 23 to 26, wherein said mammalian Treg is a human Treg and is used for treating or preventing said disease, disorder or condition in a human subject.

28. The mammalian Treg for the use of claim 27, wherein said human Treg is an allogeneic Treg.

Fig. 1

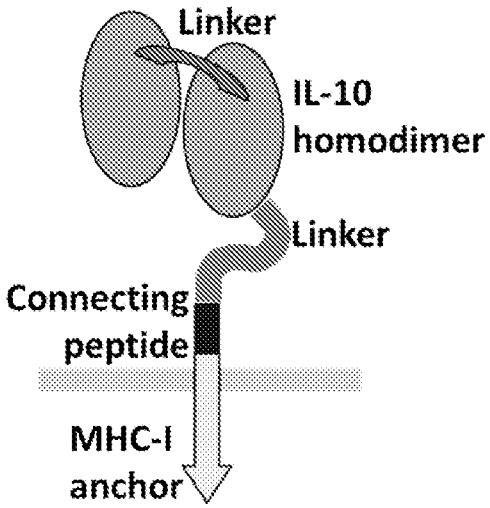


Fig. 2A

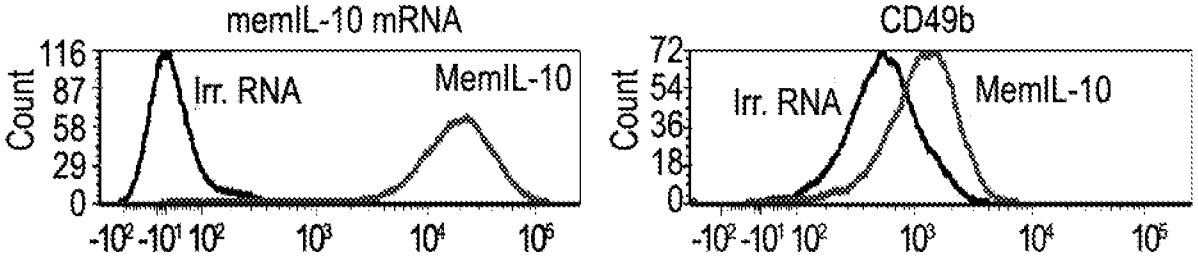


Fig. 2B

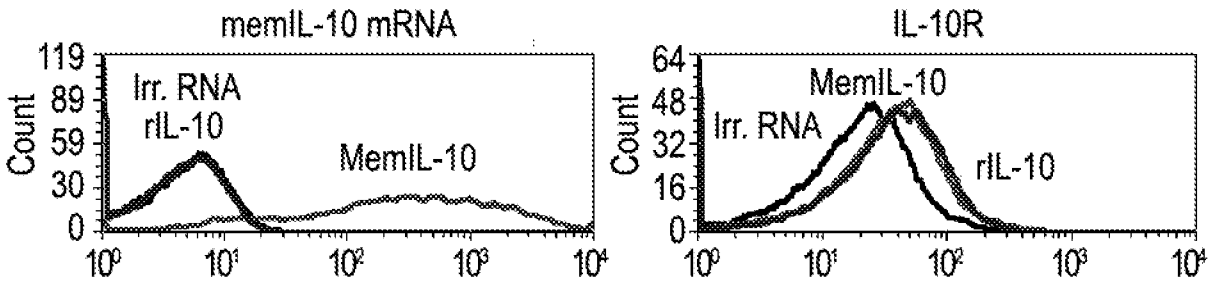


Fig. 2C

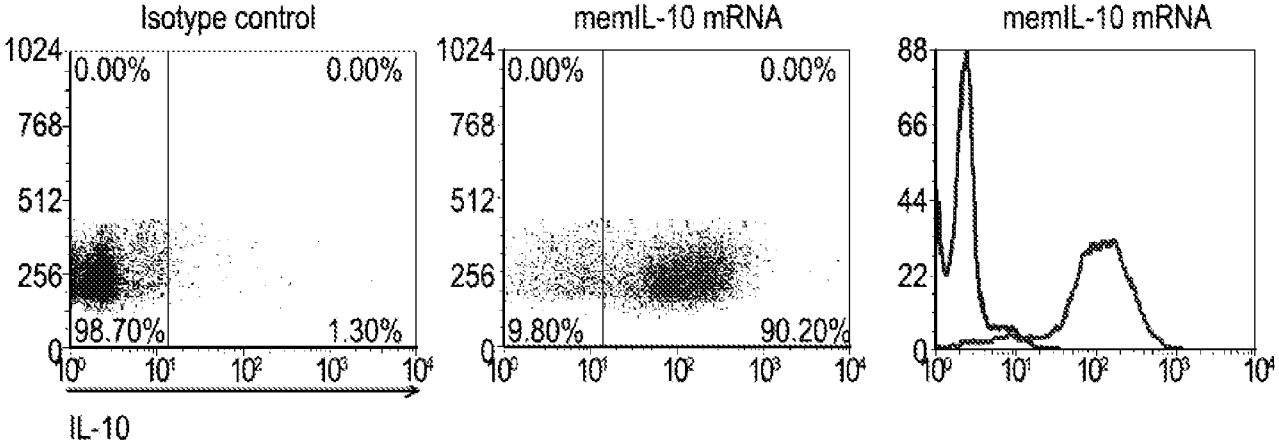

Isotype control
memIL-10 mRNA
memIL-10 mRNA
0.00%
0.00%
98.70%
1.30%
9.80%
90.20%
IL-10

