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(54) Title: METHODS AND COMPOSITIONS FOR INCREASING THE SUPPRESSIVE FUNCTION OF REGULATORY T-CELLS (TREGS)

(57) Abstract: Methods and compositions for increasing the suppressive function of regulatory T- cells (Tregs) are provided.

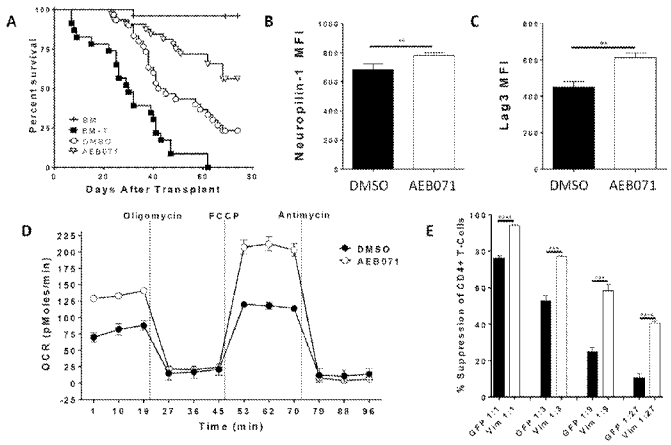

FIGURE 1
PANELS A-E

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SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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METHODS AND COMPOSITIONS FOR INCREASING THE SUPPRESSIVE FUNCTION OF REGULATORY T-CELLS (TREGS)

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under R01 HL11879, T32 AI007313, and F30 HL121873 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 62/214,680, filed September 4, 2015, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

This disclosure generally relates to T-cells and methods of engineering T-cells to reduce or eliminate graft-vs.-host-disease.

BACKGROUND

Regulatory T-cells (Tregs) play a critical role in preventing and treating autoimmune and alloimmune reactions, including graft-versus-host disease (GVHD) and solid organ transplant rejection, allergies and responses to foreign antigens (e.g., microbes, protein replacement therapy for deficiency disorders). Two recent clinical trials demonstrated that, in patients undergoing hematopoietic stem cell transplantation, adoptive transfer of Tregs significantly reduced the incidence of grades II-IV GVHD. While Tregs significantly reduced GVHD severity, they did not eliminate GVHD. One potential way to augment Treg-mediated inhibition of GVHD is to increase Treg suppressive potency. It was previously shown that Treg-specific inhibition of protein kinase C-theta (PKC-θ) enhances Treg function. However, it is unclear whether PKC-θ inhibition can boost Treg function in a systemic inflammatory condition like GVHD. Furthermore, the mechanism by which PKC-θ inhibition augments Treg function is unknown.

SUMMARY

In one aspect, a method of reducing or eliminating the vimentin protein and/or the RLTPR protein and/or the PKC-θ protein in Treg cells in provided. Such a method typically includes contacting the Treg cells with a vimentin-specific and/or a RLTPR-specific and/or a PKC-θ-specific inhibitory nucleic acid molecule.

In some embodiments, the vimentin-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 1. In some embodiments, the RLTPR-specific inhibitor nucleic acid is complementary to at least a portion of the sequence shown in SEQ ID NO: 5. In some embodiments, the PKC-θ-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 9. Representative vimentin-specific inhibitory nucleic acid molecules have the sequence shown in SEQ ID NO: 13, 14, 15 or 16.

In some embodiments, the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule is a RNAi nucleic acid molecule. In some embodiments, the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule is an antisense nucleic acid molecule. In some embodiments, the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitor nucleic acid molecule is a siRNA nucleic acid molecule.

In some embodiments, the Treg cells are contacted in vitro. In some embodiments, the Treg cells are contacted in situ. In some embodiments, the Treg cells are contacted *in vivo* in an individual who has received or is receiving a bone marrow transplant.

In some embodiments, the Treg cells exhibit a phenotype of at least one of the following: reduced PKC-θ auto-phosphorylation at Ser676; improved ability to suppress CD4+ and CD8+ Tcon proliferation; increased surface expression of Nrp1; increased surface expression of Lag3; increased basal and maximal oxygen consumption rate (OCR); increased $BoDipy_{C1\text{-}C12}$ uptake; increased expression of CD71; increased expression of CD98; increased expression of CPT1a; or reduced activity of mTORC2, compared to Tregs that lack the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule, respectively.

In another aspect, a method of increasing or augmenting the suppressor cell potency of Treg cells is provided. Typically, such a method includes reducing or eliminating vimentin and/or RLTPR and/or PKC-θ in the Treg cells.

In some embodiments, reducing or eliminating the vimentin and/or the RLTPR and/or the PKC-θ in the Treg cells comprising contacting the Treg cells with a moiety selected from the group consisting of a nucleic acid, a nuclease, an antibody, a ligand, a peptide, a drug, a chemical, or a small molecule. Representative nucleic acids include, without limitation, a vimentin-specific and/or a RLTPR-specific and/or a PKC-θ-specific inhibitory nucleic acid molecule. In some embodiments, the vimentin-specific and/or the RLTPR-specific and/or the PCK-θ-specific inhibitory nucleic acid molecule is selected from the group consisting of a RNAi nucleic acid molecule, an antisense nucleic acid molecule, and a siRNA nucleic acid molecule.

In some embodiments, the vimentin-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 1. In some embodiments, the RLTPR-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 5. In some embodiments, the PKC-θ-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 9.

In some embodiments, the method is performed in vitro. In some embodiments, the method is performed in situ. In some embodiments, the method is performed on an individual who has received or is receiving a bone marrow transplant.

In some embodiments, the Treg cells in which the vimentin, and/or RLTPR and/or PCK-θ has been reduced or eliminated exhibit a phenotype of at least one of the following: reduced PKC-θ auto-phosphorylation at Ser676; improved ability to suppress CD4+ and CD8+ Tcon proliferation; increased surface expression of Nrp1; increased surface expression of Lag3; increased basal and maximal oxygen consumption rate (OCR); increased $BoDipy_{C1-C12}$ uptake; increased expression of CD71; increased expression of CD98; increased expression of CPT1a; or reduced activity of mTORC2, compared to Tregs in which vimentin, RLTPR and/or PCK-θ, respectively, is not reduced or eliminated.

In still another aspect, a method of disrupting the structural integrity or the metabolic activity of Treg cells is provided. Typically, such a method includes reducing or eliminating vimentin and/or RLTPR and/or PKC-θ in the Treg cells.

In yet another aspect, a method of screening for compounds that increasing or augmenting the suppressor cell potency of Treg cells is provided. Typically, such a method includes contacting Treg cells with a test compound and determining whether or not the structural integrity or metabolic activity of the cell is disrupted. Disruption of the structural integrity or metabolic activity of the cell can be determined, for example, by detecting a reduction or elimination of vimentin and/or RLTPR and/or PKC-θ in the Treg cells. A test compound that disrupts the structural integrity or metabolic activity of the cell is indicative of a compound that increases or augments the suppressor cell potency of Treg cells.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Figure 1 shows that PKC- θ inhibition and vimentin siRNA treatment increase Treg function.

- Panel A shows acute GVHD. Lethally irradiated BALB/c mice were given 10e6 C57BL/6 bone marrow only (BM), or BM with 2e6 CD4+25-/CD8+25- Tcon cells without (BM+T) or with 1e6 Tregs treated with either DMSO (DMSO) or 10 μM AEB071 (AEB071) for 30 minutes. Compared to DMSO, Tregs treated with AEB071 significantly increased recipient survival (p=0.0036).

- Panel B shows Treg activation. Tregs were treated with DMSO or AEB071 as above, and activated overnight with plate bound anti-CD3/28 and IL-2. AEB071 treatment significantly increased the surface expression of Neuropilin-1.
- Panel C shows Treg activation. Tregs were treated with DMSO or AEB071 as above, and activated overnight with plate bound anti-CD3/28 and IL-2. AEB071 treatment significantly increased the surface expression of Lymphocyte activation gene 3 (Lag3).
- Panel D shows Treg metabolic activity. Tregs were treated with DMSO or AEB071 and activated as above. Oxygen consumption rate (OCR) analysis revealed that AEB071 treatment significantly increased baseline Treg OCR (time 0-19 min) and maximal OCR (time=53-70 min).
- Panel E shows Treg suppression. Tregs were transfected with vimentin siRNA (vim) or control (GFP), and activated for 36 hours. CFSE-labeled Tcon were mixed with T-cell depleted splenocytes and soluble anti-CD3 mAb. Treg:Tcon ratios of 1:1 – 1:27 were plated and CFSE dilution assessed after 3 days.

Figure 2A shows that vimentin is highly enriched in Tregs compared to conventional CD4+ T-cells (CD4 Tcon).

Figure 2B shows that, compared to the transfection control (top panel), transfection with GFP-siRNA using the transfection protocol described herein yielded 50-60% or greater transfection of Tregs.

Figure 2C shows that, compared to the control GFP-siRNA (GFP), when utilizing vimentin siRNA (vim siRNA) and the transfection protocol described herein, vimentin levels were knocked down by 15-30%.

Figure 3A shows that, in standard in vitro suppression assays, vimentin siRNA-transfected Tregs (Vim) were able to suppress proliferation of CD4 conventional T-cells significantly better than control GFP-siRNA transfected Tregs (GFP). Treg:Tcon ratios of 1:1 – 1:27 are represented with 1:1, 1:3 etc. denotations in the x-axis labels.

Figure 3B shows that, in standard in vitro suppression assays, vimentin siRNA-transfected Tregs (Vim) were able to suppress proliferation of CD8 conventional T-cells

significantly better than control GFP-siRNA transfected Tregs (GFP). Treg:Tcon ratios of 1:1 – 1:27 are represented with 1:1, 1:3 etc. denotations in the x-axis labels.

Figure 4A are histograms showing a symmetrical distribution of the labeling of the samples.

Figure 4B is a scatter plot showing the consistency between labeled samples.

Figure 5, Panels A – D, show that RLTPR siRNA significantly decreased the amount of RLTPR protein in Treg cells.

Figure 6A is a graph showing that RLTPR siRNA increased suppression of CD4+ T-cells in vitro.

Figure 6B is a graph showing that RLTPR siRNA increased suppression of CD8+ T-cells in vitro.

Figure 7A is a graph showing that RLTPR siRNA resulted in a significant increase in the expression of Foxp3 in Treg cells in vitro ($p<0.05$).

Figure 7B is a graph showing that RLTPR siRNA had no significant effect on the expression of CD25 in Treg cells in vitro.

Figure 7C is a graph showing that RLTPR siRNA significantly increased the expression of neuropilin-1 (Nrp1) in Treg cells in vitro ($p<0.0001$).

Figure 8A are photographs of the results of experiments in which Tregs were pre-treated with DMSO (control), AEB071, or vimentin siRNA, then activated. PKC-θ and vimentin staining were analyzed by confocal microscopy. Data show one experiment representative of 4 independent experiments.

Figure 8B is a representative histogram of PKC-θ phosphorylated at Ser676 after vimentin siRNA treatment, and a graph showing median fluorescent intensity (MFI) quantification. Data show one experiment representative of 2 independent experiments. n=4 replicates/group. Bars show mean ± SEM. *, $p<0.05$; **, $P<0.01$; ***, $P<0.0001$; ****, $p<0.00001$ (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 8C is a graph showing percent *in vitro* suppression of CD4+ Tcon proliferation by control and vimentin siRNA-treated Tregs in a standard *in vitro* Treg suppression assay. 1:1 – 1:9 indicates Treg:Tcon ratio. Data show one experiment representative of 3 independent experiments. n=4 replicates/group. Bars show mean ± SEM. *, $p<0.05$; **,

P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 8D shows a representative histogram of Neuropilin-1 expression, and graphs showing MFI quantifications of Nrp1, Foxp3 and CD25. Data show one experiment representative of 3 independent experiments. n=4 replicates/group. Bars show mean ± SEM. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9A is a graph showing the percent survival of recipients. Tregs were transfected with either control (non-targeting) or vimentin siRNA. Recipient mice were given BM alone, BM + Tcon (BM+T), or BM + Tcon + Tregs; Tregs pre-treated with control or vimentin siRNA. Data show one experiment representative of 3 independent experiments. n=5 mice / group / experiment. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9B is a graph showing the clinical GVHD scores (0 = no disease, 10 = most severe disease) for recipients. Tregs were transfected with either control (non-targeting) or vimentin siRNA. Recipient mice were given BM alone, BM + Tcon (BM+T), or BM + Tcon + Tregs; Tregs pre-treated with control or vimentin siRNA. Data show one experiment representative of 3 independent experiments. n=5 mice / group / experiment. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9C is a graph showing basal and maximal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured after transfection. Tregs were transfected with either control (non-targeting) or vimentin siRNA. Data show one experiment representative of 3 independent experiments. n=5 replicates / group. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9D is a representative histogram showing BoDipyC1-C12 uptake and a graph showing median fluorescent intensity (MFI) quantification of splenic Tregs from recipients on D4 after transplant. Tregs were transfected with either control (non-targeting) or vimentin siRNA, and recipients were given BM+Tcon+Tregs. Data show one experiment

representative of 3 independent experiments. n=4 replicates / group. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9E is a graph showing quantification of Glut1 and CPT1a MFI from flow cytometry analysis of splenic Tregs from recipients on D4 after transplant. Tregs were transfected with either control (non-targeting) or vimentin siRNA, and recipients were given BM+Tcon+Tregs. Data show one experiment representative of 3 independent experiments. n=4 replicates / group. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 9F are representative histograms showing Akt phosphorylation at Ser473 and Foxo3a phosphorylation (at Ser253) after control and vimentin siRNA transfection, and graphs showing corresponding MFI quantifications. Tregs were transfected with either control (non-targeting) or vimentin siRNA. Data show one experiment representative of 2 independent experiments. n=4 replicates /group. Bars show mean ± SEM. Survival differences analyzed by log-rank test. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests or one-way ANOVA with Tukey's post-test).

