Published in final edited form as:

Plast Reconstr Surg. 2019 March ; 143(3): 518e–526e. doi:10.1097/PRS.0000000000005322.

Small numbers of CD4⁺ T cells can induce development of lymphedema

Catherine L. Ly, MD¹, Daniel A. Cuzzone, MD¹, Raghu P. Kataru, PhD¹, and Babak J. Mehrara, MD¹

¹Plastic and Reconstructive Surgery Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, New York 10065

Abstract

Background: CD4⁺ T cells have been implicated in the pathology of lymphedema. Interestingly, however, there have been case reports of lymphedema development in patients with low levels of CD4⁺ T cells due to immunosuppression. In this study, we sought to delineate the effect of relative CD4⁺ T cell deficiency on the development of lymphedema in a mouse model.

Methods: A mouse model of relative CD4⁺ T cell deficiency was created through lethal total body irradiation of wild-type (WT) mice that then underwent bone marrow transplantation with progenitors harvested from CD4 knockout (CD4KO) mice (WT/CD4KO). Irradiated CD4KO mice reconstituted with WT mouse-derived progenitors (CD4KO/WT), as well as unirradiated CD4KO and WT mice were used as controls. All mice underwent tail skin and lymphatic excision to induce lymphedema and analysis was performed six weeks later.

Results: WT/CD4KO chimeras were not protected from developing lymphedema. Despite a global deficit in CD4⁺ T cells, these mice had swelling, fibrosis, inflammation, and impaired lymphatic transport function indistinguishable from that in WT and CD4KO/WT mice. In contrast, unirradiated CD4KO mice had no features of lymphedema after lymphatic injury.

Conclusions: Relatively few numbers of bone marrow and peripheral CD4⁺ T cells are sufficient to induce the development of lymphedema. These findings suggest that lymphatic injury results in expansion of CD4⁺ T cell populations in lymphedematous tissues.

Introduction

The management of secondary lymphedema, a common and morbid complication of treatment for solid tumors such as breast cancer, remains limited (1). Although several promising surgical and pharmaceutical options have been explored, conservative regimens consisting largely of compression therapy, manual lymphatic drainage, and skin care are the mainstay of treatment for a significant proportion of patients (2). The absence of a cure for

Corresponding author: Babak J. Mehrara, MD, Member, Memorial Hospital, Professor of Plastic Surgery, Weill Cornell University Medical Center, 1275 York Avenue, Suite MRI 1006, New York, New York, USA 10065, mehrarab@mskcc.org.
Author contributions

D.A.C. and B.J.M. conceived of the concept and designed the experiments. C.L.L., D.A.C., R.P.K., and B.J.M. performed the experiments and/or analyzed the data. C.L.L. and B.J.M. prepared and edited the manuscript.

lymphedema has prompted continued research into the pathophysiology of the disease to identify at-risk patients and develop targeted preventative and therapeutic interventions.

Numerous studies have found that CD4⁺ T cells play a critical role in the development of the pathologic features of lymphedema, including fibrosis, inflammation, impaired lymphangiogenesis, dysfunctional lymphatic transport, and local immunosuppression. Avraham et al., for example, used human lymphedema biopsies to demonstrate that the number of tissue-infiltrating CD4⁺ T cells is positively correlated with disease severity (3). In addition, murine studies have shown that the absence of CD4⁺ T cells either through transgenic modification (CD4 knockout or CD4KO mice) or antibody-based depletion is a potent means of preventing and treating lymphedema (4,5). Further research has found that T helper 2 (Th2) cells and T regulatory cells (Tregs) in particular are important; Th2 cells mediate fibroadipose deposition and lymphatic vessel growth and function (3,6,7), while Tregs regulate local immune responses (8,9).

Although the importance of CD4⁺ T cells has been corroborated by numerous studies, little is known about the mechanisms that lead to the accumulation of these cells in lymphedematous tissues or whether there is a threshold of cells required to promote lymphedema. Interestingly, there have been a few case reports of renal transplant patients on maintenance immunosuppressive therapy with sirolimus, an mammalian target of rapamycin (mTOR) inhibitor that hinders the proliferation of T cells, who have developed lymphedema (10,11). Similarly, others have described patients with acquired immunodeficiency syndrome (AIDS) due to human immunodeficiency virus (HIV) who have presented with lymphedema secondary to lymphatic obstruction resulting from Kaposi's sarcoma (12,13).