Fig. 2D

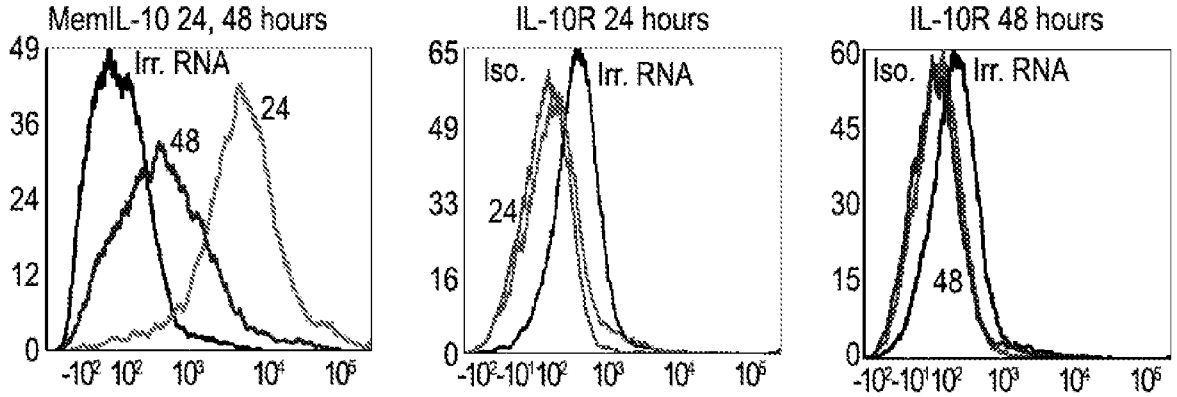

MemIL-10 24, 48 hours
IL-10R 24 hours
IL-10R 48 hours
Irr. RNA
Iso.
24
48

Fig. 3A

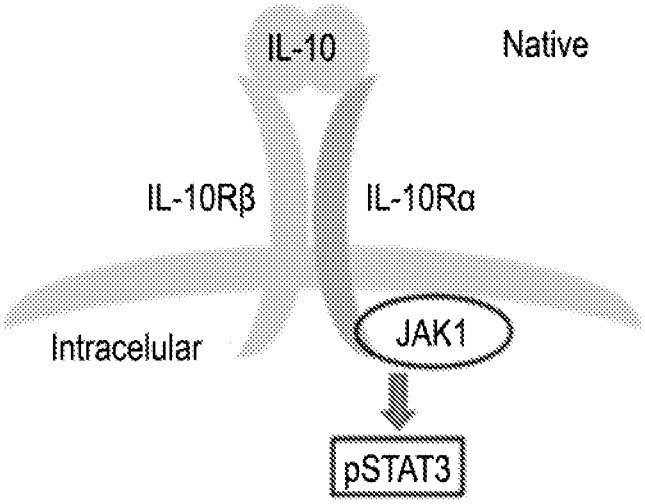

IL-10
Native
IL-10Rβ
IL-10Rα
Intracelular
JAK1
pSTAT3

Fig. 3B

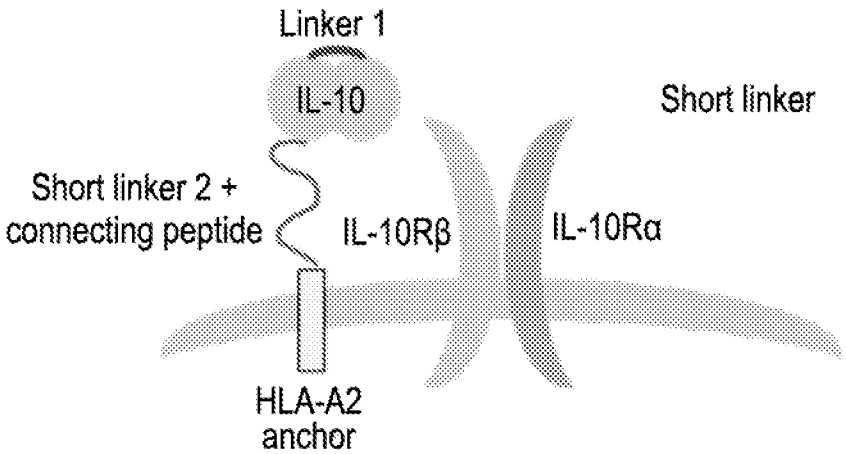

Linker 1
IL-10
Short linker
Short linker 2 +
connecting peptide
IL-10Rβ
IL-10Rα
HLA-A2
anchor

Fig. 3C

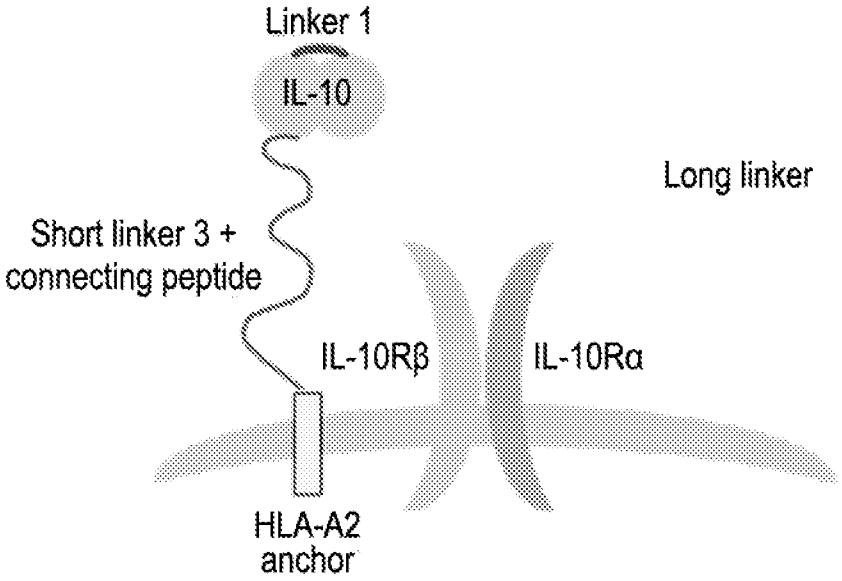

Linker 1
IL-10
Long linker
Short linker 3 +
connecting peptide
IL-10Rβ
IL-10Rα
HLA-A2
anchor

Fig. 3D

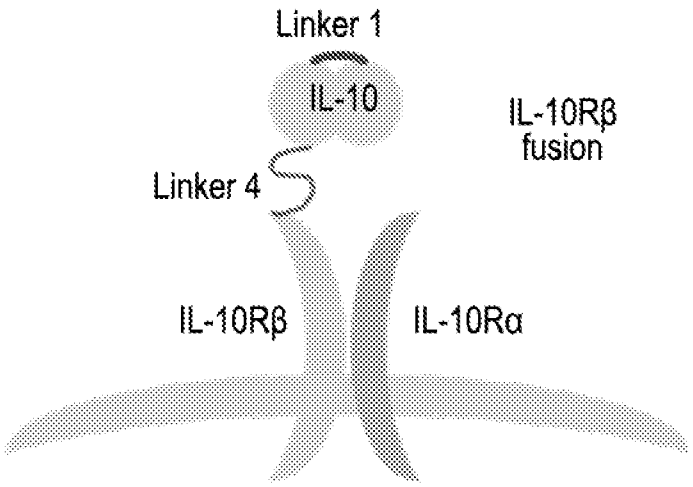

Linker 1
IL-10
IL-10Rβ
fusion
Linker 4
IL-10Rβ
IL-10Rα

Fig. 4

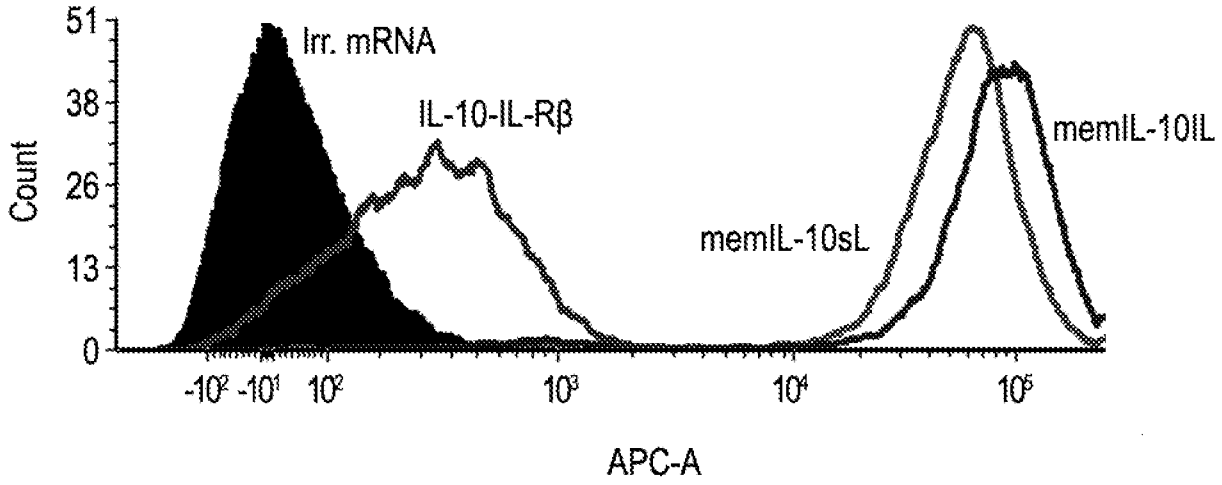

51
38
26
13
0
Count
Irr. mRNA
IL-10-IL-Rβ
memIL-10sL
memIL-10IL
-10² -10¹ 10²
10³
10⁴
10⁵
APC-A