Figure 10A is a graph showing the quantification of vimentin median fluorescent intensity (MFI) from flow cytometry analysis of purified Tregs and CD4+ Tcon. Data show one experiment representative of 4 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests).

Figure 10B is a graph showing the percent *in vitro* suppression of CD8+ Tcon proliferation in a standard *in vitro* Treg suppression assay. Data show one experiment representative of 3 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests).

Figure 10C is a graph showing MFI quantification of Lag3 expression in purified Tregs from flow cytometry analysis. Data show one experiment representative of 3 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, p<0.05; **, P<0.01; ***, P<0.0001; ****, p<0.00001 (Student's t-tests).

Figure 10D is a graph showing recipient weights from mice given only BM, BM+Tcon (BM+T), or BM+Tcon+Tregs. Data show one experiment representative of 3 independent experiments. n = 5 mice / group / experiment. Bars show mean ± SEM. *, $p<0.05$; **, $P<0.01$; ***, $P<0.0001$; ****, $p<0.00001$ (Student's t-tests).

Figure 10E are graphs showing quantifications of alpha4beta7 and CCR9 MFI, and graphs showing percent of alpha4beta7 and CCR9 positive Tregs after transfection. Data show one experiment representative of 2 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, $p<0.05$; **, $P<0.01$; ***, $P<0.0001$; ****, $p<0.00001$ (Student's t-tests).

Figure 10F are graphs showing the quantification of CD71 and CD98 MFI from splenic Tregs from recipient mice transplanted with BM+Tcon+Tregs on D4 after transplant. Data show one experiment representative of 2 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, $p<0.05$; **, $P<0.01$; ***, $P<0.0001$; ****, $p<0.00001$ (Student's t-tests).

Figure 10G are graphs showing quantifications of S6 and 4E-BP1 phosphorylation after transfection. Data show one experiment representative of 2 independent experiments. n=4 replicates / group. Bars show mean ± SEM. *, $p<0.05$; **, $P<0.01$; ***, $P<0.0001$; ****, $p<0.00001$ (Student's t-tests).

DETAILED DESCRIPTION

The present disclosure provides evidence that disrupting cytoskeletal structure in Tregs results in a release of structural components from physical constraints that is then able to increase suppressor cell potency. This effect can be direct (e.g., the molecule or compound released from the structural components within the Treg cells can exert this effect) or indirect (e.g., a molecule or compound that, upon binding to a binding partner such as, without limitation, adapter molecules, signaling molecules, enzymes, or molecules involved in degradation, recycling, mobility, metabolism, and/or differentiation, results in a release of one or more physical constraints in the Treg cells) or a combination thereof. Simply by way of example, and without being bound by any particular mechanism, such physical constraints within the Treg cells may occur at a cellular level (e.g., on the membrane of the cell, involving one or more cell-to-cell communication mechanisms) or at an intracellular level

(e.g., on one or more organelles). This is the first evidence that there are structurally-based processes that occur within Treg cells that regulate their suppressor potency.

This phenomenon (e.g., the occurrence of structurally-based processes within Treg cells that regulate their suppressor potency) can be used in methods of increasing or augmenting the function (e.g., suppressor potency) of Treg cells. As described herein, increasing or augmenting the function of Treg cells can be accomplished by reducing or eliminating any of the vimentin protein, the RLTPR protein, or the PKC-θ protein, or a combination thereof.

Vimentin is a type III intermediate filament protein, and is the major cytoskeleton protein in mesenchymal cells. Vimentin plays a significant role in maintaining the position of organelles within the three-dimensional cell, but, at the same time, vimentin is a dynamic protein that allows for some of the structural flexibility exhibited by cells. The human vimentin nucleic acid sequence is shown in SEQ ID NO: 1, and the encoded protein is shown in SEQ ID NO: 2. The mouse vimentin nucleic acid sequence is shown in SEQ ID NO: 3, and the encoded protein is shown in SEQ ID NO: 4.

RLTPR is also known as CARMIL2 and is an adaptor protein that links PKC-θ with CD28. RLTPR knock-out mice exhibit a phenotype similar to CD28 knock-out mice, underscoring the importance of RLTPR in CD28/PKC-θ signaling (see, Liang, 2013, Nat. Immunol., 14(8):858-66). In addition, RLTPR also links protein kinase C-theta (PKC-θ) with the intermediate filament, vimentin (see, Liang, 2009, Mol. Biol. Cell., 20(24):5290-305). The human RLTPR nucleic acid sequence is shown in SEQ ID NO: 5, and the encoded protein is shown in SEQ ID NO: 6. The mouse RLTPR nucleic acid sequence is shown in SEQ ID NO: 7, and the encoded protein is shown in SEQ ID NO: 8.

Protein kinase C-theta (PKC-θ; also known as PRKCQ) is a member of the PKC family of serine- and threonine-specific protein kinases. PKC-θ is a calcium-independent and phospholipid-dependent protein kinase. The human PKC-θ nucleic acid sequence is shown in SEQ ID NO: 9, and the encoded protein is shown in SEQ ID NO: 10. The mouse PKC-θ nucleic acid sequence is shown in SEQ ID NO: 11, and the encoded protein is shown in SEQ ID NO: 12.

A nucleic acid encoding vimentin from human is shown in SEQ ID NO: 1, and a nucleic acid encoding vimentin from mouse is shown in SEQ ID NO: 3. In addition, a

nucleic acid encoding RLTPR from human is shown in SEQ ID NO: 5, and a nucleic acid encoding RLTPR from mouse is shown in SEQ ID NO: 7. Further, a nucleic acid encoding PKC-θ from human is shown in SEQ ID NO:9, and a nucleic acid encoding PKC-θ from mouse is shown in SEQ ID NO:11. Unless otherwise specified, nucleic acids referred to herein can refer to DNA and RNA, and also can refer to nucleic acids that contain one or more nucleotide analogs or backbone modifications. Nucleic acids can be single stranded or double stranded, and linear or circular, both of which usually depend upon the intended use.

As used herein, an "isolated" nucleic acid molecule is a nucleic acid molecule that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the organism from which the isolated nucleic acid molecule is derived (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). Such an isolated nucleic acid molecule is generally introduced into a vector (e.g., a cloning vector, or an expression vector) for convenience of manipulation or to generate a fusion nucleic acid molecule, discussed in more detail below. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule.

The sequence of the vimentin polypeptide from human is shown in SEQ ID NO: 2, and the sequence of the vimentin polypeptide from mouse is shown in SEQ ID NO: 4. In addition, the sequence of the RLTPR polypeptide from human is shown in SEQ ID NO: 6, and the sequence of the RLTPR polypeptide from mouse is shown in SEQ ID NO: 8. Further, the sequence of the PKC-θ polypeptide from human is shown in SEQ ID NO:10, and the sequence of the PKC-θ polypeptide from mouse is shown in SEQ ID NO:12. As used herein, a "purified" polypeptide is a polypeptide that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered "purified" when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, or 99%) by dry weight, free from the polypeptides and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is "purified."

Nucleic acids can be isolated using techniques well known in the art. For example, nucleic acids can be isolated using any method including, without limitation, recombinant

nucleic acid technology, and/or the polymerase chain reaction (PCR). General PCR techniques are described, for example in *PCR Primer: A Laboratory Manual*, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995. Recombinant nucleic acid techniques include, for example, restriction enzyme digestion and ligation, which can be used to isolate a nucleic acid. Isolated nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides.

Polypeptides can be purified from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. A polypeptide also can be purified, for example, by expressing a nucleic acid in an expression vector. In addition, a purified polypeptide can be obtained by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Nucleic acids can be detected using any number of amplification techniques (see, e.g., *PCR Primer: A Laboratory Manual*, 1995, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188) with an appropriate pair of oligonucleotides (e.g., primers). A number of modifications to the original PCR have been developed and can be used to detect a nucleic acid. Nucleic acids also can be detected using hybridization.

Polypeptides can be detected using antibodies. Techniques for detecting polypeptides using antibodies include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. An antibody can be polyclonal or monoclonal. An antibody having specific binding affinity for a polypeptide can be generated using methods well known in the art. The antibody can be attached to a solid support such as a microtiter plate using methods known in the art. In the presence of a polypeptide, an antibody-polypeptide complex is formed.

Detection (e.g., of an amplification product, a hybridization complex, or a polypeptide) is oftentimes accomplished using detectable labels. The term "label" is intended to encompass the use of direct labels as well as indirect labels. Detectable labels include enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.

A construct, sometimes referred to as a vector, containing a nucleic acid (e.g., a coding sequence or a RNAi nucleic acid molecule) is provided. Constructs, including expression constructs (or expression vectors), are commercially available or can be produced by recombinant DNA techniques routine in the art. A construct containing a nucleic acid can have expression elements operably linked to such a nucleic acid, and further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene). A construct can encode a chimeric or fusion polypeptide (i.e., a first polypeptide operatively linked to a second polypeptide). Representative first (or second) polypeptides are those that can be used in purification of the other (i.e., second (or first), respectively) polypeptide including, without limitation, 6xHis tag or glutathione S-transferase (GST).

Expression elements include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an expression element is a promoter sequence. Expression elements also can include introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid. Expression elements can be of bacterial, yeast, insect, mammalian, or viral origin, and vectors can contain a combination of elements from different origins. As used herein, operably linked means that a promoter or other expression element(s) are positioned in a vector relative to a nucleic acid in such a way as to direct or regulate expression of the nucleic acid (e.g., in-frame).

Constructs as described herein can be introduced into a host cell. Many methods for introducing nucleic acids into host cells, both *in vivo* and *in vitro*, are well known to those skilled in the art and include, without limitation, electroporation, calcium phosphate precipitation, polyethylene glycol (PEG) transformation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer. As used herein, "host cell" refers to the particular cell into which the nucleic acid is introduced and also includes the progeny or potential progeny of such a cell. A host cell can be any prokaryotic or eukaryotic cell. For example, nucleic acids can be introduced into bacterial cells such as *E. coli*, or into insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

A number of methods are known in the art that can be used to reduce or eliminate vimentin and/or RLTPR and/or PKC-θ polypeptides. For example, RNA interference

(RNAi) nucleic acid molecules, nucleases (e.g., CRISPR, TALENs, megaTALs, meganucleases, zinc finger nucleases); antibodies (e.g., Fab, Fab2, chimeric, humanized); or ligands, peptides, drugs, chemicals, or small molecules that competitively bind vimentin or RLTPR or PKC-θ, that down-regulate vimentin or RLTPR or PKC-θ expression (transcription of DNA into RNA or translation of RNA into protein), that increase vimentin or RLTPR or PKC-θ degradation, or that cause intracellular depletion (e.g., by secretion) of vimentin or RLTPR or PKC-θ, can be used to reduce or eliminate vimentin and/or RLTPR and/or PKC-θ.

RNA interference (RNAi), also referred to as post-transcriptional gene silencing (PTGS), is known in the art and, as indicated herein, can be used to reduce or eliminate vimentin and/or RLTPR and/or PKC-θ polypeptides. RNAi is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Without being bound by theory, it appears that, in the presence of an antisense RNA molecule that is complementary to an expressed message (i.e., a mRNA), the two strands anneal to generate long double-stranded RNA (dsRNA), which is digested into short (< 30 nucleotide) RNA duplexes, known as small interfering RNAs (siRNAs), by an enzyme known as Dicer. A complex of proteins known as the RNA Induced Silencing Complex (RISC) then unwinds siRNAs, and uses one strand to identify and thereby anneal to other copies of the original mRNA. RISC cleaves the mRNA within the complementary sequence, leaving the mRNA susceptible to further degradation by exonucleases, which effectively silences expression of the encoding gene.

Several methods have been developed that take advantage of the endogenous machinery to suppress the expression of a specific target gene and a number of companies offer RNAi design and synthesis services (e.g., Life Technologies, Applied Biosystems). In some instances, the use of RNAi can involve the introduction of long dsRNA (e.g., greater than 50 bps) or siRNAs (e.g., 12 to 23 bps) that have complementarity to the target gene, both of which are processed by the endogenous machinery. Alternatively, the use of RNAi can involve the introduction of a small hairpin RNA (shRNA); shRNA is a nucleic acid that includes the sequence of the two desired siRNA strands, sense and antisense, on a single strand, connected by a "loop" or "spacer" nucleic acid. When the shRNA is transcribed, the two complementary portions anneal intra-molecularly to form a "hairpin," which is

recognized and processed by the endogenous machinery. Irrespective of the particular type used (e.g., dsRNA, siRNA or shRNA), such RNAi nucleic acid molecules can be referred to as "specific inhibitory nucleic acid molecules" (e.g., a vimentin-specific inhibitory nucleic acid molecule, a RLTPR-specific inhibitory nucleic acid molecule, a PKC-θ-specific inhibitory nucleic acid molecule).

A RNAi nucleic acid molecule as described herein includes a nucleic acid molecule that is complementary to at least a portion of a target mRNA (i.e., a vimentin or a RLTPR or a PKC-θ mRNA); this nucleic acid molecule typically is referred to as an "antisense strand". Generally, the antisense strand includes at least 12 contiguous nucleotides of the DNA sequence (e.g., the vimentin nucleic acid sequence shown in SEQ ID NO: 1 or 3; the RLTPR nucleic acid sequence shown in SEQ ID NO: 5 or 7; or the PKC-θ nucleic acid sequence shown in SEQ ID NO: 9 or 11); it would be appreciated that the antisense strand has the "RNA equivalent" sequence of the DNA (e.g., uracils instead of thymines; ribose sugars instead of deoxyribose sugars).