To test the hypothesis that even small numbers of functional CD4⁺ T cells can induce pathologic changes of lymphedema, we treated wild-type mice with total body irradiation (TBI) and performed with bone marrow transplantation (BMT) with progenitors harvested from CD4KO mice. This treatment resulted in subtotal ablation of bone marrow-derived CD4⁺ T cell progenitors (14). We then compared the development of lymphedema following tail lymphatic disruption in these animals with irradiated CD4KO mice transplanted with wild-type (WT) bone marrow.

Methods

Animals

The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center, which adheres to the National Institutes of Health Guide for Care and Use of Laboratory Animals and operates under guidelines put forth by the Animal Welfare Act. Female C57BL/6J (WT; #00664) and B6.129S2-*Cd4tm1Mak/J* (CD4KO; #002663) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free, temperature- and light-controlled environment. Female mice were chosen because the mouse tail model of lymphedema utilized in this study and described below has been shown to be effective and reproducible in female mice (15). All experiments were conducted using mice aged 8–12 weeks. When indicated, anesthesia was induced with isoflurane (Henry Schein Animal

Health; Dublin, OH). The depth of anesthesia was monitored every 15 minutes by tail pinching and observation of respiratory rate. Prior to tissue harvest, animals were euthanized by carbon dioxide asphyxiation as recommended by the American Veterinary Medical Association. A minimum of five animals were utilized for each experiment.

Bone marrow transplantation

TBI and BMT were performed using standard techniques (Fig. 1a) (16). Briefly, recipient WT and CD4KO mice underwent TBI with 950 rad using a cesium-137 Gammacell 40 exactor (Nordion; Ottawa, Canada). This dose of radiation was chosen because it has been shown to result in maximal bone marrow ablation without severe irradiation-induced toxicity (17). Twenty-four hours later, bone marrow was then flushed from the tibiae and femurs of donor WT and CD4KO mice using a 20-gauge needle on a 1-cc syringe filled with phosphate-buffered solution (PBS; Sigma-Aldrich) with 0.5 M ethylenediaminetetraacetic acid (EDTA; Santa Cruz Biotechnology; Dallas, TX) and 2% fetal calf serum (FCS; Sigma-Aldrich) and filtered through a 70 μ m cell strainer (Falcon; San Jose, CA) for bone marrow transplantation. Approximately 5×10^6 cells were infused into the irradiated recipients via tail vein injection to create WT/CD4KO and CD4KO/WT chimeras. All chimeras recovered for four weeks prior to further experimentation. Mice were monitored closely for adverse side effects such as radiation-induced sickness and manifestations of graft failure; only one mouse was noted to not tolerate the procedure well and was excluded from the study accordingly.

Surgical model of lymphedema

The well-described mouse tail model of lymphedema was chosen for this study because it has been shown to reliably result in edema, fibrosis, inflammation, and impaired lymphatic transport function, all of which are key characteristics of the disease (18). In this model, a 3-mm portion of skin and subcutaneous tissue are excised 2 cm from the base of the tail to allow for exposure of the deep lymphatic collecting vessels. After 5 μ L of 3% Evans blue (Sigma-Aldrich; St. Louis, MO) is injected into the distal tail to aid in the visualization of the lymphatic vasculature, the lymphatic vessels are ligated with care not to injure nearby blood vessels. All animals that underwent this procedure were housed individually during the recovery period and monitored closely. Two mice were found to have developed tail necrosis post-operatively, likely due to inadvertent vasculature injury, and were therefore excluded from the study.

Tail volume measurements

Tail volume was assessed pre-operatively and then weekly following skin and lymphatic excision. Serial tail circumference (C) was measured using digital calipers every 1 cm starting at the surgical site going distally toward the tip of the tail and inputted into the truncated cone formula to calculate tail volume (V): $V = 1/4\pi (C_1C_2 + C_2C_3 + C_3C_4)$.

Analysis of lymphatic function

Lymphatic transport function was assessed using lymphoscintigraphy per previously published methods (7,19). Briefly, 50 μ L of technetium-99m sulfur colloid (^{99m}Tc ; Nuclear

Diagnostic Products; Rockaway, NJ) was intradermally injected 1 cm from the tip of the tail, after which images were obtained using an X-SPECT camera (Gamma Medica; Northridge, CA). Region-of-interest analysis was performed using ASIPro Software (CTI Molecular Imaging; Knoxville, TN) to determine decay-adjusted uptake of ^{99m}Tc in the sacral lymph nodes, which are the closest draining lymph nodes to the tail, over the course of 60 minutes.