Fig. 5A

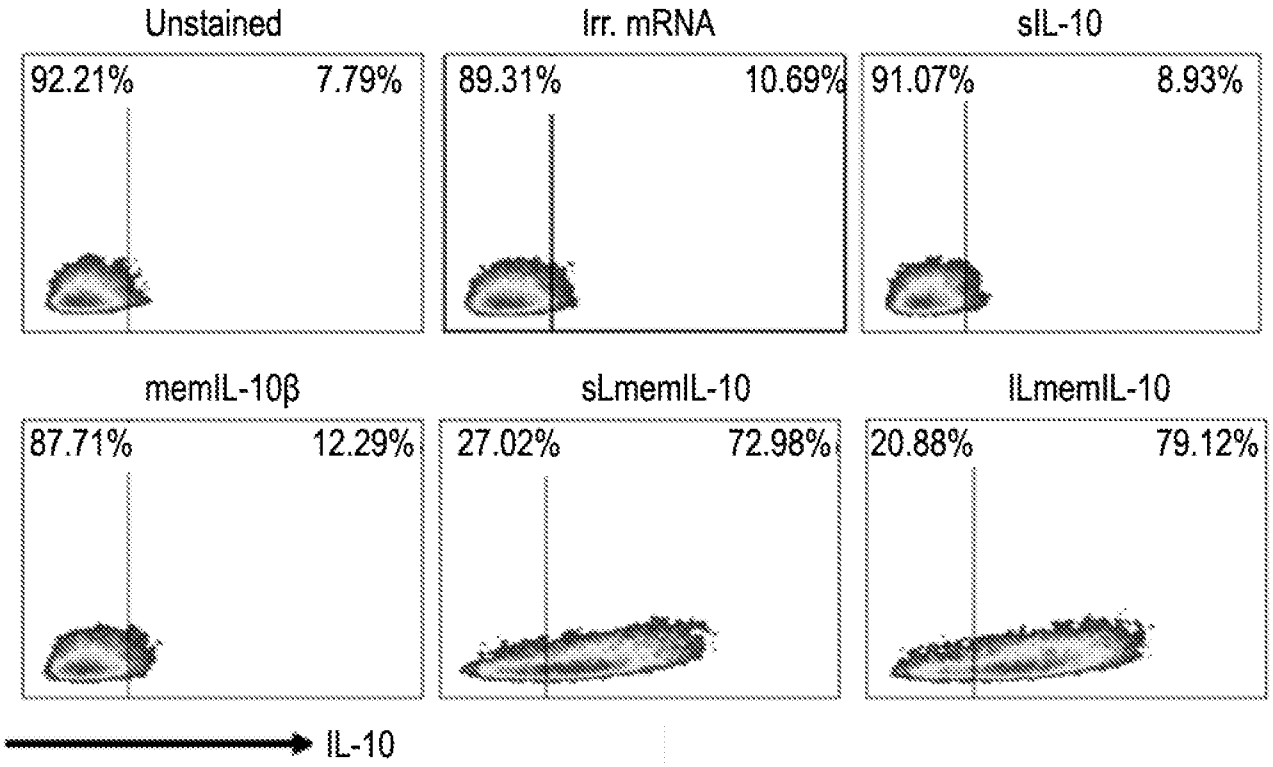

Unstained
92.21%
7.79%
Irr. mRNA
89.31%
10.69%
sIL-10
91.07%
8.93%
memIL-10β
87.71%
12.29%
sLmemIL-10
27.02%
72.98%
lLmemIL-10
20.88%
79.12%
IL-10

Fig. 5B

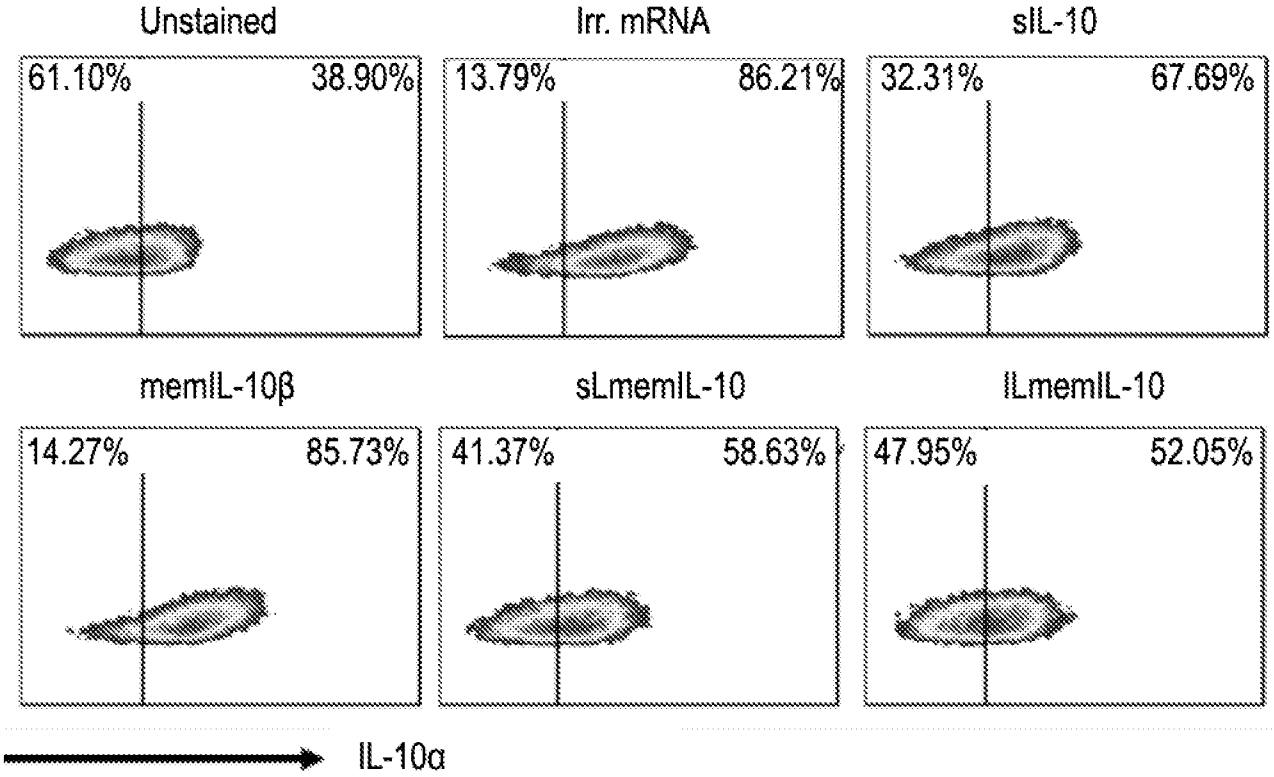

Unstained
61.10%
38.90%
Irr. mRNA
13.79%
86.21%
sIL-10
32.31%
67.69%
memIL-10β
14.27%
85.73%
sLmemIL-10
41.37%
58.63%
lLmemIL-10
47.95%
52.05%
IL-10α

