Presence of Donor Lymph Nodes Within Vascularized Composite Allotransplantation Ameliorates VEGF-C-mediated Lymphangiogenesis and Delays the Onset of Acute Rejection

Radu Olariu, MD,[1,2] Catherine Tsai, MD,[1,2] Mai Abd El Hafez, PhD,[1,3] Anastasia Milusev, MSc,[1] Yara Banz, PhD,[4] Ioana Lese, MD,[1,2] Jonathan I. Leckenby, MD,[5] Mihai Constantinescu, PhD,[1,2] Robert Rieben, PhD,[1] Esther Vögelin, PhD,[1,2] and Adriano Taddeo, PhD[1,2,6]

Background. The lymphatic system plays an active role in modulating inflammation in autoimmune diseases and organ rejection. In this work, we hypothesized that the transfer of donor lymph node (LN) might be used to promote lymphangiogenesis and influence rejection in vascularized composite allotransplantation (VCA). **Methods.** Hindlimb transplantations were performed in which (1) recipient rats received VCA containing donor LN (D:LN$^+$), (2) recipient rats received VCA depleted of all donor LN (D:LN$^-$), and (3) D:LN$^+$ transplantations were followed by lymphangiogenesis inhibition using a vascular endothelial growth factor receptor-3 (VEGFR3) blocker. **Results.** Our data show that graft rejection started significantly later in D:LN$^+$ transplanted rats as compared to the D:LN$^-$ group. Moreover, we observed a higher level of VEGF-C and a quicker and more efficient lymphangiogenesis in the D:LN$^+$ group as compared to the D:LN$^-$ group. The presence of donor LN within the graft was associated with reduced immunoactivation in the draining LN and increased frequency of circulating and skin-resident donor T regulatory cells. Blocking of the VEGF-C pathway using a VEGFR3 blocker disrupts the lymphangiogenesis process, accelerates rejection onset, and interferes with donor T-cell migration. **Conclusions.** This study demonstrates that VCA LNs play a pivotal role in the regulation of graft rejection and underlines the potential of specifically targeting the LN component of a VCA to control graft rejection.

(*Transplantation* 2021;105: 1747–1759).

INTRODUCTION

Aside from its recognized role in the regulation of fluid balance, lipid metabolism, and immune cell trafficking to lymph nodes (LN), the lymphatic system plays an active role in modulating inflammation in autoimmune diseases and organ rejection.[1-3] Lymphangiogenesis specifically describes the formation of new lymphatic vessels from preexisting vessels and occurs in many experimental and clinical conditions including transplantation. This process is primarily mediated by the signaling of the vascular endothelial growth factor (VEGF)-C through its receptor VEGFR3 that is mainly expressed in lymphatic endothelial cells (LECs) after embryogenesis.[4-6] Other mediators include VEGF-D, which also bind to VEGFR3, fibroblast growth factors, ephrin-B2, and hyaluronic acid.[5] Studies in solid organ transplantation have shown that lymphangiogenesis plays an organ-specific,

Received 16 June 2020. Revision received 14 October 2020.

Accepted 12 November 2020.

[1] *Department for BioMedical Research, University of Bern, Bern, Switzerland.*

[2] *Department of Plastic and Hand Surgery, Inselpital, University Hospital, University of Bern, Bern, Switzerland.*

[3] *Pharmacology and Toxicology Department, Faculty of Pharmacy, October University for Modern Sciences and Arts, Giza, Egypt.*

[4] *Institute of Pathology, University of Bern, Bern, Switzerland.*

[5] *University of Rochester Medical Center, School of Medicine and Dentistry, Rochester, MN.*

[6] *Institute of Virology and Immunology, Mittelhäusern, Switzerland.*

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs through the Reconstructive Transplant Research Program under Award No. W81XWH 17-1-0686 to A.T. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense. The work was also supported partially by funds from the American Foundation for Surgery of the Hand (Award 1404 to A.T.).

The authors declare no conflicts of interest.

R.O. designed the surgical model and performed the surgery with C.T., I.L., and J.I.L. C.T., M.A., and A.M. performed the in vivo and ex vivo evaluations. Y.B. performed all histological processing and evaluation. J.I.L., M.A.C., R.R., and E.V. participated in data interpretation and provided experimental support. A.T. designed the study; provided the funding; interpreted data; was responsible for the primary undertaking, completion, and supervision of all experiments; and wrote the article. All authors interpreted data, revised the article, and gave their final approval.

R.O. and C.T. contributed equally to this work.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Correspondence: Adriano Taddeo, PhD, Current address: Institute of Virology and Immunology, Mittelhäusern 3147, Switzerland. (adriano.taddeo@ivi.admin.ch).

ISSN: 0041-1337/21/1058-1747

DOI: 10.1097/TP.0000000000003601

<zdoi;10.1097/TP.0000000000003601>

heterogeneous role in graft rejection, driving immunity versus tolerance according to demand.[5,7,8] Lymphangiogenesis may propagate the detrimental immune response toward the graft as shown in heart, cornea, and pancreatic islet transplantations.[9-11] Alternatively, it may contribute to the removal of hyaluronic acid and inflammatory cells through improved graft drainage, preventing rejection of lung transplants or chronic skin inflammation.[12,13] Recent studies have given rise to the idea that LN may work as a "peripheral tolerance hub" owing to the functional organization of LN stromal cells (LNSCs) and LECs.[13,14] Indeed, these cells may promote tolerance in 2 ways: indirectly by guiding cellular localization and directly through the expression of genes encoding peripheral tissue antigens and through presentation of these on major histocompatibility complex (MHC I) and II molecules (reviewed in Hirosue and Dubrot[15]). Accordingly, it has been shown that peripheral tissue antigen presentation mediated by LEC and LNSCs may induce antigen-specific tolerance and regulatory T cells (T_{reg}) expansion in mouse models.[15,16] This makes the lymphatic system an attractive target for immunotherapeutic interventions aiming to eradicate tumors, reestablish tolerance in autoimmune disease, promote better vaccination responses, and induce tolerance of transplanted grafts.[1,17,18]

Notably, little is known about the process of lymphatic reconstitution and how this process influences graft rejection in vascularized composite allotransplantation (VCA), and more importantly, the possibility of using lymphatic-targeted therapies to promote VCA tolerance is completely unexplored. VCA, including hand and face transplantation, is emerging as a treatment option for complex functional deficiency and extensive tissue loss or destruction not amenable to conventional reconstruction.[19,20] Different therapies are currently under investigation for immune regulation and tolerance induction in VCA: costimulatory blockade, promotion of mixed chimerism, and regulatory cell transfer.[21,22] However, due to the specific immunobiological challenges associated with VCA, we believe there is little chance that single pieces of the tolerance puzzle taken in isolation can be successful in the clinical management of VCA. A vascularized composite allograft comprises different tissue types such as skin, bone, bone marrow, muscle, nerves, blood vessels, and others. In this graft, antigens are transferred through the lymphatic circulation from the different graft tissues to the specific draining LN. These LNs may be either of donor or recipient origin depending on the tissue and the modalities of the surgical operation.[23] In this work, we hypothesized that lymphangiogenesis and the transfer of donor LN may be used to influence the immunobiology of the VCA rejection process. Moreover, we characterized lymphatic reconstitution in VCA and identified a relevant pathway in the lymphangiogenic process of VCA recipients.

MATERIALS AND METHODS

Experimental Design

The Brown-Norway to Lewis rat hindlimb transplantation model was used to test whether the transfer of donor LN within the VCA may influence the rejection process. Lewis rats received either grafts with intact, vascularized inguinal and popliteal LN, or fully lymphadenectomized grafts.[24] After transplantation, rats were observed and rejection was graded macroscopically until full rejection occurred (experiment endpoint, Figure 1A). To assess the lymphangiogenic process, rats underwent daily lymphography with near-infrared imaging starting from postoperative day 2 (POD2). Whole blood was sampled and analyzed on POD7. At the endpoint, tissue analysis (eg, LN, skin, blood) was performed using flow cytometry, histology, and immunofluorescence. All the experimental procedures were approved by the Veterinary Office of the Canton Bern (approval number: BE52/16), and the animals were treated according to the Animal Welfare Act and Ordinance of the Swiss Animal Welfare Legislation during the entire experiment. Detailed material and methods are available in the Supplemental Material (SDC, http://links.lww.com/TP/C93) that may be found in the online version of this article.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 8. Unless noted otherwise, the results are expressed as means ± SD. Onset of rejection, allograft survival, indocyanine green (ICG) suture line crossing, and track appearance were examined using Kaplan-Meier analysis, and groups were compared using the log-rank test. A 2-tailed *t* test was used to compare 2 groups; 1-way analysis of variance with Tukey's multiple comparisons test was used to compare means of >2 groups; paired *t* tests and Fisher exact test were used when appropriate as reported in the text and figure legends. Statistical significance was defined as $P < 0.05$.

