Tertiary lymphoid organs are aggregates of immune and stromal cells including high endothelial venules and lymphatic vessels that resemble secondary lymphoid organs and can be induced at nonlymphoid sites during inflammation. The function of lymphatic vessels within tertiary lymphoid organs remains poorly understood. During lung transplant tolerance, Foxp3\(^+\) cells accumulate in tertiary lymphoid organs that are induced within the pulmonary grafts and are critical for the local downregulation of alloimmune responses. Here, we showed that tolerant lung allografts could induce and maintain tolerance of heterotopic donor-matched hearts through pathways that were dependent on the continued presence of the transplanted lung. Using lung retransplantation, we showed that Foxp3\(^+\) cells egressed from tolerant lung allografts via lymphatics and were recruited into donor-matched heart allografts. Indeed, survival of the heart allografts was dependent on lymphatic drainage from the tolerant lung allograft to the periphery. Thus, our work indicates that cellular trafficking from tertiary lymphoid organs regulates immune responses in the periphery. We propose that these findings have important implications for a variety of disease processes that are associated with the induction of tertiary lymphoid organs.
Lymphatic drainage from bronchus-associated lymphoid tissue in tolerant lung allografts promotes peripheral tolerance


Departments of Surgery, Medicine, and Pathology & Immunology, Washington University in St. Louis, St. Louis, Missouri, USA. Department of Surgery, Northwestern University, Chicago, Illinois, USA. Department of Surgery, University of Maryland, Baltimore, Maryland, USA.

Tertiary lymphoid organs are aggregates of immune and stromal cells including high endothelial venules and lymphatic vessels that resemble secondary lymphoid organs and can be induced at nonlymphoid sites during inflammation. The function of lymphatic vessels within tertiary lymphoid organs remains poorly understood. During lung transplant tolerance, Foxp3+ cells accumulate in tertiary lymphoid organs that are induced within the pulmonary grafts and are critical for the local downregulation of alloimmune responses. Here, we showed that tolerant lung allografts could induce and maintain tolerance of heterotopic donor-matched hearts through pathways that were dependent on the continued presence of the transplanted lung. Using lung retransplantation, we showed that Foxp3+ cells egressed from tolerant lung allografts via lymphatics and were recruited into donor-matched heart allografts. Indeed, survival of the heart allografts was dependent on lymphatic drainage from the tolerant lung allograft to the periphery. Thus, our work indicates that cellular trafficking from tertiary lymphoid organs regulates immune responses in the periphery. We propose that these findings have important implications for a variety of disease processes that are associated with the induction of tertiary lymphoid organs.

Introduction

Tertiary lymphoid organs can be induced in nonlymphoid tissues during states of chronic inflammation or antigen exposure and are structurally similar to secondary lymphoid organs. One such example is bronchus-associated lymphoid tissue, a pulmonary tertiary lymphoid organ that can be induced in murine and human lungs during a variety of inflammatory conditions, including respiratory infections, autoimmune diseases, chronic obstructive pulmonary disease, cancers, and pulmonary transplantation (1). Tertiary lymphoid organs are hallmarked by the development of high endothelial venules, which are specialized vascular structures that facilitate the entry of leukocytes into tertiary lymphoid organs (2). In addition, multiple studies have documented the existence of lymphatic vessels within tertiary lymphoid organs, including bronchus-associated lymphoid tissues, in both mice and humans (3, 4). Although the patterns and functions of lymphatic flow in secondary lymphoid tissues have been the focus of many studies, relatively little is known about the role of lymphatic vessels in tertiary lymphoid organs. Afferent lymphatics transport soluble or cell-associated antigens to secondary lymphoid organs where their encounter with lymphocytes initiates adaptive immune responses. The importance of afferent lymphatics in tertiary lymphoid organs, however, remains to be established. It has been suggested that the continuous presence of antigen in tertiary lymphoid organs, such as autoantigen in autoimmune diseases or alloantigen in transplanted organs, obviates the need for antigen transport to these sites (5). Efferent lymphatics promote the egress of lymphocytes from secondary lymphoid organs and it has been reported that CD4+ T cells make up a large fraction of lymphocytes in efferent lymph (6). Several studies have documented the presence of lymphocytes within lymphatic vessels of tertiary lymphoid organs, suggesting that cellular trafficking occurs through these channels (5, 7).