A RNAi nucleic acid molecule can be, for example, 12 to 500 nucleotides in length (e.g., 12 to 50, 12 to 45, 12 to 30, 15 to 47, 15 to 38, 15 to 29, 16 to 53, 17 to 44, 17 to 38, 18 to 36, 19 to 49, 20 to 60, 20 to 40, 25 to 75, 25 to 100, 28 to 85, 30 to 90, 12 to 100, 12 to 300, 12 to 450, 15 to 70, 15 to 150, 16 to 275, 17 to 74, 17 to 162, 17 to 305, 18 to 60, 18 to 75, 18 to 250, 18 to 400, 20 to 35, 20 to 60, 20 to 80, 20 to 175, 20 to 225, 20 to 325, 20 to 400, 20 to 475, 25 to 45, 25 to 65, 25 to 100, 25 to 200, 25 to 250, 25 to 300, 25 to 350, 25 to 400, 25 to 450, 30 to 280, 35 to 250, 200 to 500, 200 to 400, 250 to 450, 250 to 350, or 300 to 400 nucleotides in length).

In some embodiments, the antisense strand (e.g., a first nucleic acid) can be accompanied by a "sense strand" (e.g., a second nucleic acid), which is complementary to the antisense strand. In the latter case, each nucleic acid (e.g., each of the sense and antisense strands) can be between 12 and 500 nucleotides in length (e.g., between 12 to 50, 12 to 45, 12 to 30, 14 to 47, 15 to 38, 16 to 29, 17 to 53, 17 to 44, 17 to 38, 18 to 36, 19 to 49, 20 to 60, 20 to 40, 25 to 75, 25 to 100, 28 to 85, 30 to 90, 12 to 100, 13 to 300, 14 to 450, 16 to 70, 16 to 150, 16 to 275, 17 to 74, 17 to 162, 17 to 305, 18 to 60, 18 to 75, 18 to 250, 18 to 400, 20 to 35, 20 to 60, 20 to 80, 20 to 175, 20 to 225, 20 to 325, 20 to 400, 20 to 475, 25 to 45, 25 to 65, 25 to 100, 25 to 200, 25 to 250, 25 to 300, 25 to 350, 25 to 400, 25 to 450, 30 to

280, 35 to 250, 200 to 500, 200 to 400, 250 to 450, 250 to 350, or 300 to 400 nucleotides in length).

In some embodiments, a spacer nucleic acid, sometimes referred to as a loop nucleic acid, can be positioned between the sense strand and the antisense strand. In some embodiments, the spacer nucleic acid can be an intron (see, for example, Wesley et al., 2001, The Plant J., 27:581-90). In some embodiments, although not required, the intron can be functional (i.e., in sense orientation; i.e., spliceable) (see, for example, Smith et al., 2000, Nature, 407:319-20). A spacer nucleic acid can be between 20 nucleotides and 1000 nucleotides in length (e.g., 25-800, 25-600, 25-400, 50-750, 50-500, 50-250, 100-700, 100-500, 100-300, 250-700, 300-600, 400-700, 500-800, 600-850, or 700-1000 nucleotides in length).

In some embodiments, a construct can be produced by operably linking a promoter to a DNA region, that, when transcribed, produces an RNA molecule capable of forming a hairpin structure; and a DNA region involved in transcription termination and polyadenylation. It would be appreciated that the hairpin structure has two annealing RNA sequences, where one of the annealing RNA sequences of the hairpin RNA structure includes a sense sequence identical to at least 15 consecutive nucleotides of a vimentin or a RLTPR or a PKC-θ nucleotide sequence, and where the second of the annealing RNA sequences includes an antisense sequence that is identical to at least 15 consecutive nucleotides of the complement of the vimentin or the RLTPR or the PKC-θ nucleotide sequence. In addition, as indicated herein, the DNA region can include an intron (e.g., a functional intron). When present, the intron generally is located between the two annealing RNA sequences in sense orientation such that it is spliced out by the cellular machinery (e.g., the splicesome). Such a construct can be introduced into one or more plant cells to reduce the phenotypic expression of a vimentin or a RLTPR or a PKC-θ nucleic acid (e.g., a nucleic acid sequence that is normally expressed in a Treg cell).

In some embodiments, a construct (e.g., an expression construct) can include an inverted-duplication of a segment of a target nucleic acid sequence, where the inverted-duplication includes a nucleotide sequence substantially identical to at least a portion of the target nucleic acid and the complement of a portion of the target nucleic acid. It would be appreciated that a single promoter can be used to drive expression of the inverted-duplication

nucleic acid, and that the inverted-duplication typically contains at least one copy of the portion of the target nucleic acid in the sense orientation. Such a construct can be introduced into one or more Treg cells to delay, inhibit or otherwise reduce the expression of the target nucleic acid in the Treg cells.

Representative siRNA nucleic acid molecules directed toward vimentin are shown in SEQ ID NOs: 13, 14, 15, and 16. It would be appreciated by the skilled artisan that the region of complementarity, between the antisense strand of the RNAi and the mRNA or between the antisense strand of the RNAi and the sense strand of the RNAi, can be over the entire length of the RNAi nucleic acid molecule, or the region of complementarity can be less than the entire length of the RNAi nucleic acid molecule. For example, a region of complementarity can refer to, for example, at least 12 nucleotides in length up to, for example, 500 nucleotides in length (e.g., at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 28, 30, 35, 49, 50, 60, 75, 80, 100, 150, 180, 200, 250, 300, 320, 385, 420, 435 nucleotides in length up to, e.g., 30, 35, 36, 40, 45, 49, 50, 60, 65, 75, 80, 85, 90, 100, 175, 200, 225, 250, 280, 300, 325, 350, 400, 450, or 475 nucleotides in length). In some embodiments, a region of complementarity can refer to, for example, at least 12 contiguous nucleotides in length up to, for example, 500 contiguous nucleotides in length (e.g., at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 28, 30, 35, 49, 50, 60, 75, 80, 100, 150, 180, 200, 250, 300, 320, 385, 420, 435 nucleotides in length up to, e.g., 30, 35, 36, 40, 45, 49, 50, 60, 65, 75, 80, 85, 90, 100, 175, 200, 225, 250, 280, 300, 325, 350, 400, 450, or 475 contiguous nucleotides in length).

It would be appreciated by the skilled artisan that complementary can refer to, for example, 100% sequence identity between the two nucleic acids. In addition, however, it also would be appreciated by the skilled artisan that complementary can refer to, for example, slightly less than 100% sequence identity (e.g., at least 95%, 96%, 97%, 98%, or 99% sequence identity). In calculating percent sequence identity, two nucleic acids are aligned and the number of identical matches of nucleotides (or amino acid residues) between the two nucleic acids (or polypeptides) is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides (or amino acid residues)) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both nucleic acids up to the full-length size of the shortest nucleic acid. It also will be appreciated that a single

nucleic acid can align with more than one other nucleic acid and hence, can have different percent sequence identity values over each aligned region.

The alignment of two or more nucleic acids to determine percent sequence identity can be performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, Nucleic Acids Res., 31(13):3497-500. ClustalW calculates the best match between a query and one or more subject sequences (nucleic acid or polypeptide), and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the default parameters can be used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters can be used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of polypeptide sequences, the following parameters can be used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For multiple alignment of polypeptide sequences, the following parameters can be used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

The skilled artisan also would appreciate that complementary can be dependent upon, for example, the conditions under which two nucleic acids hybridize. Hybridization between nucleic acids is discussed in detail in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sections 7.37-7.57, 9.47-9.57, 11.7-11.8, and 11.45-11.57). Sambrook et al. disclose suitable Southern blot conditions for oligonucleotide probes less than about 100 nucleotides (Sections 11.45-11.46). The Tm between a nucleic acid that is less than 100 nucleotides in length and a second nucleic acid can be calculated using the formula provided in Section 11.46. Sambrook et al. additionally disclose Southern blot conditions for oligonucleotide

probes greater than about 100 nucleotides (see Sections 9.47-9.54). The Tm between a nucleic acid greater than 100 nucleotides in length and a second nucleic acid can be calculated using the formula provided in Sections 9.50-9.51 of Sambrook et al.

The conditions under which membranes containing nucleic acids are prehybridized and hybridized, as well as the conditions under which membranes containing nucleic acids are washed to remove excess and non-specifically bound probe, can play a significant role in the stringency of the hybridization. Such hybridizations and washes can be performed, where appropriate, under moderate or high stringency conditions. For example, washing conditions can be made more stringent by decreasing the salt concentration in the wash solutions and/or by increasing the temperature at which the washes are performed. Simply by way of example, high stringency conditions typically include a wash of the membranes in 0.2X SSC at 65°C.

In addition, interpreting the amount of hybridization can be affected, for example, by the specific activity of the labeled oligonucleotide probe, by the number of probe-binding sites on the template nucleic acid to which the probe has hybridized, and by the amount of exposure of an autoradiograph or other detection medium. It will be readily appreciated by those of ordinary skill in the art that although any number of hybridization and washing conditions can be used to examine hybridization of a probe nucleic acid molecule to immobilized target nucleic acids, it is more important to examine hybridization of a probe to target nucleic acids under identical hybridization, washing, and exposure conditions. Preferably, the target nucleic acids are on the same membrane. A nucleic acid molecule is deemed to hybridize to a nucleic acid, but not to another nucleic acid, if hybridization to a nucleic acid is at least 5-fold (e.g., at least 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 50-fold, or 100-fold) greater than hybridization to another nucleic acid. The amount of hybridization can be quantified directly on a membrane or from an autoradiograph using, for example, a PhosphorImager or a Densitometer (Molecular Dynamics, Sunnyvale, CA).

A construct (also known as a vector) containing a RNAi nucleic acid molecule is provided. Constructs, including expression constructs, are described herein and are known to those of skill in the art. Expression elements (e.g., promoters) that can be used to drive expression of a RNAi nucleic acid molecule are known in the art and include, without limitation, constitutive promoters such as, without limitation, the cassava mosaic virus

(CsMVM) promoter, the cauliflower mosaic virus (CaMV) 35S promoter, the actin promoter, or the glyceraldehyde-3-phosphate dehydrogenase promoter, or tissue-specific promoters such as, without limitation, root-specific promoters such as the putrescine N-methyl transferase (PMT) promoter or the quinolinate phosphosibosyltransferase (QPT) promoter. It would be understood by a skilled artisan that a sense strand and an antisense strand can be delivered to and expressed in a target cell on separate constructs, or the sense and antisense strands can be delivered to and expressed in a target cell on a single construct (e.g., in one transcript). As discussed herein, a RNAi nucleic acid molecule delivered and expressed on a single strand also can include a spacer nucleic acid (e.g., a loop nucleic acid) such that the RNAi forms a small hairpin (shRNA).

Treg cells can be contacted *in vitro*, *in situ*, or *in vivo* with any of the moieties discussed herein (e.g., nucleic acids, nucleases, antibodies, ligands, peptides, drugs, chemicals, or small molecules) using any number of methods known to those skilled in the art. For example, Treg cells can be contacted with any of the moieties discussed herein (e.g., a nucleic acid (e.g., a vimentin-specific and/or a RLTPR-specific and/or a PKC-θ-specific inhibitory nucleic acid molecule; e.g., one or more RNAi molecules)) *in vitro*, *in situ*, or *in vivo*.

For example, one or more nucleic acids can be attached to or contained within a carrier such as, without limitation, liposomes, nanoparticles, or antibodies. Such carriers can be delivered to an individual (e.g., a patient) using routine cellular therapies, and such carriers can be targeted to Treg cells using one or more Treg targeting moieties such as, for example, cytokines that preferentially activate Tregs such as IL2; or the use of one or more moieties that specifically binds to a ligand that is preferentially expressed by Tregs such as neuropillin-1, lag3, TIGIT, CD39, CD73, IL10R, ST2, PD-1, CTLA4, CD49d, GITR, GARP, FR4.

The methods described herein can be applied to an individual who has received or is receiving a bone marrow transplant or a solid organ transplant. Alternatively, the methods described herein can be applied to an individual in order to treat or mitigate the symptoms of an autoimmune disease, or to induce tolerance to one or more foreign antigens (for example, in cases of enzyme therapy, gene therapy, antibody therapy, or drug therapy). Further, the

methods described herein can be applied to an individual in order to treat or mitigate the symptoms of one or more allergic reactions.

Following contact with one or more of the moieties described herein, the Treg cells (e.g., Treg cells in which the vimentin, and/or RLTPR and/or PCK-θ has been reduced or eliminated) typically exhibit at least one of the following phenotypes (relative to Tregs in which vimentin, RLTPR and/or PCK-θ is not reduced or eliminated (e.g., relative to Tregs that lack the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule)): reduced PKC-θ auto-phosphorylation at Ser676; improved ability to suppress CD4+ and CD8+ Tcon proliferation; increased surface expression of Nrp1; increased surface expression of Lag3; increased basal and maximal oxygen consumption rate (OCR); increased BoDipy$_{C1\text{-}C12}$ uptake; increased expression of CD71; increased expression of CD98; increased expression of CPT1a; or reduced activity of mTORC2.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

EXAMPLES

Example 1—PKC-θ Inhibition and Treg Function

Using a mouse MHC class I/II disparate acute GVHD model, it was found that freshly isolated Tregs treated for 30 minutes with 10 μM of the clinically available PKC-θ inhibitor, AEB071, suppressed GVHD mortality (Figure 1A) and severity significantly better than DMSO-treated Tregs. As Tregs exert much of their protective effect against GVHD early in the course of the disease, proliferation of GVHD-causing conventional T-cells (Tcon) on D4 after transplant was analyzed. A significant reduction in Tcon proliferation in mice given AEB071 treated Tregs was observed compared to DMSO treated Tregs. Multi-photon microscopy on D4 was performed after transplant using TEα-GFP Tcon, CD11c-eYFP antigen presenting cells (APCs) and wild-type Tregs. Compared to DMSO, AEB071-

treated Tregs significantly increased Tcon velocity and displacement from APCs. Increased velocity and displacement are indicative of decreased Tcon-APC interactions, suggesting reduced priming when AEB071-treated Tregs are present.