RESULTS

Donor Lymph Node Transfer Delays Graft Rejection

To evaluate the effects of donor LN transfer on VCA rejection, 2 kinds of hindlimb transplantations were performed (n = 8/group): (1) recipient rats receiving VCA containing native donor LN (D:LN$^+$) and (2) recipient rats receiving VCA depleted of all donor LN (D:LN$^-$). Two blinded investigators graded clinical rejection macroscopically (Figure 1A). As shown in Figure 1B, acute rejection onset (ie, time before the appearance of the first signs of rejection in the graft, with rats reaching grade 1) was significantly delayed in rats that received grafts containing donor LN (D:LN$^+$) as compared to D:LN$^-$ (median rejection onset time 5.0 and 4.5 d, respectively; $P = 0.033$). Overall graft survival did not change significantly between the groups (median survival time 8 and 8.5 d in D:LN$^+$ versus D:LN$^-$, respectively; $P = 0.499$) (Figure 1C). Histopathological rejection grading based on Banff working classification, including analysis of the vascular damage,[25] confirmed rejection and the end points for all the groups without significant differences in histopathology (Figure S1, SDC, http://links.lww.com/TP/C93).

Donor Lymph Node Transfer Promotes Lymphangiogenesis

Lymphography analysis showed that on POD2, the lymphatic drainage was almost absent with a strong lymphostasis of the ICG dye at the injection site for the 2 groups (Figure 2A). Interestingly, in rats receiving grafts containing donor LN (D:LN$^+$), we observed drainage of the dye into the recipient draining basins on POD3. Moreover, in this group, we could visualize the appearance of segmental drainage with visible lymphatic tracks on the following day. However, lymphatic drainage was disrupted by the rejection process and the lymphatic tracks were lost after POD6

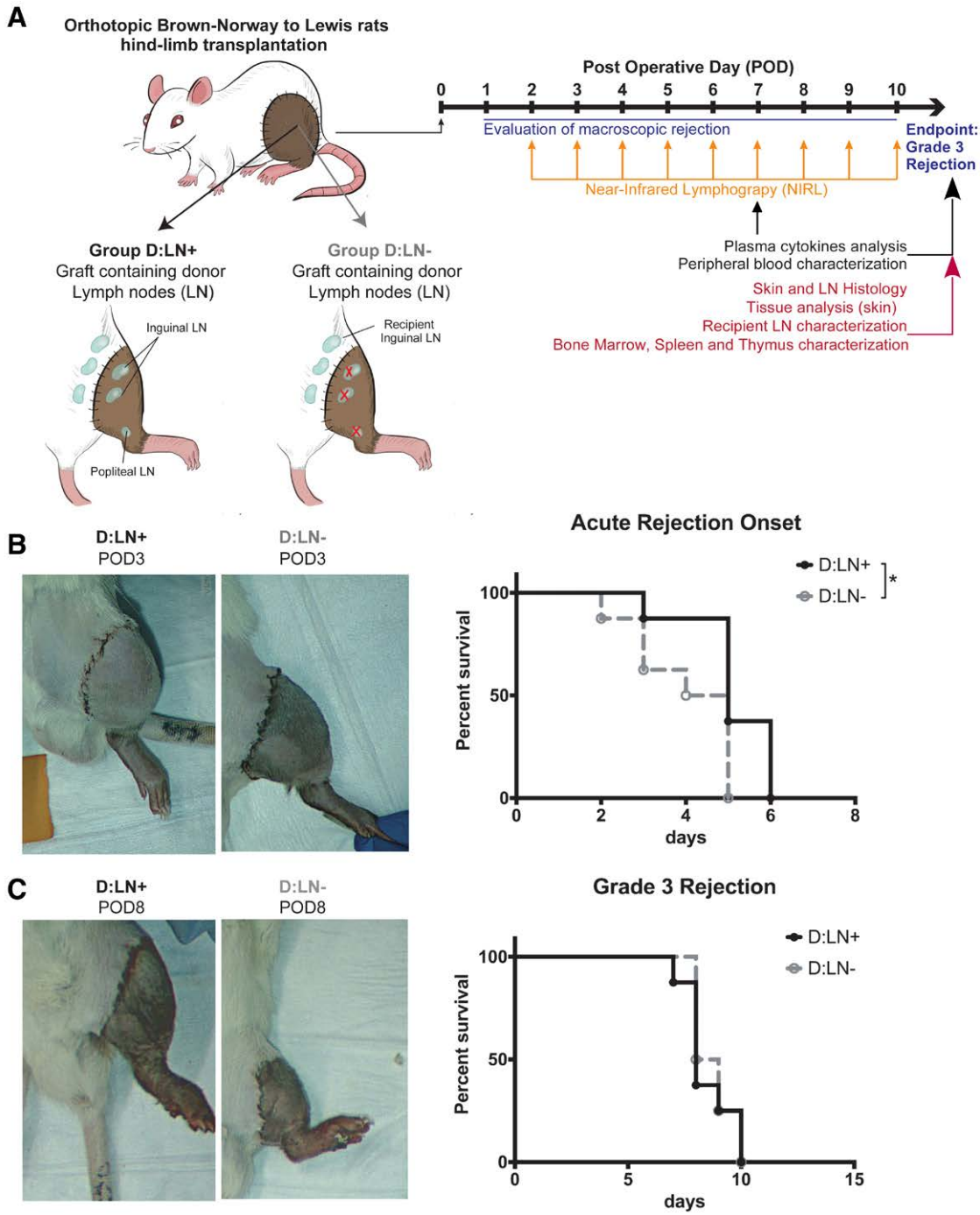

FIGURE 1. Donor LN transfer delay rejection of vascularized composite allotransplants. A, Schematic representation of the experimental design with recipients receiving either graft containing donor LNs (D:LN$^+$, n = 8) or grafts depleted of donor LN (D:LN$^-$, n = 8) and timeline of the analyses performed. Rejection was graded macroscopically as follows: 0 = no rejection, 1 = erythema and edema, 2 = epidermolysis and exudation, and 3 = desquamation, necrosis, and mummification. The rats were euthanized once grade 3 was reached. B, Representative pictures of graft rejection at POD3 in D:LN$^+$ and D:LN$^-$ recipients and onset of acute rejection (ie, time before showing signs of rejection, reaching grade 1) represented with Kaplan-Meier survival curves or rejection grading. C, Representative pictures of graft rejection at POD8 in D:LN$^+$ and D:LN$^-$ and overall graft survival (ie, time to grade 3 rejection) represented with Kaplan-Meier survival curves. *$P < 0.05$ calculated by the Mantel-Cox test. LN, lymph node; POD, postoperative day.

(Figure 2A). Accordingly, lymphatic track score increased only transiently between POD3 and POD6 (Figure 2B). In rats receiving grafts depleted of donor LN (D:LN$^-$), we observed crossing of the suture line significantly later than in the D:LN$^+$ group (median crossing day: POD6 in D:LN$^-$ and POD3 in D:LN$^+$, $P = 0.0253$) and a later appearance of draining lymphatic tracks (median appearance: POD7 in D:LN$^-$ and POD4 in D:LN$^+$, $P = 0.0253$). Notably, despite lymphatic reconstitution, all grafts demonstrated persistent slow lymphatic egress and ICG patterning did not return to that found in the native limbs (Figure 2A and Figure S2, SDC, http://links.lww.com/TP/C93).

