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(54) Title: P-SELECTIN INHIBITION TO TREAT HUMAN LYMPHEDEMA

(57) Abstract: Provided are therapeutic methods for treating a patient with lymphedema, or for prophylactically treating an individual susceptible to lymphedema, by blocking P-selectin activity.

P-SELECTIN INHIBITION TO TREAT HUMAN LYMPHEDEMA

CROSS REFERENCE TO OTHER APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/451,488 filed March 10, 2023, the contents of which are hereby incorporated by reference in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with Government support under contract HL141105 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Lymphedema is a chronic state of lymphatic vascular insufficiency characterized by regionally impaired immunity, interstitial edema, adipose deposition, and fibrotic remodeling. The condition affects around 100-200 million individuals globally but has no effective pharmacological therapies. Secondary lymphedema is the predominant form of the disease, resulting from acquired lymphatic vascular damage arising after parasitic infection, oncologic conditions, cancer therapy, chronic venous disease, and trauma. Research from the past two decades suggests that pathologically skewed $CD4^+$ T cell differentiation promotes preclinical lymphedema. While blocking Th2 immunity or inflammation, in general, improves skin histopathology, this therapy has been ineffective for reducing limb volumes.

[0004] The lymphatic vasculature is an integral circulatory system and crucially influences disease pathophysiology. Lymphedema is the prototypical lymphatic vascular disorder that afflicts nearly 200 million individuals globally with no approved drugs. A better understanding of lymphedema pathobiology can identify much-needed therapeutic targets. While inflammation is increasingly recognized as a disease-promoting pathology in lymphedema, how uncontrolled abnormal immune responses develop in this disease remains poorly understood.

[0005] In lymphedema, the lymphatic transport capacity is not sufficient to offset the lymphatic load. This causes the normal volume of interstitial fluid formation to exceed the rate of lymphatic return, resulting in the accumulation of interstitial fluid, enriched by the content of high molecular weight proteins. The result is a high-protein edema, or lymphedema, with protein concentrations of 1.0-5.5 g/mL. The high oncotic pressure in the interstitium favors the continued accumulation of water. Accumulation of interstitial fluid leads to massive dilatation of the remaining outflow tracts, along with valvular incompetence that causes reversal of flow from subcutaneous tissues into the dermal plexus. A marked inflammatory reaction is initiated. Macrophage activity is increased, resulting in destruction of elastic fibers and production of fibrosclerotic tissue. Tissue inflammation in lymphedema may reflect either an active or passive consequence of impaired immune traffic.

[0006] Surgical treatment is palliative, not curative, and it does not obviate the need for continued medical therapy. Moreover, it is rarely indicated as the primary treatment modality. Many surgical procedures have been advocated, but none of the surgical interventions has a clearly documented favorable long-term results.

[0007] Improved treatment of lymphedema is of great clinical and scientific interest. The present invention addresses this need by providing new methods for treating lymphedema.

SUMMARY OF THE INVENTION

[0008] Compositions and methods for the treatment of lymphedema are provided. In the methods of the invention, therapeutic compositions are administered to an individual suffering from lymphedema, or at risk of developing lymphedema, which therapeutic compositions block P-selectin activity. In some embodiments the agent that blocks P-selectin activity is an antibody. In some embodiments the antibody binds to and inhibits the activity of P-selectin. In some embodiments an antibody binds to and inhibits the activity of the P-selectin glycoprotein ligand-1 (PSGL-1). In some embodiments the agent that blocks P-selectin activity is a small molecule, including without limitation heparin and derivatives thereof.

[0009] In some embodiments, an effective dose of an agent that blocks P-selectin activity is administered to an individual having lymphedema, including without limitation established lymphedema, for a period of time sufficient to decrease or reverse tissue pathology of the affected (lymphedematous) tissue relative to an untreated control group. Treatment may be continued as required for maintenance of the therapeutic benefit. Where required, maintenance therapy may be maintained at the same dosage and schedule as the initial treatment, or may be achieved by transitioning to an alternative maintenance schedule, e.g. at a lower dose, less frequent dose, and the like. In some embodiments, the treating physician can determine that the treatment is efficacious by verifying a change in the architecture of the affected tissue. The tissue may be assayed by any number of means as described herein to verify therapeutic benefit, if visual inspection alone is insufficient. A convenient measure of efficacy in some applications is dermal thickness, although those of skill in the art will understand upon contemplation of this disclosure that various indicia can be used to monitor treatment and determine efficacy. The time period for treatment may be about 1 week, about 2 weeks, about 3 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 6 months, or longer.

[0010] In some embodiments of the invention, an individual with established lymphedema is treated with an effective dose of an agent that blocks P-selectin activity for a period of time sufficient to stabilize or reverse the disease. Such individuals may have pathological alterations of the skin characteristic of established disease, e.g. stage 1, 2 or 3 lymphedema. The methods of treatment according to the present invention can result in reversal of disease

conditions, exemplified by partial or complete reversal of skin pathologies in affected tissues. The therapy may be maintained after partial or complete reversal of skin pathologies.

[0011] In some embodiments, the volume of the affected limb(s), is measured over the time period, e.g. to monitor treatment, determine efficacy, etc. In some embodiments, where the volume is largely due to fluid accumulation, more typical of very early stages of the disease, volume may decrease by 10 milliliters to 100 milliliters or more from the affected tissue following treatment.

[0012] In some embodiments, an effective dose of an agent that blocks P-selectin activity is provided to an individual susceptible to lymphedema, including without limitations individuals that have undergone surgery or radiation for cancer. As noted, secondary, or acquired lymphedema, is frequently acquired as a result of cancer treatment and is much more common than primary lymphedema.

[0013] In some embodiments, therefore, the individual to be treated has been, or is being treated for cancer, but has not yet developed lymphedema. In other embodiments, the patient has lymphedema (stage 0 to 3) as a result of cancer therapy, e.g. surgery or radiotherapy or other therapy damaging to the lymphatic system. In some embodiments, the individual has been treated with surgery, which may be as a result of cancer diagnosis and treatment, or may be other surgeries affecting the lymph nodes that can cause lymphedema treatable in accordance with the invention. In such embodiments, treatment with an agent that blocks P-selectin activity may commence immediately following surgical wound healing, or may commence at a time following surgical wound healing, including after some or even substantial wound healing has occurred, e.g., 3-14 days after surgery, but before a patient has been diagnosed as having stage 0 lymphedema.

[0014] In some embodiments the individual has been treated with radiotherapy, which itself may follow surgery for cancer therapy. In such embodiments, treatment with an agent that blocks P-selectin activity may commence immediately during radiotherapy, following radiotherapy, or may commence at a time following radiotherapy, including after some or even substantial wound healing has occurred, as above, but before a patient has been diagnosed as having stage 0 lymphedema.

[0015] The time period in which to see treatment benefit may be about 1 week, about 2 weeks, about 3 weeks, about 1 month, about 2 months, about 3 months, about 4 months, or more. In some embodiments, the volume and/or structure of the affected or at risk tissue, i.e. the lymphedematous tissue, is measured or otherwise assayed or assessed at various points over the time period, e.g. at least at the beginning and some designated endpoint for testing. In some embodiments, the architecture of the affected tissue is assayed by bioimpedence or dermal thickness measurements or by histological assessment. In some embodiments, the

architecture of the affected tissue after treatment resembles or more closely resembles the architecture of unaffected tissue.

[0016] In other aspects, the compounds provided by the invention are used in the manufacture of a medicament for the treatment or prevention of lymphedema, wherein said medicament is an agent that blocks P-selectin activity. In various embodiments, the medicament is formulated for oral administration, including both immediate release and sustained release pharmaceutical formulations. In all of these embodiments, the invention provides unit dose forms of the medicament.

[0017] The methods can further comprise continuing the therapy if the therapy is determined to be efficacious. The methods can comprise maintaining, tapering, reducing, or stopping the administered amount of a compound or compounds in the therapy if the therapy is determined to be efficacious. The methods can comprise increasing the administered amount of a compound or compounds in the therapy if it is determined not to be efficacious or likely to be more efficacious if dosing is increased in daily amount or via a change in the administration schedule. Alternatively, the methods can comprise stopping therapy if it is determined not to be efficacious.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIGS. 1A-1K. S1PR1 signaling of LECs is reduced in both mouse and human lymphedema skin. (A) Acquired lymphedema was surgically induced in the tails of C57BL/6J mice through thermal ablation of lymphatic trunks. Skin incision, alone, was performed in sham surgery groups. (B) Real-time RT-qPCR analysis of *Sphk1* mRNA levels in tail skin from control sham surgery mice or animals subjected to lymphatic surgery. (C) Representative immunofluorescence (IF) staining of SPHK1 (red) and LYVE1 (green) of the skin tissues harvested from control or lymphedema mice. DAPI (blue) stains for the nucleus. Scale bar = 40 μm. (D) Quantification of the SPHK1 staining intensity comparing groups shown in B. (E) Real-time RT-qPCR analysis of *S1pr1* mRNA levels in tail skin from control sham surgery mice or lymphedema mice. (F and G) Flow cytometry histograms show the mean fluorescence intensity of S1PR1 on LEC ($Gp38^{+}CD31^{+}$) population. Representative (F) and compiling data (G) are shown. (H and I) The serum concentration of S1P in mouse (H) and human (I) lymphedema. (J) Representative IF staining of Gp38 (green) and S1PR1 (red) of the human skin from healthy control or lymphedema. DAPI (blue) stains for the nucleus. Scale bar = 50 μm. (K) Quantification of the S1PR1 intensity comparing groups shown in J. Data are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ by the Mann-Whitney test.

[0019] FIGS. 2A-2L. LEC-specific *S1pr1* deficiency exaggerates tissue swelling, augments cutaneous skin thickness, promotes lymphatic leakage, and reduces lymphatic drainage. (A)

Schematic diagram of the experimental protocol. (B) Quantification of tail volume changes over time of WT and *S1pr1*^{LECKO} mice after lymphatic surgery. (C) Representative photographs of tails 21d following lymphatic surgery. (D) A cartoon showing the cross-section view of mouse-tail skin with or without lymphedema. (E-G) H&E staining of tail cross-section 21d following lymphatic surgery. Representative images (E) are shown. Black arrows indicate lymphatic vessel areas. Double-headed black arrows demonstrate cutaneous thickness. Quantification of cutaneous thickness (F) and lymphatic vessel luminal areas (G) are shown. Scale bar = 1 mm. (H and I) Immunofluorescent images of LYVE1 (red) of tail skin 21d after surgery. Representative image (H) and quantification of the LYVE1 area data (I) are shown. DAPI (blue) stains the nucleus. Scale bar = 100 μm. (J) A cartoon showing one side of the mouse tail with two-line lymphatic trunks. (K and L) Near-infrared imaging 21d following lymphatic surgery after ICG injection near the tip of the tail. Representative images (K) are shown. White arrows denote surgical sites. Red arrows indicate ICG drainage. Yellow arrows indicate ICG leakage. Quantification of leakage (L) is shown. Data are presented as the mean ± SEM; * $p < 0.05$ and ** $p < 0.01$ by the Mann-Whitney test. ICG; indocyanine green.

[0020] FIGS. 3A-3I. LEC *S1pr1* deficiency promotes CD4 T cell infiltration following lymphatic surgery. (A) Flow cytometric gating scheme for determining tail skin immune cell populations. Flow cytometric analysis was performed d21 after lymphatic surgery. (B-G) Quantification of $CD45^+$ cells (B), $CD8^+$ T cells, (C), $CD4^+$ T cells (D), $CD4^+$IFN-γ^+ Th1 cells (E), $CD4^+$IL-4^+ Th2 cells (F), and $Foxp3^+CD25^+CD4^+$ Treg cells (G) in tail skin. (H) Representative IF staining of CD4 (red) and LYVE1 (green) of the lymphedema mouse tail skin from WT or *S1pr1*^{LECKO} mice. DAPI (blue) stains for the nucleus. Arrows indicate CD4 T cells surrounding lymphatic vessels. Scale bar = 50 μm. (I) Quantification of the CD4 T cell staining presented in H. Data are presented as mean ± SEM; * $p < 0.05$ and ** $p < 0.01$ by the Mann-Whitney test. ns: non-significant.

[0021] FIGS. 4A-4F. Decreased S1PR1 signaling in LECs promotes T cell activation. (A) Schematic diagram of LEC ($Gp38^+CD31^+$) purification. (B) Representative flow cytometry sorting strategy for the isolation of LECs from cultured lymph node stromal cells. (C) Timeline of co-culture with purified LECs and naïve CD4 T cells activating with to α-CD3/28 Abs. LECs to T cell ratio was 1:5. (D-F) Flow cytometric analysis was performed d4 after co-culture. Representative flow cytometric plots and quantification of IFN-γ^+CD44^+ in $CD4^+$ T cells (D), IL-4^+CD44^+ in $CD4^+$ T cells (E), and $Foxp3^+CD25^+$ in $CD4^+$ T cells (F). Data are presented as mean ± SEM; * $p < 0.05$ and ** $p < 0.01$ by the Mann-Whitney test.

[0022] FIGS. 5A-5L. Abnormal lymphatic S1PR1 signaling activates lymphocytes through direct cell-cell contact. (A) Timeline of the co-culture of purified naïve CD4 T cells and HDLECs. (B) sh*Ctr* or sh*S1PR1*-treated HDLECs were co-cultured with purified human naïve CD4 T cells activating with α-CD3/28 Abs. LEC to T cell ratio was 1:5. (C and D) After 3 days

of culture, human CD4 T cells were intracellularly stained for IFN-γ and IL-4. Representative flow cytometric plots and quantification of IFN-γ$^+$CD44$^+$ in CD4$^+$ T cells (C), IL-4$^+$CD44$^+$ in CD4$^+$ T cells (D). (E to I) T cell-related cytokines were detected in supernatant from co-cultured cells. (J) HDLECs treated with sh*Ctr* or sh*SPR1* were co-cultured with human naïve CD4 T cells in 0.4 μm membrane pore trans-well in the presence of α-CD3/28 antibody for 3 days. (K and L) Representative flow cytometric plots (K) and compiling data (L) of intracellular IFN-γ$^+$ and IL-4$^+$ cells in the CD4$^+$ T cell population cultured in a trans-well system. Data are presented as mean ± SEM; * $p < 0.05$ and ns (no significant) by the Mann-Whitney test for C, D, and L; by Wilcoxon matched-pairs signed rank test for E, F, G, H, and I.

[0023] FIGS. 6A-6I. The effects of abnormal S1PR1 signaling on lymphatic biology. (A and B) Timeline of HDLEC treatment and harvesting time point. RNA was extracted from HDLEC without (A) or with (B) purified naïve CD4 T cell co-culture. (C and D) Volcano plot identifying genes significantly up-regulated (red) in sh*S1PR1*-treted HDLECs versus sh*Ctr*- treated HDLECs. (C) 18 upregulated genes from HDLECs without CD4 T cell co-culture. (D) 121 upregulated genes from HDLECs with CD4 T cell co-culture. (E) Venn diagram shows data summary of differentially up-regulated genes from RNA-seq data comparing sh*S1PR1*-treated HDLECs with or without CD4 T cell co-culture. Threshold of false discovery rate < 0.05. (F) KEGG pathway enrichment analysis of differentially up-regulated gene between sh*Ctr*-treated HDLEC and sh*S1PR1*-treated HDLEC with CD4 T cell co-culture. Most significantly upregulated pathways are shown. (G) Timeline of M.O.I. dependence sh*S1PR1* treatment to HDLECs. (H and I) P-selectin fluorescence intensity in HDLECs after *S1PR1* knock-down was evaluated by flow cytometric analysis. Representative (H) and compiling data (I) are shown. Data are presented as the mean ± SEM; * $p < 0.05$ compared with the sh*Ctr*-treated HDLECs group; by Mann-Whitney test. KEGG; Kyoto Encyclopedia of Genes and Genomes. M.O.I.; multiplicity of infection.

[0024] FIGS. 7A-7H. Blocking P-selectin decreases CD4 T cell activation and lymphedema. (A) Timeline of the co-culture of purified naïve CD4 T cells and HDLEC. α-human P-selectin Ab (Waps12.2) was added to sh*S1PR1*-treated HDLECs 1h before purified memory CD4 T cell co-culture with HDLECs at day 4. (B and C) Flow cytometric analysis was performed d3 after co-culture. Quantification of IFN-γ$^+$CD44$^+$ in CD4$^+$ T cells (B), IL-4$^+$CD44$^+$ in CD4$^+$ T cells (C). Data B and C are presented as the mean ± SEM; * $p < 0.05$ compared with the sh*Ctr*-treated HDLEC group; # $p < 0.05$ compared with sh*S1pr1*-treated HDLEC group; by the Mann-Whitney test. (D) Schematic diagram of the experimental protocol. 5 mg/kg anti-mouse P-selectin Ab (RB40.34.4) or Isotype IgG control (Iso IgG) was retro-orbital-i.v. injected into WT or *S1pr1*^{LECKO} mice 1 day before lymphedema surgery and the tail size of animals was measured at days 0, 7, 14, and 21. (E and F) Quantification of tail volume changes (E). Representative photographs of tail skin on day 21 after surgery (F). (G) Quantification of IFN-

$\gamma^{+}CD44^{+}$ in $CD4^{+}$ T cells. (H) Quantification of $IL\text{-}4^{+}CD44^{+}$ in $CD4^{+}$ T cells. Data in E, G, and H are presented as mean ± SEM; * $p < 0.05$, and ** $p < 0.01$ compared with the *S1pr1*^{LECKO} + Iso Ctr group; by the Mann-Whitney test.

[0025] FIG. 8. LEC S1PR1 signaling in lymphedema pathogenesis. Graphic abstract showing how abnormal lymphatic endothelial cell (LEC) S1P signaling can induce T cell activation and lymphedema after lymphatic injury. Lymphatic injury caused LEC S1P-S1PR1 signaling reduction. These changes collectively induce P-selectin expression on LECs resulting in CD4 T cell overactivation.

[0026] FIG. 9. Tail volume was calculated through a digital photographic technique. Mouse-tail volume was calculated through a digital photographic technique pre-operatively, and post-operatively on d7, d14, and d21 using an Olympus D520 Zoom digital camera at SHQ resolution at a fixed distance from the subject (37cm). All images were processed and analyzed in Adobe Photoshop CS6.

[0027] FIG. 10. Decreased SPHK1 expression in lymphatic vessels from mice with lymphedema. Representative immunofluorescent staining of SPHK1 (green) in *Prox1-Cre*^{ERT2}-*tdTomato* mouse tail-skin. Tissues were harvested on d21 following sham surgery (control) or lymphedema surgery. DAPI (blue) stains nuclei; white arrows indicate co-staining; scale bar = 20μm. Note that different magnifications were used to better illustrate lymphatic structures in the control and lymphedema groups.

[0028] FIGS. 11A-11C. *Prox1-cre*^{ERT2} effectively mediates *S1pr1* deletion in LECs. (A) Flow cytometric analysis was performed 3 weeks after tamoxifen treatment. Flow cytometric gating scheme for the determination of LEC population. The singlet is singe cell gating for doublet discrimination. (B and C) S1PR1 fluorescence intensity in LEC ($Gp38^{+}CD31^{+}$) in the tail skin from WT (red) and *S1pr1*^{LECKO} (blue) mice was quantified by flow cytometric analysis. Representative (B) and compiling data (C) are shown. Data are presented as mean ± SEM; * $p < 0.05$ by the Mann-Whitney test.

[0029] FIGS. 12A-12B. *S1pr1*^{LECKO} does not affect wound healing following sham surgery. (A) Tail volume measurements of WT and *S1pr1*^{LECKO} on 7d, 14d, and 21d following sham surgery. (B) Representative tail image 21d following sham surgery. Data are presented as mean ± SEM; ns (no significant) by the Mann-Whitney test.

[0030] FIGS. 13A-13C. No dorsal edema pattern in mouse embryos lacking lymphatic specific *S1pr1*. (A) Strategy for injecting pregnant mice (*Prox-1-Cre*^{ERT2} x *S1pr1*$^{fl/-}$ mated with *Prox-1-Cre*^{ERT2} x *S1pr1*$^{fl/-}$) with tamoxifen. (B) Arrow shows edema formation observed in the dermis of the back. (C) Freshly harvested embryos in E17.5 were imaged under a brightfield microscope.

[0031] FIG. 14. Flow cytometry analysis strategy for T cell population profiling in tail skin. Representative flow cytometric gating scheme for determining T cell populations in the tail skin. IFN-γ^+CD4$^+$ for Th1, IL-4$^+$CD4$^+$ for Th2, and Foxp3$^+$CD25$^+$ for Treg.

[0032] FIGS. 15A-15C. IL-4$^+$ tissue-resident T memory (TRM) cells are increased in *S1pr1*^{LECKO} lymphedema mouse groups. (A) Representative flow cytometric gating scheme for determining IL-4-producing TRM cell populations in the tail skin. (B and C) Flow cytometric analysis was performed d21 after lymphatic surgery. Representative flow cytometric plots (B) and quantification of IL-4$^+$CD103$^+$ in CD69$^+$CD4$^+$ TRM cells in tail tissue skin. Data are presented as mean ± SEM; * $p < 0.05$ by the Mann-Whitney test.

[0033] FIGS. 16A-16C. sh*S1PR1* trans-infection does not affect HDLEC viability. (A) Timeline of M.O.I.-dose dependent effects of sh*S1PR1* treatment on HDLECs. (B) Green fluorescent protein (GFP) tagged lentiviral sh*S1PR1*-treated HDLECs following 3 days of culture. GFP and brightfield (BF) were imaged with fluorescence microscopy. Scale bar = 100 μm. (C) *S1PR1* mRNA level of HDLECs were analyzed by using Real-time RT-qPCR. Data is presented as mean ± SEM; $p < 0.01$ compared with the sh*Ctr* (M.O.I. = 0) by the Mann-Whitney test.

[0034] FIGS. 17A-17G. Increased S1P signaling alleviates lymphedema development. (A) Schematic diagram of the experimental protocol. 10 mg/kg 4-DP or vehicle (PBS) was injected i.p. every day after tail surgery. (B) Quantification of tail volume changes over time of vehicle or 4-DP treated mice after lymphatic surgery. (C) Representative photographs of tails 21d following lymphatic surgery. (D and E) Immunofluorescent images of LYVE1 (green) of tail skin 21d after surgery. Representative image (D) and quantification of the LYVE1 areas (E) are shown. DAPI (blue) stains the nucleus. Scale bar = 50 μm. (F and G) Flow cytometric analysis was performed d21 after lymphatic surgery. Representative flow cytometric plots (F) and quantification of IL-4$^+$CD44$^+$ in CD4$^+$ T cells in tail tissue skin (G). Data are presented as mean ± SEM; * $p < 0.05$ and *** $p < 0.001$ by the Mann-Whitney test.

[0035] FIGS. 18A-18C. Decreased CLA$^+$CD4 T cell population in lymphedema tail skin after anti P- selectin Ab treatment. (A) Memory CD4 T cells were isolated from PBMC and cultured with or without α-CD3/28 antibody for 3 days. Flow cytometric plots of CLA$^+$, IFN-γ^+, and IL-4$^+$ CD44$^+$ in CD4$^+$ T cells were shown. (B and C) Representative flow cytometric plots (B) and quantification (C) of CLA$^+$CD44$^+$ in CD4$^+$ T cells in tail skin. Data are presented as mean ± SEM; * $p < 0.05$ compared with the *S1pr1*^{LECKO} + Iso Ctr group; by the Mann-Whitney test.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0036] The lymphatic vascular system is crucial for the regulation of tissue fluid homeostasis, immune function, inflammatory response, fat absorption and tissue disposition. Lymphatic

dysfunction, either due to gene mutations, developmental disorders, or much more commonly secondary to damage to the lymphatic anatomy, can lead to lymphedema, a debilitating condition characterized by chronic tissue edema, impaired regional immunity, and regional accumulation of subcutaneous fat. In particular, there are no therapies currently approved and known to prevent lymphedema from occurring or, once it has occurred (stage 0 to 3), slow or stop its progression, much less reverse the incredibly debilitating and quality of life destroying manifestations in those patients with the most severe forms of established disease. The present invention provides methods and compositions that can be used to prevent and treat lymphedema and slow, stop, and even reverse its progression, and therefore represents a significant advance in the important goal of reducing the impact of this disease in patients and on our health care system.