We and others have observed that tertiary lymphoid organs are induced in transplanted tissues and organs, sites where alloantigen is continuously present for the life of the graft (8). In contrast to many studies that have suggested that tertiary lymphoid organs are deleterious and promote graft rejection, we have shown that Foxp3+ T lymphocytes are located within bronchus-associated lymphoid tissue that is induced in tolerant mouse lung allografts (9–11). Furthermore, we have demonstrated that selective depletion of Foxp3+ cells from tolerant grafts triggers antibody-mediated rejection, thereby establishing that Foxp3+ cells suppress alloimmune responses locally (12). In this study, we employ a mouse lung retransplant model to show that Foxp3+ cells migrate from tolerant lung allografts to the periphery via lymphatic channels. The induction of peripheral tolerance depends on lymphatic drainage from the tolerant lung allograft.
culopathy (Figure 1, B, C, E, and F). Next, we wanted to assess whether the presence of a tolerant pulmonary allograft was necessary to maintain peripheral tolerance. We therefore transplanted BALB/c hearts into B6 mice that had been engrafted with BALB/c lungs at least 30 days prior while receiving perioperative costimulatory blockade. Thirty days after heart transplantation we removed the tolerant pulmonary allograft. While most hearts stopped beating, a few cardiac grafts continued to beat weakly during the observation period (Figure 1A). These grafts had evidence of cardiac allograft vasculopathy (Figure 1, D and G). Compared with control grafts we observed a lower density of B cells, but a higher density of T lymphocytes in hearts that were rejected after either early or late removal of the tolerant lung allograft (Supplemental Figure 1, A–D; supplemental material available online with this article; https://doi.org/10.1172/JCI136057DS1). Removal of the lung allograft before or after heart transplantation was also associated with a significantly lower cardiac allograft density of Foxp3+ cells as well as a decrease in the ratio of Foxp3+ cells to overall T lymphocytes (Supplemental Figure 1, E–G). Thus, donor-specific tolerance in the periphery cannot be induced or maintained in the absence of a tolerant lung allograft.

Our findings provide insights into the function of lymphatic vessels in tertiary lymphoid organs and may have important implications for a variety of disease processes.

Results

**Induction and maintenance of peripheral tolerance after lung transplantation depends on the presence of the tolerant pulmonary allograft.** We have previously reported that tolerance develops after lung transplantation, as evidenced by long-term acceptance of donor-matched, but not third-party, hearts by recipients of tolerant pulmonary allografts (12). We wanted to investigate whether the continued presence of the tolerant lung allograft was required for the induction of tolerance. To examine this, we transplanted BALB/c lungs into B6 recipients that received perioperative costimulatory blockade, a regimen that we have previously shown results in tolerance induction after pulmonary transplantation (10). At least 30 days later, BALB/c hearts were transplanted into these B6 hosts. When the lung allografts were removed 24 hours before engraftment of the heart, the cardiac grafts were rejected (Figure 1A). We observed intimal thickening and often luminal occlusion of coronary arteries in rejected grafts, consistent with the development of cardiac allograft vasculopathy (Figure 1, B, C, E, and F). Next, we wanted to assess whether the presence of a tolerant pulmonary allograft was necessary to maintain peripheral tolerance. We therefore transplanted BALB/c hearts into B6 mice that had been engrafted with BALB/c lungs at least 30 days prior while receiving periop-erative costimulatory blockade. Thirty days after heart transplantation we removed the tolerant pulmonary allograft. While most hearts stopped beating, a few cardiac grafts continued to beat weakly during the observation period (Figure 1A). These grafts had evidence of cardiac allograft vasculopathy (Figure 1, B, C, E, and F).
took advantage of our lung retransplant model. We have previously reported that tolerant lung allografts survive long term after retransplantation into nonimmunosuppressed recipients (10). We transplanted BALB/c lungs into B6 Foxp3-GFP recipients that received perioperative costimulatory blockade. At least 30 days later these tolerant grafts were retransplanted into nonimmunosuppressed B6 mice. Twenty-one days after lung retransplantation BALB/c hearts were engrafted into these mice (Figure 3A). Six days after heart transplantation, we observed recruitment of Foxp3-GFP cells to the transplanted hearts where many Foxp3+ cells had entered the graft tissue (Figure 3B and Supplemental Video 2). These findings indicated that Foxp3+ cells within the transplanted heart originated from the tolerant lung allograft. At this time point, we continued to detect GFP+ cells in the lung graft (Figure 3C). We observed that the majority of these cells expressed Ki-67 in cardiac allografts, which is consistent with their expansion. While we also observed their proliferation in lung allografts, the percentage of Ki-67-expressing cells was higher in the transplanted hearts (Supplemental Figure 2, A–C). These cells expressed high levels of intracellular CTLA-4 in both compartments (Supplemental Figure 2, D and E). Notably, such BALB/c heart allografts survived long term (Figure 3, D and E). In addition to Foxp3+ cells, we were able to detect other T lymphocyte populations, such as CD8+ T cells in transplanted hearts that have originated from the tolerant lung allografts. Most of these CD8+ T cells were CD44+CD62L−, consistent with an effector memory phenotype (Supplemental Figure 3, A and B). Of note, while we observed that some CD8+ T cells in tolerant lung allografts expressed CD103, a marker that is characteristic of nonmigratory tissue-resident memory cells, CD103 expression was largely absent on CD8+ T cells in cardiac grafts that had originated from the lung grafts (13) (Supplemental Figure 3, C and D). In contrast to donor-matched BALB/c cardiac grafts, hearts derived from a third-party (CBA mice) were acutely rejected when transplanted into B6 recipients that had received tolerant BALB/c lung allografts 21 days prior (Supplemental Figure 4). We also assessed whether Foxp3+ cells migrate from tolerant BALB/c lung allografts into syngeneic B6 heart grafts. Only a small percentage of CD4+ T cells that were present in syngeneic B6 hearts 6 days after transplantation expressed Foxp3, very few of which were derived from the original host (Supplemental Figure 5).