AEB071 vs DMSO treatment of Tregs resulted in augmented expression of the suppressive molecules, Neuropilin-1 (Nrp1) and Lymphocyte activation gene 3 (Lag3), after in vitro activation (Figure 1B and 1C) and in Tregs isolated from acute GVHD mice. Antibody blockade of Nrp1 and Lag3 in in vitro trans-well suppression assays reduced the effect of AEB071 treatment, suggesting that these molecules play a role in enhancing Treg function after PKC-θ inhibition. Flow cytometry analysis of phosphorylated proteins in activated Tregs revealed that PKC-θ inhibition resulted in reduced phosphorylation of the mTORC2 target, FoxO3a, but not mTORC1 targets, S6 and 4E-BP1. In addition, the mTORC2-specific phosphorylation site on Akt, serine 473, was reduced, whereas the mTORC1-specific phosphorylation site, threonine 308, was unaltered. Together, these data suggest reduced mTORC2 activity. Reduced phosphorylation increases FoxO3a nuclear translocation, which may result in increased Nrp1 and Lag3 expression, since FoxO3a has binding sites in both gene promoters. As both mTORC1 and mTORC2 are involved in T-cell metabolism, the effect of AEB071 treatment on Treg oxygen consumption rate (OCR) was investigated. Compared to DMSO, AEB071 treatment significantly increased Treg baseline and maximal OCRs after activation (Figure 1D). Increased OCR has been associated with increased Treg function.

To identify additional alterations in phosphorylated proteins after PKC-θ inhibition, a phosphoproteomic screen was performed using in vitro-expanded human Tregs treated with AEB701 or DMSO. Significant alterations in phosphorylation sites on 72 proteins was observed, including reduced phosphorylation of an adaptor molecule that links PKC-θ to the intermediate filament, vimentin. It was found that vimentin is highly upregulated in Tregs compared to Tcon and that, in Tregs, vimentin interacts with PKC-θ after activation. AEB071 treatment reduced the interaction between vimentin and PKC-θ. As with AEB071 treatment, vimentin siRNA significantly increased Treg suppression in vitro compared to control-transfected Tregs (Figure 1E), and augmented expression of Nrp1 and Lag3. AEB071-treatment of vimentin siRNA transfected Tregs did not further augment Treg function, suggesting an overlapping mechanism.

In summary, the data presented herein demonstrates that PKC-θ interacts with mTORC2 and vimentin to modulate multiple aspects of Treg function, and that a brief incubation of Tregs with a PKC-θ inhibitor or a reduction in vimentin protein levels may be viable methods to enhance the efficacy of Treg therapeutics.

Example 2—T-Cell Purification from Lymph Nodes

Lymph nodes were harvested into a gentle MACS C tube containing PBS with at least 2% FBS. The tissue was homogenized one or two times on the Miltenyi GentleMACS dissociator using the Spleen-1 protocol, inverting the tube between runs.

The tissue was spun at 1200 RPM for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in MACS buffer (PBS containing 2% FBS and 1 mM EDTA). The solution was filtered through a 70 μM cell strainer into a 50 mL tube. The original C-tube was washed with additional MACS buffer and put through the strainer. The cells were counted at least three different times, and resuspended at 100 x 10e6 cells/mL in a 50 mL tube.

FCS was added to the cells at 50 μL /mL and a biotin-labeled antibody (e.g., anti-CD4, CD8, CD19, B220, CD11b, CD11c, anti-CD25, anti-NK1, anti-gamma delta TCR) was added and incubated for 10 minutes at room temperature. RapidSphere Magnetic Particles were added at 35 μL/mL and incubated for 10 minutes at room temperature. If the volume of cells after the RapidSpheres were added was between 1 and 10 mL, then 25 mL of MACS buffer was added and the sample was mixed gently. If the volume of cells after the RapidSpheres were added was between 11 and 40 mL, then 50 mL of MACS buffer was added and the sample was mixed gently. The samples were incubated on the magnet for 5 minutes at room temperature.

The negative fraction (e.g., the purified cells) were pipetted off and transferred to a different 50 mL tube. Cells were stained with anti-CD4, anti-CD8, and anti-CD25 antibodies to test for purity, and then counted at least three times. If the purity is not ideal, the sample can be placed in the magnet for another 5 minutes and the purity re-checked.

Example 3—T-Cell Purification from Spleen

The spleen was gently harvested into a MACS C tube containing PBS and at least 2% FBS. The tissue was homogenized 1 to 2 times on the Miltenyi GentleMACS dissociator using the Spleen-1 protocol, inverting the tube between runs. The tubes were spun at 1200 RPM for 10 minutes at 4°C.

The supernatant was removed and resuspended in 0.5 – 1 mL ACK lysis buffer (per spleen), and incubated for 1 minute. The C-tube was filled with MACS/PBS+2%FBS, and the solution was filtered through a 100 μM cell strainer into a 50 mL tube. The C-tube was washed with additional MACS buffer and put through the strainer. The sample was spun at 1200 rpm for 10 minutes at 4°C, the supernatant was removed, and the cells resuspended in MACS buffer. The cells were counted as least three times, and resuspended at 100 x 10e6 cells/mL in a fresh 50 mL tube.

FCS was added at 50 μL / mL, antibodies labeled with biotin were added (anti-CD19, B220, CD11b, CD11c, anti-CD4, CD8, CD25, NK1.1, DX5, gamma delta TCR), and incubated for 10 minutes at room temperature. RapidSphere Magnetic Particles were added at 55 μL/mL, and the sample was incubated for 10 minutes at room temperature. If the volume of cells after adding the RapidSpheres was between 1 and 10 mL, then 25 mL of MACS buffer was added and the sample gently mixed. If the volume of cells after adding the RapidSpheres was between 11 and 40 mL, then 50 mL of MACS buffer was added and the sample gently mixed. The samples were incubated on the magnet for 5 minutes at room temperature.

The negative fraction (e.g., purified cells) was pipetted off and transferred to another 50 mL tube. The cells were stained with anti-CD4, anti-CD8, and anti-CD25 to test for purity, then counted. If the purity is not ideal, the cells were placed in contact with the magnet for another 5 minutes, and their purity checked.

Example 4—T-Cell Purification Using CD25 Positive Selection

Cells were resuspended at 100 x 10e6 cells / mL. A selection antibody (anti-CD25 labeled with phycoerythrin (PE)) was added at 0.1 μl / 10e6 cells (2 μg / mL), and the sample was incubated for 10 minutes at 4°C. The cells were washed one time with MACS buffer, spun at 1500 RPM for 5 minutes at 4°C, and the supernatant was removed.

Cells were resuspended at 0.8 mL / 100 x 10e6 cells, and 1 µl / 10e6 of anti-PE micro beads were added and incubated for 15 minutes at 4°C. Cells were washed one time with MACS buffer, spun at 1500 RPM for 5 minutes at 4C, and the supernatant was removed. Cells were resuspended in a volume to bring the cells to 200 x 10e6 cells / mL.

A MS or LS column were placed on a magnet with a filter on top. The filter was washed with 500 µL MS or 2 mL LS buffer, and the column was loaded with the cells. The column was washed five times with 500 µL MS or 3 mL LS buffer. The column was removed from the magnet and the column was loaded with 1 mL MS or 5 mL LS buffer. The column was flushed with a plunger into a 15 mL tube. The cells were spun down, resuspended in 1 mL, and the steps above were repeated with a second MS/LS column. The cells were stained with anti CD4/8 to test for purity and counted at least three times.

Example 5—Amaxa Transfection (Nucleofection) Protocol for Mouse T-Cells

The number of cells was calculated to determine the number of cuvettes needed (e.g., 2 – 5.5 M cells per cuvette). After determining the number of cuvettes needed, 2 mL of fully supplemented Amaxa media with 300 IU / mL recombinant human IL-2 for each cuvette was warmed in a 12 well warm plate. After the cells are counted, they are pelleted by centrifugation at 1500 RPM for 5 minutes at 30°C.

As much of the supernatant as possible was removed, and cells were re-suspended in 100 µL of room temperature Amaxa Nucleofector solution per sample. Each sample contained about 2 – 5.5 x 10e6 healthy CD4+ T-cells in 100 µL of RT Nucleofector Solution. It would be appreciated that transfection will kill cell that are not healthy. To ensure healthy cells, the cells were rested for 2 – 4 hours to overnight in RPMI-c with 300 IU / mL recombinant human IL-2 at 37°C prior to transfection.

Cell solutions were aliquoted into Amaxa cuvettes as follows: 10 µL of vim siRNA was added to a final concentration of 5 µM, or 5 µL of Amaxa GFP was added to a final concentration of 2.5 µg per sample, then 100 µL of cells were added to each cuvette, and each cuvette was capped. A mock control cuvette using no GFP/siRNA also was included.

Cuvettes were loaded into the Amaxa Nucleofector II machine and transfected using Nucleofector Program X-001 for mouse CD4 T-cells. After transfection, the transfer pipettes provided by Amaxa were used to mix about 200-300 µL of warmed media with the cell

solution in the cuvette, and the cells were gently transferred into a 12 well plate. The cells were allowed to rest in the incubator for 4 – 5 hours.

While the cells were resting, the same volume of new Amaxa media supplemented with 300 IU / mL rhIL-2 was warmed. After 4-5 hours, the cells were removed from each well, and spun down at 1500 RPM for 5 minutes at 37°C. As much of the supernatant as possible was removed, and the pellet was re-suspended in the warmed media containing rhIL-2. Cells were placed in a 12 well plate that had been coated with 10 µg/mL anti-CD3/28 the day before the experiment and allowed to sit overnight at 37°C, and spun for 5 min at 500 RPM at 37°C. The cells were placed in an incubator.

24 hours later, 1 ml of fresh media, with an additional 300 IU / mL of recombinant human IL-2, was added to each well. 48 hours later, the cells were ready for use. The cells were collected from the wells, spun down at 1500 RPM for 5 minutes at 30°C, and counted. Samples were taken to check transfection efficiency.

Example 6—Vimentin siRNA Transfection Methods

Regulatory T-cells (Tregs) were purified from lymph nodes and spleens of C57Bl/6 mice using a two-step process: Step 1: CD4 negative selection – selection of CD4+ T-cells was accomplished using eBioscience biotinylated Ab (anti-CD19, B220, CD8, NK1.1, gamma delta TCR) and StemCell technologies streptavidin RapidSpheres; and Step 2: CD25 positive selection – after CD4+ negative selection, CD25 positive selection was completed using eBiosceince anti-CD25 PE Ab and Miltenyi anti-PE microbeads.

Once purified, Tregs were brought to a volume of about 1e6 / mL in complete media with 300 IU / mL recombinant human IL-2, and the cells were rested for 2 hours at 37°C in an incubator in a 24-well plate. After resting, Tregs were counted and split into 2 groups: one for transfection of control GFP siRNA and one for transfection of vimentin siRNA. After being split, Tregs were diluted in Amaxa Nucleofection Solution at a concentration of 5.5e6 / 100 µL and contacted with either 2.5 µg of control GFP plasmid or 5 µM vimentin siRNA per 100 µL.

A mixture of 4 siRNA oligonucleotides were combined in equal ratios such that the final concentration of the siRNA mixture is 5 µM. The siRNA oligonucleotides that were used have the following sequences: siRNA 1: CCA GAG AGA GGA AGC CGA A (SEQ ID

NO: 13); siRNA 2: AGG AAG AGA UGG CUC GUC A (SEQ ID NO: 14); siRNA 3: GUC UUG ACC UUG AAC GGA A (SEQ ID NO: 15); and siRNA 4: AAG CAG GAG UCA AAC GAG U (SEQ ID NO: 16).

Cells were then placed in Lonza cuvettes (100 µL per cuvette) and electroporated using program X-001 in a Lonza Nucleofector II machine. After transfection, cells were placed in 2 mL of warmed complete Amaxa media (5% FBS, Pen/Strep, 10 µL/mL of Lonza media supplement) in a 12-well plate and incubated at 37°C for 4 hours. After 4 hours, cells were removed from the wells, spun down (1500 RPM for 5 minutes), then diluted in 2 mL of warmed Amaxa complete media supplemented with 300 IU / mL recombinant human IL-2 and plated on a 12-well placed coated with anti-CD3 and anti-CD28 (10 µg / mL of each antibody). 24 hours after transfection (2D), 1 mL of Lonza complete media with 300 IU rhIL-2 was added to each well. 48 hours after transfection (3D), cells were removed from the wells, counted and used in functional studies.

Example 7—Vimentin siRNA Results

The experiments described herein demonstrated that vimentin siRNA significantly decreased vimentin levels in Tregs and that vimentin siRNA treatment increased the in vitro suppressive function in Tregs.

Figure 2A shows that vimentin is highly enriched in Tregs compared to conventional CD4+ T-cells (CD4 Tcon). Figure 2B shows that, compared to the transfection control (top panel), transfection with GFP-siRNA using the transfection protocol described herein yielded 50-60% or more transfection of Tregs. Figure 2C shows that, compared to control GFP-siRNA (GFP), vimentin levels were knocked down by 15-30% in the presence of vimentin siRNA (vim siRNA). In total, the protocol described herein yielded 50-60% Tregs transfected with siRNA, and a 15-30% reduction in vimentin levels in transfected cells.

In standard *in vitro* suppression assays, vimentin siRNA-transfected Tregs (Vim) were able to suppress proliferation of both CD4 conventional T-cells (Figure 3A) and CD8 conventional T-cells (Figure 3B) significantly better than the control GFP-siRNA-transfected Tregs (GFP). Treg : Tcon ratios of 1:1 – 1:27 are represented with the 1:1, 1:3, etc., denotations in the x-axis labels.

Example 8—Suppression Assay

On day 3 after transfection, CD4/8 Tcon and T-cell depleted splenoctyes were isolated for a suppression assay. CD4/CD8 Tcon were purified from spleen of CD45.1 C57BL/6 mice using negative selection with eBioscience biotinylated Ab (anti-CD19, B220, NK1.1, gamma-delta TCR) and StemCell technologies streptavidin RapidSphere technology. Once isolated, Tcon were labeled with CFSE (2.5 μM) for 5 minutes at room temperature with constant agitation.