Definitions

[0037] *P-selectin* (CD62P) is a type-1 transmembrane protein that functions as a cell adhesion molecule (CAM) on the surfaces of activated endothelial cells, which line the inner surface of blood vessels, and activated platelets. The extracellular region of P-selectin is composed of three different domains: a C-type lectin-like domain in the N-terminus, an EGF-like domain and a complement-binding protein-like domains having nine short consensus repeats. The gene encoding human P-selectin is located on chromosome 1q21-q24, spans > 50 kb and contains 17 exons. The reference human protein sequence may be accessed at Genbank, NP_002996.

[0038] P-selectin is constitutively expressed in megakaryocytes and endothelial cells. It is initially sorted into the membranes of secretory granules. When the megakaryocytes and endothelial cells are activated, P-selectin is rapidly translocated to the plasma membrane from the granules. Increased levels of P-selectin mRNA and protein are also induced by inflammatory mediators.

[0039] The primary ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on almost all leukocytes, although P-selectin also binds to heparan sulfate and fucoidans. PSGL-1 is situated on various hematopoietic cells such as neutrophils, eosinophils, lymphocytes, and monocytes, in which it mediates tethering and adhesion of these cells.

[0040] Inhibitors of P-selectin are known and used in the art; and include antibodies that bind to and inhibit P-selectin, and antibodies that bind to and inhibit PSGL-1. For example, the antibody crizanlizumab-tmca (Adakveo, Novartis) is FDA approved; and inclacumab is a fully human monoclonal antibody designed to bind to and selectively inhibit P-selectin, which is currently in a Phase 3 clinical trial. Other antibodies can be selected, or generated for this purpose.

[0041] In addition to antibodies, small molecules that inhibit P-selectin are known and used. Small molecule inhibitors include, for example glycomimetics such as GMI-1070 (Rivipansel), which is a pan-selectin antagonist that inhibits both P- and E-selectin. Sialyl Lewis X mimetics include, for example, bimosiamose, which is a sialyl Lewis X mimetic that inhibits the interaction between P-selectin and its ligands.

[0042] Glycosulfopeptides mimicking truncated N-terminal PSGL1 monomers, like GSnP-6, act as inhibitors. Alternatively, a pegylated glycomimetic of the N terminus of PSGL-1, PEG40-GSnP-6 (P-G6) is a highly potent P-selectin inhibitor with a favorable pharmacokinetic profile.

[0043] Low-dose unfractionated heparin can block P-selectin, as do pentosan polysulfate sodium (PPS); and phosphomannopentaose sulfate (PI-88). Sevuparin is a modified form of heparin and a P-selectin and L-selectin inhibitor. Semisynthetic hexasaccharide mimics of maltohexaose sulfate in which a central glycosidic bond is substituted by a hydrolase-resistant C-C bond (see Vismara et al. (2011) Neoplasia. 13(5):445-52) are also inhibitors.

[0044] Other small molecule inhibitors include the small-molecule oral P-selectin inhibitor 3S-1,2,3,4-tetrahydro-β-carboline-3-methyl aspartyl ester (THCMA); and 2-(4-Chlorobenzyl)-3-hydroxy-7,8,9,10-tetrahydrobenzo[H]quinoline-4-carboxylic acid (PSI-697).

[0045] In some embodiments, a P-selectin blocking agent is an antibody. The antibody may be, without limitation, crizanlizumab or inclacumab. The antibody may compete for binding with crizanlizumab or inclacumab. In other embodiments a P-selectin blocking agent is a small molecule, including without limitation, THCMA, PSI-697, GMI-1070 (Rivipansel), bimosiamose, GSnP-6, P-G6, PPS, PI-88, and Sevuparin.

[0046] The terms “active agent” and “therapeutic agent” refer a compound, also referred to as a drug, that exerts a preventive or therapeutic effect on a disease or disease condition. Active agent can refer not only to a single active agent but also to a combination of two or more different active agents.

[0047] “Alleviate” and “ameliorate” are equivalent to “treat” and refer to or mean a process by which the severity of a sign or symptom of a disorder is decreased. Importantly, a sign or symptom can be alleviated without being eliminated. Therapeutically effective dosages are expected to decrease the severity of, and so alleviate and ameliorate, a sign or symptom of disease.

[0048] “As-needed,” in “as-needed administration,” means that a formulation is administered to a patient when symptoms are observed, or when symptoms are expected to appear, or at any time that the patient and/or treating physician deems it appropriate to treat (therapeutically or prophylactically) undesirable symptoms (e.g., symptoms arising from a disease).

[0049] “Combination therapy” and “co-therapy” means the administration of a first active agent and at least a second, different active agent as part of a specific treatment regimen intended

to provide the beneficial effect from the co-action of the at least two active agents. The beneficial effect of the combination may include, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents.

[0050] Administration of therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected). Combination therapy is not intended to encompass the administration of two or more different therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily results in a combination therapy of the invention. Combination therapy includes administration of at least two different therapeutic agents in a sequential manner, wherein each therapeutic agent is administered at a different time, as well as administration of at least two different therapeutic agents in a substantially simultaneous manner. When administered in combination, each component can be administered at the same time or sequentially in any order at different points in time. Thus, each component can be administered separately but sufficiently closely in time so as to provide the desired therapeutic effect.

[0051] "Concomitant administration" of two therapeutic agents means administration of the agents at such time that both will have a therapeutic effect. Such concomitant administration may involve concurrent (i.e. at the same time), prior, or subsequent administration of the agents. A person of ordinary skill in the art would have no difficulty determining the appropriate timing, sequence and dosages of administration for particular drugs and compositions of the present invention.

[0052] Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single capsule having a fixed ratio of each therapeutic agent or in separate capsules for each of the therapeutic agents. Sequential or substantially simultaneous administration of each therapeutic agent can be effected by any appropriate route, including, but not limited to, oral routes, intravenous routes, intramuscular routes, and direct absorption through mucous membrane tissues. The two different therapeutic agents can be administered by the same route or by different routes. For example, a first therapeutic agent of the combination selected may be administered by intravenous injection while the second therapeutic agent of the combination may be administered orally. Alternatively, for example, all therapeutic agents may be administered orally or all therapeutic agents may be administered by intravenous injection. The sequence in which the therapeutic agents are administered is not critical, unless otherwise stated.

[0053] Combination therapy also includes the administration of the different therapeutic agents as described above in further combination with other biologically active ingredients and non-drug therapies (e.g., surgery or physical therapy). Where a combination therapy comprises a non-drug treatment, the non-drug treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic

agents and non-drug treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the non-drug treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

[0054] As used herein, the term "correlates," or "correlates with," and like terms, refers to a statistical association between instances of two events, where events include numbers, data sets, and the like. For example, when the events involve numbers, a positive correlation (also referred to herein as a "direct correlation") means that as one increases, the other increases as well. A negative correlation (also referred to herein as an "inverse correlation") means that as one increases, the other decreases.

[0055] "Dosage unit" refers to physically discrete units suited as unitary dosages for the particular individual to be treated. Each unit can contain a predetermined quantity of active compound(s) calculated to produce the desired therapeutic effect(s) in association with the required pharmaceutical carrier. The specification for the dosage unit forms can be dictated by (a) the unique characteristics of the active compound(s) and the particular therapeutic effect(s) to be achieved, and (b) the limitations inherent in the art of compounding such active compound(s).

[0056] "Dosage form" means any form of a pharmaceutical composition for administration to a subject (typically a human or animal of veterinary interest suffering from a disease or condition to be treated). "Dose" refers to an amount of active agent. "Unit dosage form" refers to a dosage form that contains a fixed amount of active agent. A single tablet or capsule is a unit dosage form. Multiple unit dosage forms can be administered to provide a therapeutically effective dose. A dosage form can include a combination of dosage forms.

[0057] "Effective amount" and "therapeutically effective amount" refers to a nontoxic but sufficient amount of an active agent to achieve a desired therapeutic effect.

[0058] "Controlled release" refers to a drug-containing formulation or unit dose form thereof from which release of the drug is not immediate, i.e., with a controlled release formulation, administration does not result in immediate release of all of the drug administered into an absorption pool. The term is used interchangeably with "nonimmediate release" as defined in Remington: The Science and Practice of Pharmacy, Nineteenth Ed. (Easton, PA: Mack Publishing Company, 1995). In general, controlled release formulations include sustained release and delayed release formulations.

[0059] "Sustained release" and "extended release" means a drug formulation that provides for gradual release of a drug over an extended period of time, and typically, although not necessarily, results in substantially constant blood levels of a drug over an extended time period.

[0060] "Delayed release" refers to a drug formulation that, following administration to a patient, provides a measurable time delay before drug is released from the formulation into the patient's body.

[0061] Percentages and ratios used herein, unless otherwise indicated, are by weight.

[0062] "Pharmaceutically acceptable" means not biologically undesirable, i.e., the material may be incorporated into a pharmaceutical composition administered to a patient without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. When the term "pharmaceutically acceptable" is used to refer to a pharmaceutical carrier or excipient, it is implied that the carrier or excipient has met the required standards of toxicological and manufacturing testing or that it is included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug Administration.

[0063] "Pharmaceutically acceptable salts" mean derivatives of an active agent produced by making acid or base salts thereof. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines, alkali or organic salts of acidic residues such as carboxylic acids, and the like. Pharmaceutically acceptable salts include the conventional non-toxic salts or the quaternary ammonium salts of the parent compound formed, for example, from non-toxic inorganic or organic acids.

[0064] Pharmaceutically acceptable salts include those formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like.

[0065] Pharmaceutically acceptable salts include solvent addition forms (solvates) or crystal forms (polymorphs) as defined herein, of the same salt.

[0066] "Pharmacologically active" (or "active") as in a "pharmacologically active" derivative or analog, refers to a derivative or analog having the same type of pharmacological activity as the parent compound of approximately equivalent in degree.

[0067] "Preventing" and "prevent" means avoiding the onset of a clinically evident disease progression altogether or slowing the onset of a pre-clinically evident stage of a disease in individuals at risk. Prevention includes prophylactic treatment of those at risk of developing a disease.

[0068] "Sign" means an indication of disease and includes conditions that can be observed by a doctor, nurse, or other health care professional.

[0069] "Small molecule" as used herein refers to molecules, whether naturally-occurring or artificially created (e.g., via chemical synthesis) that have a relatively low molecular weight. Preferred small molecules are biologically active in that they produce a local or systemic effect

in animals, preferably mammals, more preferably humans. In certain preferred embodiments, the small molecule is a drug and the small molecule is referred to as "drug molecule" or "drug" or "therapeutic agent". The small molecule can have a MW less than or equal to about 5 kDa. In other embodiments, the drug molecule has a MW less than or equal to about 1.5 kDa.

[0070] "Subject in need thereof refers to a human or other mammal suitable for treatment with an active agent. A subject in need thereof may have a disease or be at an increased risk, relative to the general population, of developing a disease.

[0071] "Symptom" means a sign or other indication of disease, illness, or injury. Symptoms may be felt or noticed by the individual experiencing them or by others, including by non-health-care professionals.

[0072] "Treating" and "treat" describes the management and care of a patient for the purpose of combating a disease, condition, or disorder and includes the administration of an active agent to alleviate the symptoms or complications of a disease, condition or disorder, or to eliminate the disease, condition or disorder.

[0073] "Antibodies", also referred to as immunoglobulins, conventionally comprise at least one heavy chain and one light, where the amino terminal domain of the heavy and light chains is variable in sequence, hence is commonly referred to as a variable region domain, or a variable heavy (VH) or variable light (VH) domain. The two domains conventionally associate to form a specific binding region, although as well be discussed here, a variety of non-natural configurations of antibodies are known and used in the art. In some embodiments an antibody specifically binds to and inhibits the activity of P-selectin.

[0074] A "functional" or "biologically active" antibody or antigen-binding molecule is one capable of exerting one or more of its natural activities in structural, regulatory, biochemical or biophysical events. For example, a functional antibody or other binding molecule may have the ability to specifically bind an antigen and the binding may in turn elicit or alter a cellular or molecular event such as signaling transduction or enzymatic activity. A functional antibody or other binding molecule may also block ligand activation of a receptor or act as an agonist or antagonist. The capability of an antibody or other binding molecule to exert one or more of its natural activities depends on several factors, including proper folding and assembly of the polypeptide chains.

[0075] The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, monomers, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), heavy chain only antibodies, three chain antibodies, single chain Fv, nanobodies, etc., and also include antibody fragments, so long as they exhibit

the desired biological activity (Miller et al (2003) Jour. of Immunology 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species.

[0076] The term antibody may reference a full-length heavy chain, a full length light chain, an intact immunoglobulin molecule comprising both full-length heavy and light chains; or an immunologically active portion of any of these polypeptides, i.e., a polypeptide that comprises an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin disclosed herein can be of any type (e.g., IgG, IgE, IgM, IgD, and IgA), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule, including engineered subclasses with altered Fc portions that provide for reduced or enhanced effector cell activity. The immunoglobulins can be derived from any species. In one aspect, the immunoglobulin is of largely human origin.

[0077] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a beta-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al (1991) Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md.). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

[0078] The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region may comprise amino acid residues from a "complementarity determining region" or "CDR", and/or those residues from a "hypervariable loop". "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

[0079] Variable regions of interest include 3 CDR sequences, which may be obtained from available antibodies with the desired specificity, or may be obtained from antibodies developed for this purpose. One of skill in the art will understand that a number of definitions of the CDRs

are commonly in use, including the Kabat definition (see "Zhao et al. A germline knowledge based computational approach for determining antibody complementarity determining regions." Mol Immunol. 2010;47:694–700), which is based on sequence variability and is the most commonly used. The Chothia definition is based on the location of the structural loop regions (Chothia et al. "Conformations of immunoglobulin hypervariable regions." Nature. 1989;342:877–883). Alternative CDR definitions of interest include, without limitation, those disclosed by Honegger, "Yet another numbering scheme for immunoglobulin variable domains: an automatic modeling and analysis tool." J Mol Biol. 2001;309:657–670; Ofran et al. "Automated identification of complementarity determining regions (CDRs) reveals peculiar characteristics of CDRs and B cell epitopes." J Immunol. 2008;181:6230–6235; Almagro "Identification of differences in the specificity-determining residues of antibodies that recognize antigens of different size: implications for the rational design of antibody repertoires." J Mol Recognit. 2004;17:132–143; and Padlanet al. "Identification of specificity-determining residues in antibodies." Faseb J. 1995;9:133–139., each of which is herein specifically incorporated by reference.

[0080] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

[0081] The antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al (1984) Proc. Natl. Acad. Sci. USA, 81:6851-6855). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape etc) and human constant region sequences.

[0082] An "intact antibody chain" as used herein is one comprising a full length variable region and a full length constant region. An intact "conventional" antibody comprises an intact light chain and an intact heavy chain, as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, hinge, CH2 and CH3 for secreted IgG. Other isotypes, such as IgM or IgA may have different CH domains. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variants thereof. The intact antibody may have one or more "effector functions" which refer to those biological activities attributable to the Fc constant region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis (ADCP); and down regulation of cell surface receptors. Constant region variants include those that alter the effector profile, binding to Fc receptors, and the like.

[0083] Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes." There are five major classes of intact immunoglobulin antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Ig forms include hinge-modifications or hingeless forms (Roux et al (1998) J. Immunol. 161:4083-4090; Lund et al (2000) Eur. J. Biochem. 267:7246-7256; US 2005/0048572; US 2004/0229310). The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called κ and λ, based on the amino acid sequences of their constant domains.

[0084] "Lymphedema", as used herein, is edema of a region or regions of the body due to lymphatic maldevelopment (primary lymphedema) or to obstruction, disruption or dysfunction (secondary or acquired lymphedema) of lymphatic vessels. Symptoms and signs may comprise varying degrees of brawny, fibrous, non-pitting edema in one or more regions of the body.

[0085] Primary lymphedemas are constitutional and relatively less common than the secondary forms. They vary in phenotype and patient age at presentation. The methods of the invention are applicable to these primary forms, although it will be understood by one of skill in the art that treatment may be more efficacious in some forms than others due to the differing disease etiologies. Primary forms of lymphedema include, without limitation Milroy's disease, Meige disease, (lymphedema praecox), lymphedema distichiasis, lymphedema tarda, etc., as

well as other genetic syndromes having prominent lymphedema, such as Turner's syndrome and Hennekam syndrome.

[0086] For example, congenital lymphedema appears at birth or within months thereafter, and may be due to lymphatic aplasia or hypoplasia. Milroy's disease is an autosomal dominant familial form of congenital lymphedema attributed to flt4 gene mutations and associated with edema and, sometimes, diarrhea and/or hypoproteinemia due to a protein-losing enteropathy caused by intestinal lymphangiectasia. Lymphedema distichiasis is an autosomal dominant familial form of lymphedema praecox attributed to mutations in a transcription factor gene (*FOXC2*) and associated with extra eyelashes (distichiasis), and edema of legs, arms, and sometimes the face. Lymphedema tarda occurs after age 35. Both familial and sporadic forms exist; the genetic basis of both is unknown. Clinical findings are similar to those of lymphedema praecox but may be less severe. Hereditary lymphedema type II (Meige disease, lymphedema praecox) develops around puberty or shortly thereafter in most individuals. This is the most common type of primary lymphedema. In addition to lymphedema of the legs, other areas of the body such as the arms, face and larynx may be affected. Some individuals may develop yellow nails. Lymphedema is prominent in some other genetic syndromes, including Turner syndrome; yellow nail syndrome, characterized by pleural effusions, chronic lung disease, lymphedema and yellow nails; and Hennekam syndrome, a rare congenital syndrome of generalized lymphatic abnormality, facial anomalies, and intellectual disability. The methods and compositions of the invention may be used to treat any of these primary lymphedemas and their symptoms.

[0087] Secondary (acquired) lymphedema is far more common than primary. It is most commonly caused by surgery (especially lymph node dissection, typically for staging and treatment of cancers), radiation therapy (especially axillary or inguinal), trauma, lymphatic obstruction by a tumor, and, in developing countries, lymphatic filariasis. The methods and compositions of the invention may be used to treat any of these secondary lymphedemas and their symptoms.

[0088] In some embodiments, a P-selectin blocking agent is administered to treat patients with established secondary leukemia, which may be contracted as a result of cancer therapy. It has been estimated that more than 15% of cancer survivors experience secondary lymphedema. Surgical removal of lymph nodes or therapeutic radiation of lymph nodes increases the risk of lymphedema. After axillary intervention, 15% to 30% of breast cancer survivors experience clinically relevant lymphedema, but other types of cancer and their associated treatments may cause secondary lymphedema as well. The incidences of lymphedema associated with other malignancies (cancers) were as follows: soft tissue sarcoma 30%, lower extremity melanoma 28%, gynecologic cancer 20%, genitourinary cancer 10%, and head and neck cancers 3%. Lymphedema may also result from increased lymph

production in patients with chronic venous insufficiency, congestive heart failure, and other causes of venous hypertension. The methods of the invention are applicable to all such secondary lymphedema patients.

[0089] The cardinal sign of acquired lymphedema is soft-tissue edema, graded in 4 stages. The term "established lymphedema" may refer generically to any of stages 1-3 of the disease, including without limitation the more advanced stages of the disease, e.g. stage 2 and stage 3, where structural changes in affected tissue are observed. In stage 0, the affected region is physically normal, but lymphatic insufficiency can be demonstrated through clinical assessment. In stage 1, the edema is pitting, and the affected area often returns to normal after elevation of the affected limb(s). In stage 2, the edema is pitting, and chronic soft-tissue inflammation causes structural changes in the tissues that accompany the pitting edema. In stage 3, the edema is brawny and irreversible, largely because of chronic soft-tissue structural changes.

[0090] Treatment with the methods of the invention may be prophylactic, i.e., treatment commences before onset of stage 0 disease, where the individual may be at risk of developing lymphedema due to medical procedures that confer such risk. Prophylactic treatment may also follow successful treatment of the disease: this is also referred to as "maintenance therapy" herein. Treatment itself is any administration of a P-selectin blocking agent as described herein, and may commence at any time after the onset of stage 0 lymphedema, e.g. where treatment stabilizes or reverses patient condition to a non-symptomatic state. Treatment with the methods of the invention may commence following onset of stage 1 lymphedema. Treatment with the methods of the invention may commence following onset of stage 2 lymphedema. Treatment with the methods of the invention may commence following onset of stage 3 lymphedema.

[0091] The swelling that can accompany disease progression can be unilateral or bilateral, and may worsen when the weather is warm, before menstruation occurs, following physical exertion, and/or after the limb remains for a long time in a dependent position. It can affect any part of a limb (isolated proximal or distal) or the entire extremity, or the face, head and neck, trunk, breast or genitalia; it can restrict range of motion. Disability and emotional distress can be significant, especially when lymphedema results from medical or surgical treatment. Skin changes are common and include hyperkeratosis, hyperpigmentation, lichenification, verrucae, papillomas, and fungal infections. The methods of the invention include methods to treat any and all of these conditions and symptoms.

[0092] Lymphangitis or cellulitis may develop, for example, when bacteria traverse the skin barrier, which is abnormal in lymphedema. Lymphangitis is frequently streptococcal, causing erysipelas; sometimes it is staphylococcal. The affected limb becomes red and feels hot; red streaks may extend proximally from the point of entry, and lymphadenopathy may develop.

Rarely, the skin breaks down. Rarely, long-standing lymphedema leads to lymphangiosarcoma (Stewart-Treves syndrome), usually in postmastectomy patients and in patients with filariasis. The methods of the invention include methods to treat any and all of these conditions and symptoms.

[0093] Without treatment, cellular overgrowth, adipose deposition and fibrosis promote the progressive anatomic distortion and loss of function of the affected areas. Additionally, impaired trafficking of antigen-presenting cells in lymph hampers local immune surveillance of the lymphedematous region(s) to the draining lymph nodes. Thus, there is chronic inflammation, infection, and hardening of the skin that, in turn, results in further lymph vessel damage and distortion of the shape of the affected body parts. Moreover, there is a high degree of dysfunction due to physical factors such as a decrease in joint mobility causing reduced amplitude of movements, increased leg weight, increased pain, and impaired ability to perform day-to-day tasks. The methods of the invention include methods to treat any and all of these conditions and symptoms.

[0094] Pathological skin changes associated with lymphedema include an increase in cellularity of layers of the skin, accumulation of glycoproteins, loss of elasticity, and subdermal increase in adipose layer. The methods of the invention include methods to treat any and all of these conditions and symptoms.

[0095] Those of skill will thus appreciate that the methods disclosed herein are applicable to the treatment and prevention of lymphedema including its signs and symptoms such as those associated with the following clinical indicia of lymphedema. A number of clinical indicia can be used to diagnose lymphedema and to monitor the effectiveness of therapy, including treatment with the compositions and methods of the present invention. The disclosure provides methods of determining efficacy of a lymphedema treatment in a subject in need thereof by (a) measuring an endpoint of a clinical indication in a patient, where the endpoint is measured after treatment has started, (b) comparing the endpoint of the clinical indication to a baseline or reference, where the baseline or reference is measured in the same subject or a similar subject population before treatment is begun, and (c) determining the efficacy of the lymphedema treatment based on the comparison step.