Lymphatic drainage from bronchus-associated lymphoid tissue facilitates exit of Foxp3+ cells and mediates peripheral tolerance. Having shown that Foxp3+ cells exit the tolerant pulmonary allograft, we next set out to examine how cells migrate from the bronchus-associated lymphoid tissue in the transplanted lung to the periphery. In previous work we demonstrated that high endothelial venules, specialized vessels through which cells can enter secondary or tertiary lymphoid organs, form in bronchus-associated lymphoid tissue within tolerant lung allografts (10, 14). Consistent with previous reports that lymphatic vessels can also form in tertiary lymphoid organs, we observed many vascular structures that stained for VEGFR-3 within the bronchus-associated lymphoid tissues of tolerant lung allografts (Figure 4A) (3). We detected VEGFR-3 staining only in luminal cells. Interestingly, we observed Foxp3+ cells within the lumina of these lymphatic vessels.

Peribronchial lymphatic vessels are severed as part of the lung transplant procedure. To evaluate at what time point after lung
transplantation lymphatic drainage from the pulmonary graft to the periphery is reestablished, we transplanted BALB/c lungs into B6 mice that received perioperative costimulatory blockade. Lymphatic drainage from the lung allograft was evaluated 21 days after transplantation by instilling a fluorescent dye into the bronchus of the graft. We observed accumulation of fluorescent dye in the draining mediastinal lymph nodes, albeit at somewhat lower levels when compared with naive nontransplanted mice (Figure 4B). However, when we administered fluorescent dye into the graft bronchus 7 days after transplantation, there was virtually no detectable dye in the draining lymph nodes (Figure 4B). These findings are consistent with previously published reports in large animals and more recently in mice demonstrating that lymphatic drainage from pulmonary grafts is reestablished approximately 2 to 3 weeks after the lung transplant procedure (15, 16). No differences in the density of the fluorescent dye were detected in the lungs among these conditions (Figure 4C).
Figure 5. Peripheral tolerance is not induced in the absence of lymphatic drainage from the tolerant lung allograft. (A) Schematic diagram depicting experimental transplant model. Intravital 2-photon imaging of (B) retransplanted (Retxp) tolerant BALB/c lung allograft (n = 3) and (C) BALB/c cardiac allograft (n = 3) 6 days after transplantation of BALB/c heart into B6 mice into which a tolerant BALB/c lung was retransplanted 7 days prior (Foxp3+ cells: green; quantum dot–labeled vessels: red; second harmonic generation [SHG]: blue). The BALB/c lung had been transplanted into a B6 Foxp3 IRES-GFP mouse (treated with perioperative costimulatory blockade) at least 30 days before the retransplant procedure. (D) Kaplan-Meier survival curves of BALB/c hearts that were transplanted into nonimmunosuppressed B6 mice that received tolerant BALB/c pulmonary allografts (●) 7 (n = 8) or (▲) at least 21 days (n = 7) before cardiac transplantation. The BALB/c hearts had been originally engrafted into B6 mice that received perioperative costimulatory blockade and then retransplanted at least 30 days later. (▲) Depicts the survival of BALB/c hearts that were transplanted into DT-treated B6 Foxp3-DTR mice that received perioperative costimulatory blockade. (E) Histological appearance (H&E) of rejected BALB/c hearts after transplantation into B6 mice into which a tolerant BALB/c lung allograft was retransplanted 7 days prior. Scale bars: 30 μm (B and C) and 100 μm (E).