Fig. 5C

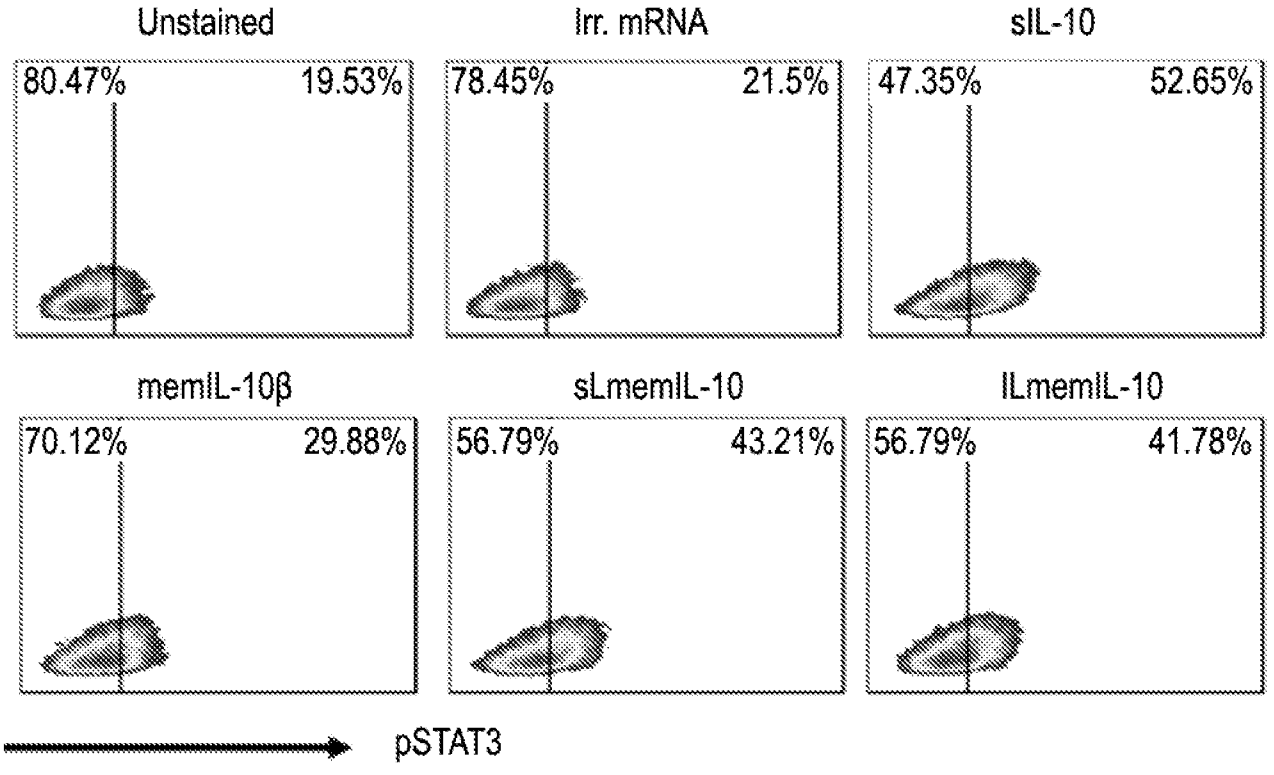

Unstained
80.47%
19.53%
Irr. mRNA
78.45%
21.5%
sIL-10
47.35%
52.65%
memIL-10β
70.12%
29.88%
sLmemIL-10
56.79%
43.21%
lLmemIL-10
56.79%
41.78%
pSTAT3

Fig. 6A

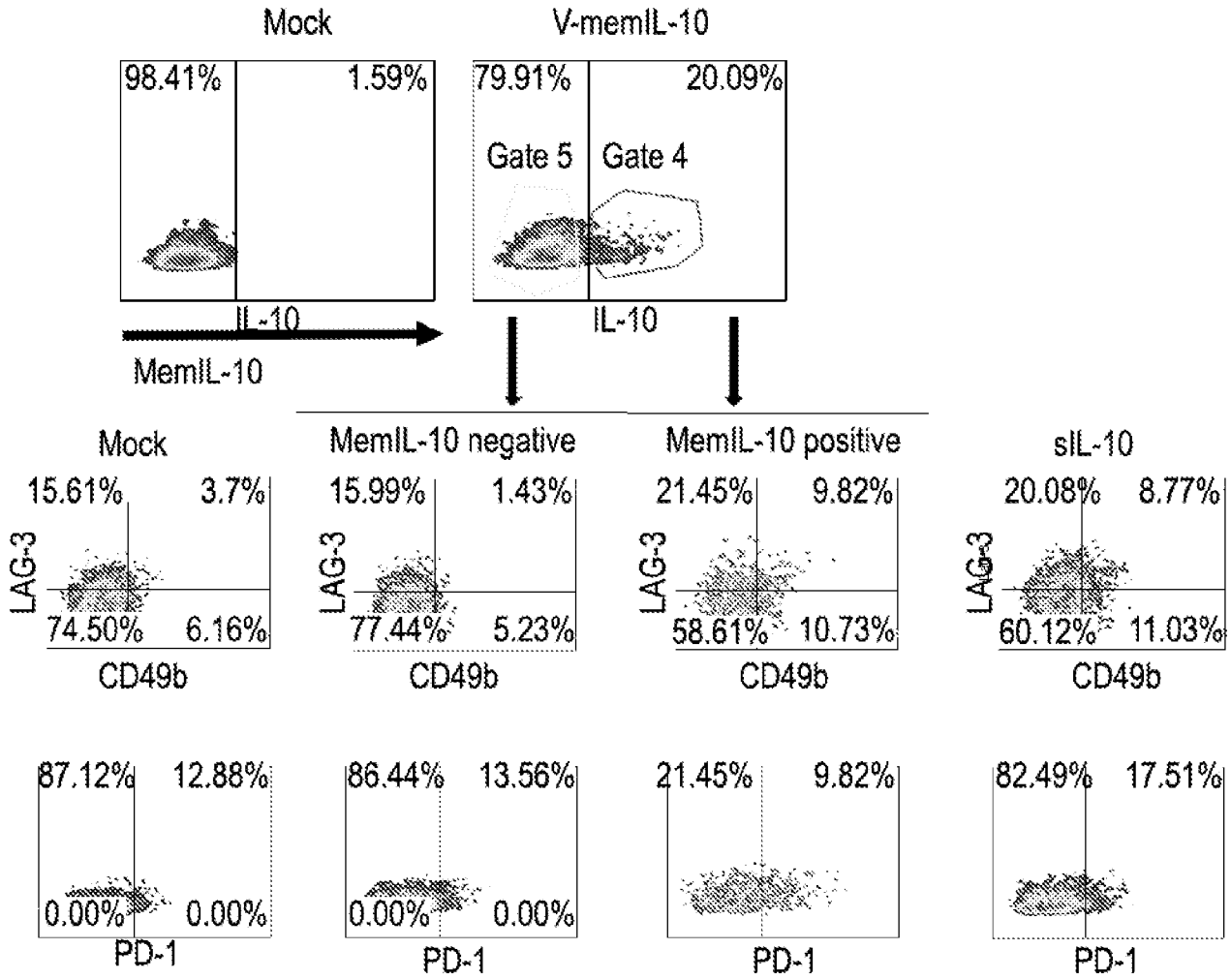

Mock
V-memIL-10
98.41%
1.59%
79.91%
20.09%
Gate 5
Gate 4
IL-10
IL-10
MemIL-10
Mock
MemIL-10 negative
MemIL-10 positive
sIL-10
15.61%
3.7%
74.50%
6.16%
15.99%
1.43%
77.44%
5.23%
21.45%
9.82%
58.61%
10.73%
20.08%
8.77%
60.12%
11.03%
LAG-3
CD49b
87.12%
12.88%
0.00%
0.00%
86.44%
13.56%
0.00%
0.00%
21.45%
9.82%
82.49%
17.51%
PD-1