[0096] Analysis of clinical indicia may include measurement of dermal thickness; change of lymphedema volume of leg/arm/ hand; change of stagnation of fluid at level of shoulder/ trunk; change of extracellular fluid in arm; change of thickness and reflectivity of cutis and subcutis of arm/ shoulder/ trunk; change of elasticity of skin and subcutaneous tissue of arm; change of lymphatic architecture and function; change of venous circulation in arm/ trunk; number of episodes of erysipelas.

[0097] When imaging is used to diagnose lymphedema or assess disease state or progression, the most common modality for diagnosis is indirect radionuclide

lymphoscintigraphy. This procedure requires subcutaneous injection of an appropriate radiolabeled tracer, for example ^{99m}Tc-antimony sulfide colloid or ^{99m}Tc-labeled human serum albumin. Criteria for the diagnosis of lymphatic dysfunction include: (1) delayed, asymmetric or absent visualization of regional lymph nodes; (2) asymmetric visualization of lymphatic channels; (3) collateral lymphatic channels; (4) dermal backflow (5) interrupted vascular structures; and (6) visualization of the lymph nodes of the deep lymphatic system. The presence of "dermal back-flow" is considered abnormal. It is interpreted to represent the extravasation of lymph fluid from the lymphatics into the interstitium as a result of lymphatic and/or venous hypertension. Beyond lymphoscintigraphy, magnetic resonance imaging and computerized axial tomography have clinical utility. These imaging techniques permit objective documentation of the structural changes caused by lymphedema. Recent advances in the magnetic resonance approach have improved the visualization of lymphatic vascular anomalies in both nonenhanced and contrast-enhanced applications (see, for example, Pankaj et al. (2013) World J Surg Oncol. 2013; 11: 237). As an alternative, bioelectrical impedance has been used to detect and monitor upper limb lymphedema (see Ridner et al. (2009) Lymphat Res Biol. 7(1): 11–15), which uses characteristics of frequency-dependent current flow to quantify changes in extracellular fluid. In various embodiments, such technology is used to monitor the progress of therapy of a patient treated in accordance with the invention or to identify a patient that may benefit from such treatment.

[0098] The effectiveness of treatment by the methods of the invention will be evidenced by improvement in disease symptoms and pathology. Individuals being treated and medical practitioners may choose to evaluate success, monitor the course of treatment, adjust dosage and timing, etc. by any convenient indicia. It is anticipated that the invention will first find application in treatment of established lymphedema but as efficacy and safety are demonstrated in more and more patients, physicians will treat patients at earlier and earlier stages of the disease, finding significant use, eventually, as a prophylaxis in most patients with a significant probability of developing lymphedema, with the acceptable probability for prophylactic treatment lowering as a drug is proved safe in more and more patients (i.e., the risks of therapy still are far outweighed by the benefits of preventing the disease even in patient populations at relatively low but still measurable risk of developing the disease).

[0099] In some embodiments of the invention, e.g., treatment of patients with established disease, improvements in the architecture of the skin provide a convenient method for assessing treatment success. For example, dermal thickness reflects the architectural changes in lymphedema. See, for example, Mellor et al., Breast J 2004;10:496–503; Hacard et al. Skin Res Technol 2014; 20: 274–81, each herein specifically incorporated by reference. For example, dermal thickness may be measured with factory calibrated skinfold calipers, such as Lange skinfold calipers, Model EQ0014921. In some embodiments, a treatment

provided herein is efficacious if, after a period of time from the onset of treatment (e.g., 2 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months or longer), there is a decrease in dermal thickness of at least one affected region (e.g., limb) as compared to the dermal thickness of the at least one affected region prior to the onset of treatment. The decrease in dermal thickness observed with successful treatment may be a decrease of at least about 1 mm, at least about 2.5 mm, at least about 5 mm, at least about 7.5 mm, at least about 10 mm, and may be at least about 12.5 mm, at least about 15 mm, or more. Alternative measurements for determining a correction of pathologies of skin architecture may include, for example, dermal ultrasound, DEXA scanning, direct biopsy, visual inspection, etc. Dermal thickness and architecture, e.g. presence of hyperkeratosis, dermal collagen, and adipose deposition in an affected limb may be monitored.

[00100] In some embodiments, a change in the volume of the affected limb is measured as a measure of treatment success, i.e., the volume declines with successful treatment. Volume can be measured by any of a number of methods in the art, e.g. circumferential measurements, water displacement volumetry, etc. For example, an assessor may use a standardized tape measure for circumference measurements taken every 2-6 cm, and calculating the volume by, for example, the truncated cone method. Successful treatment may reduce, or decrease, the volume of lymphedematous body parts (both the fluid and tissue components). In some instances, volume is decreased 2-fold or more after treatment, i.e. as compared to the volume before treatment, for example, 2-fold or more, 3-fold or more, 4-fold or more, sometimes 5-fold or more, 10-fold or more, 15-fold or more, in some instances 20-fold or more, 50 fold-or more, etc. In other words, the volume is decreased by about 50 milliliters or more, 100 milliliters or more, 200 milliliters or more, 300 milliliters or more, 400 milliliters or more, 500 milliliters or more. In some instances, the volume is restored to normal volume, i.e. the volume prior to the onset of the lymphedema, e.g. the volume of the unaffected bilateral tissue.

[00101] *Patient Selection*. In another aspect, the present disclosure provides methods for selecting patients likely to benefit from the therapies of the disclosure, as well as methods for determining whether a patient is responding to such therapy. In some embodiments a patient is selected for treatment when a diagnosis of confirmed lymphedema is made, where the lymphedema may be stage 0, stage 1, stage 2 or stage 3. Diagnosis may be made on the basis of any of the clinical indicia described above.

[00102] In other embodiments, an individual at risk of developing lymphedema is treated prophylactically to prevent the development of lymphedema by the methods of the invention. In some such embodiments, the individual at risk of developing lymphedema has been treated for cancer. Such individuals can be stratified for risk of developing lymphedema by a medical

professional. Cancers associated with a high degree of risk following treatment include, for example, breast cancer, soft tissue sarcoma, melanoma, ovarian cancer, prostate cancer, lymphoma, head and neck cancer.

[00103] The present disclosure provides a variety of methods that enable the treating physician to treat patients individually, reflecting each patient's individual risk of developing lymphedema. Thus, some patients, at low risk of developing lymphedema, may not be treated until symptoms manifest or persist for some period of time. Other patients, however, may be treated prophylactically, before symptoms appear or can be detected by clinicians (e.g. before Stage 0). These methods are further elaborated below.

[00104] Most individuals with lymphedema today have it as a result of cancer therapy involving surgery and/or radiation that damages or destroys lymph nodes. For patients at a low risk of developing lymphedema associated with surgery, i.e., patients whose surgery involved only or less than about 4 lymph nodes with sentinel node technique, the treating physician may delay any treatment unless and until lymphedema symptoms manifest, i.e., the physician likely will not use the drug in a prophylactic mode in such patients unless and until safety and benefit has been demonstrated in greater numbers of patients. However, there is a much higher risk associated with nodal sampling of greater than 4 lymph nodes or with radiotherapy of lymph nodes, or both, so some patients receiving these procedures may well, in accordance with the invention, be treated in a prophylactic mode.

[00105] The risk of developing lymphedema, and timing of treatment, can be further stratified based on the treatment received by the individual. A low risk is associated with surgery only of less than about 4 lymph nodes with sentinel node technique. A much higher risk is associated with nodal sampling of greater than 4 lymph nodes; or with radiotherapy of lymph nodes, or both. Thus, in some embodiments a person having undergone nodal sampling of greater than 4 lymph nodes; or with radiotherapy of lymph nodes, or both is prophylactically treated with an effective dose of a P-selectin blocking agent according to the methods of the invention, or is treated after development of established lymphedema.

[00106] Where treatment is performed prophylactically after surgical treatment, initiation of treatment with a P-selectin blocking agent in accordance with the invention may be delayed for some period of time after surgery to ensure that the preventive treatment does not slow or delay wound healing. Treatment may commence immediately following surgical wound healing, or at a period of time following some or substantial surgical wound healing, e.g. after at least about 1 day, and more typically after about at least 3 days or more days, including about 1 week to 2 weeks, at which time wound healing is substantially complete in most patients, although again, prophylactic therapy may begin at any time prior to Stage 0, including after about 3 weeks post-surgery.

[00107] Where treatment is performed prophylactically in a patient treated without surgery, e.g. a patient treated with radiotherapy, chemotherapy, or a combination thereof, believed to put the patient at such risk for lymphedema that prophylactic therapy is required, the lymphedema prophylaxis may commence at any time during or following therapy, e.g. at the time of treatment, after about 1 day, about 3 days, about 1 week, about 2 weeks, about 3 weeks, or more. However, again, as such treatments are causing "wounds" requiring healing, the physician may defer the initiation of treatment precisely as described above to minimize any potential for the prophylactic therapy to interfere with wound healing.

[00108] While patients with established lymphedema can readily be diagnosed by visual means, and while cancer patients can be stratified regarding how likely they are to develop leukemia based on the treatments they receive, the invention provides other methods for diagnosing lymphedema and otherwise identifying patients likely to benefit from the treatment methods of the present invention.

[00109] The development of secondary (acquired) lymphedema is associated with insufficient reparative lymphangiogenesis and lymphatic vessel remodeling. Lymphangiogenesis is a complex cellular process characterized by proliferation, migration, and differentiation of lymphatic endothelial cells.

[00110] Therapeutic agents useful in the methods of the invention will also have the effect of partially or completely restoring the architecture of the tissue, i.e. skin, toward or to its condition pre-lymphedema. In other words, the architecture of the affected tissue will more closely resemble the architecture of normal, unaffected tissue, after treatment than before. Most notably, the dermis and subdermis return to a normal or more normal thickness, and inflammatory cell infiltrates and accumulated glycoproteins and collagen are reduced or absent. The architecture of the tissue may be assayed by any convenient means as described herein, e.g. skinfold thickness, biopsy, etc., if necessary or helpful to assess the efficacy of treatment or need to continue or alter therapy.

[00111] Therapeutic agents can serve as the active ingredient in pharmaceutical compositions formulated for the treatment of lymphedema, including a propensity for lymphedema. The compositions can also include various other agents to enhance delivery and efficacy. The compositions can also include various agents to enhance delivery and stability of the active ingredients.

Methods

[00112] An effective dose of an agent that blocks P-selectin activity can be formulated as provided herein for use in the methods of the disclosure. In some embodiments, a therapeutically effective dose leads to sustained serum levels of anti-P-selectin agent (e.g.,

an anti-P-selectin antibody) of about 40 μg/ml or more (e.g, about 50 μg/ml or more, about 60 μg/ml or more, about 75 μg/ml or more, about 100 μg/ml or more, about 125 μg/ml or more, or about 150 μg/ml or more). In some embodiments, a therapeutically effective dose leads to sustained serum levels that range from about 40 μg/ml to about 300 μg/ml (e.g, from about 40 μg/ml to about 250 μg/ml, from about 40 μg/ml to about 200 μg/ml, from about 40 μg/ml to about 150 μg/ml, from about 40 μg/ml to about 100 μg/ml, from about 50 μg/ml to about 300 μg/ml, from about 50 μg/ml to about 250 μg/ml, from about 50 μg/ml to about 200 μg/ml, from about 50 μg/ml to about 150 μg/ml, from about 75 μg/ml to about 300 μg/ml from about 75 μg/ml to about 250 μg/ml, from about 75 μg/ml to about 200 μg/ml, from about 75 μg/ml to about 150 μg/ml, from about 100 μg/ml to about 300 μg/ml, from about 100 μg/ml to about 250 μg/ml, or from about 100 μg/ml to about 200 μg/ml).

[00113] Examples of suitable doses of an antibody include, but are not necessarily limited to a range from about 0.05 mg/kg to about 50 mg/kg (e.g., from about 0.1 mg/kg to about 40 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 20 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 5 mg/kg, from about 0.5 mg/kg to about 10 mg/kg, from about 0.5 mg/kg to about 7.5 mg/kg, from about 0.5 mg/kg to about 5 mg/kg, from about 0.5 mg/kg to about 4 mg/kg, from about 0.5 mg/kg to about 3 mg/kg, from about 1 mg/kg to about 10 mg/kg, from about 1 mg/kg to about 7.5 mg/kg, from about 1 mg/kg to about 5 mg/kg, from about 1 mg/kg to about 4 mg/kg, from about 1 mg/kg to about 3 mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 7.5 mg/kg, or about 10 mg/kg).

[00114] Examples of suitable doses of small molecule agent include, but are not necessarily limited to a range from about 0.05 mg/kg to about 50 mg/kg (e.g., from about 0.1 mg/kg to about 40 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 20 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 5 mg/kg, from about 0.5 mg/kg to about 10 mg/kg, from about 0.5 mg/kg to about 7.5 mg/kg, from about 0.5 mg/kg to about 5 mg/kg, from about 0.5 mg/kg to about 4 mg/kg, from about 0.5 mg/kg to about 3 mg/kg, from about 1 mg/kg to about 10 mg/kg, from about 1 mg/kg to about 7.5 mg/kg, from about 1 mg/kg to about 5 mg/kg, from about 1 mg/kg to about 4 mg/kg, from about 1 mg/kg to about 3 mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 7.5 mg/kg, or about 10 mg/kg).

[00115] Agents may be administered in a dose comparable to the dosage of the agent for other indications. For example, a clinically approved dose of crizanlizumab is 5 mg/kg based on patient's actual body weight and may be given with or without hydroxyurea. Non-antibody agents will be administered at a dose suitably adjust for the agent, for example sevuparin was

administered in clinical trials at 18 mg/kg per day; PSI-697 was administered at a flat dose of 600 mg/day orally, etc.

[00116] In addition to active agents, such therapeutic compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers of diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can include other carriers, adjuvants, or non-toxic, nontherapeutic, nonimmunogenic stabilizers, excipients and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents, wetting agents and detergents.

[00117] The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. When the pharmaceutical composition includes a polypeptide, the polypeptide can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (*e.g.,* increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or complexing agents include sulfate, gluconate, citrate and phosphate. The polypeptides of a composition can also be complexed with molecules that enhance their *in vivo* attributes. Such molecules include, for example, carbohydrates, polyamines, amino acids, other peptides, ions (*e.g.,* sodium, potassium, calcium, magnesium, manganese), and lipids.

[00118] Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990).

[00119] The therapeutic compositions, also referred to herein as pharmaceutical compositions, can be administered for prophylactic and/or therapeutic treatments as described herein. For previously untested compositions, toxicity and therapeutic efficacy of the active ingredient can be determined according to standard pharmaceutical procedures in cell cultures and/or experimental animals, including, for example, determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred.

[00120] The data obtained from cell culture and/or animal studies can be used in formulating a range of dosages for humans. The dosage of the active ingredient typically lines within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized.

[00121] The pharmaceutical compositions described herein can be administered in a variety of different ways. Examples include administering a composition containing a pharmaceutically acceptable carrier via oral, intranasal, rectal, topical, intraperitoneal, intravenous, intramuscular, subcutaneous, subdermal, transdermal, and intrathecal methods.

[00122] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

[00123] Formulations suitable for enteral administration, such as, for example, administration topically (e.g., as solutions, lotions, creams, paste, emulsions, suspensions, etc.), orally, rectally, vaginally, or by inhalation, include capsules, liquid solutions, emulsions, suspensions, and elixirs. For example, if prepared for topical applications, the compositions may comprise a biocompatible organic solvent, e.g. an isopropyl ester such as isopropyl myristate and isopropyl palmitate; a polar lipid, e.g. lecithin, phosphatidylcholine, etc., a surfactant, e.g. docusate sodium, docusate sodium benzoate, docusate calcium, tween 80, polysorbate 80; water; and/or urea (present at a concentration of about 5 to 20% by mass of the final composition). In some instances, a topical formulation will comprise an enhancer for skin penetration, such as SEPA 09. Examples of topical formulations may be found in, e.g. U.S. Pat. No 5,654,337, U.S. Pat. No. 5,093,133, U.S. Pat. No. 5,210,099, U.S. Pat. No. 3,957,971, U.S. Pat. No. 5,016,652, the complete disclosures of which are incorporated herein by reference.

[00124] The components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (e.g., at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade). Moreover, compositions intended for in vivo use are usually sterile. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

The therapeutic methods of the invention may be combined with one or more additional therapies for the treatment of lymphedema. Prior to the present invention, few pharmacologic therapies have been found to be effective in the treatment of lymphedema. Case reports have suggested, however, that chronic lymphedematous changes (eg, elephantiasis nostra verrucosa [ENV]) can be treated with oral and topical retinoids. These therapies are thought to help normalize keratinization and decrease inflammatory and fibrotic changes. Other pharmaceutical agents of interest include, for example, ketoprofen; doxycycline; sodium selenite; tacrolimus; cyclophosphamide; VEGF-C; retinoic acid agonists such as 9-cis retinoic acid (RA), etc.

[00125] Topical emollients and keratolytics, such as ammonium lactate, urea, and salicylic acid, have been recommended to improve secondary epidermal changes.

[00126] Patients experiencing recurrent lymphangitis or cellulitis may require long-term, prophylactic treatment with antimicrobial agents such as penicillin, cephalexin, or erythromycin.

[00127] Filariasis has been treated with the anthelmintic agents diethylcarbamazine and albendazole.

[00128] Mobilizing fluid may utilize elevation and compression, exercise, massage, pressure bandages, intermittent pneumatic compression, drainage of lymphatics, complex decongestive therapy, and the like. Surgical soft-tissue reduction, lymphatic reanastomoses, and formation of drainage channels may also be included in a therapeutic program.

EXPERIMENTAL

[00129] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

[00130] Human and experimental lymphedema tissues exhibited decreased LEC S1P signaling through S1PR1. LEC *S1pr1* loss-of-function exacerbated lymphatic vascular insufficiency, tail swelling, and increased CD4 T cell infiltration in mouse lymphedema. LECs, isolated from *S1pr1*^{LECKO} mice and co-cultured with CD4 T cells, resulted in augmented

lymphocyte differentiation. Inhibiting S1PR1 signaling in human dermal LECs (HDLECs) promoted T helper type 1 and 2 (Th1 and Th2) cell differentiation through direct cell contact with lymphocytes. HDLECs with dampened S1P signaling exhibited enhanced P-selectin, an important cell adhesion molecule expressed on activated vascular cells. *In vitro*, P-selectin blockade reduced the activation and differentiation of Th cells co-cultured with sh*S1PR1*-treated HDLECs. P-selectin-directed antibody treatment improved tail swelling and reduced Th1/Th2 immune responses in mouse lymphedema.

[00131] In this study, we showed that LEC S1PR1 expression was low in both clinical and pre-clinical lymphedema skin. Using LEC-specific *S1pr1* loss-of-function transgenic mouse lines (*S1pr1*^{LECKO}), we found that lymphatic *S1pr1* deficiency exacerbates lymphedema. Cell culture studies demonstrated that LECs with reduced S1PR1 skewed T cell differentiation towards T helper 1/2 (Th1/Th2) phenotypes in a contact-dependent manner. Bulk mRNA-Sequencing analysis revealed that S1P signaling deficiency enhanced LEC expression of P-selectin, an adhesion molecule regulating T cell trafficking and activity. Blocking P-selectin attenuated Th1/Th2 differentiation induced by *S1PR1*-deficient LECs in culture; anti-P-selectin antibody treatment decreased tail swelling and reduced Th1/Th2 cell population in lymphedema skin of *S1pr1*^{LECKO} mice. Collectively, we describe how lymphedema-associated immune dysregulation may be linked to reduced LEC S1P signaling and how P-selectin inhibitors, already approved for use in other diseases, are effective in this otherwise refractory condition.

Methods

[00132] *Mice*. All mice were purchased from Jackson Laboratory. Detailed mice information: C57BL/6J (B6, JAX:000664), Prox1tm3(cre/ERT2)Gco/J (Prox1-Cre-ER^{T2}, JAX:022075), B6.129S6(FVB)-S1pr1tm2.1Rlp/J (S1P$^{1\,loxp}$, JAX:019141), and B6.Cg *Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (Ai14(RCL-tdT)-D, JAX:007914) To generate lymphatic endothelial-specific *S1pr1*-deficient transgenic strain, mice expressing *Prox1-Cre-ER*^{T2} were crossed with *S1PR1*^{loxp} mice. *Prox1-Cre-ER*^{T2} alone littermates were used as WT controls. To generate lymphatic endothelial-specific *tdTomato* reporter mice (*Prox1-Cre-ER*^{T2}*-tdTomato*), *Prox1- Cre-ER*^{T2} mice were crossed with *tdTomato* mice. 250 mg/kg tamoxifen was subcutaneously injected to 8 weeks mice for 3 consecutive days to activate the Cre-loxP system.

[00133] *Study approval*. All human tissues were approved by the Stanford University Institutional Review Board (protocol 7781). Adult patients with primary and acquired lymphedema were assessed in this study. Control samples were obtained from the healthy contralateral limb of the same patient. All animal studies were approved by the VA Palo Alto Institutional Animal Care and Use Committee (IACUC). Human buffy coats were purchased from Stanford Blood Center.

[00134] *The mouse-tail model of acquired lymphedema.* Lymphedema was induced through ablation of major lymphatic trunks on both sides of the tail and dermal lymphatic capillaries. Tail skin incision was made ~2 cm from the mouse tail base. For surgical sham controls, skin incision alone was performed. Disease progression was quantified by volumetric and histopathological measurements. Lymphatic vessel functions were analyzed with a near-infrared (NIR) imaging system.

[00135] *Tail volume measurement.* Mice tail images were taken through digital photographic technique preoperatively (D0) and postoperatively (D7, 14, and 21) using an Olympus D-520 Zoom digital camera at a fixed distance from the subject (37cm). Digital photographic tail images were quantified using Adobe Photoshop ruler tool. Tail volumes were derived from measurement of the tail diameter using the truncated cone approximation method (Fig 9).

[00136] *Quantification of S1P with liquid chromatography tandem mass spectrometry (LC-MS/MS).* Briefly, S1P was separated from serum with an ACE reverse phase C18 HPLC column. Formic acid 0.5% in 5mM NH_4Ac (A) and formic acid 0.5% in acetonitrile/water (9/1) (B) were used as the mobile phase. Lipids were detected with positive multiple reaction monitor (MRM) scanning at 330.20/264.40 m/z for S1P using a Sciex API-4000 MS/MS combined with a Shimazu 20A HPLC system. The internal standard for this method was Carbutamide. The quantification limit was 1 ng/ml. The calibration range was 1-2000ng/ml. The accuracy of the standards and control samples ranged from 88.7% to 120%. The information of patients was described in **Table 1**.

[00137] *Lymphatic drainage and leakiness test by NIR imaging.* The lymphatic vessel transportation function and leakage were characterized by using a NIR lymphatic imaging system and dye quantification at day 21. The collecting lymphatic function was tracked throughout the procedure by imaging the dynamics of transport of a 10 µl ICG (10mM) injected intradermally, at the tip of the mouse tail. NIR images were taken 10 minutes after dye injection using a custom NIR imaging system as described in detail in the previous study. ImageJ was used to verify ICG leakage intensity. Relative intensity of leaked ICG was calculated and presented.

[00138] *Immunofluorescence (IF) staining.* Surgery sites of 10 µm tail frozen sections were used for immunohistochemistry. Tail tissues were snap-frozen in O.C.T. compound solution (Fisher Healthcare) for IF staining and paraffin-embedded for hematoxylin and eosin (H&E) staining. Anti-LYVE1 (1:50; LSBio) Ab for lymphatic vessel and anti-CD4 (1:50; Abcam) Ab for CD4 T cell were stained at 4°C overnight. Secondary antibodies were labeled with the Alexa Fluor 488 or Cy3 for 1h at room temperature (1:400; Invitrogen). Photomicrographs were acquired using LEICA DMi8 or Zeiss LSM710. ImageJ was used to quantify for lymphatic areas.