Next, we wanted to evaluate whether the reestablishment of lymphatic drainage from the tolerant lung allograft was critical for the induction of peripheral tolerance. To this end, we transplanted BALB/c lungs into B6 Foxp3-GFP recipients that received perioperative costimulatory blockade. At least 30 days later these tolerant lungs were retransplanted into nonimmunosuppressed B6 mice. Seven days after lung retransplantation BALB/c hearts were engrafted into these mice (Figure 5A). Although Foxp3-GFP cells were present in the lungs, we did not observe Foxp3-GFP cells within the cardiac allografts 6 days after heart transplantation (Figure 5, B and C). In contrast to the long-term survival of cardiac allografts that were engrafted 21 days after lung retransplantation, hearts that were transplanted 7 days after lung retransplantation were rejected (Figure 5, D and E). We next wanted to determine if pharmacologically induced lymphangiogenesis could facilitate peripheral tolerance in this model. Here, we employed VEGF-C156S, a VEGF-C mutant with exclusive binding affinity for VEGFR-3, a receptor that is expressed on lymphatic endothelial cells and mediates lymphatic sprouting (17). Although VEGFR-3 expression has been observed by immunostaining on sprouting blood vessels in monkeys and humans, most studies indicate that VEGF-C156S does not promote angiogenesis in adults (18–21). Given that our vascularized lung transplant model establishes blood flow at the time of engraftment, any potential effect of VEGF-C156S on new blood vessel formation is not likely to impact cell migration. Also, VEGF-C156S did not increase the chemotaxis of regulatory T cells compared with vehicle control (Supplemental Figure 6). We treated B6 recipients with VEGF-C156S for 7 days after receiving a tolerant BALB/c lung allograft and then transplanted BALB/c hearts into these mice. In contrast to our observations without VEGF-C156S treatment (Figure 5C), administration of VEGF-C156S resulted in the infiltration of lung allograft–derived Foxp3-GFP cells into the cardiac allografts (Figure 6, A–C), which was associated with their long-term survival (Figure 6, D and E).

Finally, we wanted to evaluate whether disruption of lymphatic growth prevented induction of peripheral tolerance after lung transplantation. Here, we employed an anti–VEGFR-3 antibody that we and others have shown to inhibit lymphatic regrowth (22, 23). We transplanted BALB/c lungs into B6 Foxp3-GFP recipients that received perioperative costimulatory blockade. At least 30 days later these tolerant lungs were retransplanted into nonimmunosuppressed B6 hosts that received treatment with anti–VEGFR-3. Twenty-one days later we transplanted BALB/c hearts into these mice. While Foxp3-GFP cells were present in the lung allografts, in contrast to control conditions (Figure 3B), we did not observe GFP+ cells in the cardiac allografts 6 days after heart transplantation (Figure 7, A–C). All hearts stopped beating, with histological evidence of rejection (Figure 7, D and E), similarly to heart grafts implanted 7 days after lung retransplantation (Figure 5, D and E). The lung allografts did not have evidence of rejection (Supplemental Figure 7, A and B). However, the size of their bronchus-associated lymphoid tissue was increased compared with mice that did not receive treatment with anti–VEGFR-3 (Supplemental Figure 7C). Moreover, many lymphatic vessels were filled with cells, including Foxp3+ cells (Supplemental Figure 7D).