Fig. 6B

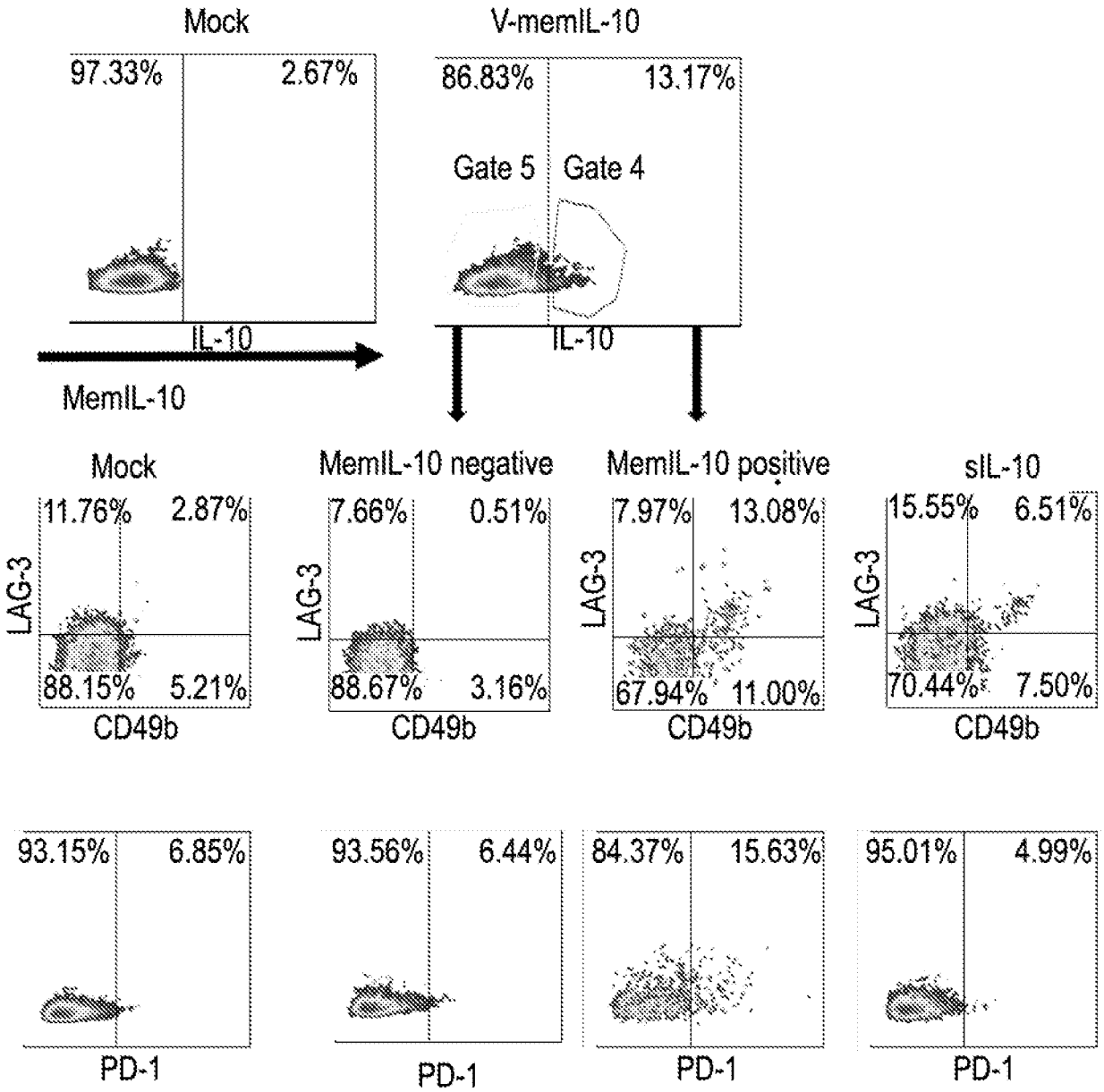

Mock
V-memIL-10
97.33%
2.67%
86.83%
13.17%
Gate 5
Gate 4
IL-10
IL-10
MemIL-10
Mock
MemIL-10 negative
MemIL-10 positive
sIL-10
LAG-3
11.76%
2.87%
88.15%
5.21%
CD49b
7.66%
0.51%
88.67%
3.16%
7.97%
13.08%
67.94%
11.00%
15.55%
6.51%
70.44%
7.50%
93.15%
6.85%
93.56%
6.44%
84.37%
15.63%
95.01%
4.99%
PD-1