Responder Tcon were provided with 0.75 μg/mL soluble anti-CD3 mAb. This 0.75 μg/mL was equivalent to a 3X concentration such that the final concentration of anti-CD3, once Teff were mixed with stimulator splenocytes and Tregs, was 0.25 μg/mL.

Splenocytes from 1 CD45.1 C57BL/6 mouse were depleted of T-cells and NK cells using eBioscience biotinylated mAb (anti-CD4, CD8, NK1.1, gamma delta TCR) and StemCell technologies streptavidin RapidSphere technology. These TCD splenocytes were used as stimulator cells.

Ratios of 0:1, 1:1, 1:3, 1:9 and 1:27 Treg:Teff were made. Four replicates of each ratio were made and plated in a 96-well, round bottom plate. After 3 days in culture, the cells from each well were harvested, washed with PBS and then stained with antibodies for flow cytometry analysis of CFSE dilution (proliferation) of CD4 and CD8 Tcon. The antibodies used were as follows: CD4 – BV510, CD8 – PE-ef610, CD25 – BV605, Nrp1 – PerCP-ef710, Lag3 – APC, Fixable viability dye – APC-ef780, Foxp3 – PE-Cy7, and Vimentin – PE.

Example 9—Phosphoproteomic Screen with Human Treg Cells

Utilizing mass spectroscopy, a total of 12,452 phosphorylation sites were quantified from human Tregs. In order to determine significant differences between DMSO-treated and AEB071-treated Tregs, two experiments were performed concurrently:

- Forward experiment: This experiment utilized heavy isotope-labeled Tregs treated with AEB071 and medium isotope-labeled Tregs treated with DMSO.

- Reverse experiment: medium isotope-labeled Tregs treated with AEB071 and heavy isotope-labeled Tregs treated with DMSO.

After processing the raw spectra with Max Quant software, the positively identified phosphopeptides were analyzed using Persus software. Contaminating peptides and reverse database hits were filtered out and the peptide intensity values and normalized H/M ratios were log2 transformed. The significances of the individual H/M ratios calculated using the Significance B test with a false discovery rate of 0.05 for the forward and reverse experiment separately.

Relying only on the Sig B test, the overlap between the two data sets was low with only 15% overlap. In order to improve the overlap between the two data sets, the data was filtered based on fold change (<1.5), consistency of ratios, phospho site localization probability (<75%), and the MaxQuant score (<75). By filtering the results, the overlap improved to 60%. See Table 1.

Table 1.

Significant Category	Sig B Test Only	Sig B Test and Filters
Forward Experiment	380	96
Reverse Experiment	448	92
Either For or Rev	723	117
Both	105 (105/772=15%)	72 (72/117=62%)

Histograms show a nice symmetrical distribution centered at 0 (Log2 transformed data), indicating equal amounts of samples and labeling overall. See Figure 4A. The scatter plot provides an indication of the consistency between samples. The red crosses are consistent between samples and, for the most part, cluster in the upper left quadrant as expected, since the inhibitor should decrease the levels of phosphorylation. See Figure 4B.

The results of the phosphoproteomic screen pointed toward the PKC- θ adapter and interaction partner, RLTPR. The RLTPR protein had significantly reduced phosphorylation at the PKC-theta consensus site: IGVSRGS(ph)GGAEGK (SEQ ID NO:17), with the phosphorylation site on the serine at residue 1226 indicated by the (ph) after the amino acid.

Example 10—RLTPR siRNA Transfection Methods and Results

Tregs were contacted with either 2.5 μg of control GFP plasmid or 1 μM, 5 μM, or 10 μM RLTPR siRNA per 100 μL. Cells were then placed in Lonza cuvettes (100 μL per cuvette) and electroporated using program X-001 in a Lonza Nucleofector II machine. After transfection, cells were placed in 2 mL of warmed complete Amaxa media (5% FBS,

Pen/Strep, 10 μL/mL of Lonza media supplement) in a 12-well plate and incubated at 37°C for 4 hours. After 4 hours, cells were removed from the wells, spun down (1500 RPM for 5 minutes), then diluted in 2 mL of warmed Amaxa complete media supplemented with 300 IU / mL recombinant human IL-2 and plated on a 12-well placed coated with anti-CD3 and anti-CD28 (10 μg / mL of each antibody). 24 hours after transfection (2D), 1 mL of Lonza complete media with 300 IU rhIL-2 was added to each well. 48 hours after transfection (3D), cells were removed from the wells and counted. The cells contacted with the 10 μM of RLTPR siRNA were used in functional studies.

Figures 5A – 5D show that RLTPR siRNA significantly decreased RLTPR protein levels in Tregs. Results also demonstrated that RLTPR siRNA treatment (at 10 μM) increased in vitro suppressive function (see Figures 6A and 6B) and augmented Nrp1 (see Figure 7C).

Example 11—PKC-θ Inhibition Alters PKC-θ Localization and Vimentin Interaction

Since PKC-θ localization and function appear to be linked, experiments were performed to understand whether PKC-θ inhibition modulated PKC-θ localization. Consistent with previous work, PKC-θ accumulated at the distal pole of control Tregs after 5 minutes of anti-CD3/CD28 mAb activation (Figure 8A, top). It was also found that PKC-θ was tightly associated with the intermediate filament, vimentin (Figure 8A, top), a molecule that was found to be more highly expressed in Tregs compared to CD4+ Tcons (Figure 10A). In contrast to controls, in AEB071-pre-treated Tregs, PKC-θ was dispersed throughout the cell after activation, and the overlap between PKC-θ and vimentin was reduced (Figure 8A, middle). Since PKC-θ inhibition dispersed PKC-θ and reduced PKC-θ/vimentin overlap, experiments were performed to determine whether modifying vimentin levels with siRNA might result in comparable changes. Indeed, vimentin knockdown by as little as 30% resulted in a similar pattern as AEB071: dispersed PKC-θ, and reduced PKC-θ/vimentin overlap (Figure 8A, bottom). These data demonstrate that inhibition of PKC-θ or vimentin alters PKC-θ localization and increases Treg function, and that PKC-θ/vimentin interactions may be important for PKC-θ trafficking.

Example 12—Vimentin siRNA Reduces PKC-θ Activity and Augments Treg Function

To better characterize whether PKC-θ/vimentin interactions were facilitating PKC-θ function, PKC-θ activity and Treg suppressive function were analyzed after treatment with vimentin siRNA. Using phosphoflow, it was noted that, similar to treatment with AEB071, PKC-θ auto-phosphorylation at Ser676 was significantly reduced in vimentin siRNA-treated Tregs compared with controls (Figure 8B). Furthermore, vimentin siRNA-treated Tregs were significantly better at suppressing CD4+ and CD8+ Tcon proliferation in standard *in vitro* suppression assays compared to control Tregs (Figure 8C, Figure 10B). This increased Treg function correlated with a significant increase in surface expression of Nrp1 (Figure 8D) and Lag3 (Figure 10C). All other Treg suppressive molecules remained unchanged (Figure 8D, and other data not shown).

To determine whether vimentin siRNA treatment would augment the ability of Treg to suppress GVHD, control- and vimentin siRNA-treated Tregs were compared using the GVHD model. Recipients given vimentin siRNA-treated Tregs had significantly increased survival and reduced GVHD severity compared with controls (Figure 9A-B, Figure 10D). As with AEB071 treatment, vimentin siRNA did not increase GI homing molecule expression (Figure 10E). In combination, these results suggest that vimentin plays a key role in coordinating PKC-θ activity, and that vimentin knockdown in Tregs results in a similar functional enhancement as direct PKC-θ inhibition.

Example 13—Vimentin siRNA Augments Treg Metabolic Activity and Reduces mTORC2 Function

Since PKC-θ inhibition modulated Treg metabolism and mTORC2 function, experiments were performed to determine whether vimentin siRNA might have a similar effect. As with PKC-θ inhibition, treatment with vimentin siRNA significantly increased basal and maximal OCR, but did not alter ECAR (Figure 9C). In GVHD, vimentin siRNA-treated Treg also had increased $BoDipy_{C1\text{-}C12}$ uptake, and augmented expression of CD71, CD98, and CPT1a, but not Glut1 (Figure 9D-E, Figure 10F). $BoDipy_{C1\text{-}C12}$ uptake was also increased *in vitro*. Since mTORC2 signaling was reduced after PKC-θ inhibition, it was hypothesized that mTORC2 activity also could be reduced after treatment with vimentin siRNA. Consistent with this hypothesis, phosphoflow analysis demonstrated reduced

phosphorylation of Akt at Ser473 and Foxo3a (Figure 9F), but no change in the phosphorylation of S6 or 4E-BP-1 (Figure 10G), in vimentin siRNA-treated Tregs versus controls. Together, these data demonstrate that reducing vimentin levels alters Treg metabolism (e.g., vimentin siRNA increases Treg function) and mTORC2 activity in a manner similar to PKC-θ inhibition.

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

WHAT IS CLAIMED IS:

1. A method of reducing or eliminating the vimentin protein and/or the RLTPR protein and/or the PKC-θ protein in Treg cells, comprising:

contacting the Treg cells with a vimentin-specific and/or a RLTPR-specific and/or a PKC-θ-specific inhibitory nucleic acid molecule.

2. The method of claim 1, wherein the vimentin-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 1.

3. The method of claim 1, wherein the RLTPR-specific inhibitor nucleic acid is complementary to at least a portion of the sequence shown in SEQ ID NO: 5.

4. The method of claim 1, wherein the PKC-θ-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 9.

5. The method of claim 1, wherein the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule is a RNAi nucleic acid molecule.

6. The method of claim 1, wherein the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule is an antisense nucleic acid molecule.

7. The method of claim 1, wherein the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitor nucleic acid molecule is a siRNA nucleic acid molecule.

8. The method of claim 1, wherein the vimentin-specific inhibitory nucleic acid molecule has the sequence shown in SEQ ID NO: 13, 14, 15 or 16.

9. The method of claim 1, wherein the Treg cells are contacted in vitro.

10. The method of claim 1, wherein the Treg cells are contacted in situ.

11. The method of claim 1, wherein the Treg cells are contacted *in vivo* in an individual who has received or is receiving a bone marrow transplant.

12. The method of claim 1, wherein the Treg cells exhibit a phenotype of at least one of the following:

reduced PKC-θ auto-phosphorylation at Ser676;
improved ability to suppress CD4+ and CD8+ Tcon proliferation;
increased surface expression of Nrp1;
increased surface expression of Lag3;
increased basal and maximal oxygen consumption rate (OCR);
increased BoDipy$_{C1\text{-}C12}$ uptake;
increased expression of CD71;
increased expression of CD98;
increased expression of CPT1a; or
reduced activity of mTORC2,

relative to Tregs that lack the vimentin-specific and/or the RLTPR-specific and/or the PKC-θ-specific inhibitory nucleic acid molecule.

13. A method of increasing or augmenting the suppressor cell potency of Treg cells, comprising:

reducing or eliminating vimentin and/or RLTPR and/or PKC-θ in the Treg cells.

14. The method of claim 13, wherein reducing or eliminating the vimentin and/or the RLTPR and/or the PKC-θ in the Treg cells comprising contacting the Treg cells with a

moiety selected from the group consisting of a nucleic acid, a nuclease, an antibody, a ligand, a peptide, a drug, a chemical, or a small molecule.

15. The method of claim 14, wherein the nucleic acid is a vimentin-specific and/or a RLTPR-specific and/or a PKC-θ-specific inhibitory nucleic acid molecule.

16. The method of claim 15, wherein the vimentin-specific and/or the RLTPR-specific and/or the PCK-θ-specific inhibitory nucleic acid molecule is selected from the group consisting of a RNAi nucleic acid molecule, an antisense nucleic acid molecule, and a siRNA nucleic acid molecule.

17. The method of claim 15, wherein the vimentin-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 1.

18. The method of claim 15, wherein the RLTPR-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 5.

19. The method of claim 15, wherein the PKC-θ-specific inhibitory nucleic acid molecule is complementary to at least a portion of the sequence shown in SEQ ID NO: 9.

20. The method of claim 13, wherein the method is performed in vitro.

21. The method of claim 13, wherein the method is performed in situ.

22. The method of claim 21, wherein the method is performed on an individual who has received or is receiving a bone marrow transplant.

23. The method of claim 13, wherein the Treg cells in which the vimentin, and/or RLTPR and/or PCK-θ has been reduced or eliminated exhibit a phenotype of at least one of the following:

reduced PKC-θ auto-phosphorylation at Ser676;

improved ability to suppress CD4+ and CD8+ Tcon proliferation;
increased surface expression of Nrp1;
increased surface expression of Lag3;
increased basal and maximal oxygen consumption rate (OCR);
increased $BoDipy_{C1\text{-}C12}$ uptake;
increased expression of CD71;
increased expression of CD98;
increased expression of CPT1a; or
reduced activity of mTORC2,

relative to Tregs in which vimentin, RLTPR and/or PCK-θ is not reduced or eliminated.