Flow cytometry

Single-cell suspensions of splenic and bone marrow cells were obtained using modifications of previously published methods (4). Spleens underwent mechanical dissociation followed by enzymatic digestion using an 8:2:1 mixture of dispase II, collagenase D, and DNase I (all from Roche Diagnostics; Indianapolis, IN). Erythrocytes in both the spleens and bone marrow were lysed with RBC lysis buffer (eBioscience; San Diego, CA) prior to antibody incubation. After incubation with Fc receptor block (rat anti-mouse monoclonal antibody to CD16/CD32; 14-0161-85, eBioscience) to reduce non-specific staining, cell suspensions were stained with rat anti-mouse monoclonal antibodies to CD3 (17A2; #17-0032-80) and CD4 (GK1.5; #11-0041-82) from eBioscience, in addition to 4,6-diamidino-2-phenylindole (DAPI; #D1306, Molecular Probes/Invitrogen; Eugene, OR) viability stain. Single-stain compensation samples were created using UltraComp eBeads™ (#01-2222-42, Affymetrix, Inc.; San Diego, CA). Data were obtained using a BD Fortessa flow cytometry analyzer (BD Biosciences; San Jose, CA) with BD FACS Diva and analyzed with FlowJo software (Tree Star; Ashland, OR).

Immunohistochemistry

Histologic analysis was performed per prior protocols (4). Briefly, after euthanasia, mouse tail cross-sections were harvested 1 cm from the surgical site and immediately fixed in 4% paraformaldehyde (Affymetrix; Cleveland, OH). The specimens then underwent two weeks of decalcification with 0.5 M EDTA before paraffin embedding. For analysis, paraffin blocks were cut into 5- μm sections then rehydrated.

Hematoxylin and eosin staining was performed using standard techniques. Cut sections were incubated with Mayer's hematoxylin (Lillie's Modification; Dako North America; Carpinteria, CA) followed by eosin Y solution (Thermo Fisher Scientific; Waltham, MA), alcohol-based dehydration, and alcohol extraction with xylene (Sigma-Aldrich) before mounting with VectaMount Permanent Mounting Medium (Vector Laboratories, Inc.; Burlingame, CA). Fibroadipose tissue deposition was quantified in standardized H&E-stained tail cross-sections by measuring the width of tissues bounded by reticular dermis to deep fascia in four quadrants by two blinded reviewers.

For immunofluorescent staining, heat-mediated antigen retrieval was accomplished using sodium citrate (Sigma-Aldrich) in a 90°C water bath. Non-specific binding was blocked with a solution of 20% donkey serum (Sigma-Aldrich) in PBS, after which the cut sections were incubated overnight at 4°C with rabbit polyclonal collagen I (1:100; #ab34710; Abcam; Cambridge, MA) and goat polyclonal LYVE-1 (1:400; #2125-LY; R&D Systems; Minneapolis, MN). After washing with PBS, the sections were then incubated with corresponding fluorescent-labeled secondary antibodies (1:1000; Life Technologies;

Carlsbad, CA) for five hours and 4,6-diamidino-2-phenylindole (DAPI; 1:1000; #D1306; Molecular Probes/Invitrogen; San Jose, CA) for ten minutes at room temperature. Once staining was complete, all sections were mounted with Mowiol (Sigma-Aldrich) and then scanned using a Mirax slide scanner (Zeiss; Munich, Germany). Analysis was performed with Panoramic Viewer (3D Histech; Budapest, Hungary) and type I collagen deposition was quantified as a ratio of positively stained dermis and subcutaneous tissues within a fixed area to total tissue area using Fiji (ImageJ, National Institutes of Health; Bethesda, MD) (20). A minimum of four high-powered fields (40x) was assessed per sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.; San Diego, CA). One- or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was utilized for comparison of multiple groups at a single time point or over time, respectively, with the Brown-Forsythe test to confirm equality of group variances. Data are presented as mean and standard deviation and $P < 0.05$ was considered significant.