Discussion
In this study, we took advantage of a temporal requirement for the reestablishment of lymphatic drainage from lungs after transplantation to show that immune cells exit tolerant pulmonary grafts via lymphatics. Retransplantation of tolerant lung allografts has allowed us to uncover that efferent lymphatic flow from tertiary lymphoid organs can regulate immune responses in the periphery. We show that long-term survival of cardiac
Figure 6. Stimulation of lymphangiogenesis promotes induction of peripheral tolerance after lung transplantation. Intravital 2-photon imaging of (A) retransplanted (Retxp) tolerant BALB/c lung allograft (n = 2) and (B) BALB/c cardiac allograft (n = 2) 6 days after transplantation of BALB/c heart into a B6 mouse into which a tolerant BALB/c lung was retransplanted 7 days prior and received VEGF-C156S between the time of lung retransplantation and heart transplantation (Foxp3+ cells; green; quantum dot–labeled [q-dot–labeled] vessels; red; second harmonic generation [SHG]; blue). The BALB/c lung had been originally transplanted into a B6 Foxp3 IRES-GFP mouse (treated with perioperative costimulatory blockade [CSB]) at least 30 days before the retransplant procedure. (C) Quantification of density of Foxp3-GFP cells in BALB/c hearts (up to 5 separate areas/graft were examined) 6 days after transplantation (Txp) into B6 recipients of tolerant BALB/c lung allografts, that received no treatment or were treated with VEGF-C156S for 7 days before cardiac transplantation. Data are expressed as mean ± SEM. Mann-Whitney U test was used to compare the means. (D) Kaplan-Meier survival curves of BALB/c hearts that were transplanted into immunosuppressed B6 mice, (●) untreated (n = 7) or (▲) treated with VEGF-C156S (n = 4), that received tolerant BALB/c pulmonary allografts 7 days before cardiac transplantation. The BALB/c lungs had been originally engrafted into B6 mice that received perioperative costimulatory blockade and then retransplanted at least 30 days later. (E) Histological appearance (H&E) of long-term-surviving BALB/c hearts after transplantation into VEGF-C156S–treated B6 mice into which a tolerant BALB/c lung allograft was retransplanted 7 days prior. Scale bars: 30 μm (A and B) and 100 μm (E).

The stimulation of lymphangiogenesis in allografts depends on Foxp3+ cells and that the survival of donor-matched hearts is associated with their infiltration with Foxp3+ cells that originate from tolerant lung allografts. In this setting, peripheral tolerance cannot be induced in the absence of lymphatic drainage from the lung allograft.

Most tertiary lymphoid organs in transplanted tissues or organs have been associated with the promotion of immune responses that are deleterious to the graft. For example, tertiary lymphoid organs have been observed in mouse cardiac allografts that have evidence of acute or chronic rejection (9). Moreover, lymphoid neogenesis in rat aortic allografts has been shown to contribute to the generation of a local humoral alloimmune response (24). Similarly, most studies have linked lymphangiogenesis in murine and human allografts, irrespective of their association with tertiary lymphoid organs, to the propagation of immune responses that mediate rejection (25–27). Lymphatic vessel density is increased in the myocardium of rat cardiac allografts that undergo chronic rejection (28). Interestingly, inhibition of VEGF-3 resulted in prolongation of rat heart allograft survival when recipients received immunosuppression with low-dose cyclosporine, which may in part be related to decreased trafficking of antigen-presenting cells via lymphatic vessels from the graft to the draining secondary lymphoid organs. Similar roles have been proposed for lymphatic vessels in islet, tracheal, and corneal allografts, where anti-VEGF-3–mediated inhibition of lymphangiogenesis results in attenuation of the immune response and prevention of tissue destruction (29–31). However, evidence has emerged that lymphangiogenesis in allografts is not necessarily harmful and may in fact promote the resolution of inflammation. Lungs differ from other organ and tissue grafts, as alloimmune responses that regulate rejection and tolerance occur locally within the pulmonary graft and are not dependent on cellular trafficking to secondary lymphoid organs (10, 32). Thus, unlike the case for hearts, islets, and corneas, we have shown that lymphatic migration of antigen-presenting cells from pulmonary grafts to the periphery is not critical for graft rejection (33–35). In fact, promoting lymphangiogenesis through treatment with VEGF-C has been shown to attenuate acute rejection of mouse lung allografts (19). Mechanistically, lymphatic vessels facilitate the clearance of damage-associated molecular patterns, such as hyaluronan fragments that are known to accentuate inflammatory responses following lung transplantation through engagement of Toll-like receptors (36). These findings are somewhat reminiscent of previous studies that demonstrated that VEGF-C–driven activation of lymphatic vessels limits inflammation of the skin (18, 37). A more recent study showed that induction of lymphangiogenesis in murine renal allografts through doxycycline-induced expression of VEGF-C resulted in prolongation of their survival (38). Although the underlying mechanism was not explored, the authors speculated that lymphatic endothelial cells may facilitate the generation or trafficking of immunoregulatory cells. In support of this, evidence exists that lymphatic endothelial cells can suppress T cell responses directly by presenting antigen in tolerogenic fashion or indirectly by inhibiting the maturation of dendritic cells (39).