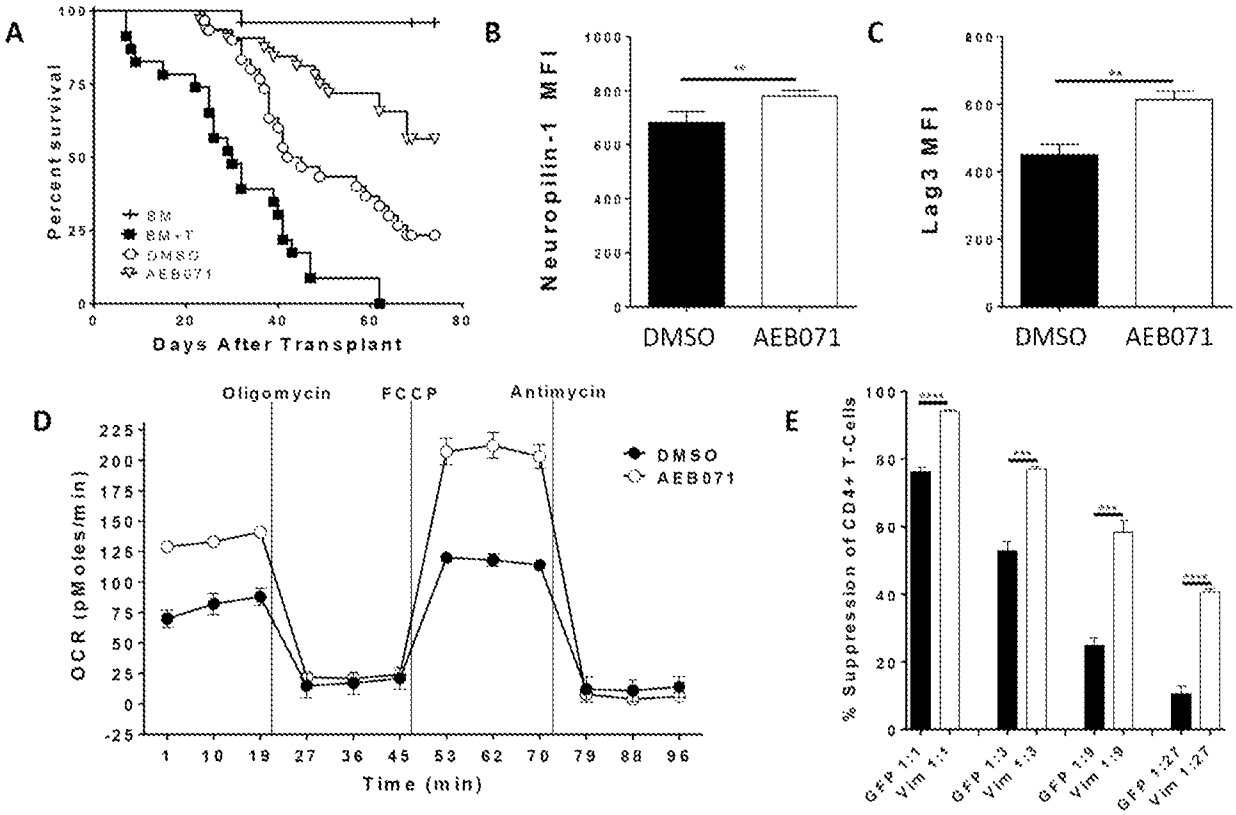


FIGURE 1
PANELS A-E

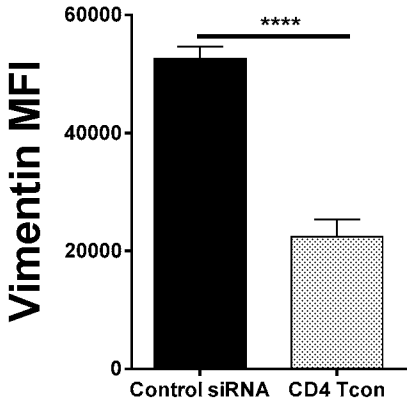
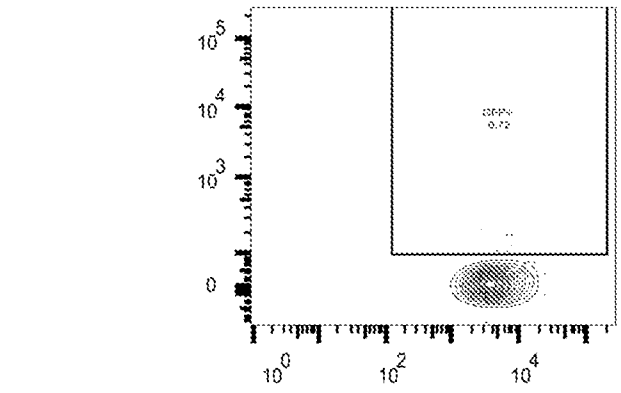


FIGURE 2A



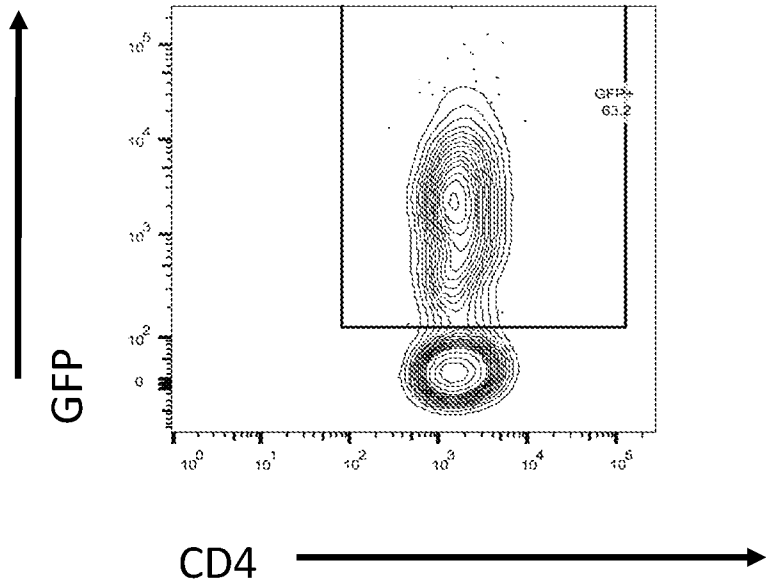


FIGURE 2B

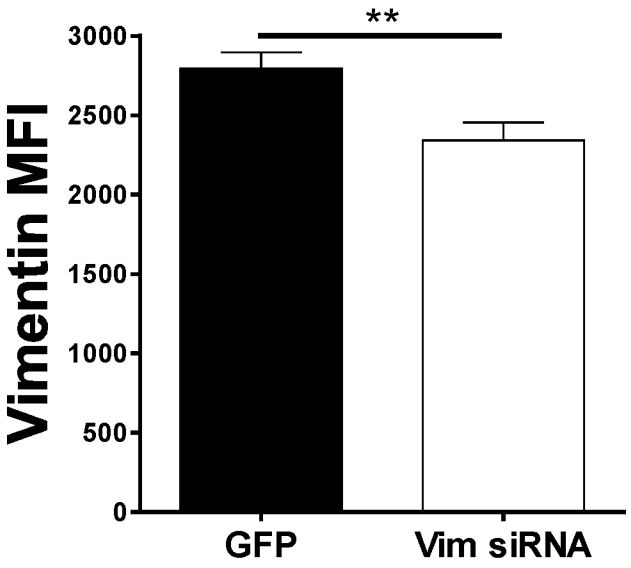
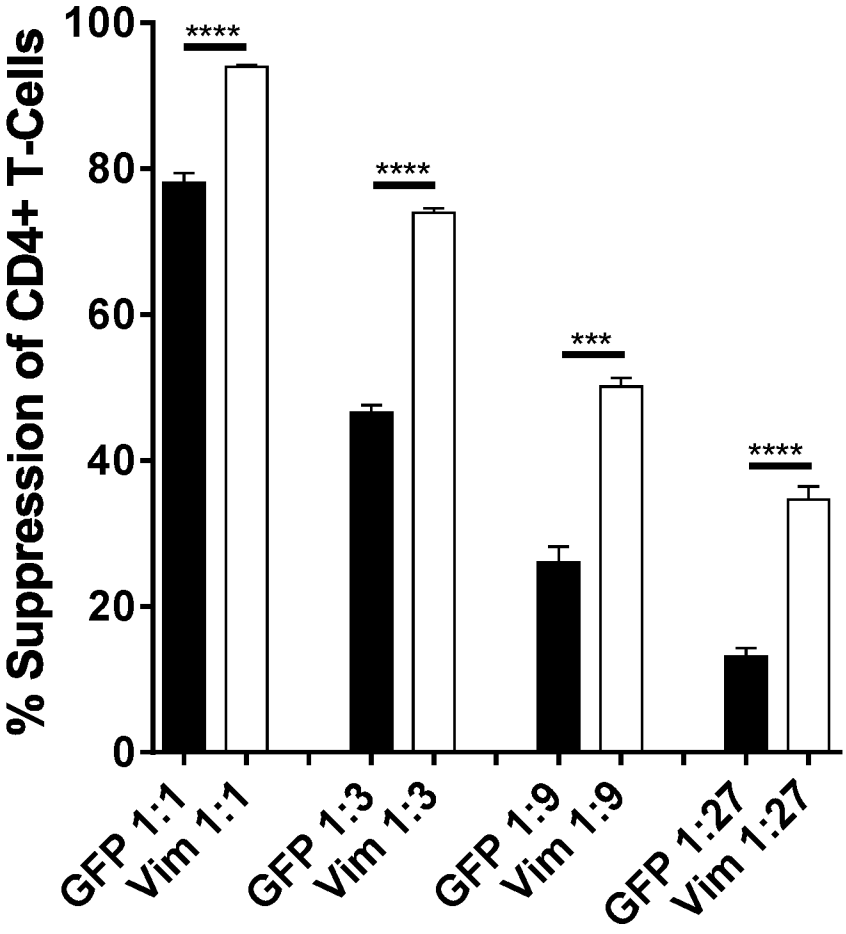


FIGURE 2C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80
60
40
20
0
****
****
***
****
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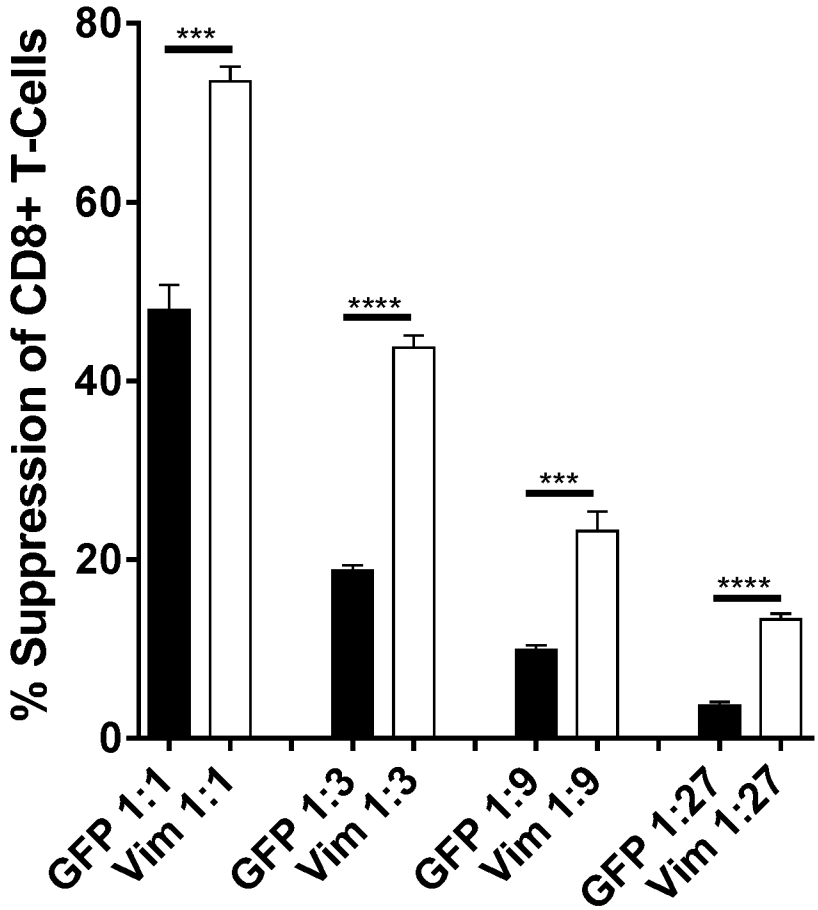
Vim 1:1
GFP 1:3
Vim 1:3
GFP 1:9
Vim 1:9
GFP 1:27
Vim 1:27

FIGURE 3A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60
40
20
0
***
****
***
****
GFP 1:1
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GFP 1:3
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GFP 1:9
Vim 1:9
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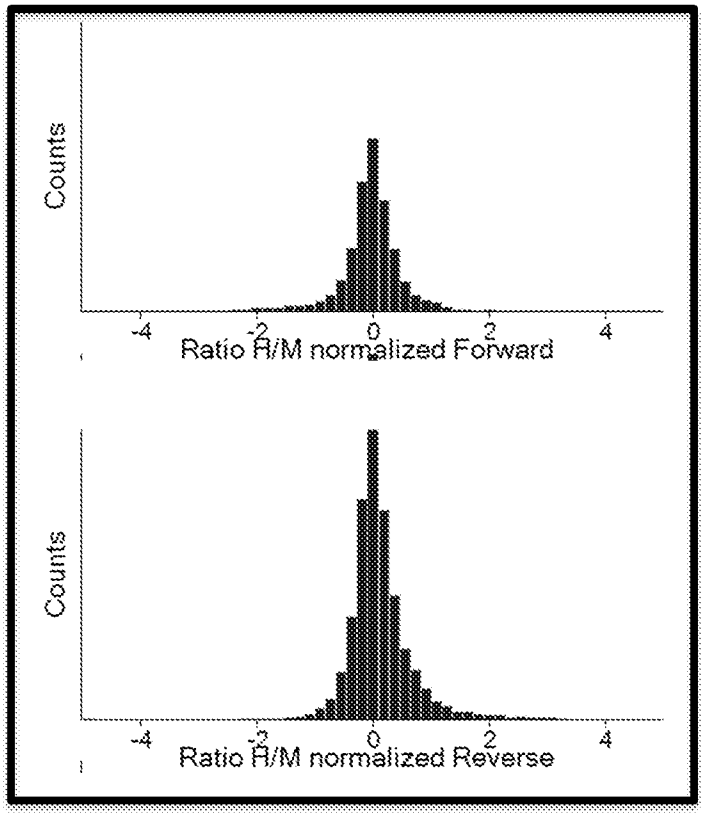
Vim 1:27