Results

WT/CD4KO chimeras have small numbers of CD4⁺ T cells

To study the effects of differing quantities of CD4⁺ T cells on the development of lymphedema, we irradiated CD4KO and WT mice then performed BMT using BM-derived progenitors from WT and CD4KO donors, respectively, to create CD4KO/WT and WT/CD4KO chimeras. One month after BMT, these animals, as well as unirradiated age- and sex-matched WT and CD4KO mice, were treated with tail skin and lymphatic excision to induce lymphedema (Fig. 1a).

We first analyzed the percentage of CD4⁺ T cells in the spleens and bone marrow of animals from the 4 experimental groups using flow cytometry (Figure 1b, 1c) (See Figure, Supplemental Digital Content 1, which shows the WT/CD4KO chimeras have small numbers of CD4⁺ T cells that proliferate in the area of lymphatic injury. Representative flow cytometric dot plots of live CD45⁺CD3⁺CD4⁺ T cells in the spleen (top), bone marrow (middle), and tail skin (bottom) six weeks after tail skin and lymphatic excision.. As expected, CD4KO mice had no CD4⁺ T cells while WT mice had large numbers of these cells (up to 20% of live cells in the spleen and more than 1% of cells in the bone marrow). CD4KO/WT mouse chimeras had similar percentage of CD4⁺ T cells in the spleen and bone marrow as compared with WT mice ($P = 0.94$ and $P = 0.28$, respectively). In contrast, WT/CD4KO chimeras had significantly decreased numbers of CD4⁺ T cells as compared with WT mice ($P = 0.0005$ for spleen and $P = 0.0015$ for bone marrow). This finding is consistent with the fact that TBI at the given dose does not completely ablate all CD4⁺ T cells, thus resulting in a small proportion of residual cells that repopulate, albeit at lower levels, the peripheral lymphoid organs and bone marrow niche.

WT/CD4KO chimeras develop lymphedema after lymphatic injury

Consistent with our previous studies (4), we found that CD4KO mice do not develop lymphedema after tail skin and lymphatic excision. In contrast, WT mice developed gross

swelling of the distal tail with a distinctive J-shaped curvature of the tail due to progressive collagen deposition (Fig. 2a). Similarly, CD4KO/WT chimeras also developed severe tail lymphedema and fibrosis. Interestingly, even though WT/CD4KO chimeras had significantly decreased numbers of peripheral and bone marrow CD4⁺ T cells, the severity of tail lymphedema in these animals was indistinguishable from WT mice. These differences were particularly apparent when we quantified tail volume changes using the truncated formula, demonstrating decreased swelling only in CD4KO mice and similar levels of tail lymphedema in WT, CD4KO/WT, and WT/CD4KO chimeras (Fig. 2b; $P < 0.0005$ for all groups starting at week 1 after surgery).

WT/CD4KO chimeras have impaired lymphatic transport function after lymphatic injury

Based on the knowledge that fibrosis and inflammation in lymphedema are closely associated with impaired lymphatic function, we next sought to determine if WT/CD4KO bone marrow chimeric mice also had a deficit in lymphatic transport capacity. To evaluate this, we assessed the transportation of the radionuclide ^{99m}Tc to the sacral lymph nodes after intradermal injection into the distal tail in all groups. As expected, CD4KO mice had markedly greater ^{99m}Tc uptake compared to all other groups, thus reflecting improved lymphatic transport function (Fig. 2c; $P < 0.05$ for indicated time points) (See Figure, Supplemental Digital Content 2, which shows the WT/CD4KO chimeras have impaired lymphatic transport function. Representative images of technetium-99m sulfur colloid (^{99m}Tc) after injection in the distal tail with arrows indicating sacral lymph nodes. In contrast, WT, CD4KO/WT, and WT/CD4KO mice all had similarly low uptake that did not change over time.

WT/CD4KO chimeras develop fibrosis after lymphatic injury

To corroborate our gross observations of fibrosis in the mouse tails six weeks after skin and lymphatic excision, we also assessed fibroadipose deposition histologically. Consistent with our prior findings, CD4KO mice had significantly less fibroadipose deposition compared to WT, CD4KO/WT, and WT/CD4KO mice (Fig. 3a, c; $P = 0.0036$, $P = 0.0003$, and $P = 0.0005$, respectively). This was corroborated with immunofluorescent staining for type I collagen deposition, which revealed the least amount of staining in CD4KO mice (Fig. 3b, d; $P < 0.0001$ for WT and CD4KO/WT and $P = 0.0001$ for WT/CD4KO compared to CD4KO mice).