Fig. 7

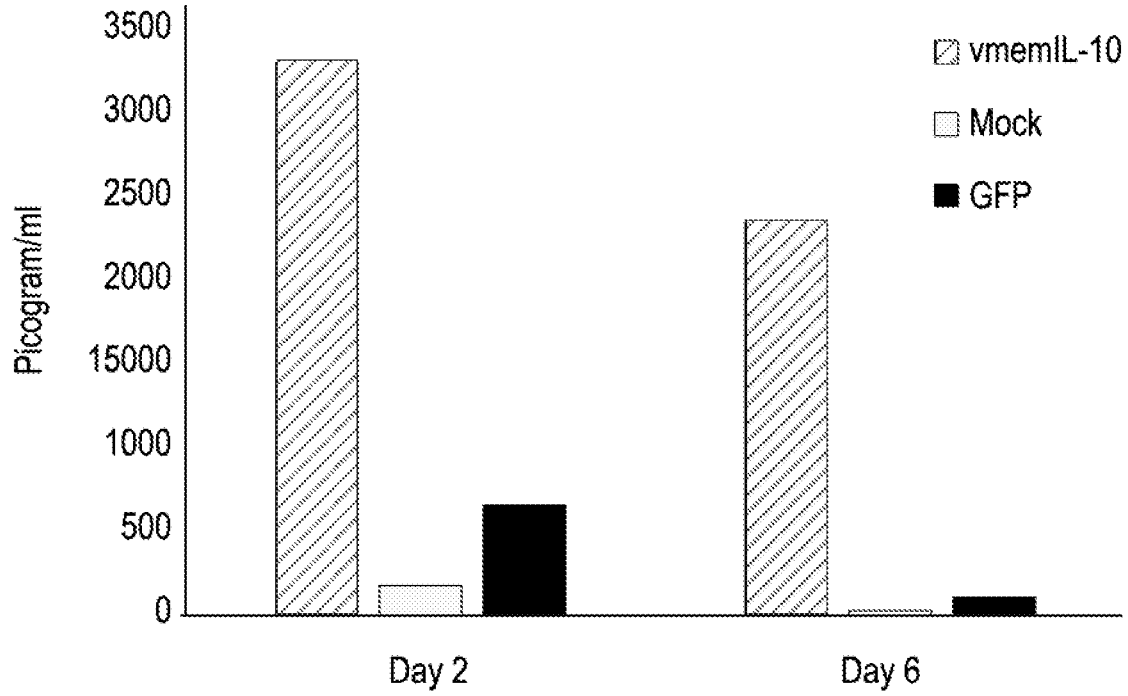

3500
3000
2500
2000
15000
1000
500
0
Picogram/ml
vmemIL-10
Mock
GFP
Day 2
Day 6

Fig. 8A

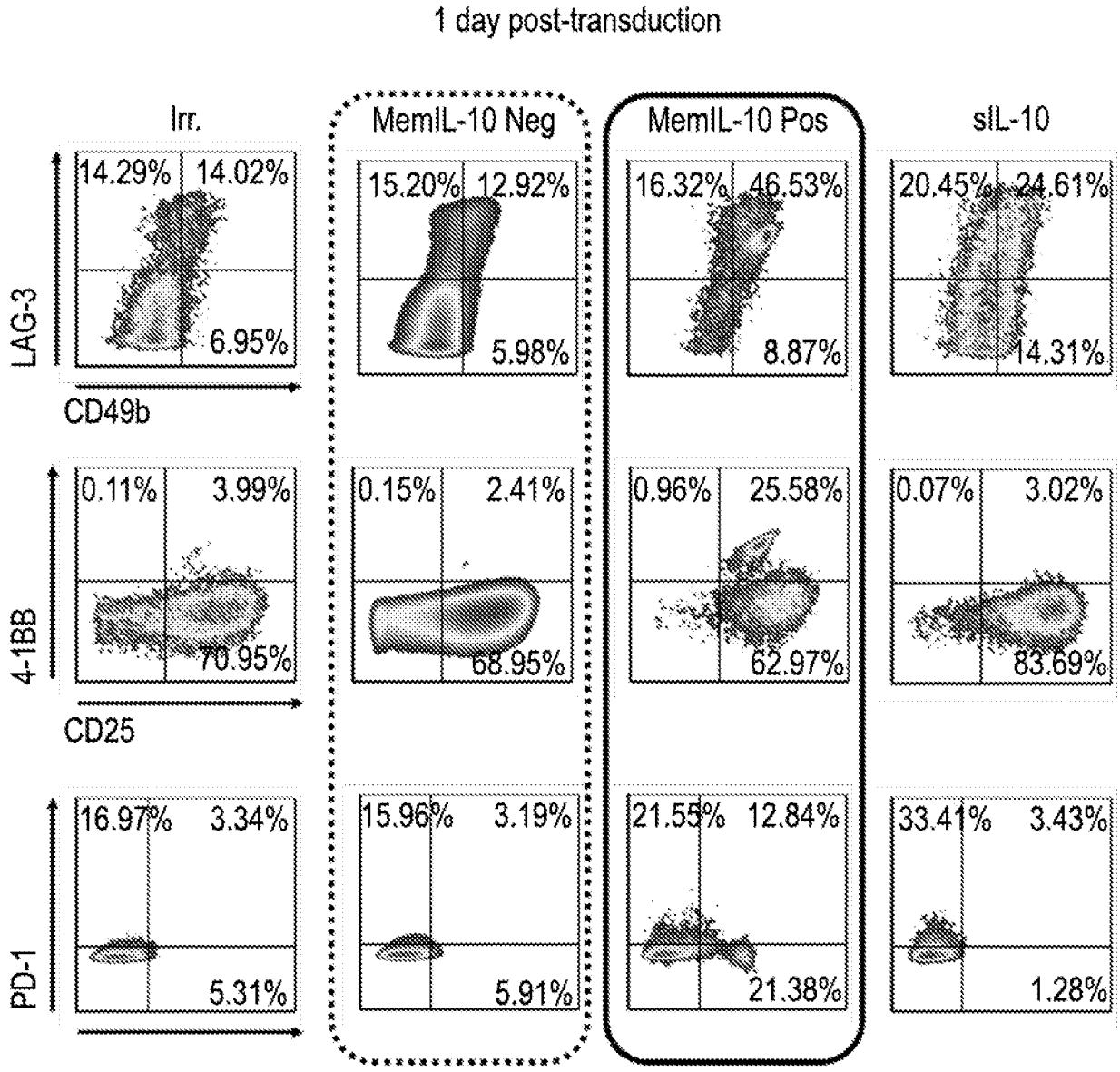

1 day post-transduction
Irr.
MemIL-10 Neg
MemIL-10 Pos
sIL-10
LAG-3
CD49b
14.29%
14.02%
6.95%
15.20%
12.92%
5.98%
16.32%
46.53%
8.87%
20.45%
24.61%
14.31%
4-1BB
CD25
0.11%
3.99%
70.95%
0.15%
2.41%
68.95%
0.96%
25.58%
62.97%
0.07%
3.02%
83.69%
PD-1
16.97%
3.34%
5.31%
15.96%
3.19%
5.91%
21.55%
12.84%
21.38%
33.41%
3.43%
1.28%