To better evaluate lymphangiogenesis, we performed immunostaining of lymphatic vessels in VCA skin at

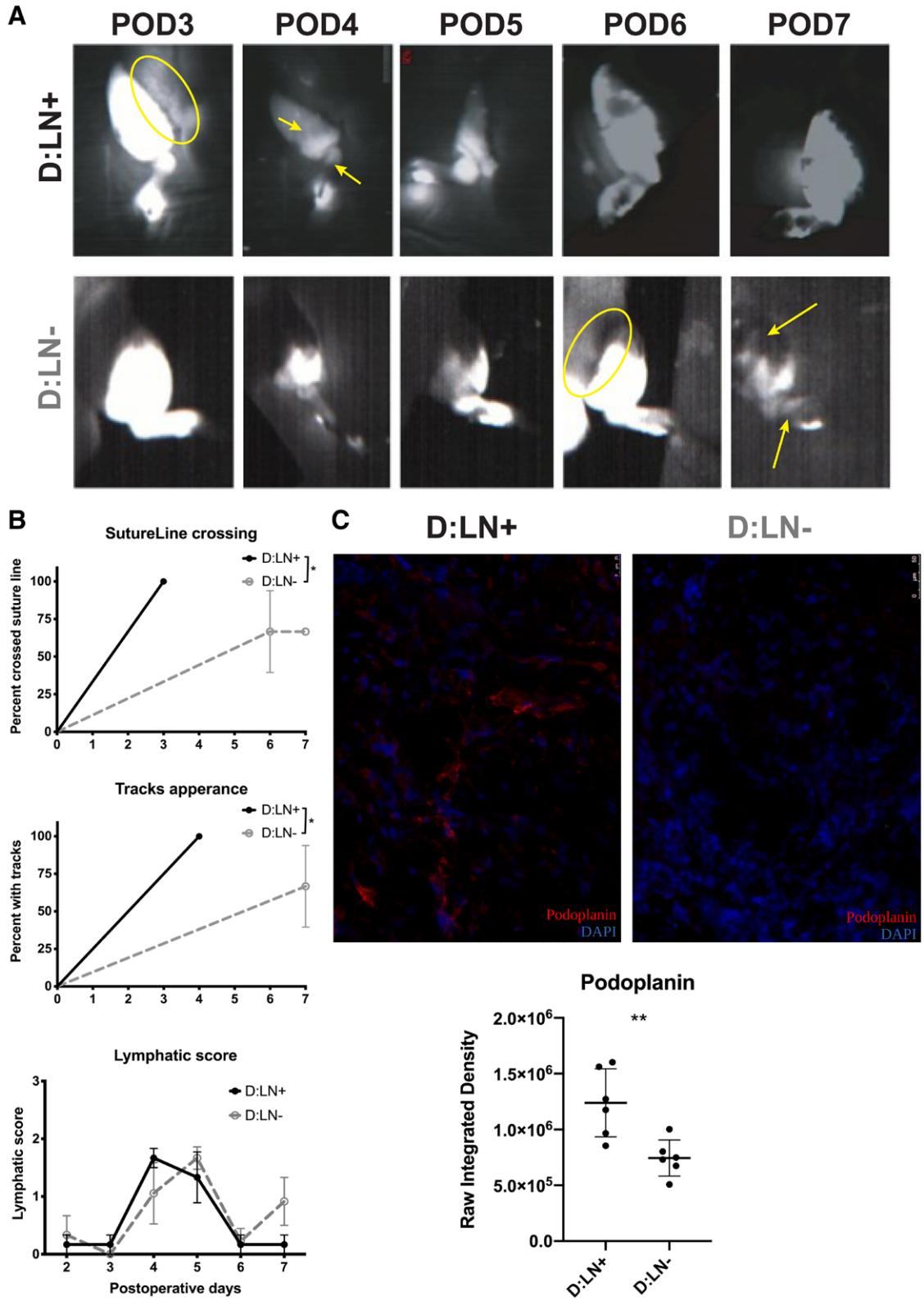

FIGURE 2. Donor lymph node transfer promotes lymphangiogenesis. Evaluation of lymphatic reconstitution after hindlimb transplantation by Visionsense VS3 Iridium analysis. A, Black/white near-infrared signal visualization after ICG injection in D:LN$^+$ (upper row) and D:LN$^-$ rats (lower row). Yellow circles represent the draining basins across the suture line and yellow arrows the appearance of lymphatic tracks. B, Analysis of the lymphatic reconstitution was performed and the day of suture line crossing or appearance of lymphatic track was indicated and the difference was analyzed by the Mantel-Cox test $^*P<0.05$. Density score of the lymphatic tracks was assessed in all the rats at the different time points as 0 = no visible tracks, 1 = low-density tracks, 2 = medium-density tracks, 3 = high-density tracks. Error bars report SE. C, Evaluation of lymphatic vessel by podoplanin expression in the VCA skin at the end point. Representative pictures in the D:LN$^+$ (left, n = 6) and D:LN$^-$ (right, n = 6) groups (podoplanin—red, DAPI—blue) and quantification raw integrated density of podoplanin expression (lower plot) are shown. Data are presented as mean and SD, $^{**}P<0.01$ by 1-way ANOVA with Tukey's multicomparisons test. ANOVA, analysis of variance; D:LN, donor lymph node; ICG, indocyanine green; VCA, vascularized composite allotransplantation.

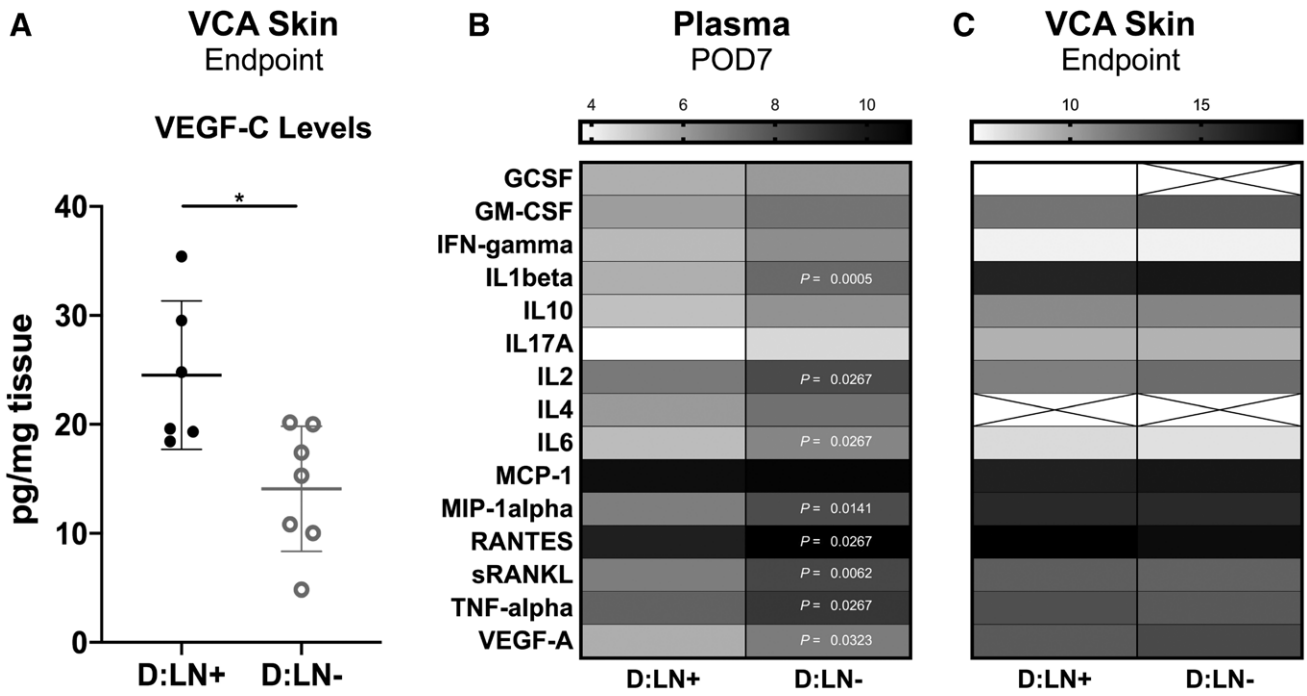

FIGURE 3. Donor LN transfer increased the levels of VEGF-C in the graft and reduced inflammatory response. A, VEGF-C concentration (pg/mg tissue) in the skin retrieved from the graft at the endpoint in rats receiving VCA containing native donor LN (D:LN$^+$, n = 6) or rats receiving VCA depleted of all donor LN (D:LN$^-$, n = 7). Data are presented as individual values with mean and SD. *$P < 0.05$ calculated by the Student *t* test. B, Heatmap showing changes in abundances of 15 absolutely quantified cytokines in plasma collected at POD7 from rats receiving VCA containing donor LN (D:LN$^+$, n = 6) and rats depleted of all the donor LN (D:LN$^-$, n = 6). Individual protein levels measured as fluorescence intensity were log2 transformed. White boxes correlate with lower protein concentration and black boxes with higher protein concentration. Numbers represent the *P* value of 2-sample *t* tests with the Holm-Sidak correction to compare the 2 groups. C, Heatmap showing changes in abundances of 15 absolutely quantified cytokines in skin retrieved at grade 3 rejection from rats of the D:LN$^+$ (n = 6) or D:LN$^-$ (n = 6) groups. Individual protein pictogram abundance per milligram of tissue was log2 transformed. Statistically significant changes were not observed. D:LN, donor lymph node; ICG, indocyanine green; POD, postoperative day; VCA, vascularized composite allotransplantation; VEGF-C, vascular endothelial growth factor-C.

rejection using the lymphatic endothelium-specific marker podoplanin. As shown in Figure 2C, podoplanin expression was significantly higher in the D:LN$^+$ group as compared to the D:LN$^-$ group (signal raw integrated density 1 238 984 ± 304 965 and 744 519 ± 161 922, respectively; $P = 0.006$). In normal skin retrieved from the contralateral recipient limb, podoplanin expression did not differ between the 2 groups, but in both groups, it was significantly lower as compared to matched transplanted skin, consistently with the elevated inflammatory status at rejection in VCA skin (Figure S3, SDC, http://links.lww.com/TP/C93).