WT/CD4KO chimeras have increased CD4⁺ T cells in their lymphedematous tails

Flow cytometric analysis of tail skin and subcutaneous tissues of WT and CD4KO/WT chimera mice six weeks following lymphatic ablation demonstrated marked infiltration of CD4⁺ T cells (comprising nearly 50% of all CD45⁺ leukocytes) as compared to similarly treated CD4KO mice (Fig. 4, $P = 0.013$ and $P = 0.007$, respectively) (See Figure, Supplemental Digital Content 1. Interestingly, despite the fact that WT/CD4KO chimeras had a 2–3-fold decrease in the percentage of peripheral and bone marrow CD4⁺ T cells, analysis of tail tissues in these animals also demonstrated a marked increase in the percentage of infiltrating CD4⁺ T cells. The percentage of CD4⁺ T cells in the lymphedematous tissues of WT/CD4KO chimeras was only 36% lower than WT animals, a difference that was not statistically significant ($P = 0.58$), suggesting that infiltrating CD4⁺

cells in these animals undergo proliferation locally. In addition, our findings collectively suggest that the presence of smaller proportions of peripheral and bone marrow derived CD4⁺ T cells are sufficient to promote the development of characteristic lymphedema features, including fibrosis, inflammation, and dysfunctional lymphatic transport.

Discussion

Despite its morbidity and prevalence, lymphedema remains without a cure. The development of effective preventative or therapeutic options not only requires a clear understanding of the pathophysiology of the disease, but also the identification of patients who are susceptible to lymphedema and would best benefit from such treatments. Recent studies have shown that CD4⁺ T cells play a critical role in development of the hallmarks of lymphedema (3–9). Interestingly, however, there have been reports of immunosuppressed patients with secondary lymphedema despite having low levels of CD4⁺ T cells (10–13). In this study, we corroborate this data by demonstrating that mice with relative CD4⁺ T cell deficiency due to TBI are not protected from developing lymphedema after lymphatic injury. Although these mice had a global deficit in CD4⁺ T cells compared to WT mice and irradiated CD4KO mice that then underwent BMT with WT mouse-derived progenitors, irradiated WT mice that were transplanted with CD4KO mouse-derived progenitors still presented with swelling, fibrosis, inflammation, and impaired lymphatic transport function that was indistinguishable from their WT and CD4KO/WT counterparts. Such results suggest that lymphatic injury promotes the expansion of CD4⁺ T cell populations in lymphedematous tissues.

Combined with the knowledge that the complete absence of CD4⁺ T cells prevents lymphedema development (4), our findings suggest that the number of CD4⁺ T cells is not as important as the presence of these critical cells. Although the elucidation of a threshold of CD4⁺ T cells would be valuable to identify at-risk patients, the specific number needed to promote the pathologic findings of lymphedema is likely dependent on a variety of factors and is, therefore, different in each patient. Such factors include baseline lymphatic function, degree of lymphatic injury (i.e., sentinel lymph node biopsy versus complete lymph node dissection), and the presence of any known independent risk factors such as obesity, radiation, or infection (21–23).

Further studies are necessary to delineate the means by which lymphedema develops in the setting of immunosuppression, but the understanding that few CD4⁺ T cells are sufficient to lead to disease is important as lymphedema may otherwise be excluded from the differential diagnosis in such patients. This is especially relevant because lymphedema is already often diagnosed at a late stage in a great proportion of patients due to its delayed presentation and its variable and sometimes unpredictable natural history (24,25). Furthermore, many promising new surgical and pharmaceutical therapies have been shown to be most effective in early-stage lymphedema; other patients with late-stage disease are often relegated to conservative treatment regimens that are largely palliative in nature (26).

In conclusion, we have further confirmed that CD4⁺ T cells are important in a well-described mouse model of lymphedema and have also shown that a relatively low number of these critical cells is sufficient to cause disease. This should be taken into consideration

when patients with known immunosuppression present with signs and symptoms suggestive of lymphedema.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank the Molecular Cytology and Flow Cytometry Cores at Memorial Sloan Kettering Cancer Center for their assistance (Core Grant P30 CA008748).