We have not observed the induction of bronchus-associated lymphoid tissue in long-term-surviving syngeneic lung grafts, indicating that severing lymphatic connections between the graft and...
existing pulmonary lymphatic vessels were destroyed in this study, which likely accounts for the drastic differences in phenotype compared with transient interruption of lymphatic flow after syngeneic lung transplantation in our previous experiments (10). It is noteworthy that treatment with anti–VEGFR-3, a regimen that we and others have shown inhibits the growth of new lymphatic vessels, results in an increase in the size of the bronchus-associated lymphoid tissue in lung allografts (22, 41). This finding, combined with our observation that many lymphatic vessels in the bronchus-associated lymphoid tissue are filled with lymphocytes after administration of anti–VEGFR-3, lends further support to the notion that these lymphatics provide flow to the periphery. In support of this, we have shown that, in addition to Foxp3+ cells, other immune cells, such as CD8+ T lymphocytes, can also migrate from tolerant lung allografts to the periphery.

Foxp3+ cells play an important role in mediating transplantation tolerance. Consistent with our observations, previous experimental studies have shown that Foxp3+ cells home to allografts during the induction of tolerance. Also, positive correlations between the presence of Foxp3+ cells in murine and human allografts and graft survival or function have been reported (42, 43). Our observations extend a previous report that demonstrated the presence of regulatory CD4+ T cells in tolerant murine skin grafts (44). Interestingly, retransplantation of tolerant skin grafts onto immunodeficient mice revealed that T cells can exit the graft and suppress alloantigen-specific immune responses in the periphery. Although migratory requirements of T lymphocytes were not directly examined, it is likely that immune cells exit skin allografts throughafferent lymphatics (45). Our finding that the presence of a tolerant lung allograft is critical to induce tolerance to donor-matched hearts is somewhat reminiscent of previous observations in miniature swine kidney transplantation and murine heart transplant models (46, 47). However, in these models, the time taken to lose tolerance after removal of the primary graft was substantially longer compared with our study. We suggest that the continuous presence of donor alloantigen within the bronchus-associated lymphoid tissue of tolerant lung allografts is necessary for the expansion, survival, and/or activation of Foxp3+ cells. We have previously reported that recipient Foxp3+ cells accumulate in tolerant lung allografts following retransplantation into nonimmunosuppressed hosts. This raises the possibility that Foxp3+ cells that reside within tertiary lymphoid organs of tolerant lung allografts may be able to confer regulatory properties upon other T cells, a process referred to as infectious tolerance (48, 49). Thus, while it is conceivable that Foxp3+ cells that originate from tolerant lung allografts promote infectious tolerance in the periphery, the rejection of heart grafts after anti–VEGFR-3 antibody–mediated inhibition of lymphatic regrowth and the rejection of third-party grafts demonstrate that

---

**Figure 7. Inhibition of lymphatic regrowth after lung transplantation prevents induction of peripheral tolerance.** Intravital 2-photon imaging of (A) retransplanted (Retxp) tolerant BALB/c lung allograft (n = 2) and (B) BALB/c cardiac allograft (n = 2) 6 days after transplantation of BALB/c heart into a B6 mouse into which a tolerant BALB/c lung was retransplanted 21 days prior and received anti–VEGFR-3 between the time of lung retransplantation and heart transplantation (Foxp3+ cells: green; quantum dot–labeled [q-dot–labeled] vessels: red; second harmonic generation [SHG]: blue). The BALB/c lung had been originally transplanted into a B6 Foxp3 IRES-GFP mouse (treated with perioperative costimulatory blockade [CSB]) at least 30 days before the retransplantation procedure. (C) Quantification of density of Foxp3-GFP cells in BALB/c hearts (up to 5 separate areas/graft were examined) 6 days after transplantation (Txp) into B6 recipients of tolerant BALB/c lung allografts that received no treatment or were treated with anti–VEGFR-3 for 21 days before cardiac transplantation. Data are expressed as mean ± SEM. Mann-Whitney U test was used to compare the means. (D) Kaplan-Meier survival curves of BALB/c hearts that were transplanted into nonimmunosuppressed B6 mice, (●) untreated (n = 7) or (△) treated with anti–VEGFR-3 (n = 3), that received tolerant BALB/c pulmonary allografts 21 days before cardiac transplantation. The BALB/c lungs had been originally engrafted into B6 mice that received perioperative costimulatory blockade and then retransplanted at least 30 days later. (E) Histological appearance (H&E) of rejected BALB/c hearts after transplantation into anti–VEGFR-3–treated B6 mice into which a tolerant BALB/c lung allograft was retransplanted 21 days prior. Scale bars: 30 μm (A and B) and 100 μm (E).
recipient regulatory T cells alone are not sufficient to mediate the survival of heart grafts.