[00139] *Real-time reverse transcription quantitative PCR (RT-qPCR).* Total RNA was isolated from mouse tail skin using the RNeasy® Fibrous Tissue Mini Kit (QIAGEN) and from HDLECs using RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA was synthesized to complementary DNA using the High-Capacity cDNA Reverse Transcription Kits (Applied biosystems) following the manufacturer's protocol. RT-PCR was performed using PowerSYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The amplification condition was set at 40 cycles at 95°C for 15s and 60°C for 60s using AMI Prism® 7900HT Sequence detection System (Applied Biosystems). The sequences of the primers used for RT-qPCR are listed in Table 2 and 3.

[00140] *$CD4^+$ T cell isolation.* Naïve $CD4^+$ T cells from the spleens of C57BL/6 WT mice were purified with Naïve $CD4^+$ T cell Isolation Kit according to the manufacturer's (Miltenyi Biotec) instructions. Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats by gradient centrifugation with histopaque (density: 1.077 g/ml). Naïve CD4 T cells were purified from PBMCs using EasySep Human Naïve $CD4^+$ T cell Isolation Kit II (STEMCELL Technologies). Memory CD4 T cells were purified from PBMCs using EasySep Human Memory $CD4^+$ T cell Isolation Kit (STEMCELL Technologies).

[00141] *LEC purification and culture.* LNs were isolated from WT and *S1pr1*^{LECKO} mice. LNs were digested with the multi-tissue dissociation Kit I and gentle MACS Octo Dissociator with Heaters (Miltenyi Biotec). Digested lymph node stromal cells were cultured in endothelial cell growth medium II supplemented with 2% fetal calf serum, growth supplements, 100 U/mL of penicillin, and 100 g/mL of streptomycin. After five days of culture, cells of $CD45^-$, $CD31^+$, and $Gp38^+$ were selected as LEC population. Cells were sorted by fluorescence-activated cell sorting (FACS) Aria III. LECs used in experiments showed over 99% purity.

[00142] *Co-culture of $CD4^+$ T cells with LECs.* Purified LECs were cultured in a 24-well plate (4×10^4/well). After overnight adhesion, 2×10^5 mouse naïve $CD4^+$ T cells and APCs along with anti-CD3 and CD28 monoclonal antibodies (BD Biosciences; 3 µg/ml) were added and cultured for 3 days in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO2 atmosphere. Commercially-purchased HDLECs (PromoCell) were cultured in a 24-well plate (4×10^4/well). After overnight adhesion, 2×10^5 human naïve $CD4^+$ T cells with ImmunoCult™ Human CD3/CD28 T cell Activator (STEMCELL Technologies) were added and cultured for 3 days in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

[00143] *Flow cytometry analysis.* Single-cell suspension prepared from mice tail skin digested with multi tissue dissociation Kit I or from *ex vivo* cultures were analyzed by intracellular cytokine staining as previously described. Prepared cells were restimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (ThermoFisher Scientific) for 5 h at 37°C

in a humidified 5% CO_2 atmosphere. After stimulation, the cells were stained with fluorescein-conjugated antibodies against Fixable Viability Dye (Invitrogen), CD4, CD8, CD25, and CD44 (BD Bioscience). Following surface staining, Foxp3, IFN-γ, IL-4, and IL-17A were intracellularly stained using fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions. Additionally, CD31, CD45, CD69, CD103, Gp38, and S1PR1 fluorescein-conjugated antibodies were used for surface staining. Isotype-matched antibodies were used as isotype controls.

[00144] *Cytokine profiling.* The supernatant co-cultured human CD4 T cell with HDLEC was collected at day 3. T cell related-cytokine assay was performed in EVE technologies.

[00145] *Analysis of bulk mRNA sequencing (bulk RNA-seq) data.* Total RNA was isolated from HDLECs using the RNeasy® Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. mRNA library was formed by ploy A enrichment and reverse transcription of cDNA. Illumina PE150 technology is processed to sequence the sample (Novogene). Sequencing reads were aligned to the Human GRCh38 reference genome according to the ENCODE uniform processing pipeline, by STAR (v2.1.3) then quantified with RSEM (v1.4.1). Differential gene expression analysis was performed in R using EBSeq package, an empirical Bayes hierarchical model for inference in RNA-seq experiments, using false discovery rate (FDR) of 0.05 to retrieve list of differentially expressed genes (DEGs). Differentially expressed genes were ranked by posterior fold change and enriched for gene sets using The Molecular Signatures Database (MSigDB) and Gene Set Enrichment Analysis (GSEA) tools developed by the Broad Institute.

[00146] *Statistics.* All data are presented as a mean ± SEM. Nonparametric Mann-Whitney test and Wilcoxon matched-pairs signed rank test were used for statistical analyses using GraphPad Prism v9.3.1. $p < 0.05$ was considered statistically significant. Error bars correspond to the mean with SEM.

Results

[00147] *Lymphedema is characterized by decreased LEC S1PR1-mediated S1P signaling.* LEC S1P signaling through S1PR1 is critical for lymphatic homeostasis and repair. To determine the relevance of LEC S1P/S1PR1 signaling in lymphedema, we used a mouse tail lymphedema model, in which tail skin incision and lymphatic trunk ablation were performed for surgical induction of lymphedema (Fig. 1A). Real-time reverse transcription quantitative PCR (RT-qPCR) analysis of *Sphk1*, the key gene involved in S1P production, demonstrated a reduction of the expression in lymphedematous mouse skin (Fig. 1B), confirming our prior microarray study. LECs are the major cell type in the skin that produces S1P, a process reliant on SPHK1 activity. LECs in lymphedema tissues express decreased SPHK1 (Fig. 1C, D, and

10). S1PR1 is the most strongly expressed S1P receptor in LECs, and S1PR1 signaling is necessary for lymphatic vessel maturation. A diminished *S1pr1* mRNA level was detected in lymphedema tail skin (Fig. 1E). More specifically, we analyzed S1PR1 expression in $Gp38^{+}CD31^{+}$ cells using flow cytometry and found significantly decreased S1PR1 expression in LECs in lymphedema skin (Fig. 1F and G). Next, we assessed S1P serum concentration in preclinical and clinical lymphedema. Decreased S1P production was detected in the lymphedema condition compared with the healthy control in both mouse and human (Fig. 1H and I). Consistent with mouse lymphedema tail skin, the expression of S1PR1 was decreased in $Gp38^{+}$ lymphatic vessel of human lymphedema skin (Fig. 1J and K). These data demonstrate that LEC S1P/S1PR1 signaling is reduced in both human and preclinical lymphedema.

[00148] *Lymphatic S1pr1 deficiency promotes lymphatic dysfunction in lymphedema.* To evaluate lymphatic-specific S1P/S1PR1 signaling in lymphedema pathogenesis, we generated LEC-specific *S1pr1*-deficient ($S1pr1^{LECKO}$) mice by crossing $S1pr1^{fl/fl}$ mice with transgenic mice expressing Cre-ERT2 under the control of the Prox-1 promoter ($Prox\text{-}1\text{-}Cre^{ERT2}$). Assessment of LEC ($Gp38^{+}CD31^{+}$) S1PR1 expression demonstrated >75% knockout efficiency (Fig. 11). $S1pr1^{LECKO}$ mice were subjected to lymphedema surgery three weeks following tamoxifen (TAM) treatment (Fig. 2A). Serial evaluation of tail volumes following lymphedema surgery showed that genetic silencing of LEC-specific *S1pr1* exacerbated tail swelling compared with WT littermate control mice (Fig. 2B and C). Sham surgery, which only involves skin incision, did not cause tail swelling in both WT and $S1pr1^{LECKO}$ mice (Fig. 12).

[00149] The mouse lymphedema model is characterized by severe cutaneous thickening and the lymphatic area increase in affected tails (Fig. 2D). H&E staining of cross-sections demonstrated increased cutaneous thickness and lymphatic area in tissues derived from $S1pr1^{LECKO}$ mice (Fig. 2E-G). Immunofluorescence (IF) staining similarly demonstrated areas of increased lymphatic capillaries identified by LYVE1 positivity in tail samples from $S1pr1^{LECKO}$ mice (Fig. 2H and I). Because the dilated lymphatic vessels have been related to decreased transport functionality of lymphatic vasculature, an evaluation of lymphatic vessel transport function was performed by using near-infrared (NIR) imaging system. The indocyanine green (ICG) was injected intradermally at the end of the mouse tail and NIR images were captured through side- and top-down views (Fig. 2J). In line with the tail volume assessment, lymphatics of $S1pr1^{LECKO}$ mice subjected to lymphedema surgery exhibited decreased lymphatic drainage and increased lymphatic leakage (Fig. 2K and L).

[00150] Together, these data show that loss of lymphatic S1P/S1PR1 signaling causes a more severe decline of lymphatic function and worsens lymphedematous tissue swelling. Pathways critical for lymphatic repair in disease conditions also play important roles in lymphatic development; however, assessment of LEC S1P/S1PR1 signaling during early embryonic stage illustrated only minimal dorsal edema (Fig. 13), indicating S1P signaling

through S1PR1 is not crucial for early lymphatic development characterized by lymphangiogenesis. This study also suggests that in pathological conditions, LEC S1PR1 signaling may regulate other biological processes beyond lymphangiogenesis.

[00151] *LEC S1pr1 deficiency increases CD4 T cell infiltration.* CD4 T cells regulate lymphedema pathology by inhibiting lymphatic vessel pumping capacity, decreasing collateral lymphatic formation, and increasing lymphatic leakage. Prior studies demonstrated that LECs regulate T cell differentiation. We hypothesized that LEC *S1pr1* deficiency skews pathological T cell differentiation in lymphedema. Flow cytometric analyses of single-cell suspension of lymphedematous tissues harvested from WT and *S1pr1*^{LECKO} mice were performed, and the T cell populations were assessed (Figs. 3A and 14). There was an increased accumulation of CD45$^+$ immune cells in the tail skin of *S1pr1*^{LECKO} as compared with WT skin following lymphedema surgery (Fig. 3B). Further analysis demonstrated that lymphedema tissue from *S1pr1*^{LECKO} mice contained significantly more CD4$^+$ T cells compared with WT littermate controls, but not CD8$^+$ T cells (Fig. 3C and D).

[00152] We focused our analysis on CD4 T cells subsets and demonstrated increased IFN-γ-producing Th1 cells and interleukin (IL)-4-expressing Th2 cells (Fig. 3E and F), and decreased Foxp3$^+$CD25$^+$ regulatory T cells (Tregs) (Fig. 3G) in lymphatic *S1pr1*-deficient lymphatic mice. Recent research shows that tissue-resident memory CD4$^+$ (T_{RM}) cells are associated with vasculitis pathogenesis. Evaluation of IL-4-producing CD103$^+$CD4$^+$ T_{RM} cells demonstrated a significant increase of this population in *S1pr1*^{LECKO} lymphedema tissues (Fig. 15), indicating CD4$^+$ T_{RM} cells comprise an important source of CD4 T cells in a more severe form of experimental lymphedema. An IF study demonstrated increased numbers of CD4 T cells located in close proximity to lymphatic vessels in lymphedema tissue from *S1pr1*^{LECKO} mice (Fig. 3H and I). These results suggest that LEC *S1pr1* deficiency may attract more CD4 T cells to lymphatic cells and promote pathological differentiation and expansion of these T cells in lymphedema.

[00153] *LEC S1P/S1PR1 signaling regulates CD4 T cell differentiation.* Emerging evidence demonstrates the LEC's ability in regulating adaptive immune responses. To assess whether LEC S1P signaling deficiency preferentially skews CD4 T cell differentiation toward certain Th pathways, LEC-dependent CD4 T cell activation and differentiation assays were performed. Mouse lymph node-LECs (mLN-LECs) were sorted by FACS by selecting CD45(-) population that expresses both Gp38 and CD31 (Fig. 4A). The purity of mLN-LECs was approximately 99% after sorting (Fig. 4B). Sorted LECs were co- cultured with mouse splenocyte-derived naïve CD4 T cells in the presence of anti-CD3/28 antibodies (Ab) stimulation for 4 days (Fig. 4C). *S1pr1*-deficient LECs significantly enhanced IFN-ɣ and IL-4 production in T cells (Fig. 4D and E), while reducing the relative Foxp3$^+$CD25$^+$ population (Fig. 4F). These results suggest

that *S1pr1*-deficient LECs preferentially promote Th1 and Th2 effector cells and suppress Treg cell differentiation.

[00154] Because LECs have tissue-specific tolerogenic properties, we tested whether LECs from peripheral tissues also exhibit immunomodulatory function. We used lentiviral vectors that express short hairpin RNA targeting *S1PR1* (sh*S1PR1*) to knockdown S1PR1 in primary human dermal LECs (HDLECs) (Fig. 16). sh*Control* (sh*Ctr*)- or sh*S1PR1*-treated HDLECs (sh*S1PR1*-HDLECs) were co-cultured for three days with naïve CD4 T cells, purified from healthy individual peripheral blood mononuclear cells (PBMCs), in the presence of anti-CD3/28 Abs (Fig. 5A and B). CD4 T cells, co-cultured with sh*S1PR1*-HDLECs, generally secreted higher amounts of the Th1 cytokine, IFN-γ, and the Th2 cytokine, IL-4, compared with T cells co-cultured with sh*Ctr*-HDLECs (Fig. 5C and D). Multiplex cytokine measurement of CD4 T cell-related cytokines, IL-2 (T cell proliferation and activation), IFN-γ (Th1), and IL-4/5/13 (Th2) showed that sh*S1PR1*-treated HDLECs significantly increased the cytokine production by $CD4^+$ T cells compared with sh*Ctr*-HDLECs (Fig. 5E to I). Co-culture of those cells in a *trans*-well system (Fig. 5J) indicated that the frequency of IFN-γ and IL-4 production by $CD4^+$ T cells was similar in sh*Ctr*- and sh*S1PR1*-HDLECs (Fig. 5K and L). Taken together, these results suggest that LECs regulate CD4 T cell differentiation via a mechanism which requires cell-cell contact.

[00155] *Increased S1P signaling alleviates lymphedema development.* Given that LEC-specific *S1pr1* deficiency significantly exacerbates lymphatic function and skews towards inflammatory CD4 immune responses, we asked whether increasing S1P signaling could restore pathological lymphatic remodeling and alleviate lymphedema. We used 4-deoxypyridoxine (4-DP), which inhibits the degradation of S1P by dampening S1P lyase activity. 10m/kg 4-DP was administered intraperitoneally (i.p.) to WT B6 mice daily following lymphedema surgery (Fig. 17A). 4-DP therapy reduced tail swelling (Fig. 17B and C) and lymphatic remodeling (Fig. 17D and E) in mice with lymphedema. Evaluation of immune cell composition in lymphedema tissue revealed significantly ameliorated Th2 immune responses in samples harvested from 4-DP treated mice (Fig. 179F and G). Together, our results indicate that LEC S1P signaling is vital during lymphatic repair and important for the control of local pathological immune responses in lymphedema.

[00156] *S1P signaling suppresses P-selectin expression in HDLECs.* To identify putative S1P-regulated targets expressed in LECs that regulate T-cell differentiation, we performed bulk RNA-sequencing to characterize the gene expression change in HDLECs following *S1PR1* knockdown. We prepared RNA extraction from *S1PR1* knockdown HDLECs with or without CD4 T cell co-culture (Fig. 6A and B). *SELP*, encoding the adhesion protein P-selectin, was the highest upregulated gene among those commonly upregulated in sh*S1PR1*- HDLECs with

or without CD4 T cells in the culture (Fig. 6C and D). Differential expression analysis showed a total of 18 upregulated genes in sh*S1PR1*-HDLECs without CD4 T cell co-culture and 111 upregulated in sh*S1PR1*-HDLECs with CD4 T cell co-culture (Fig. 6E and Table 5). *SELP* was detected in the intersection of 7 overlapping upregulated genes between sh*S1PR1*-HDLECs with or without CD4 T cell co-culture. Gene set enrichment analysis showed a significantly modulated pathway with up-regulated genes in sh*Ctr*-HDLECs versus sh*S1PR1*-HDLECs with CD4 T cell co-culture and suggested the upregulated genes by S1PR1 knockdown were enriched for genes involved in cell-cell contact and T cell activation (Fig. 6F). Flow cytometry analysis confirmed increased cell surface P-selectin protein expression in HDLECs following *S1PR1* knockdown in sh*S1PR1* multiplicity of infection (M.O.I.)-dependent manner (Fig. 6G-I).

[00157] *P-selectin blockade inhibits the activation of CD4 T cells co-cultured with S1PR1-deficient HDLECs.* Cutaneous lymphocyte-associated antigen (CLA) is a carbohydrate modification form of P- selectin glycoprotein ligand-1 (PSGL-1), which is a high affinity receptor for P-selectin and its expression is up-regulated during T cell activation. P-selectin engagement of PSGL-1/CLA expressed on T cells may regulate T cell differentiation. The CLA^+ CD4 T cell population increased following 3 days of culture in the presence of anti-CD3/28 Abs (Fig. 18A). Blocking PSGL-1/CLA signaling with P-selectin-specific antibody, Waps 12.2, suppressed IFN-γ and IL-4 expression in CD4 T cells co-cultured with sh*S1PR1*-treated HDLECs in an antibody dose-dependent manner (Fig. 7A-C). These data demonstrate that S1P signaling deficiency induces P-selectin expression which, in turn, mediates enhanced Th1 and Th2 CD4 T cell differentiation.

[00158] *Anti-P-selectin Ab alleviates lymphedema developed in $S1pr1^{LECKO}$ mice.* To assess the relevance of P-selectin in lymphedema, we evaluated the effect of blocking P-selectin in lymphedema pathogenesis. 5 mg/kg anti-mouse P-selectin Ab (RB40.34.4) was injected intravenously (i.v.; retro-orbital) into $S1pr1^{LECKO}$ mice one day before lymphedema surgery, and tail volumes were monitored weekly (Fig. 7D). $S1pr1^{LECKO}$ mice treated with RB40.34.4 showed a significant decrease in tail swelling compared to $S1pr1^{LECKO}$ mice treated with control IgG (Fig. E and F). A flow cytometry analysis of CD4 T cell populations in lymphedematous tail skin demonstrated significant decrease of IFN-γ and IL-4-producing CD4 T cells in samples derived from mice treated with anti-P-selectin (Fig. 7G and H). Additionally, lymphedema skin from anti-P-selectin Ab-treated mice contained significantly reduced CLA- expressing $CD44^+$ CD4 T cells (Fig. 18B and C). Collectively, these data demonstrate that P-selectin blockade improves lymphatic tissue swelling and pathogenic CD4 T cell accumulation in severe lymphedema.

[00159] In this study, we showed that impaired S1P signaling in LECs promotes pathogenic CD4 T cell differentiation and exacerbates lymphatic malfunction in lymphedema. These results illustrate how pathological LEC and T cell interactions can contribute to lymphedema progression and how suppressing these intercellular relationships may attenuate the disease. We first assessed key molecules involved in the S1P signaling in lymphedema conditions. LC/MS analysis demonstrated significantly decreased S1P concentrations in both clinical and preclinical serum samples. Evaluation of SPHK1, the main SPHK isoform, indicated a diminished expression of this protein in LECs, which is the major S1P producer in peripheral tissues. These data suggest that lymphedema tissues have reduced local S1P production concordant with reduced systemic levels. Additionally, lymphatic vessels in lymphedema tissue exhibited low expression levels of the main isoform of S1P receptors, S1PR1.

[00160] To evaluate the biological effects of diminished LEC S1P signaling in lymphedema, we generated the LEC-specific *S1pr1* loss-of-function mice (*S1pr1*^{LECKO}) and found that *S1pr1*^{LECKO} mice develop more severe lymphedema with exacerbated tail swelling, cutaneous thickening, increased lymphatic leakage, and aggravated tissue inflammation. Conversely, enhanced S1P signaling by systemic 4-DP treatment improved tail swelling, lymphatic vascular function, and inflammation. Together, these data indicate that LEC S1P signaling through S1PR1 is crucial for lymphatic vascular repair and the control of pathological tissue inflammation.

[00161] While existing data from both clinical and preclinical studies reveal exuberant inflammation characterized by dysregulated adaptive immune cells infiltration in lymphedema tissues, how malfunctioning adaptive immune responses propagate is not completely understood. We first compared immune cell compositions in the skin tissue of lymphedema of WT vs *S1pr1*^{LECKO} mice. Our data showed significantly increased Th1 (IFN-γ^+CD4$^+$) and Th2 (IL- 4$^+$CD4$^+$) cells but decreased immunosuppressive Treg cells (CD4$^+$CD25$^+$FoxP3$^+$) in *S1pr1*^{LECKO} mice comparing to that observed in controls. Because Th1 and Th2 cells are pathogenic in lymphedema, whereas Treg cells are demonstrably protective, our data suggest that LEC *S1pr1* deficiency exacerbates pathogenic inflammatory responses in lymphedema. Additionally, our immune profiling result demonstrated a significantly increased CD69$^+$CD103$^+$ [markers for Tissue-resident memory cells (T_{RM})], IL-4-producing CD4 T cell population in lymphedema tissue from *S1pr1*^{LECKO} mice, suggesting that LEC *S1pr1* deficiency promotes the expansion of T_{RM} population in lymphedema. T_{RM} cells play critical roles in tissue-specific immune and inflammatory diseases and are associated with vasculitis. LEC *S1pr1* deficiency-associated CD4$^+$ T_{RM} expansion may play an important pathogenic role in lymphedema progression. Interestingly, several studies have demonstrated that Th2 immune responses are more critical than Th1 immune responses in lymphedema pathogenesis, which supports our

observation that the dominancy of IL-4$^+$CD4$^+$ T_{RM} cells over IFN-γ^+CD4$^+$ T_{RM} cells in lymphedema tail skin of *S1pr1*^{LECKO} mice.

[00162] Because LECs can directly regulate T cell immunity and are important immunomodulators of peripheral tolerance, we went on to ask whether LECs with deficient S1P signaling skew T cell differentiation and enhance inflammation in lymphedema. *S1pr1*-deficient LECs isolated from LEC-specific *S1pr1* KO mice promoted mouse Th1/Th2 cell differentiation but decreased the immunosuppressive Treg cell subset. Consistently, co-culture of naïve human CD4$^+$ T cells with sh*Ctr*- or sh*S1PR1*-HDLECs illustrated that human LECs with *S1PR1* knockdown similarly enhance human Th1/Th2 T cell differentiation. Using the trans-well system, we further show that LEC-modulated skewed T cell differentiation requires cell-cell contact. Collectively, our data suggests that the loss of S1P signaling may lead to altered expression of certain cell surface proteins, which in turn, affects local T cell differentiation. While previous experiments suggested that lymphatic damage-induced CD4 T cell immunity involves dendritic cell (DC) migration to the draining lymph node, immune priming, and T cell trafficking back to the local lymphedematous tissue, our data show that in lymphedema, activation of the pathogenic adaptive immunity may be enhanced by S1P signaling-deficient local LECs.

[00163] To find genes involved in T cell reprogramming by S1P signaling-deficient LECs, we performed bulk RNA-seq analyses. Among the most significantly changed pathways is the cell adhesion signaling and upregulation of the adhesion molecule P-selectin. Blocking lymphatic P-selectin signaling reversed the effect of S1P signaling deficiency in promoting Th1/Th2 differentiation in vitro; P-selectin neutralization significantly alleviated tail swelling and tissue inflammation in severe lymphedema. While P-selectin is best known for its role in T cell rolling during immune cell extravasation, recent data indicated that P-selectin may modulate T cell activity through PSGL-1. In agreement, our data show that increased P-selectin expression in LECs can promote CD4 T cell accumulation around lymphatic vessels and directly modulate their differentiation and activation; a process that could promote abnormal immune responses in lymphedema. Blocking P-selectin provides a new approach for treating lymphedema involving T cell immunity.