Financial Disclosure Statement: None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this manuscript. This work was supported by the National Institutes of Health (NIH) R01 HL111130-01 and R21 CA194882 grants awarded to B.J.M., the NIH T32 CA9501-29 grant to C.L.L., the NIH T32 CA009685-21A1 grant to D.A.C., and the NIH/NCI P30 CA008748 (Cancer Center Support Grant) to Memorial Sloan Kettering Cancer Center.

References

1. The diagnosis and treatment of peripheral lymphedema: 2013 Consensus Document of the International Society of Lymphology. *Lymphology* 2013;46:1–11. [PubMed: 23930436]
2. Arsenio J, Kakaradov B, Metz PJ, Kim SH, Yeo GW, Chang JT Early specification of CD8+ T lymphocyte fates during adaptive immunity revealed by single-cell gene-expression analyses. *Nature immunology* 2014;15:365–372. [PubMed: 24584088]
3. Avraham T, Zampell JC, Yan A, et al. Th2 differentiation is necessary for soft tissue fibrosis and lymphatic dysfunction resulting from lymphedema. *FASEB J* 2013;27:1114–1126. [PubMed: 23193171]
4. Zampell JC, Yan A, Elhadad S, Avraham T, Weitman E, Mehrara BJ CD4(+) cells regulate fibrosis and lymphangiogenesis in response to lymphatic fluid stasis. *PLoS one* 2012;7:e49940. [PubMed: 23185491]
5. Zampell JC, Avraham T, Yoder N, et al. Lymphatic function is regulated by a coordinated expression of lymphangiogenic and anti-lymphangiogenic cytokines. *Am J Physiol Cell Physiol* 2012;302:C392–404. [PubMed: 21940662]
6. Savetsky IL, Ghanta S, Gardenier JC, et al. Th2 cytokines inhibit lymphangiogenesis. *PLoS one* 2015;10:e0126908. [PubMed: 26039103]
7. Avraham T, Daluvoy S, Zampell J, et al. Blockade of transforming growth factor-beta1 accelerates lymphatic regeneration during wound repair. *Am J Pathol* 2010;177:3202–3214. [PubMed: 21056998]
8. Gousopoulos E, Proulx ST, Bachmann SB, et al. Regulatory T cell transfer ameliorates lymphedema and promotes lymphatic vessel function. *JCI insight* 2016;1:e89081. [PubMed: 27734032]
9. Garcia Nores GD, Ly CL, Savetsky IL, et al. Regulatory T Cells Mediate Local Immunosuppression in Lymphedema. *J Invest Dermatol* 2018;138:325–335. [PubMed: 28942366]
10. Al-Otaibi T, Ahamed N, Nampoory MR, et al. Lymphedema: an unusual complication of sirolimus therapy. *Transplantation proceedings* 2007;39:1207–1210. [PubMed: 17524934]
11. Baliu C, Esforzado N, Campistol JM, Mascaro JM, Jr. Chronic lymphedema in renal transplant recipients under immunosuppression with sirolimus: presentation of 2 cases. *JAMA dermatology* 2014;150:1023–1024. [PubMed: 24898278]
12. Allen PJ, Gillespie DL, Redfield RR, Gomez ER Lower extremity lymphedema caused by acquired immune deficiency syndrome-related Kaposi's sarcoma: case report and review of the literature. *Journal of vascular surgery* 1995;22:178–181. [PubMed: 7637119]
13. Ramdial PK, Chetty R, Singh B, Singh R, Aboobaker J Lymphedematous HIV-associated Kaposi's sarcoma. *Journal of cutaneous pathology* 2006;33:474–481. [PubMed: 16872469]