FIGURE 3B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Ratio R/M normalized Forward
Counts
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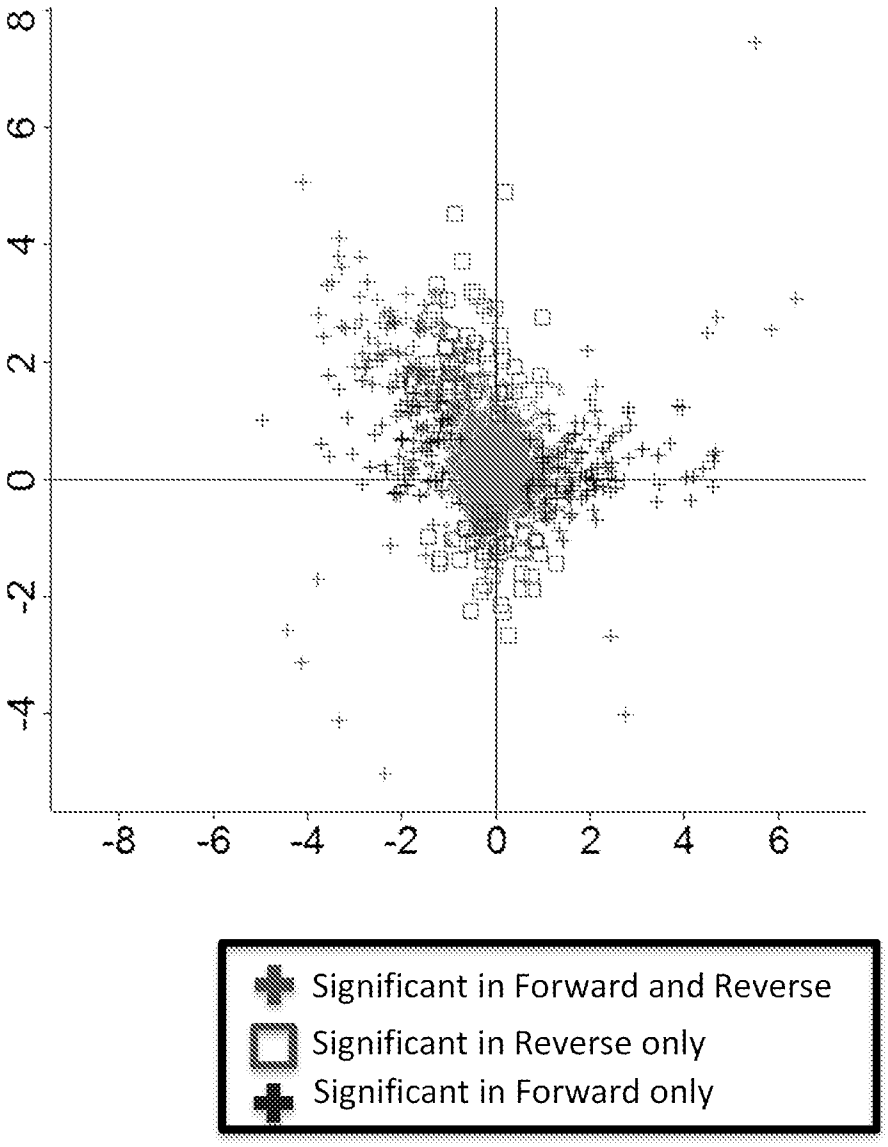
Ratio R/M normalized Reverse

FIGURE 4A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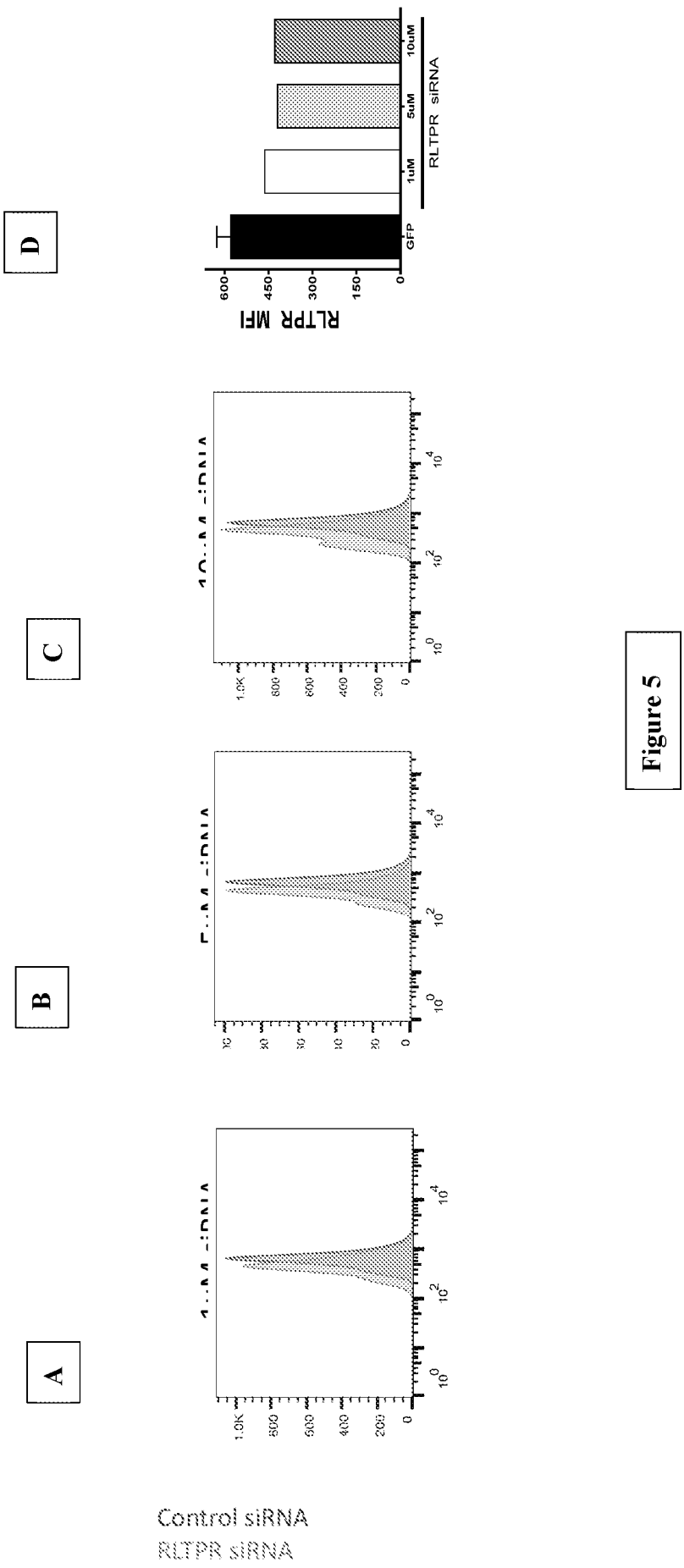
Significant in Reverse only
Significant in Forward only

FIGURE 4B


A
B
C
D
RLTPR MFI
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450
300
150
0
GFP
1uM
5uM
10uM
RLTPR siRNA
Control siRNA
RLTPR siRNA

Figure 5

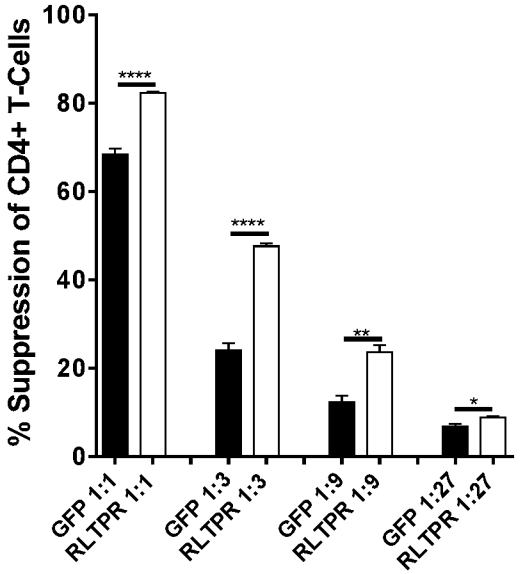


Figure 6A

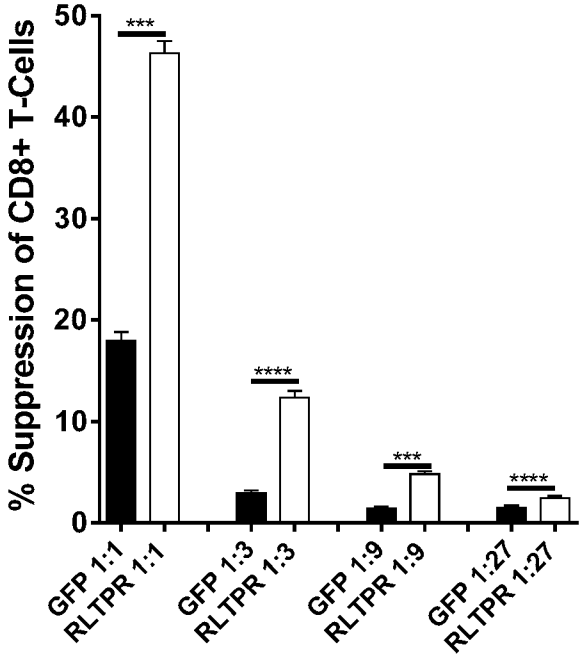


Figure 6B

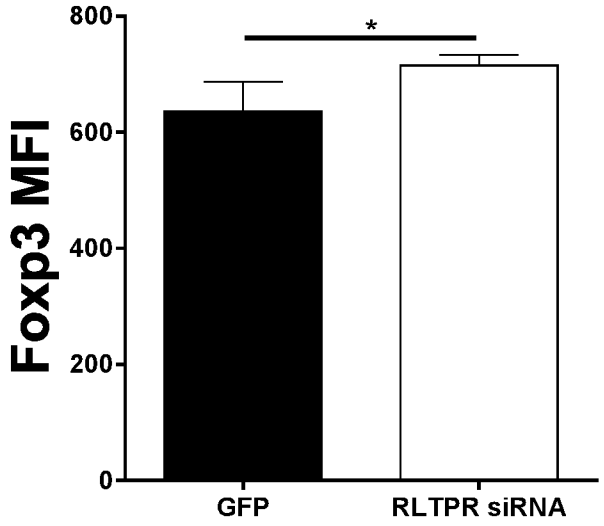


Figure 7A

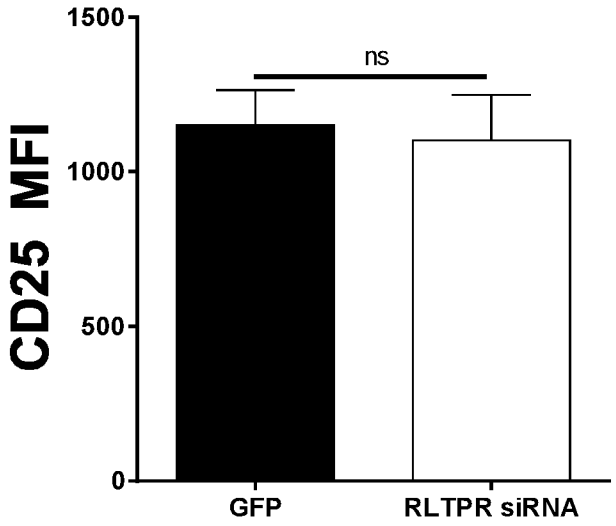


Figure 7B

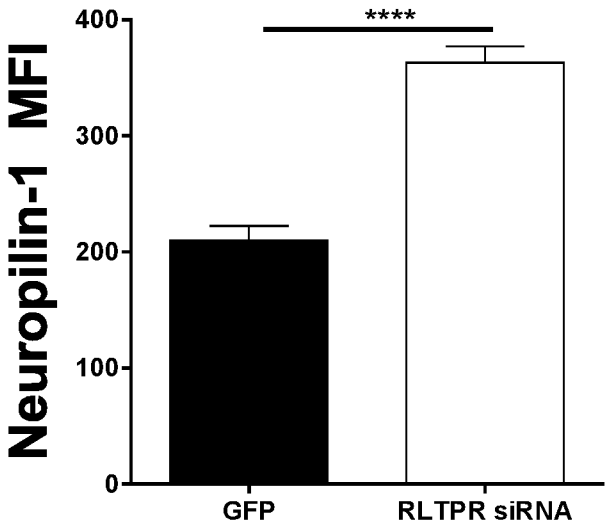
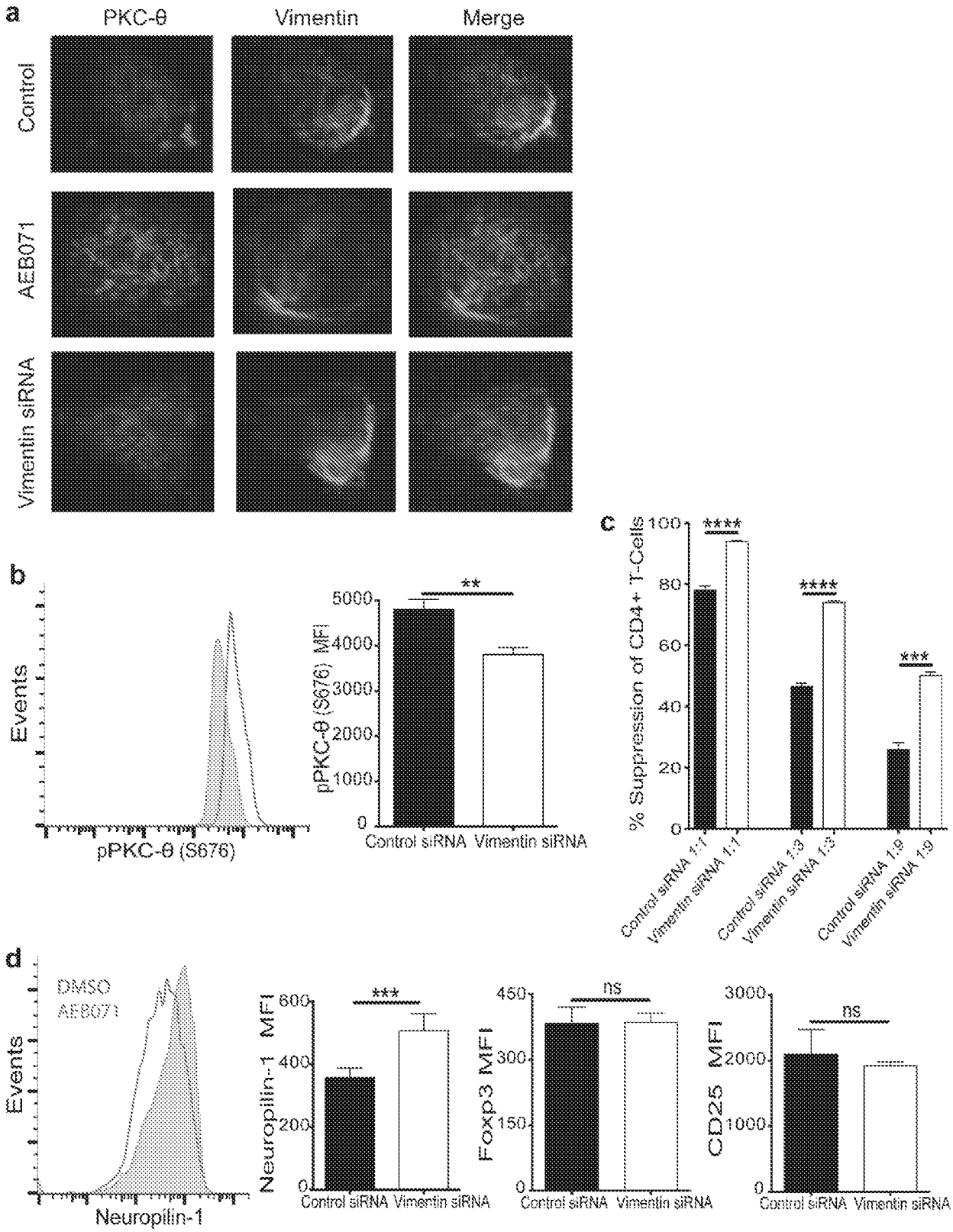