Donor Lymph Node Presence Increases VEGF-C Levels in VCA Skin

Considering the importance of the VEGF-C pathway in the lymphangiogenic process, we quantified the amount of this cytokine in VCA skin at rejection. VEGF-C was lower than the detection range in plasma at POD7 in both groups (data not shown). However, it was found at significantly higher concentrations in the VCA skin of the D:LN$^+$ group as compared to that of the D:LN$^-$ group (24.51 ± 6.81 and 14.09 ± 5.73 pg/mg tissue, respectively; Figure 3A).

To evaluate the inflammatory response following LN transfer, we measured the levels of 15 inflammatory cytokines in plasma at POD7 and in VCA skin at rejection. In the plasma collected on POD7, 8 out of the 15 measured cytokines (ie, interleukin [IL]1β, IL2, IL6, MIP-1α, RANTES, sRANKL, tumor necrosis factor-α, and VEGF-A) were present in significantly lower levels in rats receiving donor LN-containing grafts (D:LN$^+$) as compared to the group without donor LN (D:LN$^+$) (Figure 3B). This is in line with the finding that the transfer of donor LN delays graft rejection and thus inflammatory reaction. There was no difference in the amount of cytokines in the skin when comparing the 2 groups at rejection (Figure 3C), supporting the idea that at the endpoint (grade 3 rejection), inflammation was comparable between the 2 groups.

VEGFR3 Blockade Resets the Effect of Donor Lymph Node Transfer

VEGF-C is an essential stimulator of lymphangiogenesis due to its ability to activate VEGFR3 expressed on LECs.[4] Therefore, a selective blocker of the receptor VEGFR3 that inhibits VEGF-C-induced activation of VEGFR3[26] was injected intradermally in the transplanted limb daily from POD0 to verify the importance of this cytokine in delayed rejection onset and improved lymphangiogenesis observed following donor LN transfer (Figure 4A). Blocking the lymphangiogenesis process using the VEGFR3 blocker accelerated graft rejection as compared to the matched D:LN$^+$ untreated grafts (median rejection onset time 3, 5.0, and 4.5, respectively; $P = 0.021$; Figure 4B), making the rejection-free period similar to that of the D:LN$^-$ group ($P = 0.453$). Overall, graft survival and histopathological score did not change among the groups (Figures S4 and S1, respectively, SDC, http://links.lww.com/TP/C93). As expected, VEGFR3

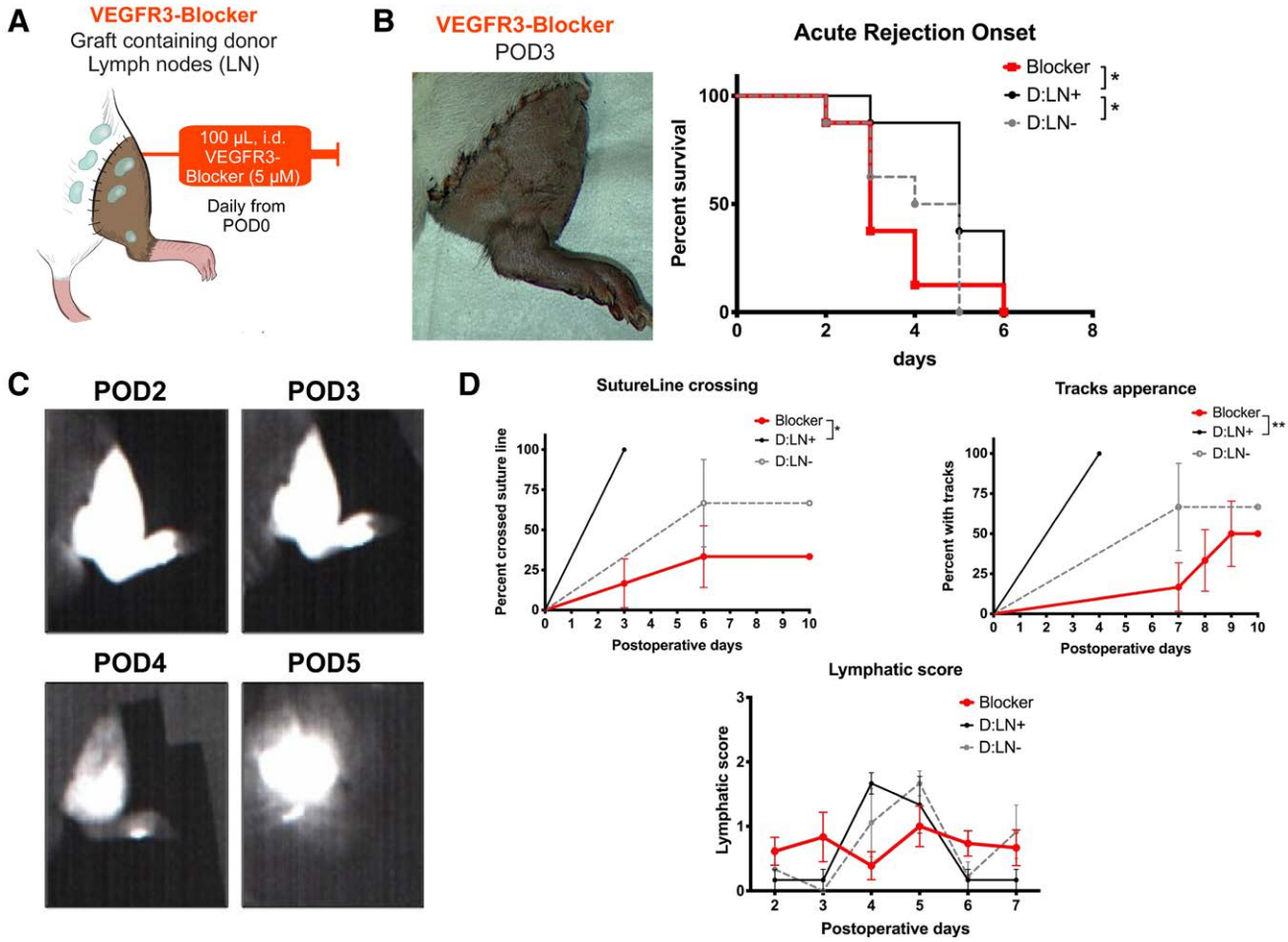

FIGURE 4. Blockage of the VEGF-C pathway resets the effects of D:LN transfer. A, Schematic description of the VEGFR3 blocker group. B, Representative pictures of graft rejection at POD 3 in rats receiving grafts containing D:LN treated with VEGFR3 blocker and onset of acute rejection (ie, time before showing signs of rejection, reaching grade 1) represented with Kaplan-Meier survival curves or rejection grading in VEGFR3 blocker group (red line) as compared to the D:LN$^+$ and D:LN$^-$ groups (from Figure 1). *$P < 0.05$ calculated by the Mantel-Cox test. C, Black/white near-infrared signal visualization after ICG injection in the VEGFR3 blocker group. D, Analysis of the lymphatic reconstitution with evaluation of suture line crossing, appearance of, and density score of the lymphatic tracks. Error bars report SE, *$P < 0.05$ by the Mantel-Cox test. D:LN, donor lymph node; ICG, indocyanine green; POD, postoperative day; VEGF-C, vascular endothelial growth factor-C; VEGFR3, vascular endothelial growth factor receptor-3.

blockade impaired the lymphangiogenesis process inducing a prolonged lymphostasis (Figure 4C). As compared to the D:LN$^+$ untreated group, VEGFR3 blockade decreased the speed and the quality of lymphatic reconstitution with a significant delay in crossing of the suture line ("undefined day of crossing" in VEGFR3 blocker versus POD3 in D:LN$^+$; $P = 0.0253$) and time of appearance of lymphatic tracks ($P = 0.0047$), making the VEGFR3 blocker group similar to the D:LN$^-$ group (Figure 4D).