References

[00164] Rockson SG. Lymphedema after Breast Cancer Treatment. *N Engl J Med.* 2018;379:1937-1944.

[00165] Rockson SG. Diagnosis and management of lymphatic vascular disease. *J Am Coll Cardiol.* 2008;52:799-806.

[00166] Kataru RP, Baik JE, Park HJ, Wiser I, Rehal S, Shin JY and Mehrara BJ. Regulation of Immune Function by the Lymphatic System in Lymphedema. *Front Immunol.* 2019;10:470.

[00167] Li CY, Kataru RP and Mehrara BJ. Histopathologic Features of Lymphedema: A Molecular Review. *Int J Mol Sci*. 2020;21.

[00168] Mehrara BJ, Park HJ, Kataru RP, Bromberg J, Coriddi M, Baik JE, Shin J, Li C, Cavalli MR, Encarnacion EM, Lee M, Van Zee KJ, Riedel E and Dayan JH. Pilot Study of Anti-Th2 Immunotherapy for the Treatment of Breast Cancer-Related Upper Extremity Lymphedema. *Biology (Basel)*. 2021;10.

[00169] Schulte-Merker S, Sabine A and Petrova TV. Lymphatic vascular morphogenesis in development, physiology, and disease. *J Cell Biol*. 2011;193:607-18.

[00170] Oliver G, Kipnis J, Randolph GJ and Harvey NL. The Lymphatic Vasculature in the 21(st) Century: Novel Functional Roles in Homeostasis and Disease. *Cell*. 2020;182:270-296.

[00171] Petrova TV and Koh GY. Biological functions of lymphatic vessels. *Science*. 2020;369.

[00172] Tewalt EF, Cohen JN, Rouhani SJ and Engelhard VH. Lymphatic endothelial cells - key players in regulation of tolerance and immunity. *Front Immunol*. 2012;3:305.

[00173] Kim KW and Song JH. Emerging Roles of Lymphatic Vasculature in Immunity. *Immune Netw*. 2017;17:68-76.

[00174] Rouhani SJ, Eccles JD, Tewalt EF and Engelhard VH. Regulation of T-cell Tolerance by Lymphatic Endothelial Cells. *J Clin Cell Immunol*. 2014;5.

[00175] Randolph GJ, Ivanov S, Zinselmeyer BH and Scallan JP. The Lymphatic System: Integral Roles in Immunity. *Annu Rev Immunol*. 2017;35:31-52.

[00176] Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, Conaway MR, Bender TP, Tung KS, Vella AT, Adler AJ, Chen L and Engelhard VH. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood*. 2012;120:4772-82.

[00177] Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, LeibundGut-Landmann S, Garbi N, Reith W, Swartz MA and Hugues S. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med*. 2014;211:1153-66.

[00178] Podgrabinska S, Kamalu O, Mayer L, Shimaoka M, Snoeck H, Randolph GJ and Skobe M. Inflamed lymphatic endothelium suppresses dendritic cell maturation and function via Mac-1/ICAM-1-dependent mechanism. *J Immunol*. 2009;183:1767-79.

[00179] Cartier A and Hla T. Sphingosine 1-phosphate: Lipid signaling in pathology and therapy. *Science*. 2019;366.

[00180] Don-Doncow N, Zhang Y, Matuskova H and Meissner A. The emerging alliance of sphingosine-1-phosphate signalling and immune cells: from basic mechanisms to implications in hypertension. *Br J Pharmacol*. 2019;176:1989-2001.

[00181] Mendelson K, Evans T and Hla T. Sphingosine 1-phosphate signalling. *Development*. 2014;141:5-9.

[00182] Blaho VA and Hla T. Regulation of mammalian physiology, development, and disease by the sphingosine 1-phosphate and lysophosphatidic acid receptors. *Chem Rev.* 2011;111:6299-320.

[00183] Blaho VA and Hla T. An update on the biology of sphingosine 1-phosphate receptors.

[00184] *J Lipid Res.* 2014;55:1596-608.

[00185] Paik JH, Skoura A, Chae SS, Cowan AE, Han DK, Proia RL and Hla T. Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. *Genes Dev.* 2004;18:2392-403.

[00186] Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI and Hla T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell.* 1999;99:301-12.

[00187] Cartier A, Leigh T, Liu CH and Hla T. Endothelial sphingosine 1-phosphate receptors promote vascular normalization and antitumor therapy. *Proc Natl Acad Sci U S A.* 2020;117:3157-3166.

[00188] Akhter MZ, Chandra Joshi J, Balaji Ragunathrao VA, Maienschein-Cline M, Proia RL, Malik AB and Mehta D. Programming to S1PR1(+) Endothelial Cells Promotes Restoration of Vascular Integrity. *Circ Res.* 2021;129:221-236.

[00189] Yanagida K and Hla T. Vascular and Immunobiology of the Circulatory Sphingosine 1-Phosphate Gradient. *Annu Rev Physiol.* 2017;79:67-91.

[00190] Geng X, Yanagida K, Akwii RG, Choi D, Chen L, Ho Y, Cha B, Mahamud MR, Berman de Ruiz K, Ichise H, Chen H, Wythe JD, Mikelis CM, Hla T and Srinivasan RS. S1PR1 regulates the quiescence of lymphatic vessels by inhibiting laminar shear stress-dependent VEGF-C signaling. *JCI Insight.* 2020;5.

[00191] Tabibiazar R, Cheung L, Han J, Swanson J, Beilhack A, An A, Dadras SS, Rockson N, Joshi S, Wagner R and Rockson SG. Inflammatory manifestations of experimental lymphatic insufficiency. *PLoS Med.* 2006;3:e254.

[00192] Zollner TM and Asadullah K. Selectin and selectin ligand binding: a bittersweet attraction. *J Clin Invest.* 2003;112:980-3.

[00193] Weiler MJ, Cribb MT, Nepiyushchikh Z, Nelson TS and Dixon JB. A novel mouse tail lymphedema model for observing lymphatic pump failure during lymphedema development. *Sci Rep.* 2019;9:10405.

[00194] Kim D, Kim M, Kim TW, Choe YH, Noh HS, Jeon HM, Kim H, Lee Y, Hur G, Lee KM, Shin K, Lee SI and Lee SH. Lymph node fibroblastic reticular cells regulate differentiation and function of CD4 T cells via CD25. *J Exp Med.* 2022;219.

[00195] Kim DE, Lee Y, Kim M, Lee S, Jon S and Lee SH. Bilirubin nanoparticles ameliorate allergic lung inflammation in a mouse model of asthma. *Biomaterials.* 2017;140:37-44.

[00196] Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, Coughlin SR, McDonald DM, Schwab SR and Cyster JG. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med.* 2010;207:17-27.

[00197] Peres M, Montfort A, Andrieu-Abadie N, Colacios C and Segui B. S1P: the elixir of life for naive T cells. *Cell Mol Immunol.* 2018;15:657-659.

[00198] Huang WC, Nagahashi M, Terracina KP and Takabe K. Emerging Role of Sphingosine-1-phosphate in Inflammation, Cancer, and Lymphangiogenesis. *Biomolecules.* 2013;3:408-34.

[00199] Garcia Nores GD, Ly CL, Cuzzone DA, Kataru RP, Hespe GE, Torrisi JS, Huang JJ, Gardenier JC, Savetsky IL, Nitti MD, Yu JZ, Rehal S and Mehrara BJ. CD4(+) T cells are activated in regional lymph nodes and migrate to skin to initiate lymphedema. *Nat Commun.* 2018;9:1970.

[00200] Gousopoulos E, Proulx ST, Scholl J, Uecker M and Detmar M. Prominent Lymphatic Vessel Hyperplasia with Progressive Dysfunction and Distinct Immune Cell Infiltration in Lymphedema. *Am J Pathol.* 2016;186:2193-2203.

[00201] Tokumoto M, Tanaka H, Tauchi Y, Tamura T, Toyokawa T, Kimura K, Muguruma K, Yashiro M, Maeda K, Hirakawa K and Ohira M. Immunoregulatory Function of Lymphatic Endothelial Cells in Tumor-draining Lymph Nodes of Human Gastric Cancer. *Anticancer Res.* 2017;37:2875-2883.

[00202] Card CM, Yu SS and Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest.* 2014;124:943-52.

[00203] Saravia J, Chapman NM and Chi H. Helper T cell differentiation. *Cell Mol Immunol.* 2019;16:634-643.

[00204] Ruterbusch M, Pruner KB, Shehata L and Pepper M. In Vivo CD4(+) T Cell Differentiation and Function: Revisiting the Th1/Th2 Paradigm. *Annu Rev Immunol.* 2020;38:705-725.

[00205] Zhu J, Yamane H and Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol.* 2010;28:445-89.

[00206] Zhang H, Watanabe R, Berry GJ, Nadler SG, Goronzy JJ and Weyand CM. CD28 Signaling Controls Metabolic Fitness of Pathogenic T Cells in Medium and Large Vessel Vasculitis. *J Am Coll Cardiol.* 2019;73:1811-1823.

[00207] Wen Z, Shen Y, Berry G, Shahram F, Li Y, Watanabe R, Liao YJ, Goronzy JJ and Weyand CM. The microvascular niche instructs T cells in large vessel vasculitis via the VEGF-Jagged1-Notch pathway. *Sci Transl Med.* 2017;9.

[00208] Cohen JN, Tewalt EF, Rouhani SJ, Buonomo EL, Bruce AN, Xu X, Bekiranov S, Fu YX and Engelhard VH. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PLoS One*. 2014;9:e87740.

[00209] Iftakhar EKI, Fair-Makela R, Kukkonen-Macchi A, Elima K, Karikoski M, Rantakari P, Miyasaka M, Salmi M and Jalkanen S. Gene-expression profiling of different arms of lymphatic vasculature identifies candidates for manipulation of cell traffic. *Proc Natl Acad Sci U S A*. 2016;113:10643-8.

[00210] Ni ZY and Walcheck B. Cutaneous lymphocyte-associated antigen (CLA) T cells up-regulate P-selectin ligand expression upon their activation. *Clin Immunol*. 2009;133:257-264.

[00211] Nakayama F, Teraki Y, Kudo T, Togayachi A, Iwasaki H, Tamatani T, Nishihara S, Mizukawa Y, Shiohara T and Narimatsu H. Expression of cutaneous lymphocyte-associated antigen regulated by a set of glycosyltransferases in human T cells: involvement of alpha1, 3-fucosyltransferase VII and beta1,4-galactosyltransferase I. *J Invest Dermatol*. 2000;115:299-306.

[00212] Aspelund A, Robciuc MR, Karaman S, Makinen T and Alitalo K. Lymphatic System in Cardiovascular Medicine. *Circ Res*. 2016;118:515-30.

[00213] Brakenhielm E and Alitalo K. Cardiac lymphatics in health and disease. *Nat Rev Cardiol*. 2019;16:56-68.

[00214] Zhou Y, Huang C, Hu Y, Xu Q and Hu X. Lymphatics in Cardiovascular Disease.

[00215] *Arterioscler Thromb Vasc Biol*. 2020;40:e275-e283.

[00216] Yeo KP, Lim HY and Angeli V. Leukocyte Trafficking via Lymphatic Vessels in Atherosclerosis. *Cells*. 2021;10.

[00217] Milasan A, Smaani A and Martel C. Early rescue of lymphatic function limits atherosclerosis progression in Ldlr(-/-) mice. *Atherosclerosis*. 2019;283:106-119.

[00218] Balasubbramanian D, Lopez Gelston CA, Rutkowski JM and Mitchell BM. Immune cell trafficking, lymphatics and hypertension. *Br J Pharmacol*. 2019;176:1978-1988.

[00219] Vieira JM, Norman S, Villa Del Campo C, Cahill TJ, Barnette DN, Gunadasa-Rohling M, Johnson LA, Greaves DR, Carr CA, Jackson DG and Riley PR. The cardiac lymphatic system stimulates resolution of inflammation following myocardial infarction. *J Clin Invest*. 2018;128:3402-3412.

[00220] Houck P, Dandapantula H, Hardegree E and Massey J. Why We Fail at Heart Failure: Lymphatic Insufficiency Is Disregarded. *Cureus*. 2020;12:e8930.

[00221] Donnan MD, Kenig-Kozlovsky Y and Quaggin SE. The lymphatics in kidney health and disease. *Nature Reviews Nephrology*. 2021.

[00222] Jiang X, Tian W, Nicolls MR and Rockson SG. The Lymphatic System in Obesity, Insulin Resistance, and Cardiovascular Diseases. *Front Physiol*. 2019;10:1402.

[00223] Jiang X, Nicolls MR, Tian W and Rockson SG. Lymphatic Dysfunction, Leukotrienes, and Lymphedema. *Annu Rev Physiol.* 2018;80:49-70.

[00224] Zampell JC, Yan A, Avraham T, Andrade V, Malliaris S, Aschen S, Rockson SG and Mehrara BJ. Temporal and spatial patterns of endogenous danger signal expression after wound healing and in response to lymphedema. *Am J Physiol Cell Physiol.* 2011;300:C1107-21.

[00225] Tian W, Jiang X, Tamosiuniene R, Sung YK, Qian J, Dhillon G, Gera L, Farkas L, Rabinovitch M, Zamanian RT, Inayathullah M, Fridlib M, Rajadas J, Peters-Golden M, Voelkel NF and Nicolls MR. Blocking macrophage leukotriene b4 prevents endothelial injury and reverses pulmonary hypertension. *Sci Transl Med.* 2013;5:200ra117.

[00226] Tian W, Rockson SG, Jiang X, Kim J, Begaye A, Shuffle EM, Tu AB, Cribb M, Nepiyushchikh Z, Feroze AH, Zamanian RT, Dhillon GS, Voelkel NF, Peters-Golden M, Kitajewski J, Dixon JB and Nicolls MR. Leukotriene B(4) antagonism ameliorates experimental lymphedema. *Sci Transl Med.* 2017;9.

[00227] Harle G, Kowalski C, Dubrot J, Brighouse D, Clavel G, Pick R, Bessis N, Niven J, Scheiermann C, Gannage M and Hugues S. Macroautophagy in lymphatic endothelial cells inhibits T cell-mediated autoimmunity. *J Exp Med.* 2021;218.

[00228] Tindemans I, van Schoonhoven A, KleinJan A, de Bruijn MJ, Lukkes M, van Nimwegen M, van den Branden A, Bergen IM, Corneth OB, van IWF, Stadhouders R and Hendriks RW. Notch signaling licenses allergic airway inflammation by promoting Th2 cell lymph node egress. *J Clin Invest.* 2020;130:3576-3591.

[00229] Gousopoulos E, Proulx ST, Bachmann SB, Scholl J, Dionyssiou D, Demiri E, Halin C, Dieterich LC and Detmar M. Regulatory T cell transfer ameliorates lymphedema and promotes lymphatic vessel function. *JCI Insight.* 2016;1:e89081.

[00230] Szabo PA, Miron M and Farber DL. Location, location, location: Tissue resident memory T cells in mice and humans. *Sci Immunol.* 2019;4.

[00231] Park CO and Kupper TS. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nat Med.* 2015;21:688-97.

[00232] Mueller SN and Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. *Nat Rev Immunol.* 2016;16:79-89.

[00233] Zundler S, Becker E, Spocinska M, Slawik M, Parga-Vidal L, Stark R, Wiendl M, Atreya R, Rath T, Leppkes M, Hildner K, Lopez-Posadas R, Lukassen S, Ekici AB, Neufert C, Atreya I, van Gisbergen K and Neurath MF. Hobit- and Blimp-1-driven CD4(+) tissue-resident memory T cells control chronic intestinal inflammation. *Nat Immunol.* 2019;20:288-300.

[00234] Turner DL, Goldklang M, Cvetkovski F, Paik D, Trischler J, Barahona J, Cao M, Dave R, Tanna N, D'Armiento JM and Farber DL. Biased Generation and In Situ Activation of Lung

Tissue-Resident Memory CD4 T Cells in the Pathogenesis of Allergic Asthma. *J Immunol.* 2018;200:1561-1569.

[00235] Ly CL, Nores GDG, Kataru RP and Mehrara BJ. T helper 2 differentiation is necessary for development of lymphedema. *Transl Res.* 2019;206:57-70.

[00236] Vokali E, Yu SS, Hirosue S, Rincon-Restrepo M, F VD, Scherer S, Corthesy-Henrioud P, Kilarski WW, Mondino A, Zehn D, Hugues S and Swartz MA. Lymphatic endothelial cells prime naive CD8(+) T cells into memory cells under steady-state conditions. *Nat Commun.* 2020;11:538.

[00237] Saxena V, Li L, Paluskievicz C, Kasinath V, Bean A, Abdi R, Jewell CM and Bromberg JS. Role of lymph node stroma and microenvironment in T cell tolerance. *Immunol Rev.* 2019;292:9-23.

[00238] Tedder TF, Steeber DA, Chen A and Engel P. The selectins: vascular adhesion molecules. *FASEB J.* 1995;9:866-73.

[00239] Ruiz M, Frej C, Holmer A, Guo LJ, Tran S and Dahlback B. High-Density Lipoprotein-Associated Apolipoprotein M Limits Endothelial Inflammation by Delivering Sphingosine-1-Phosphate to the Sphingosine-1-Phosphate Receptor 1. *Arterioscler Thromb Vasc Biol.* 2017;37:118-129.

[00240] Perry HM, Huang L, Ye H, Liu C, Sung SJ, Lynch KR, Rosin DL, Bajwa A and Okusa MD. Endothelial Sphingosine 1-Phosphate Receptor-1 Mediates Protection and Recovery from Acute Kidney Injury. *J Am Soc Nephrol.* 2016;27:3383-3393.

[00241] Ley K and Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol.* 2004;4:325-35.

[00242] Sackstein R, Schatton T and Barthel SR. T-lymphocyte homing: an underappreciated yet critical hurdle for successful cancer immunotherapy. *Lab Invest.* 2017;97:669-697.

[00243] Tinoco R, Carrette F, Barraza ML, Otero DC, Magana J, Bosenberg MW, Swain SL and Bradley LM. PSGL-1 Is an Immune Checkpoint Regulator that Promotes T Cell Exhaustion. *Immunity.* 2016;44:1470.

[00244] Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, Guthrie TH, Knight-Madden J, Alvarez OA, Gordeuk VR, Gualandro S, Colella MP, Smith WR, Rollins SA, Stocker JW and Rother RP. Crizanlizumab for the Prevention of Pain Crises in Sickle Cell Disease. *N Engl J Med.* 2017;376:429-439.

[00245] Nolo R, Herbrich S, Rao A, Zweidler-McKay P, Kannan S and Gopalakrishnan V. Targeting P-selectin blocks neuroblastoma growth. *Oncotarget.* 2017;8:86657-86670.

[00246] Li Q, Zhou C, Zhao K, Duan Y, Yue J, Liu X, Wu J and Deng S. Lymphatic endothelial sphingosine 1-phosphate receptor 1 enhances macrophage clearance via lymphatic system following myocardial infarction. *Front Cardiovasc Med.* 2022;9:872102.

Table 1. Demographics of the controls and lymphedema patients

Patient ID	Age	Sex	BMI	WHO Class (1-3)	Primary/ Secondary	Duration of Disease (year)	S1P LC-MS/MS (pg/ml)
C1	33	M	n/a	n/a	n/a	n/a	686.0
C2	35	M	n/a	n/a	n/a	n/a	511.0
C3	30	F	n/a	n/a	n/a	n/a	475.0
C4	21	M	n/a	n/a	n/a	n/a	444.0
C5	31	M	n/a	n/a	n/a	n/a	412.0
C6	32	M	n/a	n/a	n/a	n/a	404.0
C7	27	F	n/a	n/a	n/a	n/a	363.0
C8	60	M	n/a	n/a	n/a	n/a	360.0
C9	61	M	n/a	n/a	n/a	n/a	351.0
C10	84	M	n/a	n/a	n/a	n/a	254.0
P1	68	F	26.19	2	Secondary	4	200.0
P2	85	F	26.18	2	Secondary	4	193.0
P3	44	F	24.58	2	Secondary	38	185.0
P4	27	M	22.97	2	Primary	8	182.0
P5	62	F	33.08	2	Secondary	5	172.0
P6	59	F	29.27	2	Secondary	22	142.0
P7	77	F	37.68	2	Secondary	5	100.0
P8	76	F	48.53	3	Primary	childhood	85.0
P9	69	M	51.35	2	Secondary	13	72.0
P10	71	F	23.67	2	Secondary	4	46.0
Statistics	P<0.01	n/a	n/a	n/a	n/a	n/a	P<0.0001

Table 2. The sequences of the mouse-specific primers used for RT-qPCR

Gene (mouse)	Sense (5′→3′)	Antisense (5′→3′)
SPHK1	SEQ ID NO:1 CGGTGATGGTCTGATGCATG	SEQ ID NO:2 GAGGCTACACAGGGGTTTCT
S1PR1	SEQ ID NO:3 GCCACCACTTACAAGCTCAC	SEQ ID NO:4 CAAACATACTCCCTTCCCGCA
18S	SEQ ID NO:5	SEQ ID NO:6

	GGACCAGAGCGAAAGCATTTGCC	TCA ATC TCG GGT GGC TGA ACG C

Table 3. The sequences of the human-specific primers used for RT-qPCR

Gene (human)	Sense (5′→3′)	Antisense (5′→3′)
S1PR1	SEQ ID NO:7 TGCTGGCAAATTCAAGC	SEQ ID NO:8 GGGTTGTCCCCTTCGTC
18S	SEQ ID NO:9 CTGCCATTAAGGGTGTTGGC	SEQ ID NO:10 CAGCCCTCTGGTGGGTCAAT

Table 5. Differentially up-regulated gene in sh*S1pr1*-HDLECs

Without CD4 T cell co-culture	Intersection	With CD4 T cell co-culture			
STAB2	SELP	OMG	CPM	WDFY4	SLC12A5
AGTR1	RGS5	GPNMB	PCDH17	LIMCH1	COL12A1
TCF15	DIRAS3	BRME1	MMP9	CD36	KCNN4
MAILR	ADAMTS18	TOX	H2BC4	COL15A1	ZC3H12A-DT
FLRT3	ADRA1D	KIF19	ADAMTS14	OLFM1	ADGRG4
RNF185-AS1	APLN	TIPARP-AS1	DRAXIN	DCLK1	TMEM163
PITX1-AS1	IL33	CEROX1	MYRIP	LINC02015	RPLP0P6
C3AR1		SPEG	CDH11	SIRT4	CORO2B
CFI		RPAP3-DT	CEND1	RASSF5	CETP
SPTBN5		TSPAN11	MIR210HG	SELE	CHST6
TGFA		SRGAP3	AR	H2AC6	HOXD3
		GOLGA8R	TRIM73	AQP11	ZNF667-AS1
		LINC01303	NEK10	COL9A3	INKA1
		LINC02159	TMEM265	LYPD1	LINC02154
		DUT-AS1	DPEP1	NPFFR2	MAP1A
		MAN1C1	PKIB	CTXN1	LFNG
		ODAD3	NPTX2	PTGS1	PRND
		CPE	KCNE3	MAFB	SLCO2B1
		KIF1A	RN7SL3	ASS1	SORL1
		PTGIS	SLC22A17	CKB	PIK3R3
		GRM1	BMPER	SLC9A7	NPTXR
		MIR503HG	H2BC5	FOXD1	MARVELD3
		AIF1L	IL2RB	H2BC12	ZAP70
		PCDHB6	AFTPH-DT	ASRGL1	MYG1-AS1
		SOGA3	HSD17B14	CXADR	CPA4
		PCMTD1-DT	RNASE1	SELL	SNCB

Table 6. Gene set enrichment analysis comparisons between sh*Ctr*-HDLECs and sh*S1pr1*-HDLECs with CD4 T cell co-culture.