Figure 7C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PKC-θ
Vimentin
Merge
Control
AEB071
Vimentin siRNA
b
Events
pPKC-θ (S676)
pPKC-θ (S676) MFI
5000
4000
3000
2000
1000
0
**
Control siRNA
Vimentin siRNA
c
% Suppression of CD4+ T-Cells
100
80
60
40
20
0
****
****
***
Control siRNA 1:1
Vimentin siRNA 1:1
Control siRNA 1:3
Vimentin siRNA 1:3
Control siRNA 1:9
Vimentin siRNA 1:9
d
Events
DMSO
AEB071
Neuropilin-1
Neuropilin-1 MFI
600
400
200
0
***
Control siRNA
Vimentin siRNA
Foxp3 MFI
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300
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2000
1000
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Control siRNA
Vimentin siRNA

Figure 8

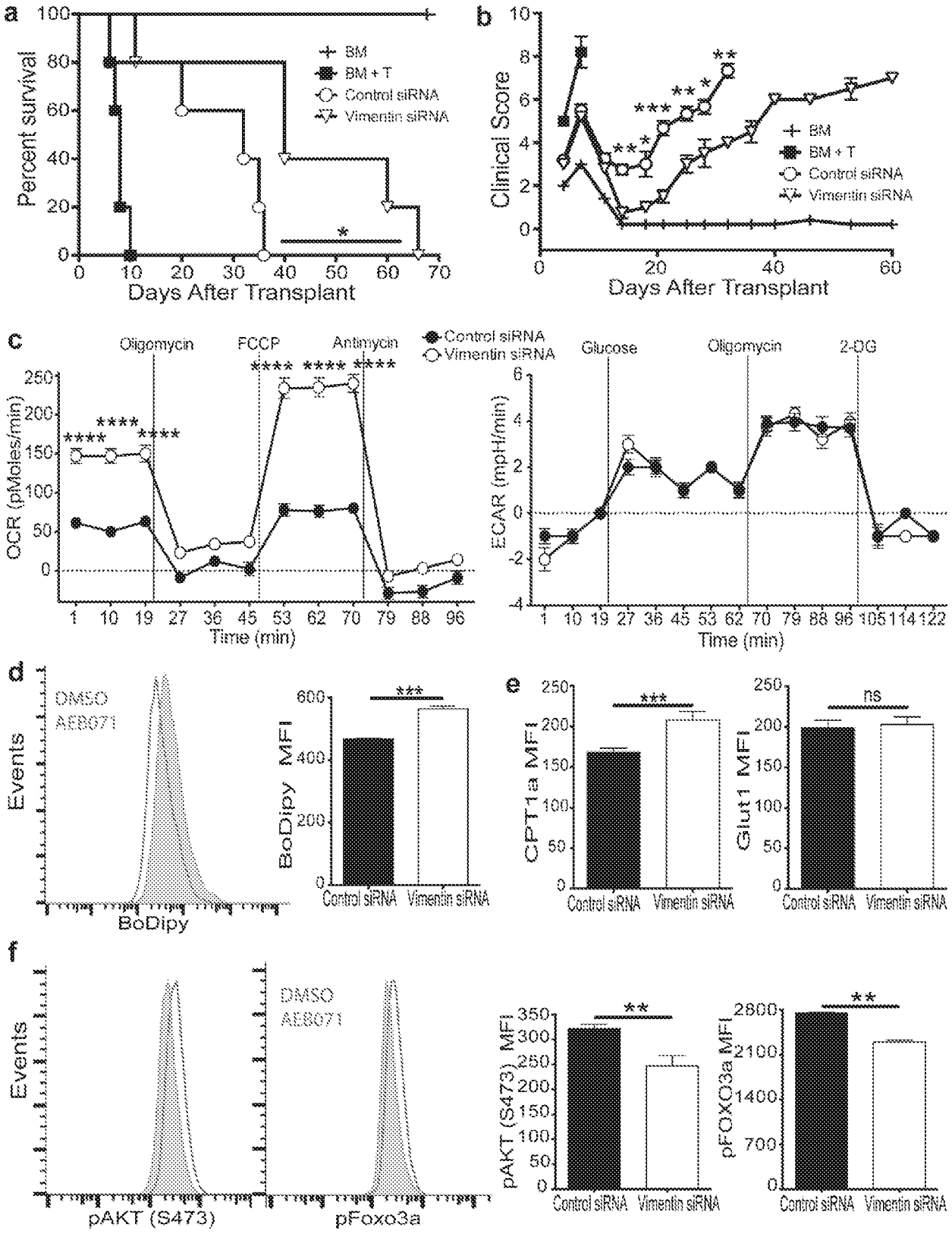
a
Percent survival
Days After Transplant
BM
BM + T
Control siRNA
Vimentin siRNA
b
Clinical Score
Days After Transplant
c
Oligomycin
FCCP
Antimycin
OCR (pMoles/min)
Time (min)
Glucose
Oligomycin
2-DG
ECAR (mpH/min)
d
DMSO
AE8071
Events
BoDipy
BoDipy MFI
e
CPT1a MFI
Glut1 MFI
ns
f
pAKT (S473)
pFoxo3a
pAKT (S473) MFI
pFOXO3a MFI

Figure 9

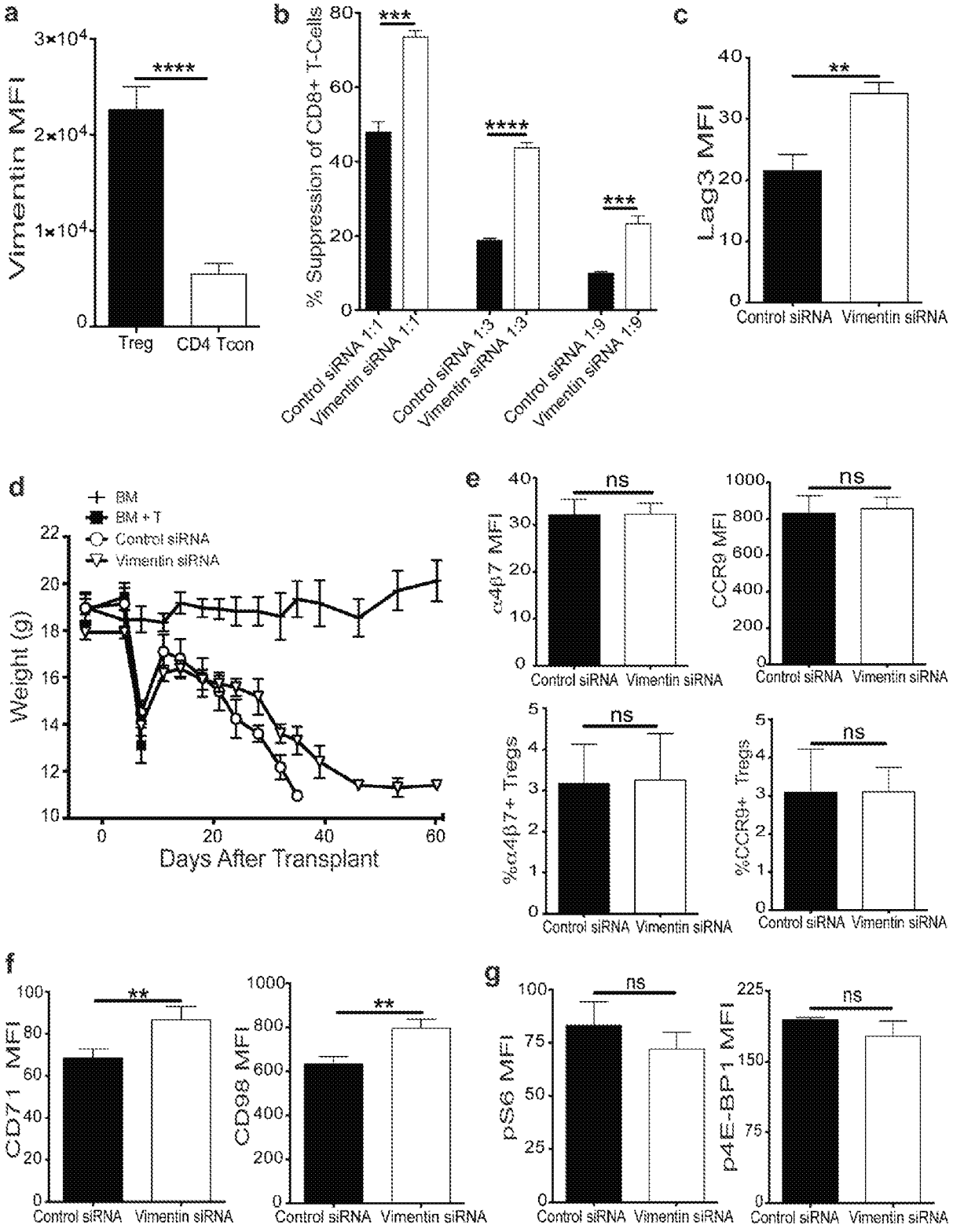
a
Vimentin MFI
3×10^4
2×10^4
1×10^4
0
****
Treg
CD4 Tcon
b
% Suppression of CD8+ T-Cells
80
60
40
20
0
***
****
***
Control siRNA 1:1
Vimentin siRNA 1:1
Control siRNA 1:3
Vimentin siRNA 1:3
Control siRNA 1:9
Vimentin siRNA 1:9
c
Lag3 MFI
40
30
20
10
0
**
Control siRNA
Vimentin siRNA
d
BM
BM + T
Control siRNA
Vimentin siRNA
Weight (g)
22
20
18
16
14
12
10
0
20
40
60
Days After Transplant
e
α4β7 MFI
CCR9 MFI
%α4β7+ Tregs
%CCR9+ Tregs
ns
Control siRNA
Vimentin siRNA
f
CD71 MFI
CD98 MFI
**
Control siRNA
Vimentin siRNA
g
pS6 MFI
p4E-BP1 MFI
ns
Control siRNA
Vimentin siRNA

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/050215

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/113(2010.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/113; A61P 37/06; A61K 35/12; A61K 31/713

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:vimentin, RLTPR, PKC-theta, regulatory T cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0142814 A1 (ZANIN-ZHOROV, ALEXANDRA et al.) 16 June 2011 See abstract; claims 1-2 and 8-12; paragraphs [0013]-[0015], [0020] and [0047]. Y	1,5-7,9-16,20-23 4,19
A		2-3,8,17-18
Y	NCBI, GenBank accession no. NM_006257.3 (14 September 2013) See the sequence.	4,19
A	LIANG, YINMING et al., 'The lymphoid lineage-specific actin-uncapping protein Rltpr is essential for costimulation via CD28 and the development of regulatory T cells', Nature Immunology, August 2013, Vol. 14, No. 8, pp. 858-866 See the whole document.	1-23
A	US 2012-0196919 A1 (BROWN, MARYANNE et al.) 02 August 2012 See abstract; claims 1 and 4-6.	1-23
A	BILLADEAU, DANIEL D. et al., 'Regulation of T-cell activation by the cytoskeleton', Nature reviews. Immunology, February 2007, Vol. 7, pp. 131-143 See the whole document.	1-23

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2016 (21.12.2016)	**21 December 2016 (21.12.2016)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/050215

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.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:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/050215

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0142814 A1	16/06/2011	None	
US 2012-0196919 A1	02/08/2012	AU 2010-241701 A1	13/10/2011
		BR PI1014775 A2	19/04/2016
		CA 2760305 A1	04/11/2010
		CN 102421435 A	18/04/2012
		EA 201101568 A1	30/05/2012
		EP 2445503 A1	02/05/2012
		JP 2012-525403 A	22/10/2012
		KR 10-2012-0005460 A	16/01/2012
		MX 2011011290 A	13/02/2012
		NZ 595331 A	30/08/2013
		WO 2010-126967 A1	04/11/2010