As expected, daily treatment with the VEGFR3 blocker that inhibits VEGF-C-induced activation of the receptor without blocking its ligand was not associated with significant changes in VEGF-C levels that remained comparable to the D:LN$^+$ group in the graft skin and in the contralateral skin (Figure S5, SDC, http://links.lww.com/TP/C93) and undetectable in plasma (data not shown).

Recipient Lymph Node Characterization After Donor Lymph Node Transfer

To understand how the presence of donor LN affected the graft draining LN, we analyzed LN retrieved from VCA recipients by histology and flow cytometry. All transplant draining LN retrieved from recipients receiving LN-containing grafts (D:LN$^+$, n = 4) showed minimal and nonspecific reactive changes at rejection with mild histopathological stigmata of immune stimulation. Conversely, draining LN from the D:LN$^-$ group (n = 3) showed a prominent increase in the presence of follicular plasma cells (Figure 5A) with a significant difference as compared to the D:LN$^+$ group (Figure 5B; $P = 0.029$ by the Fisher exact test). Draining LN from the VEGFR3 blocker group showed both the patterns with 5 LNs having only minimal changes and 2 LNs with increased presence of follicular plasma cells (Figure 5B).

To characterize the cell dynamics within the LN after donor LN transfer or VEGFR3 blockade, we analyzed the differences in the cellular composition of the draining LN from the transplanted side and of the ones collected from the contralateral side at the endpoint (ie, grade 3 rejection). As shown in Figure 5C and D, VEGFR3 blockade was associated with a significant decrease of the frequencies of donor T cells in the draining LN as compared to untreated recipient receiving LN-containing grafts (0.45 ± 0.28 and 2.94% ± 2.56% of CD3 cells, respectively). There were no

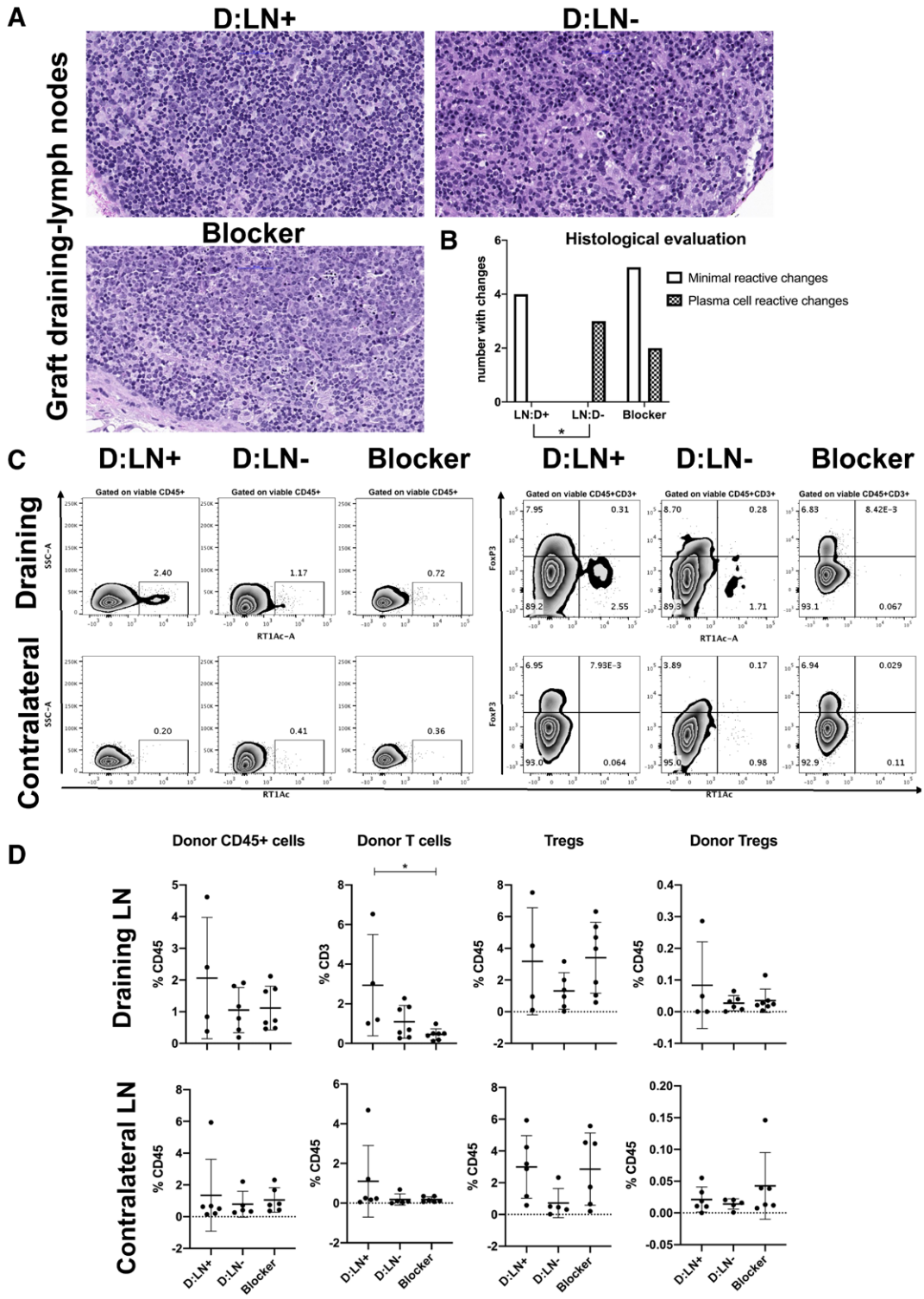

FIGURE 5. Recipients LNs characterization. A and B, Histological evaluation of draining (LN) in rats receiving VCA grafts contain donor LN (D:LN$^+$), depleted of donor LN (D:LN$^-$), or treated with VEGFR3 blocker (Blocker). A, Representative microphotographs of the histology sections of the draining LN stained with H&E showing a preserved lymph node architecture with minimal, nonspecific reactive changes in the D:LN$^+$ group (upper-left panel), prominent interfollicular plasmacytic reactive changes/plasmacytic hyperplasia in the D:LN$^-$ group (upper-right panel), and minimal reactive changes, with preserved LN architecture in the blocker group (lower-left panel). B, Summary and analysis of the findings in the 3 groups. *$P<0.05$ by the Fisher exact test. C and D, Evaluation of the cellular composition of the draining and the contralateral LN by flow cytometry. C, Representative flow cytometry for the characterization of the draining LN (upper row) or contralateral LN (lower row) in the 3 groups. Donor leukocytes were identified as RT1Ac$^+$ cells in the CD45$^+$ gate; donor T cells as CD45$^+$CD3$^+$RT1Ac$^+$, T_{reg} as CD45$^+$CD3$^+$FoxP3$^+$ cells, and donor T_{reg} as CD45$^+$CD3$^+$FoxP3$^+$RT1Ac$^+$ cells. D, Quantitative summaries of LN characterizations in the 3 groups. Data are presented as mean and SD, *$P<0.05$ by 1-way ANOVA with Tukey's multicomparisons test. ANOVA, analysis of variance; D:LN, donor lymph node; H&E, hematoxylin and eosin; POD, postoperative day; SSC, side-scatter; T_{reg}, T regulatory cell; VCA, vascularized composite allotransplantation; VEGFR3, vascular endothelial growth factor receptor-3.

differences in the frequency of T_{reg} or donor T_{reg} among the 3 groups. When we analyzed the inguinal LN retrieved from the contralateral side, we did not observe any significant change among the 3 groups (Figure 5D).