Gene Set Name	Genes in Gene Set (K)	Genes in Overlap (k)
CELL CELL SIGNALING	1679	18
CELL ADHESION	1541	16
CELLULAR_MONOVALENT INORGANIC ANION HOMEOSTASIS	11	3
CELL MOTILITY	1794	17
ANATOMICAL STRUCTURE FORMATION INVOLVED IN MORPHOGENESIS	1244	14
REGULATION OF CELLULAR LOCALIZATION	954	12
NEGATIVE REGULATION OF PROTEIN LOCALIZATION	203	6
CELLULAR ANION HOMEOSTASIS	20	3
MONOVALENT INORGANIC ANION HOMEOSTASIS	20	3
SYNAPTIC SIGNALING	761	10
CELLULAR HOMEOSTASIS	775	10
CENTRAL NERVOUS SYSTEM PROJECTION NEURON AXONOGENESIS	28	3
CELLULAR ION HOMEOSTASIS	517	8
LYMPHOCYTE ACTIVATION	841	10
PHOSPHORYLATION	1862	15
CELL VOLUME HOMEOSTASIS	32	3
LOCOMOTION	1446	13
CELL JUNCTION ORGANIZATION	707	9
T CELL ACTIVATION	549	8
CENTRAL NERVOUS SYSTEM NEURON DIFFERENTIATION	174	5

[00247] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[00248] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the

protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

WHAT IS CLAIMED:

1. A method for the treatment of lymphedema in a human patient, the method comprising:

administering to the patient an agent that inhibits P-selectin activity in an amount effective to prevent or reduce lymphedema symptoms.

2. The method of claim 1, wherein the agent that inhibits P-selectin activity is an antibody that specifically binds to P-selectin.

3. The method of claim 2, wherein the antibody is crizanlizumab or inclacumab.

4. The method of claim 1, wherein the agent that inhibits P-selectin activity is an antibody that specifically binds to P-selectin glycoprotein ligand-1 (PSGL-1).

5. The method of claim 1 wherein the agent that inhibits P-selectin activity is a small molecule drug.

6. The method of claim 5, wherein the small molecule drug is a glycomimetic; a sialyl lewis X mimetic; a glycosulfopeptide mimicking truncated N-terminal PSGL1; a heparin, or a heparin variant.

7. The method of claim 5, wherein the small molecule drug is selected from THCMA, PSI-697, GMI-1070 (Rivipansel), bimosiamose, GSnP-6, P-G6, PPS, PI-88, and Sevuparin.

8. The method of any of claims 1-7, wherein administering the agent that inhibits P-selectin activity restores architecture of lymphedematous tissue.

9. The method of claim 8, wherein architecture of the affected tissue is assayed by bioimpedance, dermal thickness measurements, histological assessment.

10. The method of any of claims 1-9, wherein the administration is continued for a period of time, if determined to be efficacious.

11. The method of claim 10, wherein determining efficacy of the lymphedema treatment comprises:

(a) measuring an endpoint of a clinical indication in a patient, where the endpoint is measured after treatment has started,

(b) comparing the endpoint of the clinical indication to a baseline or reference, where the baseline or reference is measured in the same subject or a similar subject population before treatment is begun, and

(c) determining the efficacy of the lymphedema treatment based on the comparison step.

12. The method of any of claims 1-11, wherein dermal thickness of lymphedematous tissue is reduced by at least about 2 mm following treatment.

13. The method of any of claims 1-12, wherein volume decreases by 10 milliliters to 100 milliliters or more from affected tissue following treatment.

14. The method of any of claims 1-13, wherein the lymphedema is acquired lymphedema.

15. The method of any of claims 1-14, wherein the patient is at risk for acquired lymphedema but has not yet developed clinical indicia indicative of stage 0 lymphedema.

16. The method of claim 15, wherein risk is a result of surgery or radiation treatment for cancer.

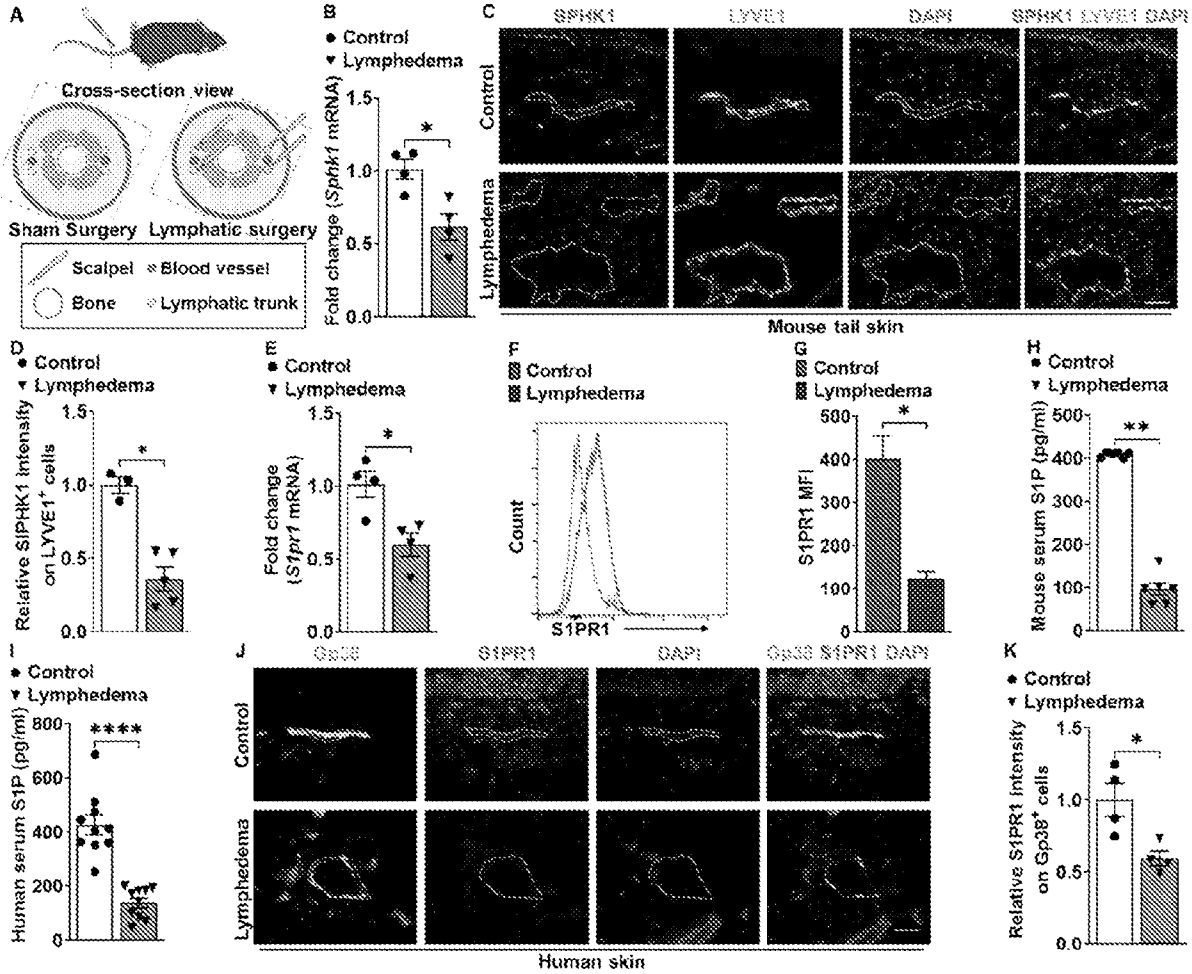
A
Cross-section view
Sham Surgery
Lymphatic surgery
Scalpel
Blood vessel
Bone
Lymphatic trunk
B
Control
Lymphedema
Fold change (Sphk1 mRNA)
C
SPHK1
LYVE1
DAPI
SPHK1 LYVE1 DAPI
Control
Lymphedema
Mouse tail skin
D
Relative SPHK1 intensity on LYVE1+ cells
E
Fold change (S1pr1 mRNA)
F
Count
S1PR1
G
S1PR1 MFI
H
Mouse serum S1P (pg/ml)
I
Human serum S1P (pg/ml)
J
Gp38
S1PR1
DAPI
Gp38 S1PR1 DAPI
Human skin
K
Relative S1PR1 intensity on Gp38+ cells

FIG. 1

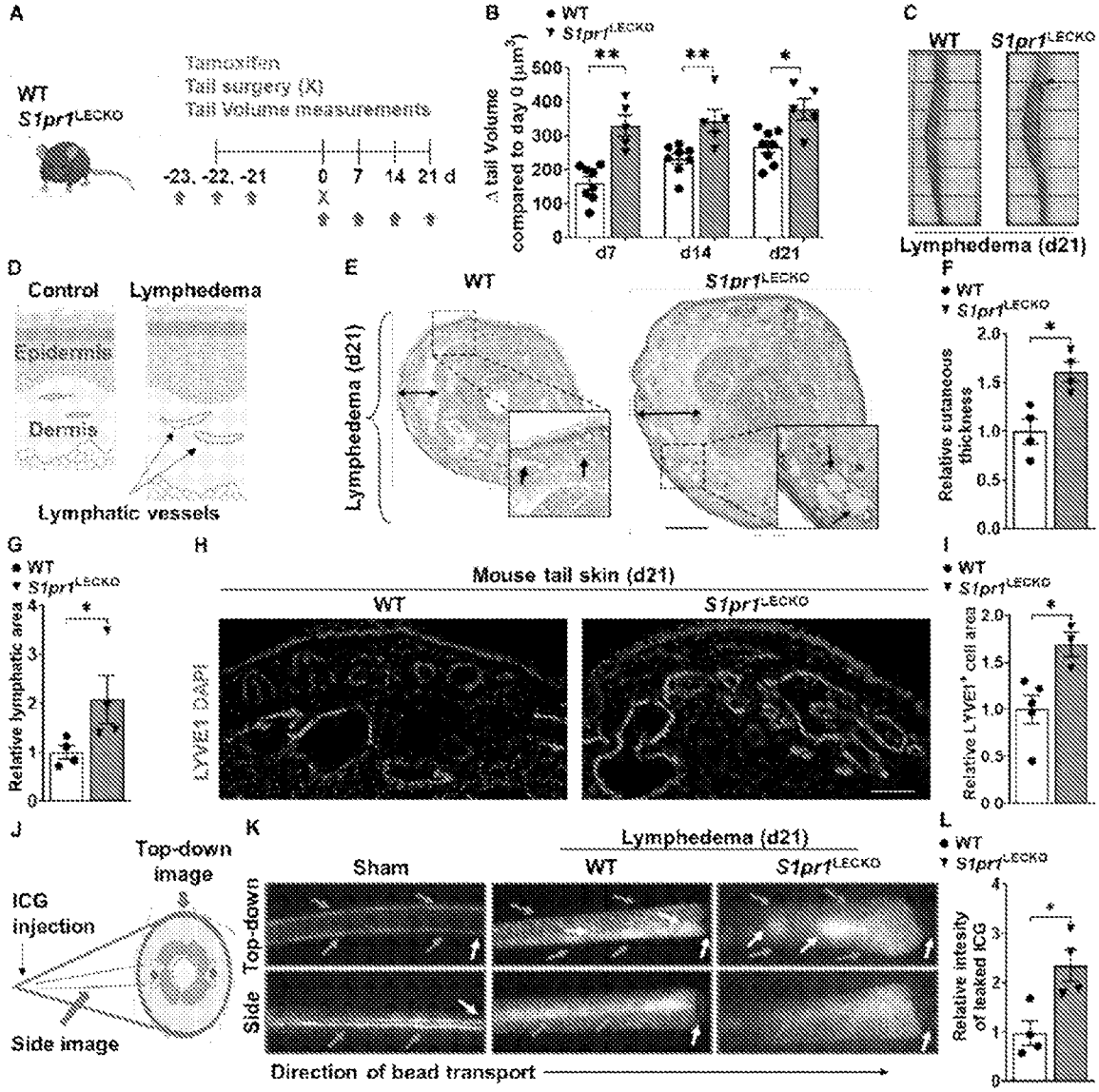
A
WT
S1pr1LECKO
Tamoxifen
Tail surgery (X)
Tail Volume measurements
-23, -22, -21
0
7
14
21 d
B
WT
S1pr1LECKO
Δ tail Volume compared to day 0 (μm3)
d7
d14
d21
C
WT
S1pr1LECKO
Lymphedema (d21)
D
Control
Lymphedema
Epidermis
Dermis
Lymphatic vessels
E
WT
S1pr1LECKO
Lymphedema (d21)
F
Relative cutaneous thickness
G
Relative lymphatic area
H
Mouse tail skin (d21)
WT
S1pr1LECKO
LYVE1 DAPI
I
Relative LYVE1+ cell area
J
Top-down image
ICG injection
Side image
K
Lymphedema (d21)
Sham
WT
S1pr1LECKO
Top-down
Side
Direction of bead transport
L
Relative intesity of leaked ICG

FIG. 2

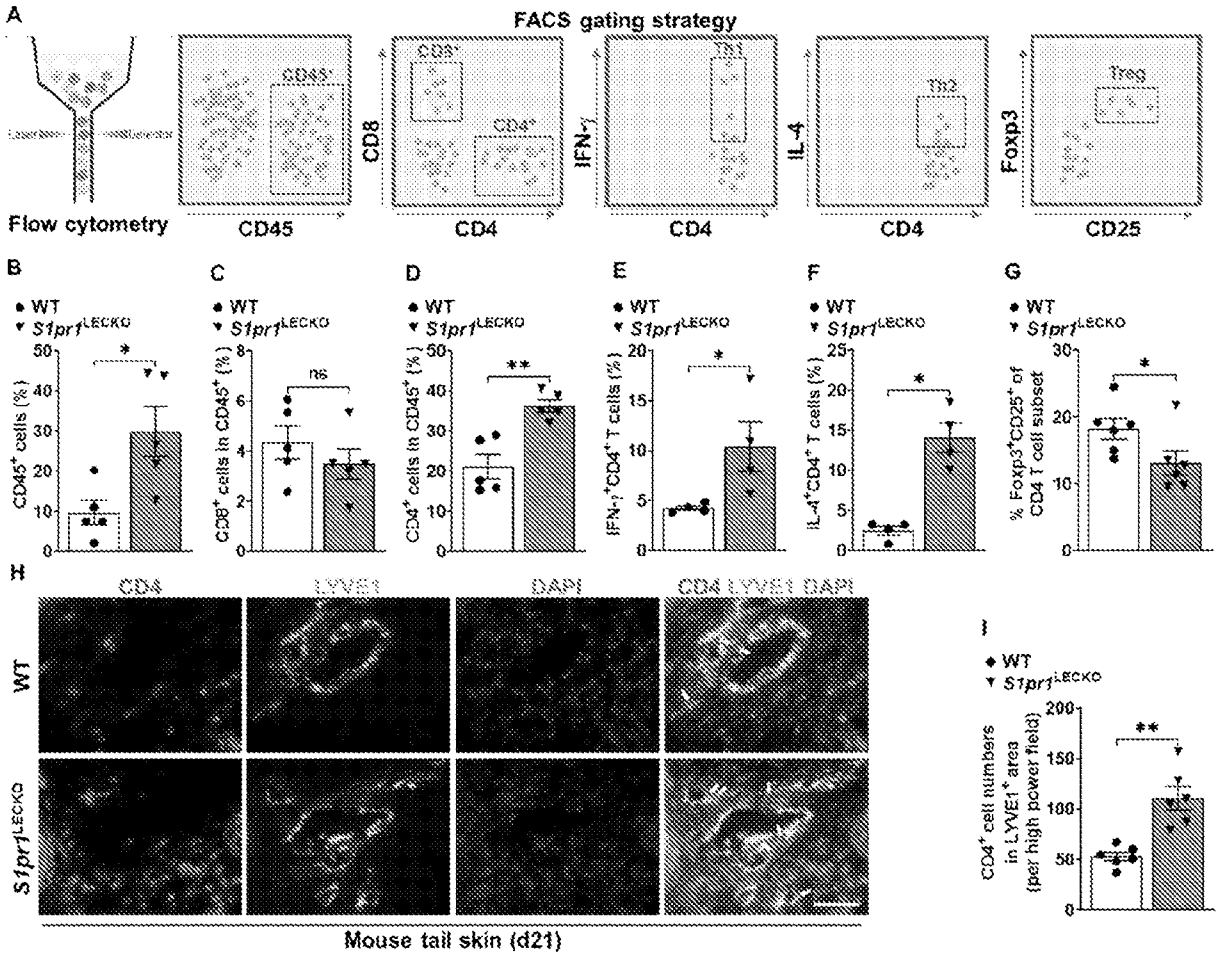
A
FACS gating strategy
Flow cytometry
CD45
CD45+
CD8
CD4
CD8+
CD4+
IFN-γ
Th1
IL-4
Th2
Foxp3
Treg
CD25
B
C
D
E
F
G
WT
S1pr1LECKO
CD45+ cells (%)
CD8+ cells in CD45+ (%)
CD4+ cells in CD45+ (%)
IFN-γ+CD4+ T cells (%)
IL-4+CD4+ T cells (%)
% Foxp3+CD25+ of CD4 T cell subset
ns
H
CD4
LYVE1
DAPI
CD4 LYVE1 DAPI
WT
S1pr1LECKO
Mouse tail skin (d21)
I
CD4+ cell numbers in LYVE1+ area (per high power field)

FIG. 3

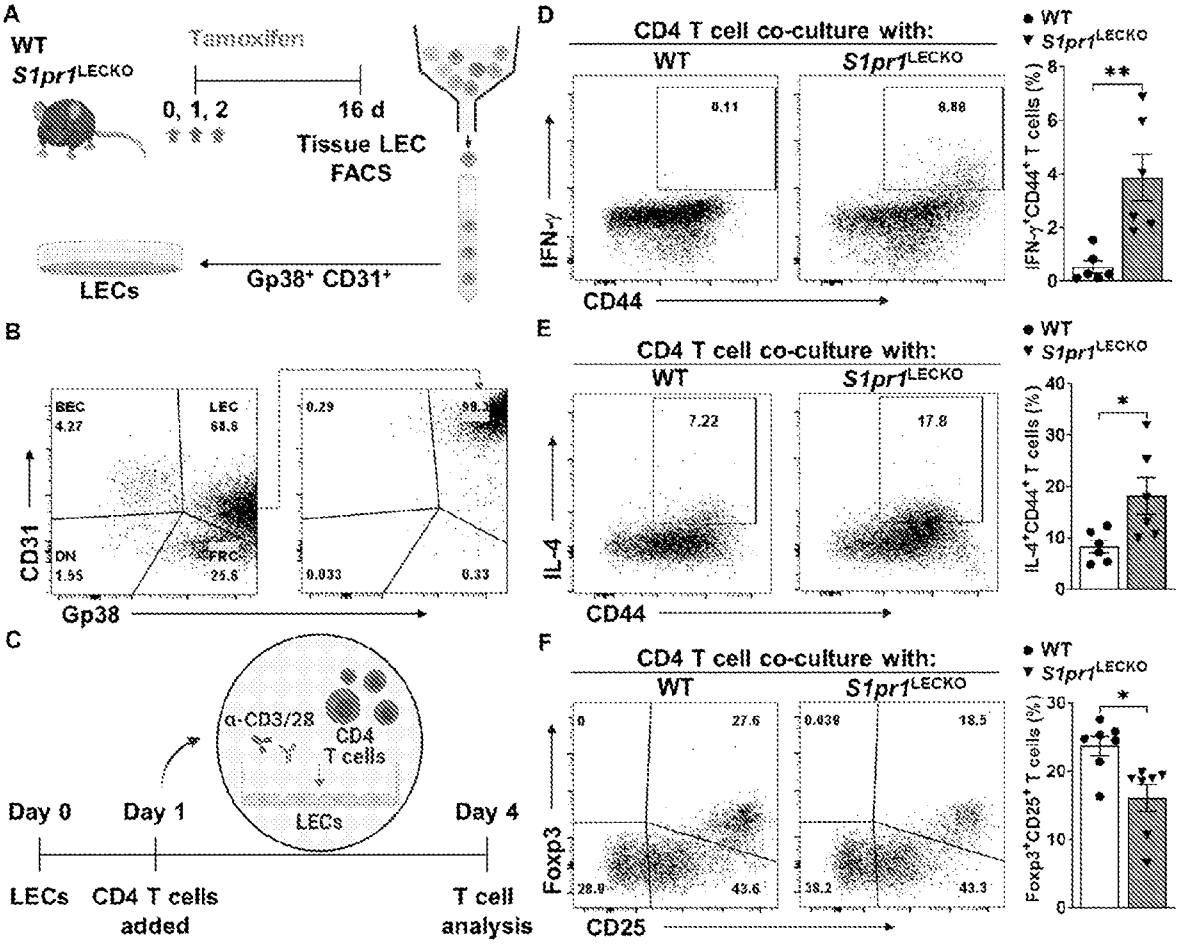
A
WT
S1pr1LECKO
Tamoxifen
0, 1, 2
16 d
Tissue LEC FACS
LECs
Gp38+ CD31+
B
BEC 4.27
LEC 68.8
DN 1.95
FRC 25.6
0.29
98.3
0.033
0.33
CD31
Gp38
C
α-CD3/28
CD4 T cells
LECs
Day 0
Day 1
Day 4
LECs
CD4 T cells added
T cell analysis
D
CD4 T cell co-culture with:
WT
S1pr1LECKO
8.11
8.88
IFN-γ
CD44
WT
S1pr1LECKO
IFN-γ+CD44+ T cells (%)
**
E
CD4 T cell co-culture with:
WT
S1pr1LECKO
7.22
17.8
IL-4
CD44
WT
S1pr1LECKO
IL-4+CD44+ T cells (%)
*
F
CD4 T cell co-culture with:
WT
S1pr1LECKO
0
27.6
0.038
18.5
28.9
43.6
38.2
43.3
Foxp3
CD25
WT
S1pr1LECKO
Foxp3+CD25+ T cells (%)
*