We have recently reported that IL-22 production by innate lymphoid cells type 3 and γδ T cells plays a critical role in inducing organized bronchus-associated lymphoid tissue in tolerant lung allografts (14). In the absence of IL-22, Foxp3-rich lymph follicles are induced in lung allografts, but B cells are not recruited to these structures and peripheral nodal addressin-expressing high endothelial venules are not formed. However, VEGFR-3+ lymphatic vessels develop in proximity to the Foxp3+ aggregates (data not shown), indicating that induction of high endothelial venules and lymphangiogenesis are regulated through different mechanisms. Furthermore, IL-22-deficient lung allograft recipients accept donor-matched cardiac allografts long term, indicating that high endothelial venules within the transplanted lungs are not necessary to mediate peripheral tolerance (data not shown). Several cell populations, including dendritic cells and macrophages, as well as soluble mediators such as IL-17 or VEGF-C have been suggested to play a role in the regulation of lymphangiogenesis during inflammation (50–53). As cellular and molecular requirements that regulate the induction of tertiary lymphoid organs may depend on the anatomical site and the nature of the inflammatory signal, future studies will need to determine how lymphangiogenesis is induced in the bronchus-associated lymphoid tissue in tolerant lung allografts (54–56).

Lymphangiogenesis has been reported in tertiary lymphoid organs surrounding tumors. These lymphatic vessels can provide avenues for metastatic spread of cancer cells. Interestingly, recent work has shown that, similar to our observations in tolerant lung allografts, Foxp3-rich tertiary lymphoid organs can also form in association with tumors (57). Therefore, our findings raise the possibility that growth of metastatic tumor deposits may be facilitated by lymphatic migration of Foxp3+ cells that recognize tumor antigens from the primary tumor site to the periphery. Similarly, one can envision that effector T cells may leave tertiary lymphoid organs that are induced in the setting of cancer or autoimmune disease and promote cytotoxic or inflammatory responses at other sites (58). Future studies will need to elucidate the chemotactic cues that promote lymphatic egress of immune cells from tertiary lymphoid organs.

**Methods**

**Mice and reagents.** C57BL/6 (B6), B6 CD45.1, BALB/c, CBA, B6 Foxp3-DTR, and B6 Foxp3-ires-GFP mice were purchased from The Jackson Laboratory. As indicated, select lung and heart transplant recipients were treated with perioperative costimulatory blockade consisting of anti-CD40 ligand (250 μg intraperitoneally [i.p.], day 0) and CTLA-4-Ig (200 μg i.p., day 2) (Bio X Cell). Diphertheria toxin (DT) (List Biological Laboratories Inc.) (500 ng i.p. days 0, 1, and 7) was given to select B6 Foxp3-DTR heart transplant recipients to deplete Foxp3+ cells. Select lung transplant recipients received treatment with anti-VEGFR-3 antibody (clone mF4-3I1) (provided by Eli Lilly) (60 mg/kg, i.p. day 0; 30 mg/kg day 2; and then q.o.d. until day 21) or VEGF-C156S (R&D Systems) (125 μg/kg, intravenously q.d. for 7 days). Six- to 10-week-old mice were used for these studies. Gender-matched mice were used for the transplant procedures.


28. Nykänen AI, et al. Targeting lymphatic vessel activation and CCL21 production by vascular endothelial growth factor receptor-3 inhibition has novel immunomodulatory and antiarterio-