Presence of Graft Donor Lymph Node Impacted Chimerism Levels

Chimerism and the numbers of T_{reg} were analyzed in blood at POD7 (ie, before full rejection) and in blood and spleen at grade 3 rejection (endpoint). At POD7, we did not observe differences in the absolute number of donor leukocytes, monocytes, granulocytes, T helper, or T cytotoxic cells in the blood (Figure 6A). The D:LN$^+$ group showed an increase in the absolute number of circulating T_{reg} of donor origin as compared to the D:LN$^-$ group (0.799 ± 0.512 and 0.202 ± 0.331 cells/μL of blood, respectively; $P = 0.021$), and this effect was reverted by VEGFR3 blockade (0.213 ± 0.213 cells/μL of blood, $P = 0.047$ versus D:LN$^+$) (Figure 6A and B). When rats reached grade 3 rejection, the number of donor T_{reg} dropped significantly in the D:LN$^+$ group (Figure 6C) but did not change in the other 2 groups (Figure S6, SDC, http://links.lww.com/TP/C93).

Chimerism level was also analyzed in the spleen at the endpoint. As shown in Figure 6D and E, we observed an increased frequency of donor B cells in rats receiving VCA containing LN as compared to the D:LN$^-$ group (0.528 ± 0.121 and 0.240% ± 0.113 % of CD45$^+$ cells, respectively; $P = 0.009$). No other differences were observed in the frequencies of the cell populations analyzed.

Total T_{reg} numbers (ie, from donor and recipient) were not significantly different among the groups in blood at POD7 and in the spleen and blood at the endpoint (Figure S7, SDC, http://links.lww.com/TP/C93).

Tertiary Lymphoid Organ Formation at Rejection Is Not Affected by LN Transfer.

Tertiary lymphoid organs (TLOs) are tissue structures that resemble secondary lymphoid tissue in their organization and that are formed following inflammation. TLOs are characterized by the presence of high endothelial venules surrounded by a cluster of B cells.[27] To investigate where TLO structures are present during VCA rejection and the effects of donor LN transfer and lymphangiogenesis blocking on TLO formation, we performed immunofluorescence analysis of the VCA skin at rejection in the 3 study groups. Consistent with the presence of inflammatory status due to VCA rejection, we identified the structure of B220$^+$ B cells organized around cells expressing a specific marker of high endothelial venule: peripheral lymph node addressin (PNAd), a cellular organization typical of TLO (Figure 7A). Quantification of TLO formation at rejection as well as quantification of the expression of the PNAd marker did not show significant differences among the 3 groups (number of TLO per section 2.0 ± 1.4, 2.6 ± 0.8, and 3.5 ± 2.4, mean gray values of PNAd expression 54.7 ± 13.3, 86.0 ± 19.5, and 92.5 ± 52.0 in D:LN$^+$, D:LN$^-$ and VEGFR3 blocker, respectively; Figure 7B and C). Accordingly, flow cytometric analysis of the VCA skin revealed that B-cell infiltration in the skin was comparable in the 3 groups (Figure 7D). However, we observed an increased frequency of donor T cells in the skin of grafts containing donor LN at rejection as compared to the D:LN$^-$ group (0.347 ± 0.293 and 0.001% ± 0.002% of CD45$^+$ cells, respectively; $P = 0.03$; Figure 8). Also, T_{reg} frequencies were higher in D:LN$^+$ as compared to both D:LN$^-$ and VEGFR3 blocker groups (0.729 ± 0.492, 0.000 ± 0.000, and 0.574% ± 0.387 % of CD45, respectively). Of these T_{reg}, a significantly higher portion was of donor origin in the D:LN$^+$ as compared to the D:LN$^-$ (24.73 ± 23.34 and 0.00% ± 0.00% of total T_{reg}, respectively; Figure 8).

DISCUSSION

In this study, we investigated the potential of manipulating the lymphatic system to influence acute VCA rejection. Using a surgical model in which we transplanted donor LN within vascularized allografts, we demonstrated that donor LN transfer delays the onset of graft rejection by improving lymphatic drainage through the secretion of VEGF-C. This is accompanied by milder immunoactivation with cues of a potential switch toward immunoregulation.

Disruption of lymphatic channels is a natural consequence of transplantation. Because surgical reconnection is not performed in most of the cases, regeneration of the severed lymphatic vessels must occur spontaneously. Studies have shown that regrowth dynamics of lymphatic vessels differ among organ transplants and models with evidence of small/superficial lymphatic reconstitution as early as 3 days after kidney and lung transplantation.[5] In line with our observation, it has been reported that a superficial lymphatic donor/recipient network is established at POD5 in animal models of VCA.[28] Here, we show that the presence of donor LN within the graft hastens the establishment of the superficial donor/recipient network to POD3 as compared to POD4 in rats receiving LN-depleted graft. Moreover, LN transfer improves lymphatic track appearance and increases the expression of the lymphatic marker podoplanin in the skin. Improved lymphangiogenesis is associated with increased concentration of VEGF-C in the skin, reduced systemic inflammation at POD7, and delayed onset of graft rejection. This observation is in apparent contrast to published studies on heart, cornea, and islet transplantations where it has been shown that lymphatic circulation is primarily harmful to allografts.[9-11] However, recent literature has started to uncover a protective role of lymphangiogenesis after solid organ transplantation. Pedersen et al[29] have shown that induced expression of VEGF-C in kidney allografts induced lymphangiogenesis and attenuated rejection prolonging recipient survival. Cui et al[12] have shown that lymphatic vessels may serve a beneficial role in lung transplantation, contributing to reduction of inflammation, in part, through facilitating pulmonary drainage and immune cell transport early after transplantation. An important consideration is that in VCA grafts, acute rejection may be initiated in situ. Indeed, unlike hearts, lungs and VCA do not strictly require lymphatic flow to draining LN to trigger rejection because they may provide a suitable environment for the activation of alloimmune responses within the graft,[30] as demonstrated by the appearance of TLOs in graft skin in the recipients of all the groups. Therefore, in these organs, lymphatic drainage seems to facilitate postsurgical edema resolution and debris clearance, reducing inflammation. In line with this hypothesis, rats of the D:LN$^+$ groups showed reduced levels of inflammatory cytokines at POD7 in plasma and reduced immune activation in the draining