FIG. 4

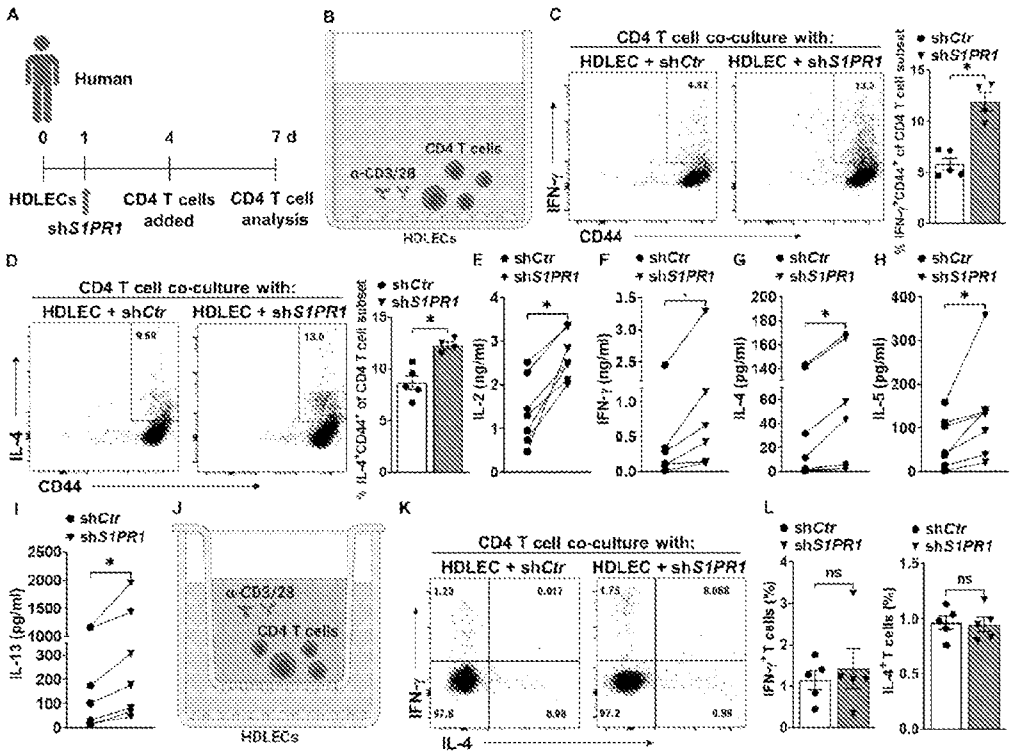
A
Human
0
1
4
7 d
HDLECs shS1PR1
CD4 T cells added
CD4 T cell analysis
B
CD4 T cells
α-CD3/28
HDLECs
C
CD4 T cell co-culture with:
HDLEC + shCtr
HDLEC + shS1PR1
IFN-γ
CD44
shCtr
shS1PR1
% IFN-γ+CD44+ of CD4 T cell subset
D
CD4 T cell co-culture with:
HDLEC + shCtr
HDLEC + shS1PR1
9.68
13.6
IL-4
CD44
shCtr
shS1PR1
% IL-4+CD44+ of CD4 T cell subset
E
shCtr
shS1PR1
IL-2 (ng/ml)
F
shCtr
shS1PR1
IFN-γ (ng/ml)
G
shCtr
shS1PR1
IL-4 (pg/ml)
H
shCtr
shS1PR1
IL-5 (pg/ml)
I
shCtr
shS1PR1
IL-13 (pg/ml)
J
α-CD3/28
CD4 T cells
HDLECs
K
CD4 T cell co-culture with:
HDLEC + shCtr
HDLEC + shS1PR1
1.23
0.017
1.75
8.088
97.8
8.98
97.2
0.98
IFN-γ
IL-4
L
shCtr
shS1PR1
IFN-γ+ T cells (%)
ns
shCtr
shS1PR1
IL-4+ T cells (%)
ns

FIG. 5

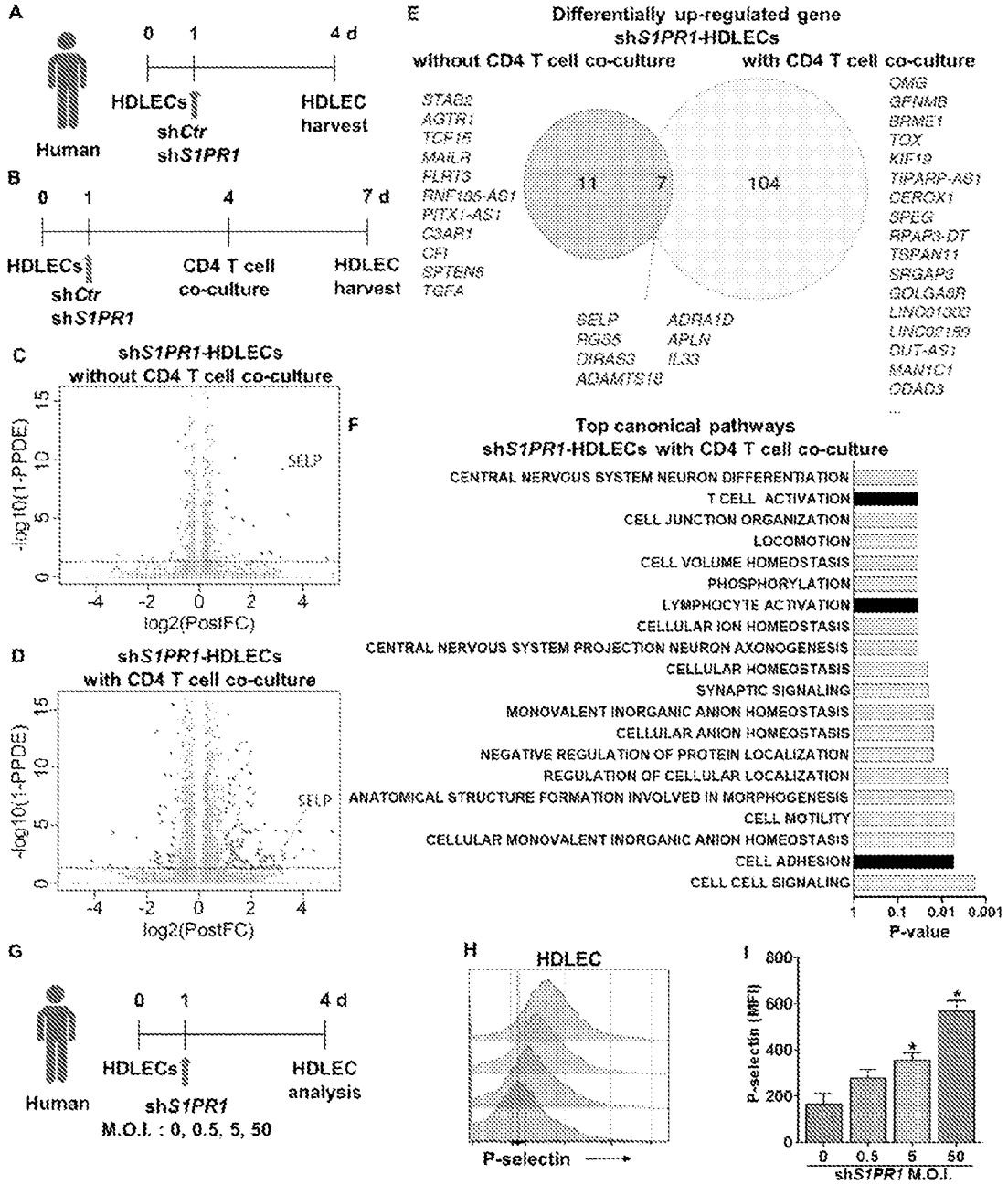
A
0
1
4 d
HDLECs
shCtr
shS1PR1
HDLEC
harvest
Human
B
0
1
4
7 d
HDLECs
shCtr
shS1PR1
CD4 T cell
co-culture
HDLEC
harvest
C
shS1PR1-HDLECs
without CD4 T cell co-culture
-log10(1-PPDE)
log2(PostFC)
SELP
D
shS1PR1-HDLECs
with CD4 T cell co-culture
-log10(1-PPDE)
log2(PostFC)
SELP
E
Differentially up-regulated gene
shS1PR1-HDLECs
without CD4 T cell co-culture
with CD4 T cell co-culture
11
7
104
STAB2
AGTR1
TCF15
MAILR
FLRT3
RNF186-AS1
PITX1-AS1
C3AR1
CFI
SPTBN5
TGFA
SELP
ADRA1D
PGS5
APLN
DIRAS3
IL33
ADAMTS18
OMG
GPNMB
BPNE1
TOX
KIF19
TIPARP-AS1
CEROX1
SPEG
RPAP3-DT
TSPAN11
SRGAP3
GOLGA8R
LINC01300
LINC02159
OUT-AS1
MAN1C1
ODAD3
...
F
Top canonical pathways
shS1PR1-HDLECs with CD4 T cell co-culture
CENTRAL NERVOUS SYSTEM NEURON DIFFERENTIATION
T CELL ACTIVATION
CELL JUNCTION ORGANIZATION
LOCOMOTION
CELL VOLUME HOMEOSTASIS
PHOSPHORYLATION
LYMPHOCYTE ACTIVATION
CELLULAR ION HOMEOSTASIS
CENTRAL NERVOUS SYSTEM PROJECTION NEURON AXONOGENESIS
CELLULAR HOMEOSTASIS
SYNAPTIC SIGNALING
MONOVALENT INORGANIC ANION HOMEOSTASIS
CELLULAR ANION HOMEOSTASIS
NEGATIVE REGULATION OF PROTEIN LOCALIZATION
REGULATION OF CELLULAR LOCALIZATION
ANATOMICAL STRUCTURE FORMATION INVOLVED IN MORPHOGENESIS
CELL MOTILITY
CELLULAR MONOVALENT INORGANIC ANION HOMEOSTASIS
CELL ADHESION
CELL CELL SIGNALING
P-value
G
0
1
4 d
HDLECs
shS1PR1
HDLEC
analysis
Human
M.O.I. : 0, 0.5, 5, 50
H
HDLEC
P-selectin
I
P-selectin (MFI)
shS1PR1 M.O.I.

FIG. 6

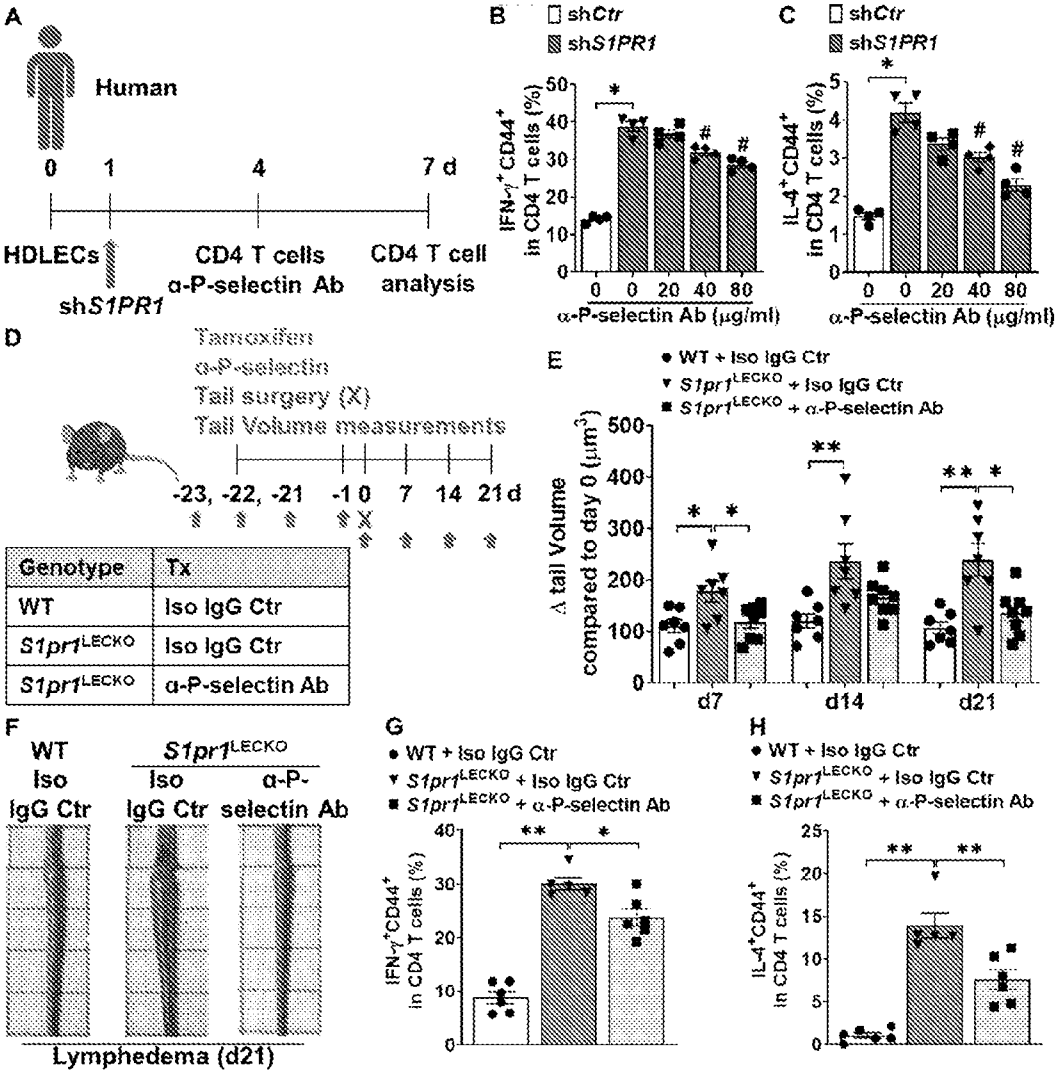
A
Human
0
1
4
7 d
HDLECs
shS1PR1
CD4 T cells
α-P-selectin Ab
CD4 T cell
analysis
B
shCtr
shS1PR1
IFN-γ+CD44+ in CD4 T cells (%)
α-P-selectin Ab (μg/ml)
C
shCtr
shS1PR1
IL-4+CD44+ in CD4 T cells (%)
α-P-selectin Ab (μg/ml)
D
Tamoxifen
α-P-selectin
Tail surgery (X)
Tail Volume measurements
-23, -22, -21
-1 0
7
14
21 d
Genotype
Tx
WT
Iso IgG Ctr
S1pr1LECKO
Iso IgG Ctr
S1pr1LECKO
α-P-selectin Ab
E
WT + Iso IgG Ctr
S1pr1LECKO + Iso IgG Ctr
S1pr1LECKO + α-P-selectin Ab
Δ tail Volume compared to day 0 (μm3)
d7
d14
d21
F
WT
Iso
IgG Ctr
S1pr1LECKO
Iso
IgG Ctr
α-P-
selectin Ab
Lymphedema (d21)
G
WT + Iso IgG Ctr
S1pr1LECKO + Iso IgG Ctr
S1pr1LECKO + α-P-selectin Ab
IFN-γ+CD44+ in CD4 T cells (%)
H
WT + Iso IgG Ctr
S1pr1LECKO + Iso IgG Ctr
S1pr1LECKO + α-P-selectin Ab
IL-4+CD44+ in CD4 T cells (%)

FIG. 7

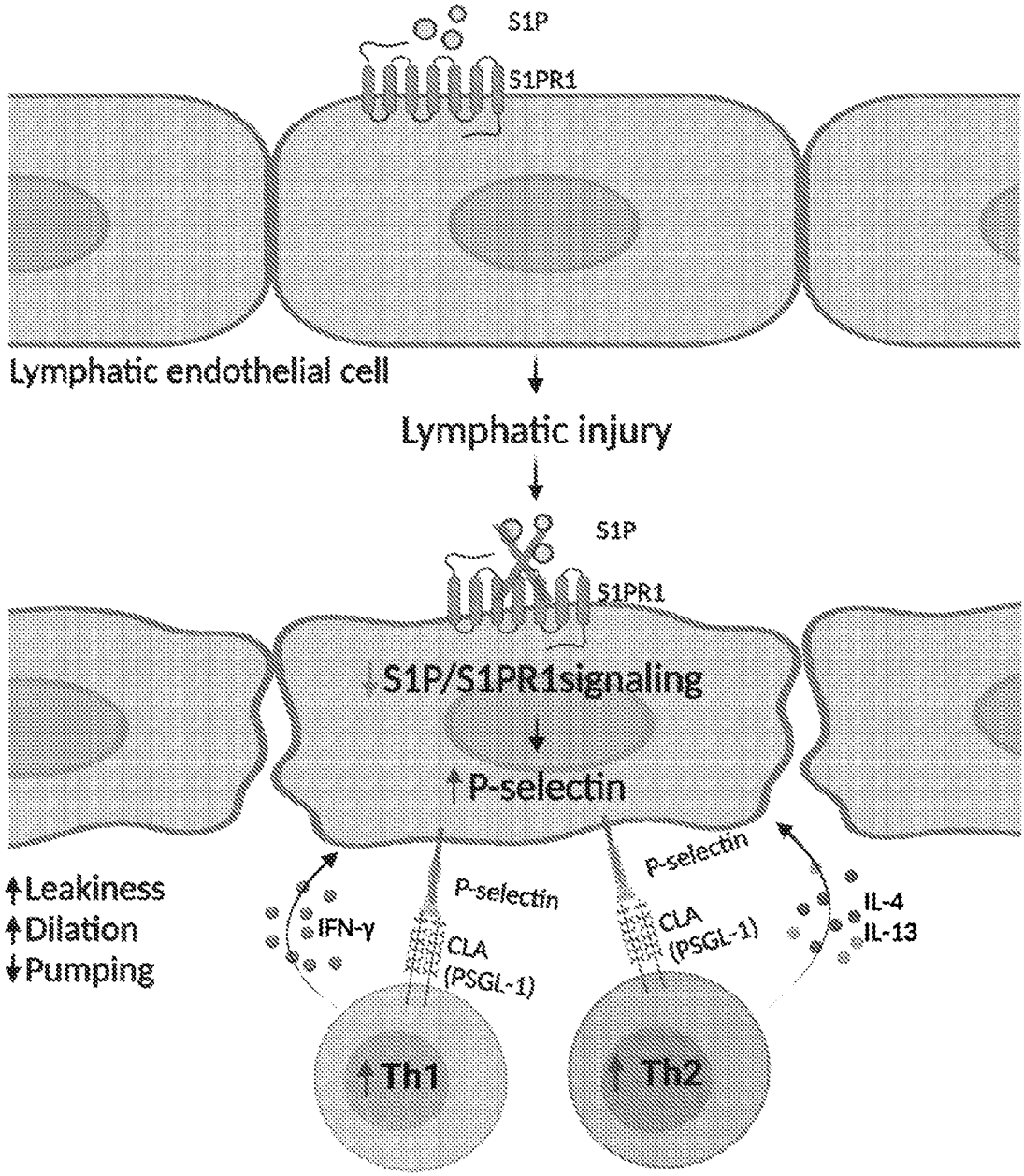
S1P
S1PR1
Lymphatic endothelial cell
Lymphatic injury
S1P
S1PR1
S1P/S1PR1signaling
↑P-selectin
↑Leakiness
↑Dilation
↓Pumping
IFN-γ
P-selectin
CLA
(PSGL-1)
P-selectin
CLA
(PSGL-1)
IL-4
IL-13
↑Th1
↑Th2

FIG. 8

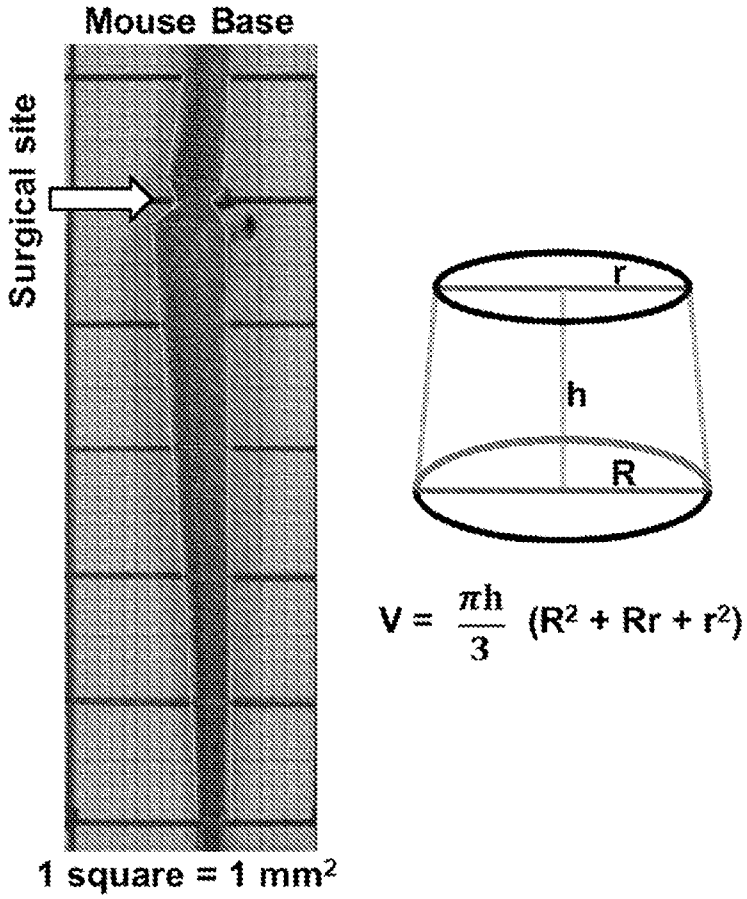
Mouse Base
Surgical site
1 square = 1 mm²
r
h
R
V = πh/3 (R² + Rr + r²)

FIG. 9

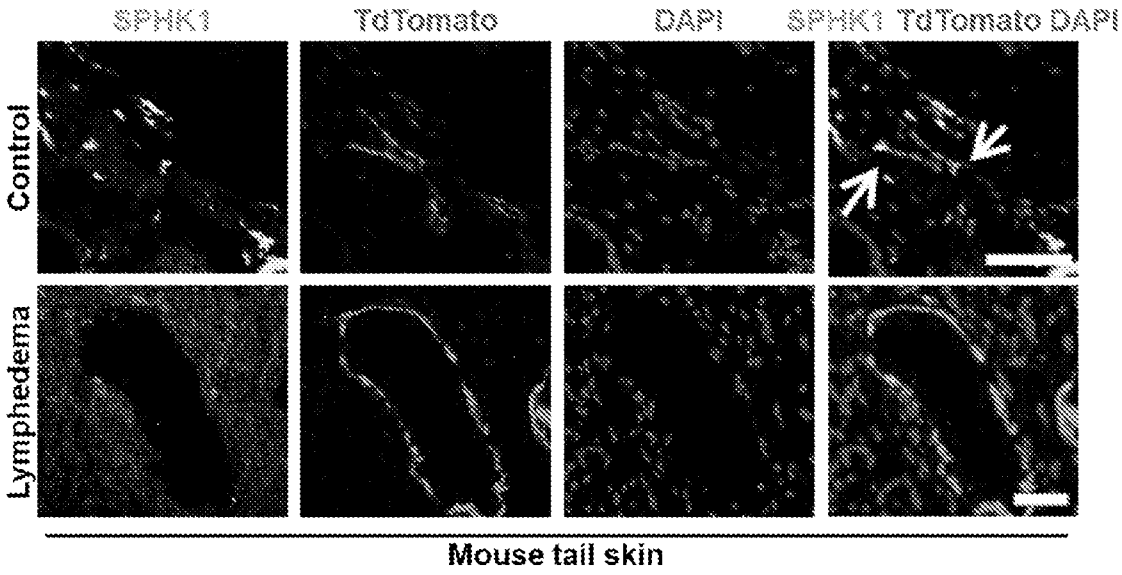
SPHK1
TdTomato
DAPI
SPHK1 TdTomato DAPI
Control
Lymphedema
Mouse tail skin

FIG. 10

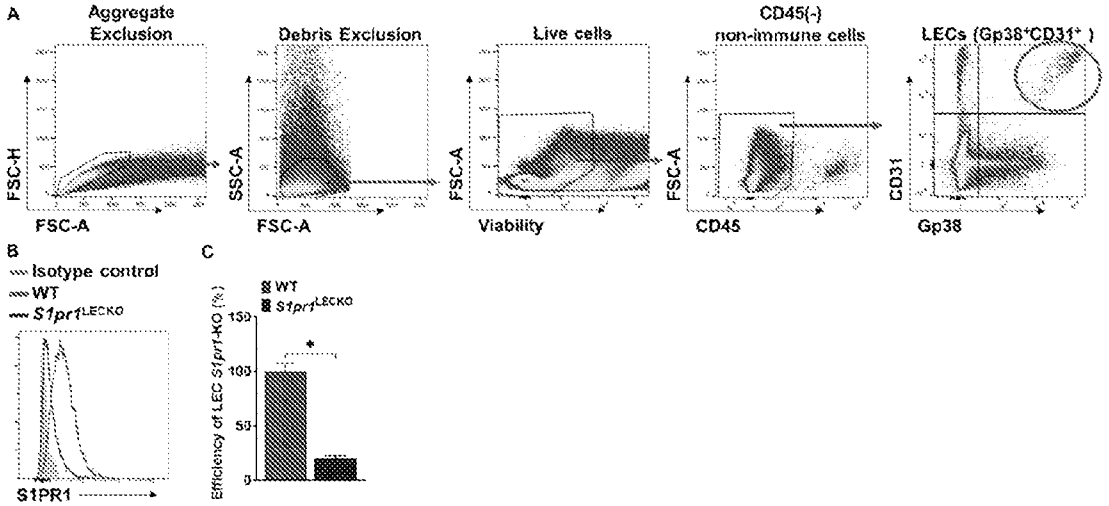
A
Aggregate Exclusion
Debris Exclusion
Live cells
CD45(-) non-immune cells
LECs (Gp38+CD31+)
FSC-H
FSC-A
SSC-A
FSC-A
FSC-A
Viability
FSC-A
CD45
CD31
Gp38
B
Isotype control
WT
S1pr1LECKO
S1PR1
C
WT
S1pr1LECKO
Efficiency of LEC S1pr1-KO (%)
150
100
50
0
*