Fig. 8B

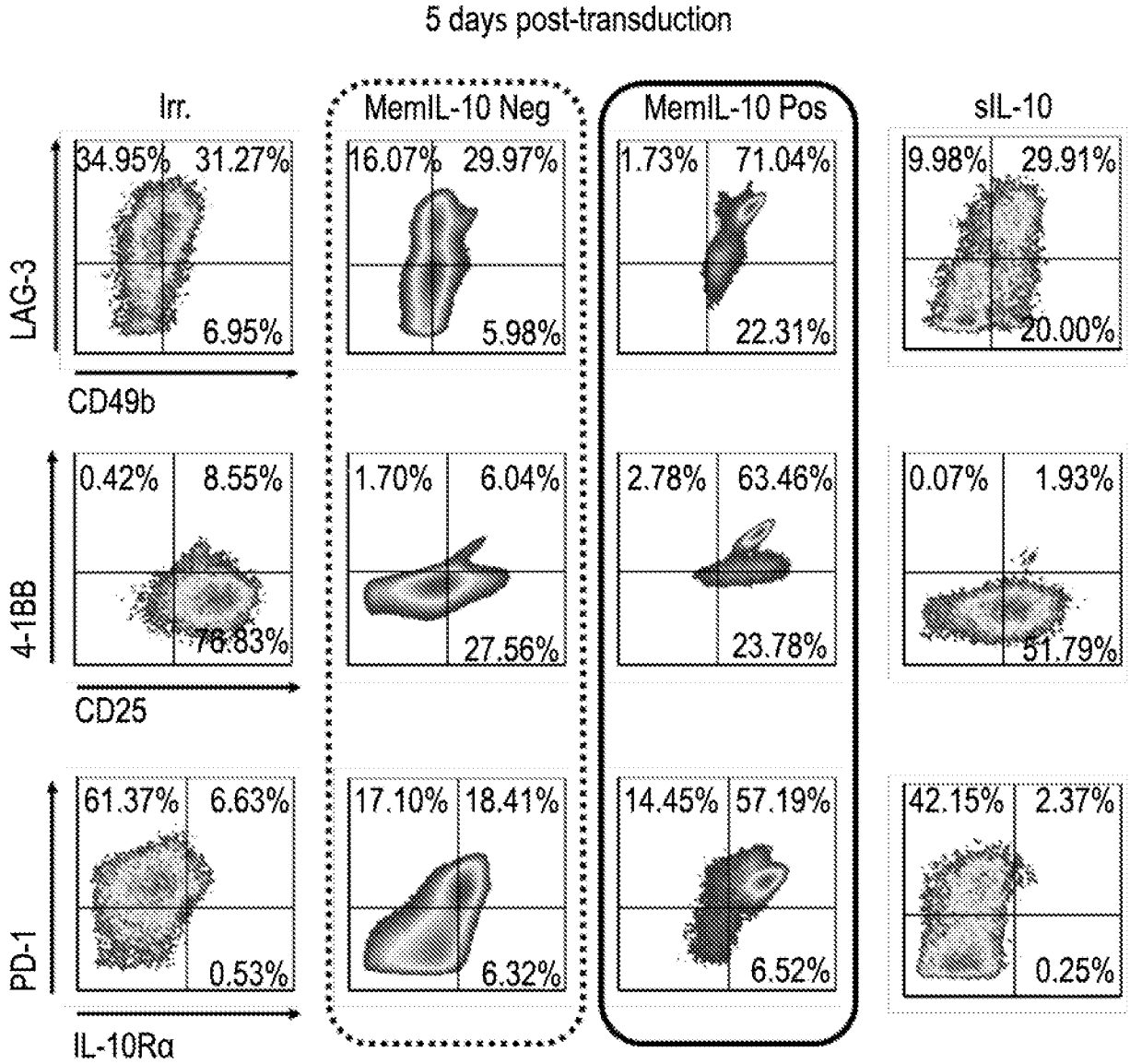

5 days post-transduction
Irr.
MemIL-10 Neg
MemIL-10 Pos
sIL-10
LAG-3
CD49b
34.95%
31.27%
6.95%
16.07%
29.97%
5.98%
1.73%
71.04%
22.31%
9.98%
29.91%
20.00%
4-1BB
CD25
0.42%
8.55%
76.83%
1.70%
6.04%
27.56%
2.78%
63.46%
23.78%
0.07%
1.93%
51.79%
PD-1
IL-10Rα
61.37%
6.63%
0.53%
17.10%
18.41%
6.32%
14.45%
57.19%
6.52%
42.15%
2.37%
0.25%

Fig. 8C

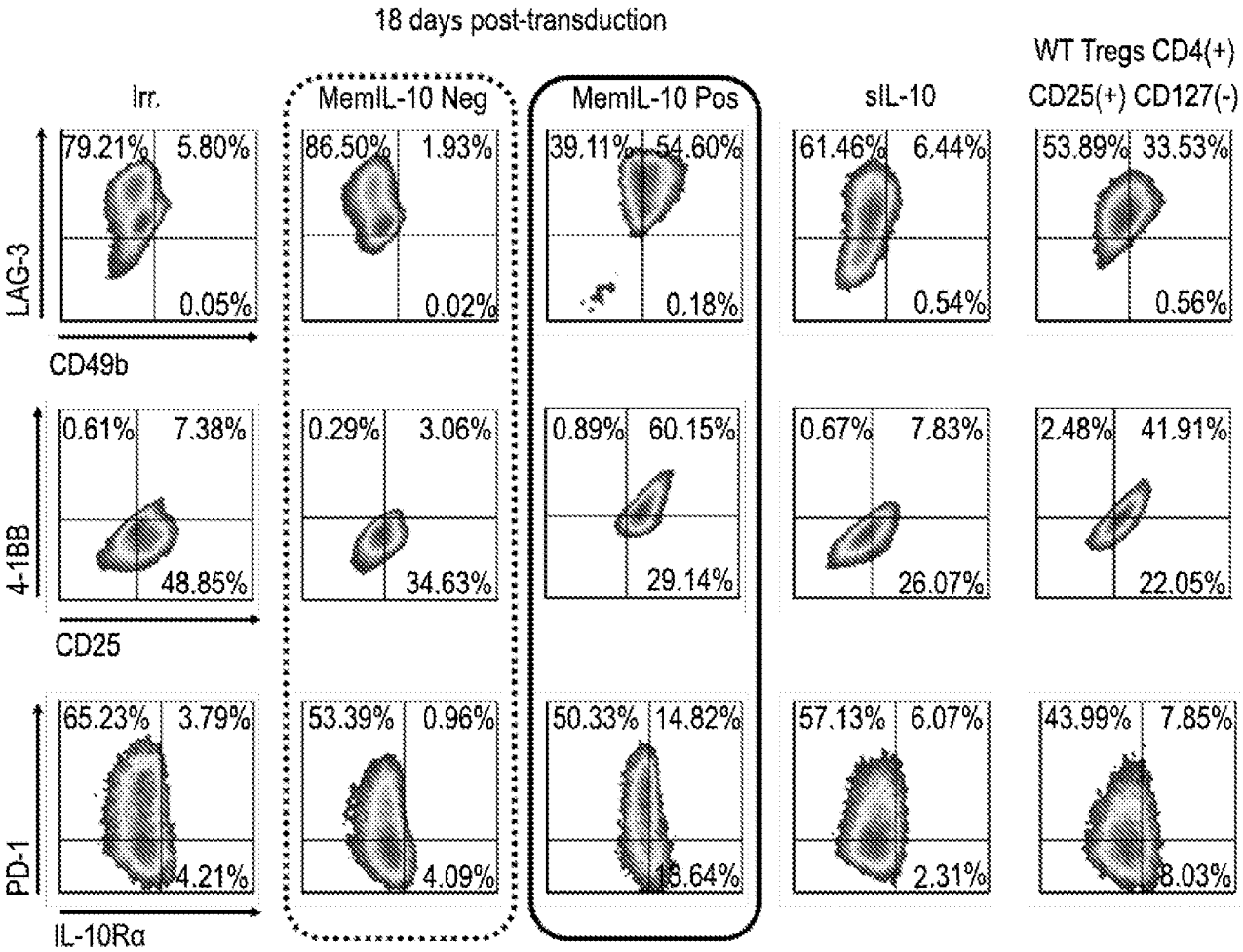

18 days post-transduction
Irr.
MemIL-10 Neg
MemIL-10 Pos
sIL-10
WT Tregs CD4(+) CD25(+) CD127(-)
LAG-3
CD49b
79.21%
5.80%
0.05%
86.50%
1.93%
0.02%
39.11%
54.60%
0.18%
61.46%
6.44%
0.54%
53.89%
33.53%
0.56%
4-1BB
CD25
0.61%
7.38%
48.85%
0.29%
3.06%
34.63%
0.89%
60.15%
29.14%
0.67%
7.83%
26.07%
2.48%
41.91%
22.05%
PD-1
IL-10Rα
65.23%
3.79%
4.21%
53.39%
0.96%
4.09%
50.33%
14.82%
18.64%
57.13%
6.07%
2.31%
43.99%
7.85%
8.03%