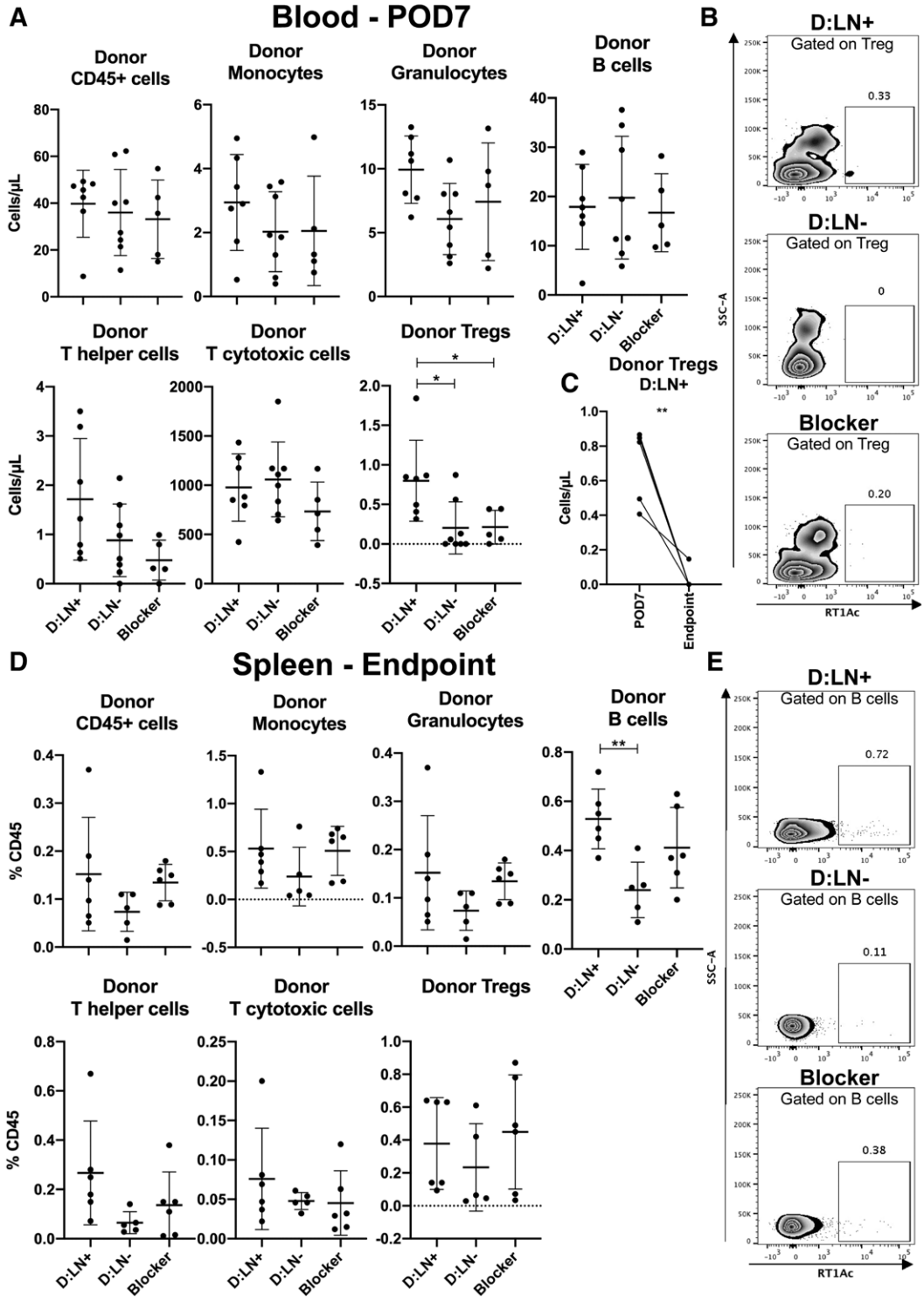

FIGURE 6. Effects of D:LN transfer on chimerism levels. A, Absolute number (expressed as cell/µL of blood) of donor leukocytes (identified as $CD45^+RT1Ac^+$ cells), donor monocytes ($CD45^+CD4^+CD3^-RT1Ac^+$), donor granulocytes ($CD45^+CD4^+CD3^-FSC^{high}SSC^{high}RT1Ac^+$), donor B cells ($CD45^+CD3^-CD4^-FSC^{low}SSC^{low}RT1Ac^+$), donor T helper cells ($CD45^+CD3^+CD4^+RT1Ac^+$), T cytotoxic cells ($CD45^+CD3^+CD4^-RT1Ac^+$), and donor T_{reg} ($CD45^+CD3^+CD4^+CD25^+FoxP3^+RT1Ac^+$) in the peripheral blood at POD7. Data are presented as single values and SD. $^*P<0.05$, by 1-way ANOVA with Tukey's multiple comparisons test. B, Representative flow cytometry plots showing the quantification of donor T_{reg} in blood at POD7 in the $D:LN^+$ (upper plot, n = 7), $D:LN^-$ (middle plot, n = 8), and VEGFR3 blocker (lower plot, n = 5) groups. C, Comparison of the absolute number of donor T_{reg} in the peripheral blood of rats receiving LN-containing grafts (n = 5) measured at POD7 and at grade 3 rejection (endpoint). Data are presented as single values. $^{**}P<0.01$ by paired *t* test. D, Frequency (expressed as % of $CD45^+$ cells) of donor leukocytes, donor monocytes, donor granulocytes, donor B cells, donor T helper cells, T cytotoxic cells, and donor T_{reg} in the spleen retrieved at the endpoint. Data are presented as single values and SD. $^{**}P<0.01$, by 1-way ANOVA with Tukey's multiple comparisons test. E, Representative flow cytometry plots showing the quantification of donor B cells in spleen at rejection in the $D:LN^+$ (upper plot, n = 6), $D:LN^-$ (middle plot, n = 5), and VEGFR3 blocker (lower plot, n = 6) groups. ANOVA, analysis of variance; D:LN, donor lymph node; FSC, forward scatter; POD, postoperative day; SSC, side-scatter; T_{reg}, T regulatory cell; VEGFR3, vascular endothelial growth factor receptor-3.

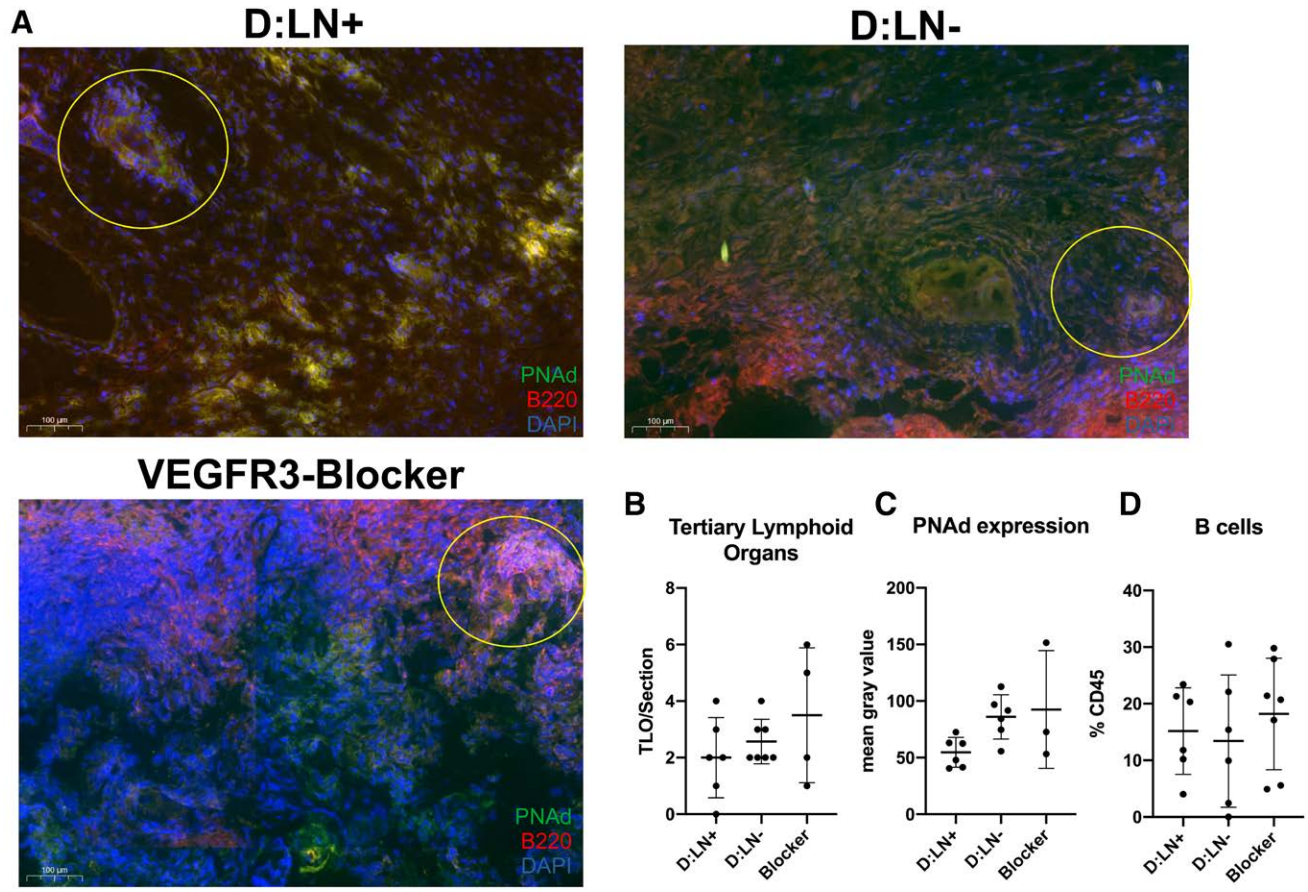

FIGURE 7. TLOs are present in VCA skin at rejection. A, Representative pictures of PNAd (green), B220 (CD45R, red), and DAPI (blue) staining in the D:LN$^+$ (n = 6), D:LN$^-$ (n = 7), and blocker (n = 4) groups. Yellow circles identify TLO. B, Quantification of TLO in a single skin section. C, Quantification of PNAd expression in the D:LN$^+$ (n = 6), D:LN$^-$ (n = 6), and blocker (n = 3) groups expressed as mean gray value. D, Frequency of skin-infiltrating B cells (identified as CD45$^+$CD3$^-$FSC^{low}SSC^{low}) quantified by flow cytometry after tissue digestion. Data are presented as mean and SD. D:LN, donor lymph node; FSC, forward scatter; PNAd, peripheral lymph node addressin; SSC, side-scatter; TLO, tertiary lymphoid organs; VCA, vascularized composite allotransplantation.