FIG. 11

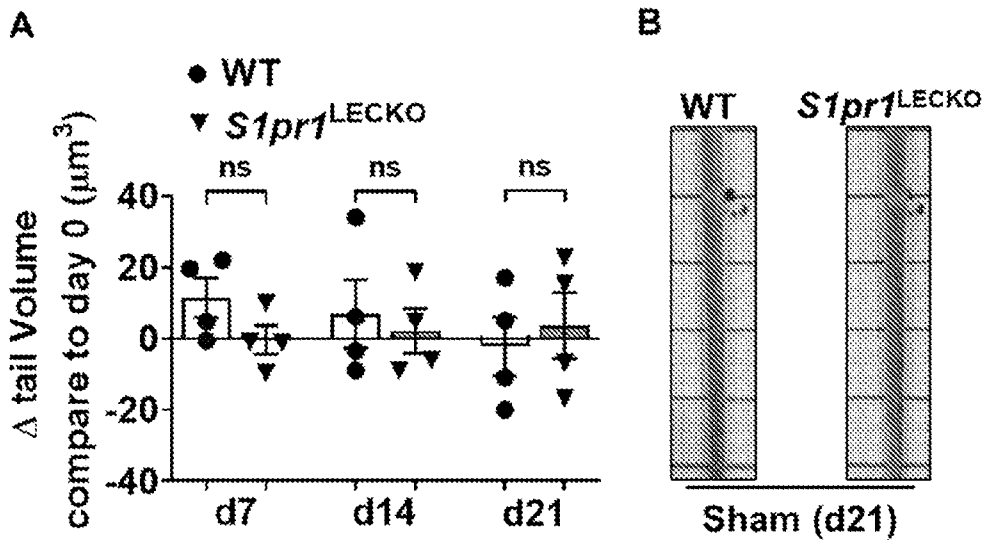
A
WT
S1pr1LECKO
ns
ns
ns
Δ tail Volume compare to day 0 (μm3)
40
20
0
-20
-40
d7
d14
d21
B
WT
S1pr1LECKO
Sham (d21)

FIG. 12

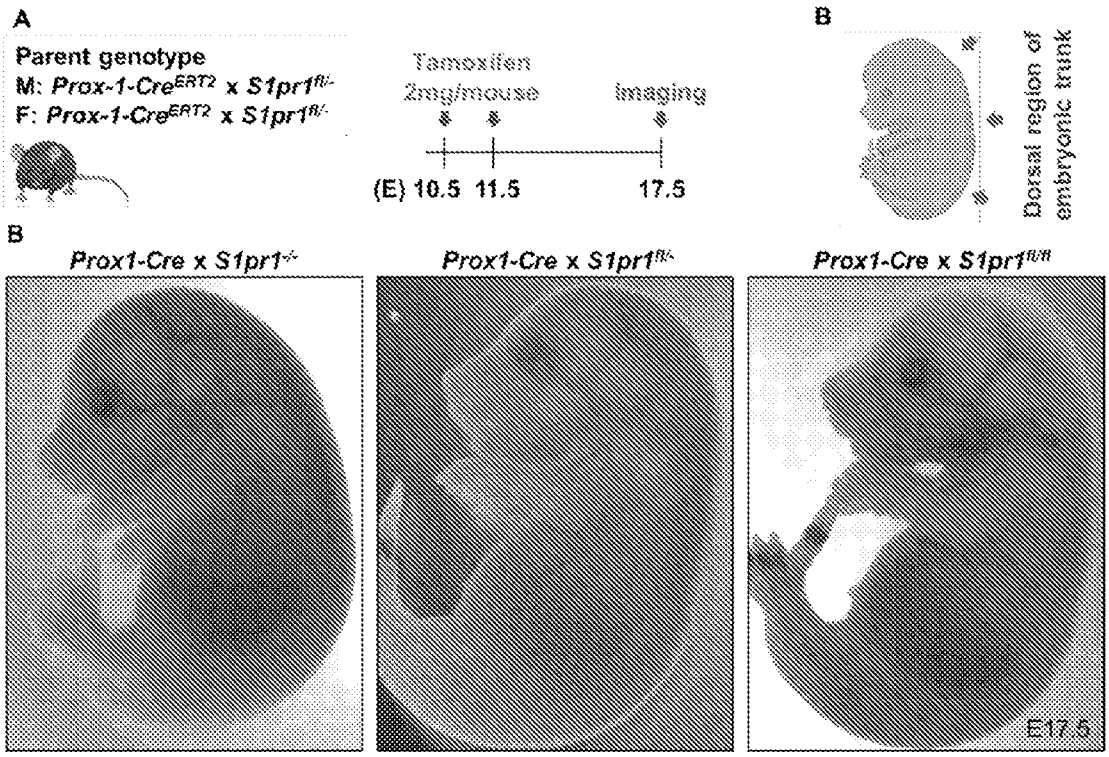
A
Parent genotype
M: Prox-1-Cre^ERT2 x S1pr1^fl/-
F: Prox-1-Cre^ERT2 x S1pr1^fl/-
Tamoxifen
2mg/mouse
Imaging
(E) 10.5 11.5 17.5
B
Dorsal region of embryonic trunk
B
Prox1-Cre x S1pr1-/-
Prox1-Cre x S1pr1fl/-
Prox1-Cre x S1pr1fl/fl
E17.5

FIG. 13

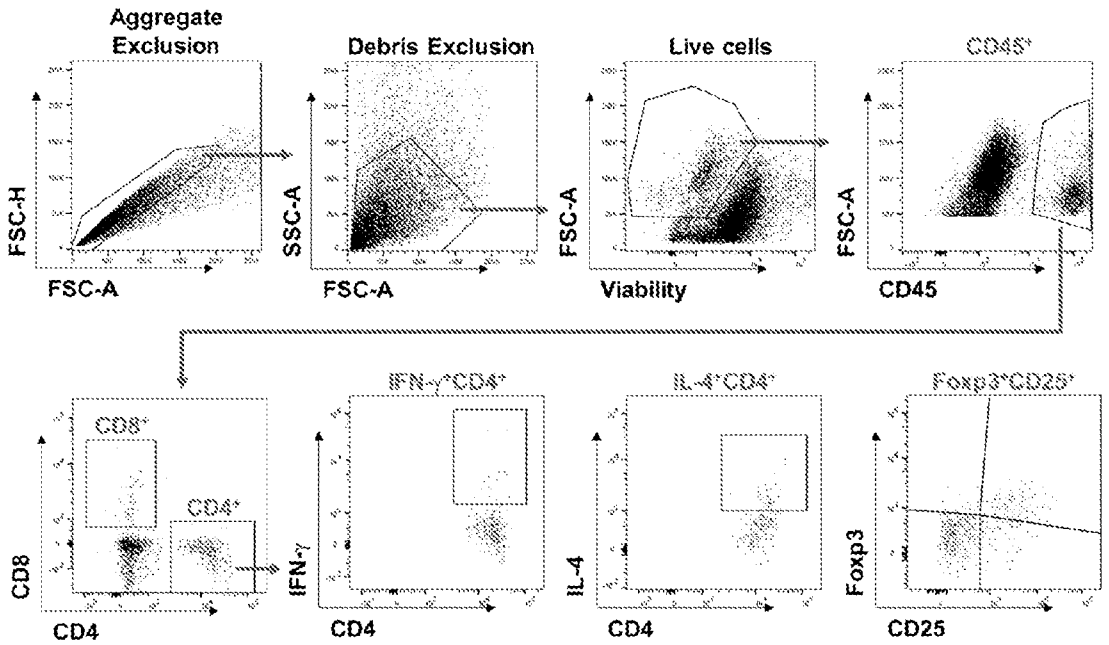
Aggregate Exclusion
FSC-H
FSC-A
Debris Exclusion
SSC-A
FSC-A
Live cells
FSC-A
Viability
CD45+
FSC-A
CD45
CD8+
CD4+
CD8
CD4
IFN-γ+CD4+
IFN-γ
CD4
IL-4+CD4+
IL-4
CD4
Foxp3+CD25+
Foxp3
CD25

FIG. 14

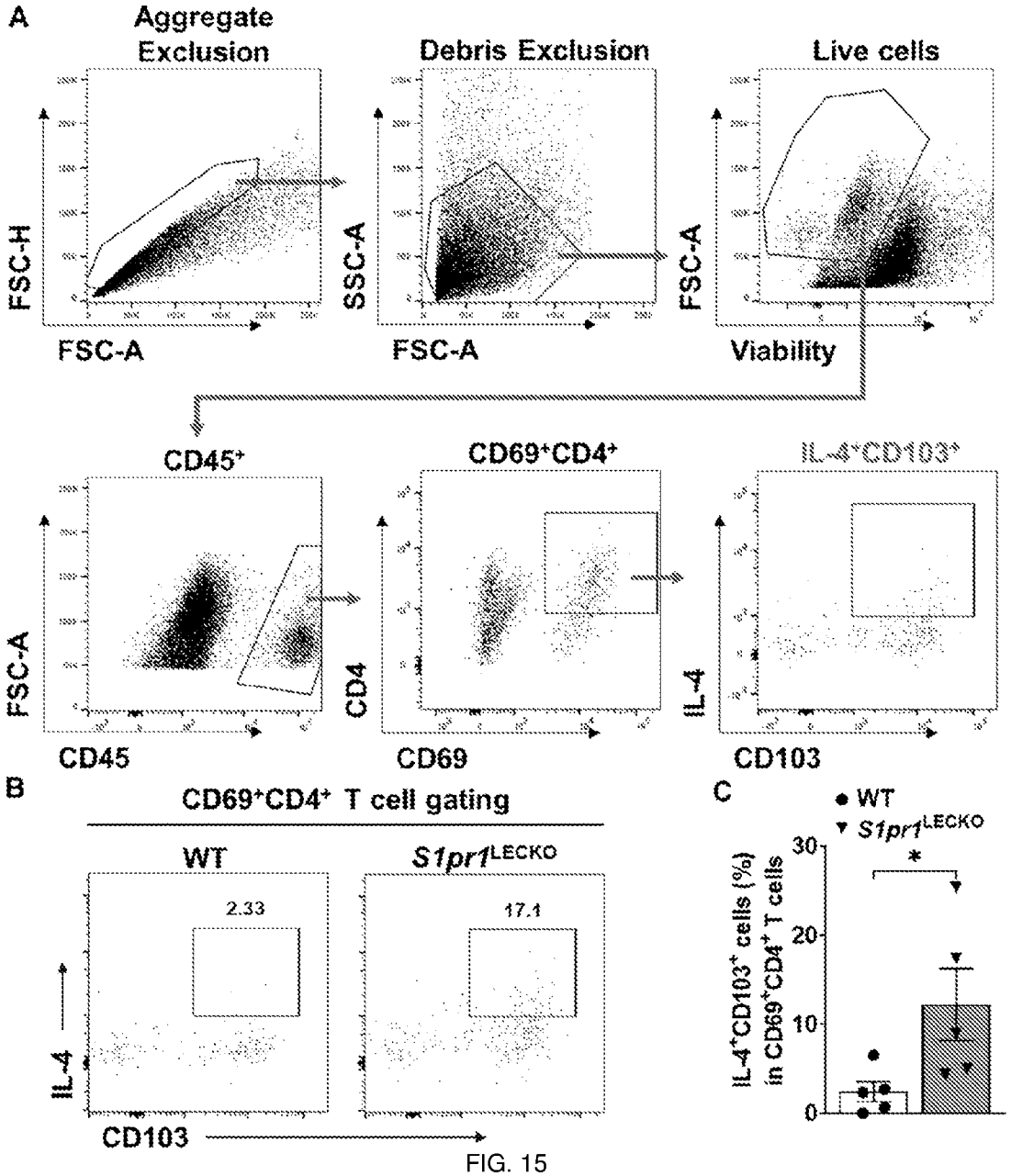
A
Aggregate Exclusion
Debris Exclusion
Live cells
FSC-H
FSC-A
SSC-A
FSC-A
FSC-A
Viability
CD45+
CD69+CD4+
IL-4+CD103+
FSC-A
CD45
CD4
CD69
IL-4
CD103
B
CD69+CD4+ T cell gating
WT
S1pr1LECKO
2.33
17.1
IL-4
CD103
C
WT
S1pr1LECKO
IL-4+CD103+ cells (%) in CD69+CD4+ T cells
30
20
10
0
*

FIG. 15

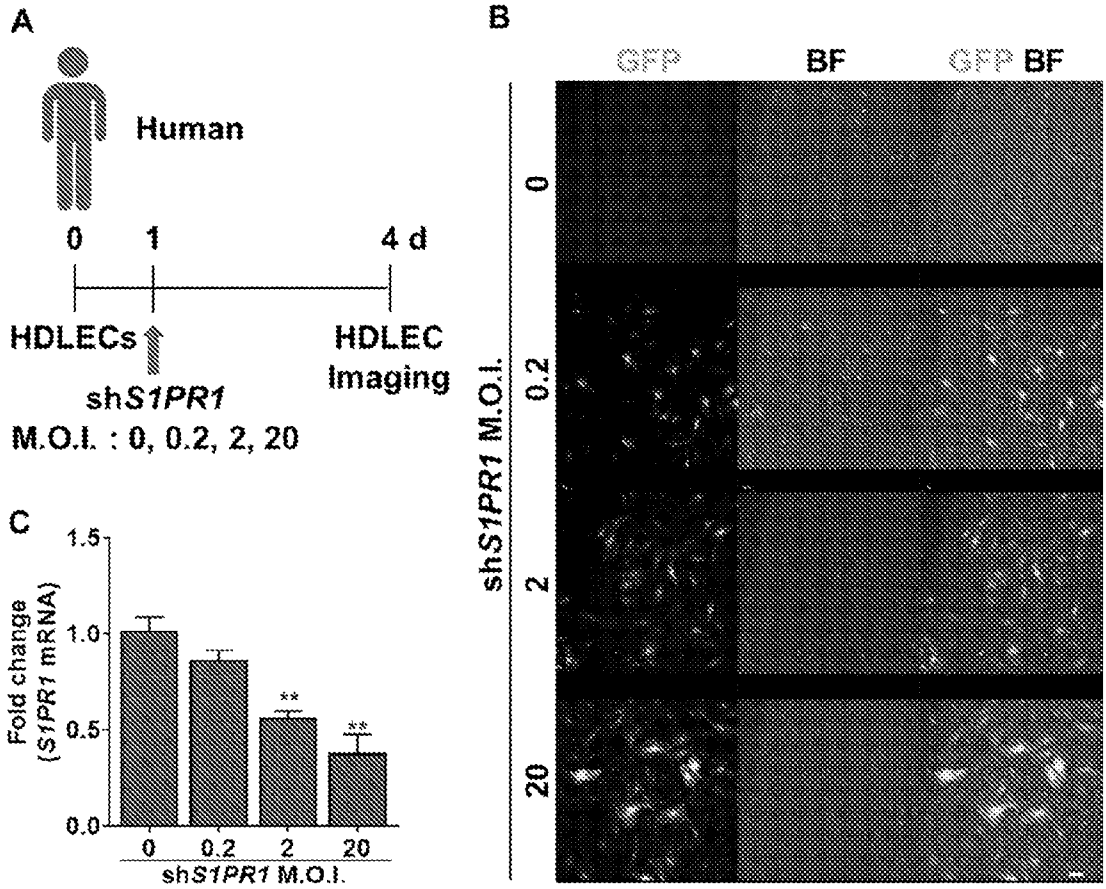
A
Human
0
1
4 d
HDLECs
HDLEC Imaging
shS1PR1
M.O.I. : 0, 0.2, 2, 20
B
GFP
BF
GFP BF
shS1PR1 M.O.I.
0
0.2
2
20
C
Fold change (S1PR1 mRNA)
1.5
1.0
0.5
0.0
**
**
0
0.2
2
20
shS1PR1 M.O.I.

FIG. 16

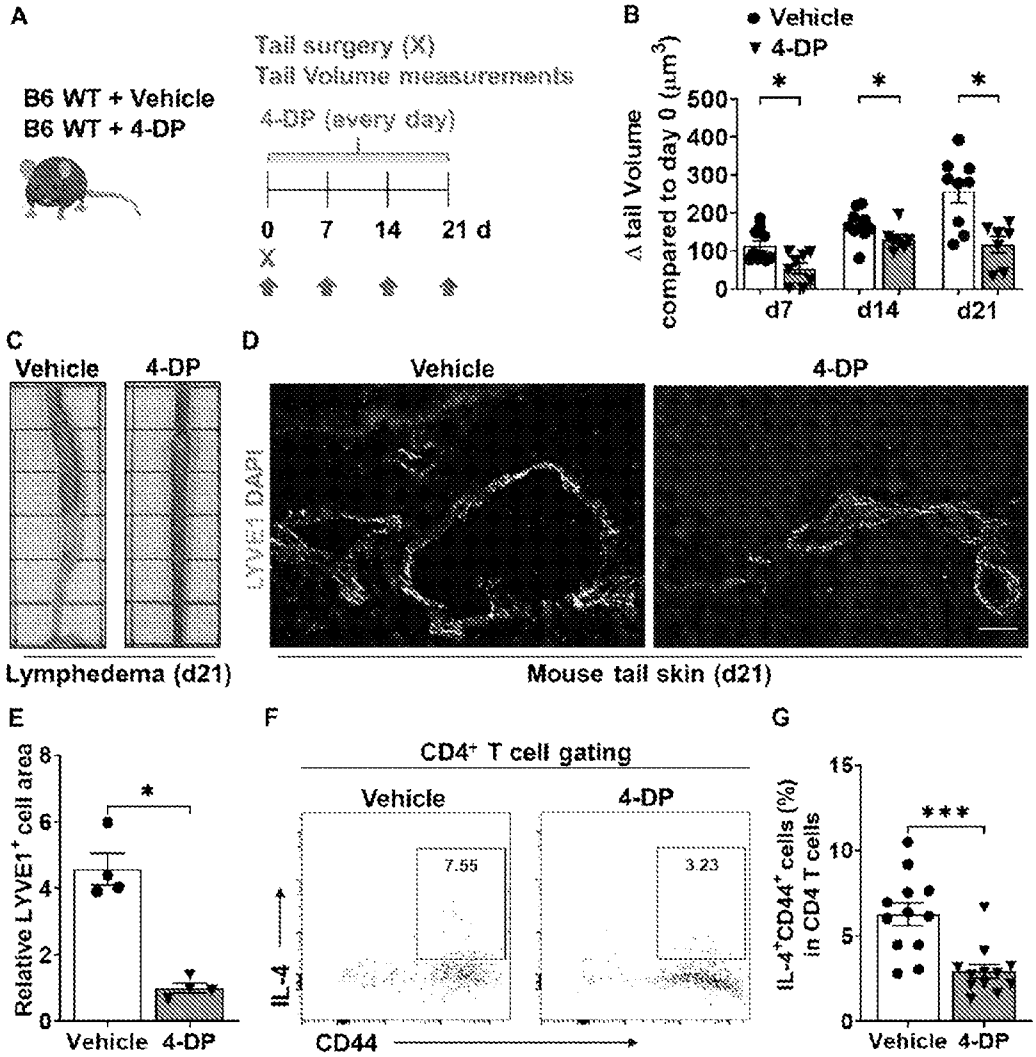
A
B6 WT + Vehicle
B6 WT + 4-DP
Tail surgery (X)
Tail Volume measurements
4-DP (every day)
0 7 14 21 d
B
Vehicle
4-DP
Δ tail Volume compared to day 0 (μm³)
500
400
300
200
100
0
d7
d14
d21
C
Vehicle
4-DP
Lymphedema (d21)
D
Vehicle
4-DP
LYVE1 DAPI
Mouse tail skin (d21)
E
Relative LYVE1⁺ cell area
8
6
4
2
0
Vehicle 4-DP
F
CD4⁺ T cell gating
Vehicle
4-DP
7.55
3.23
IL-4
CD44
G
IL-4⁺CD44⁺ cells (%) in CD4 T cells
15
10
5
0
Vehicle 4-DP

FIG. 17

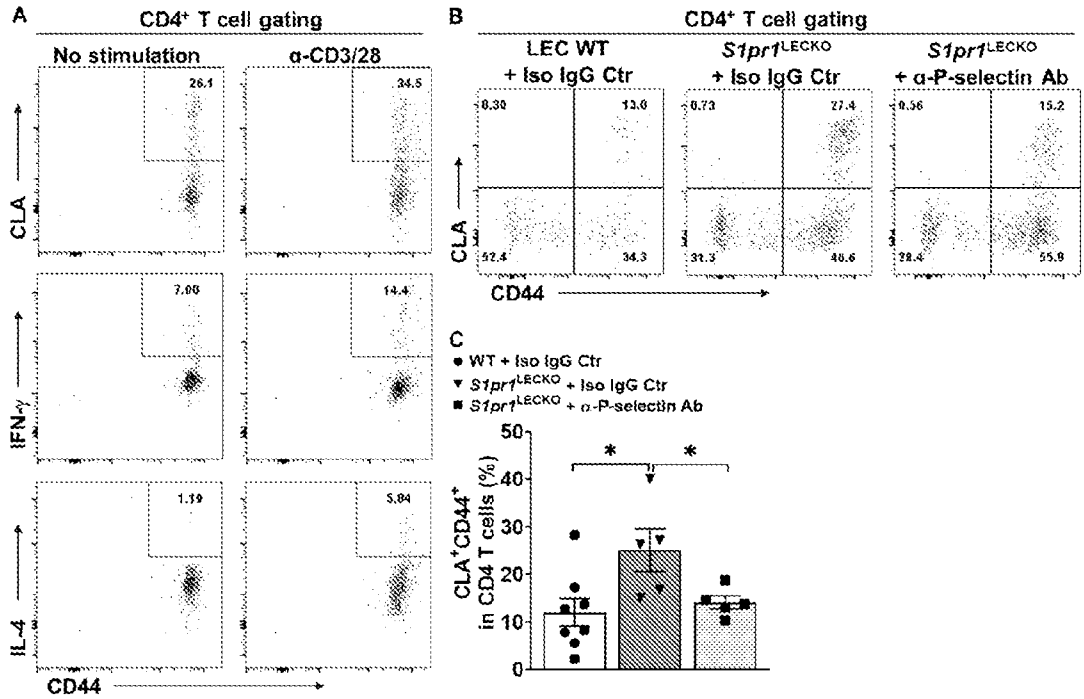
A
CD4+ T cell gating
No stimulation
α-CD3/28
CLA
IFN-γ
IL-4
CD44
26.1
34.5
7.08
14.4
1.19
5.84
B
CD4+ T cell gating
LEC WT
+ Iso IgG Ctr
S1pr1LECKO
+ Iso IgG Ctr
S1pr1LECKO
+ α-P-selectin Ab
CLA
CD44
8.30
13.8
52.4
34.3
0.73
27.4
31.3
40.6
0.56
15.2
28.4
55.8
C
WT + Iso IgG Ctr
S1pr1LECKO + Iso IgG Ctr
S1pr1LECKO + α-P-selectin Ab
CLA+CD44+ in CD4 T cells (%)
50
40
30
20
10
0
*
*

FIG. 18