Fig. 9

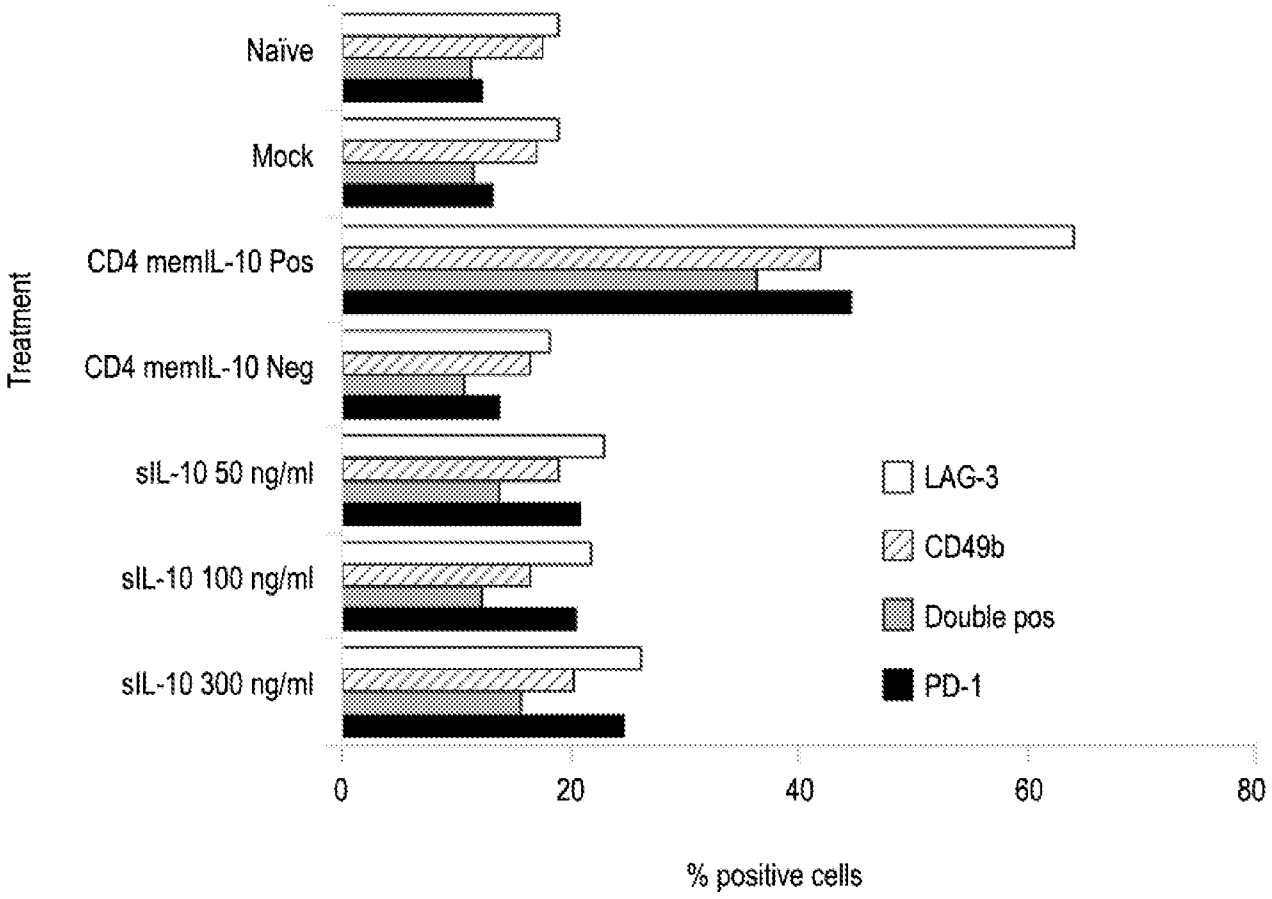
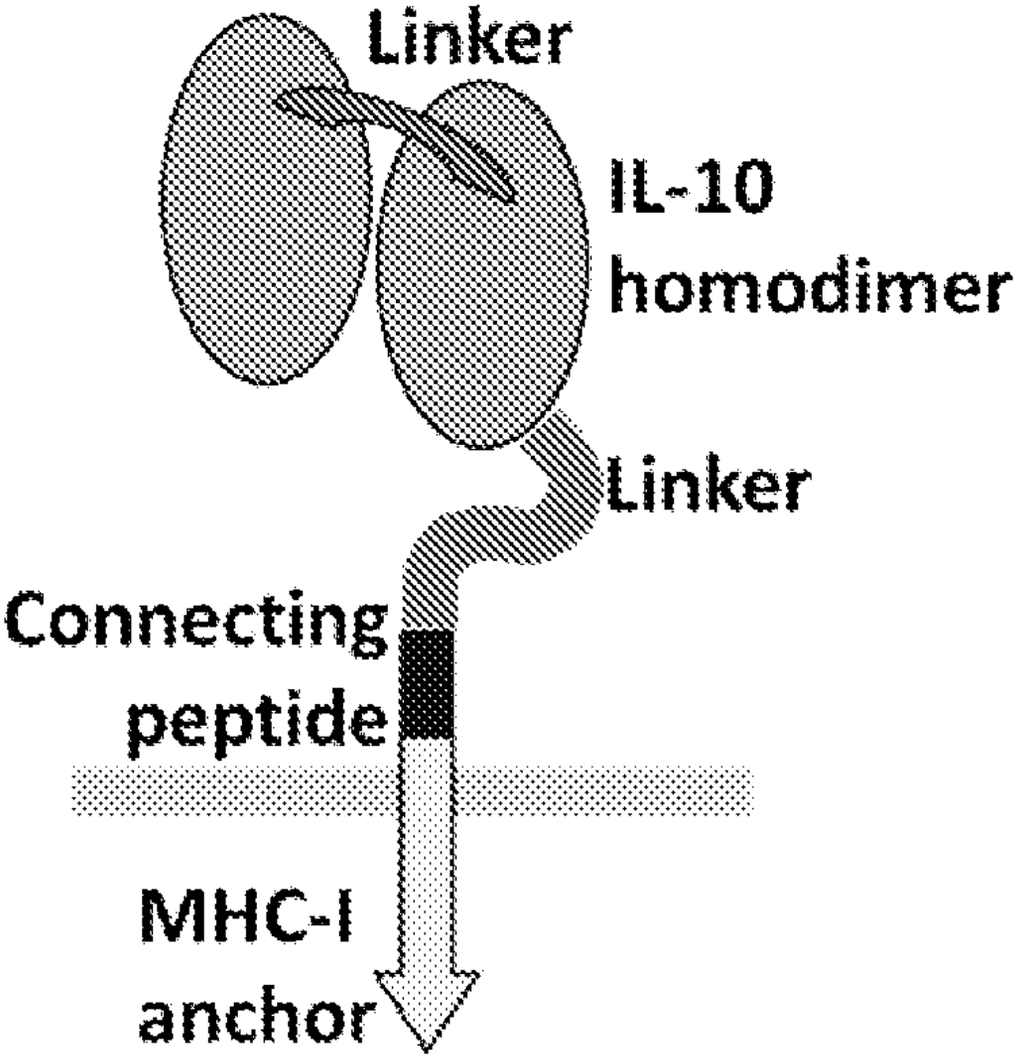

Naïve
Mock
CD4 memIL-10 Pos
CD4 memIL-10 Neg
sIL-10 50 ng/ml
sIL-10 100 ng/ml
sIL-10 300 ng/ml
Treatment
LAG-3
CD49b
Double pos
PD-1
0
20
40
60
80
% positive cells

Fig. 1


Linker
IL-10
homodimer
Linker
Connecting
peptide
MHC-I
anchor