14. Lapidot T, Singer TS, Salomon O, Terenzi A, Schwartz E, Reisner Y Booster irradiation to the spleen following total body irradiation. A new immunosuppressive approach for allogeneic bone marrow transplantation. *J Immunol* 1988;141:2619–2624. [PubMed: 3049814]
15. Tabibiazar R, Cheung L, Han J, et al. Inflammatory manifestations of experimental lymphatic insufficiency. *PLoS Med* 2006;3:e254. [PubMed: 16834456]
16. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 2012;18:883–891. [PubMed: 22635005]
17. Duran-Struuck R, Dysko RC Principles of bone marrow transplantation (BMT): providing optimal veterinary and husbandry care to irradiated mice in BMT studies. *Journal of the American Association for Laboratory Animal Science : JAALAS* 2009;48:11–22. [PubMed: 19245745]
18. Ly CL, Kataru RP, Mehrara BJ Inflammatory Manifestations of Lymphedema. *International journal of molecular sciences* 2017;18.
19. Clavin NW, Avraham T, Fernandez J, et al. TGF-beta1 is a negative regulator of lymphatic regeneration during wound repair. *American journal of physiology Heart and circulatory physiology* 2008;295:H2113–2127. [PubMed: 18849330]
20. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nature methods* 2012;9:676–682. [PubMed: 22743772]
21. Bevilacqua JL, Kattan MW, Changhong Y, et al. Nomograms for predicting the risk of arm lymphedema after axillary dissection in breast cancer. *Ann Surg Oncol* 2012;19:2580–2589. [PubMed: 22395997]
22. Kuroda K, Yamamoto Y, Yanagisawa M, et al. Risk factors and a prediction model for lower limb lymphedema following lymphadenectomy in gynecologic cancer: a hospital-based retrospective cohort study. *BMC women's health* 2017;17:50. [PubMed: 28743274]
23. Tsai RJ, Dennis LK, Lynch CF, Snetselaar LG, Zamba GK, Scott-Conner C The risk of developing arm lymphedema among breast cancer survivors: a meta-analysis of treatment factors. *Ann Surg Oncol* 2009;16:1959–1972. [PubMed: 19365624]
24. Kwan ML, Darbinian J, Schmitz KH, et al. Risk factors for lymphedema in a prospective breast cancer survivorship study: the Pathways Study. *Arch Surg* 2010;145:1055–1063. [PubMed: 21079093]
25. Tada H, Teramukai S, Fukushima M, Sasaki H Risk factors for lower limb lymphedema after lymph node dissection in patients with ovarian and uterine carcinoma. *BMC cancer* 2009;9:47. [PubMed: 19193243]
26. Dayan JH, Ly CL, Kataru RP, Mehrara BJ Lymphedema: Pathogenesis and Novel Therapies. *Annual review of medicine* 2017.

Figure 1. Lethally irradiated WT mice reconstituted with CD4KO mouse-derived progenitors have residual CD4⁺ T cells.

(a) Schematic diagram depicting total body irradiation, bone marrow transplantation, and tail skin and lymphatic excision. (b, c) Flow cytometric quantification of CD45⁺CD3⁺CD4⁺ T cells in the spleen (b) and bone marrow (c) six weeks after surgery ($n = 5-6$ per group). One-way ANOVA with Tukey's multiple comparisons test; mean (SD); ** $P < 0.01$, *** $P < 0.001$.

Figure 2. WT/CD4KO chimeric mice develop lymphedema after lymphatic injury.

(a) Representative photographs of mouse tails six weeks after tail skin and lymphatic excision. (b) Quantification of change in tail volume over time ($n = 5-9$ per group). (c) Quantification of decay-adjusted ^{99m}Tc uptake by sacral lymph nodes over time six weeks after tail skin and lymphatic excision ($n = 4$ per group). Two-way ANOVA with Tukey's multiple comparisons test; mean (SD); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. WT/CD4KO chimeras develop fibrosis after lymphatic injury.

Tissue harvest performed six weeks after tail skin and lymphatic excision. (a) Representative hematoxylin and eosin staining of tail cross-sections with brackets indicating fibroadipose thickness. (b) Immunofluorescent localization of type I collagen and LYVE-1⁺ lymphatic vessels in tail cross-sections. (c) Quantification of fibroadipose thickness ($n = 4-5$ per group). (d) Quantification of type I collagen deposition ($n = 5$ per group; 4 high-powered fields per mouse). One-way ANOVA with Tukey's multiple comparisons test; mean (SD); ** $P < 0.01$, *** $P < 0.001$.

Figure 4. WT/CD4KO chimeras have increased inflammation after lymphatic injury. Flow cytometric quantification CD45⁺CD3⁺CD4⁺ T cells in mouse tail skin six weeks after tail skin and lymphatic excision ($n = 5-6$ per group). One-way ANOVA with Tukey's multiple comparisons test; mean (SD); ** $P < 0.01$, *** $P < 0.001$.