LN. Importantly, VEGFR3 blockade resets the effect of donor LN transfer in terms of lymphatic reconstitution and onset of rejection. Previous studies have revealed the potential of targeting the lymphatic system to control inflammation by administering the prolymphangiogenic factor VEGF-C.[31] Transgenic or virus-mediated delivery of VEGF-C potently alleviated chronic skin inflammation, rheumatoid arthritis, and inflammatory bowel disease (reviewed in Schwager and Detmar[31]). Here we show that endogenous VEGF-C production by donor LN may also be used to activate the lymphatic vasculature in VCA. In addition to endogenous production of VEGF-C from transferred LN, exogenous treatment may be applied to further increase lymphangiogenesis. To this aim, it will be important to carefully select VEGF-C delivery strategies. Indeed, in a preliminary experiment, we tried to use a recombinant VEGF-C. However, daily intradermal injection of a recombinant protein (125 μg/kg) did not show improvement of the lymphangiogenic process and, most importantly, we could not find an increased VEGF-C concentration in the skin of rats receiving the recombinant protein (data not shown). The lack of effectiveness of recombinant growth factors has already been reported, and it may be due to the widespread expression of cognate receptors and to the short half-life of unmodified growth factors.[13] Therefore, the use of specifically designed fusion proteins that are able to stabilize and implement the effect of VEGF-C[13] or transgenic or viral overexpression of VEGF-C[31] may be better delivery strategies.

Notably, in transplanted rats, tissue distribution of the ICG dye as well as its clearance was never comparable to naive rats, also in the D:LN$^+$ group. Therefore, although our lymphography analysis focused only on the superficial network, it is likely that deep-channel reconnection did not occur or was greatly impaired as demonstrated in other VCA models and patients.[32,33] Further studies are warranted to evaluate strategies aimed at improving lymphatic functions in VCA. Besides LN transfer and exogenous delivery of VEGF-C, direct lymphatic vessel anastomosis at the time of the transplant has been suggested to improve fluid drainage in patients, albeit technically difficult.[33] A more practical approach is the use of topical tacrolimus. This treatment has been shown to significantly increase collateral lymphatic formation, decreased inflammation, and decreased fibrosis in preclinical mouse lymphedema.[34] Combining the advantage of site-specific immunosuppression and lymphangiogenesis and LN targeting is a promising approach in VCA.[35] Notably, our previous work on site-specific immunosuppression with localized delivery of tacrolimus was performed in recipient rats receiving LN-containing grafts.[36-39] This underlines the importance of investigating the role of the lymphatic system and the

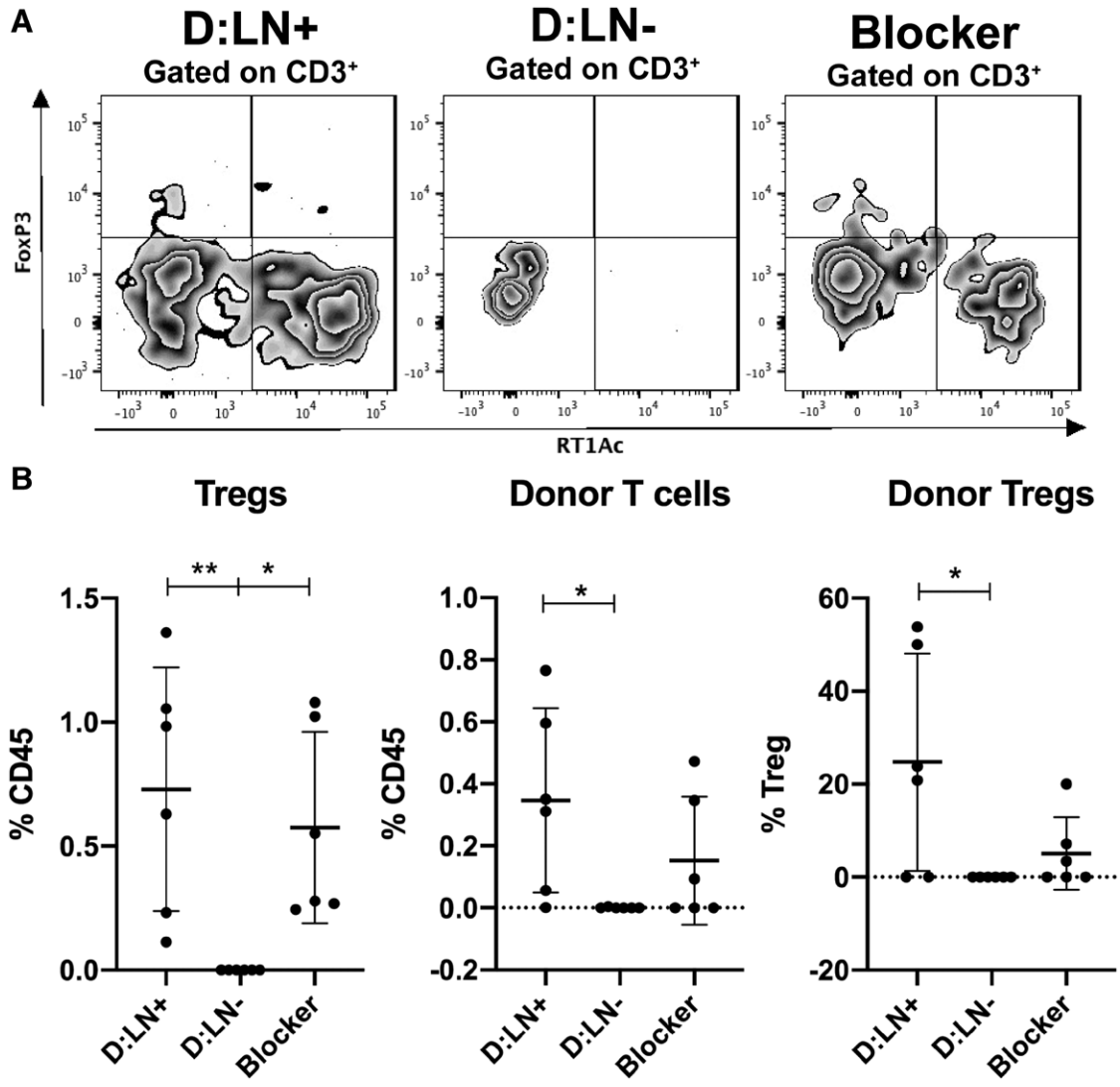

FIGURE 8. D:LN transfer increases T_{reg} infiltration in VCA skin at rejection. Flow cytometry analysis of the T-cell populations in VCA skin at rejection. A, Representative flow cytometry for the characterization of T-cell populations in the 3 groups. All plots are gated on $CD45^+CD3^+$ cells. Donor T cells were identified as $CD45^+CD3^+RT1Ac^+$, T_{reg} as $CD45^+CD3^+FoxP3^+$ cells, and donor T_{reg} as $CD45^+CD3^+FoxP3^+RT1Ac^+$ cells. B, Quantitative summaries of T-cell analysis in the 3 groups. Data are presented as mean and SD, $^*P<0.05$, $^{**}P<0.01$ by 1-way ANOVA with Tukey's multicomparisons test. ANOVA, analysis of variance; D:LN, donor lymph node; T_{reg}, T regulatory cells; VCA, vascularized composite allotransplantation.

possibility of modulating LN response with localized, targeted therapies. Notably, beside topical tacrolimus, other immunosuppressive drugs may influence the lymphatic system, with systemic rapamycin treatment showing inhibition of lymphangiogenesis in preclinical and clinical models (reviewed in Wong[5]). However, further studies are warranted to define the primary effects of immunosuppression on lymphangiogenesis and its secondary effects on LECs and LNSCs. These studies will not only define how immunosuppression may influence the lymphatic process in transplantation but may also provide new pharmacological approaches for lymphangiogenesis induction.

Considering the emerging role of the lymphatic system in modulating immunity and tolerance,[2,15] we also analyzed the immunological outcomes of donor LN transfer. Donor LN transfer is associated with an increased frequency of (1) donor T cells in the draining LN, (2) donor T_{reg} in the peripheral blood at POD7, and (3) donor B cells in the spleen. Moreover, improved lymphatic reconnection secondary to donor LN transfer seems to play a role in the migration of the donor T cells and donor T_{reg}, because the inhibition of lymphangiogenesis by VEGFR3 blockade prevents the accumulation of these cells in the draining LN and the peripheral blood, respectively. This is not surprising, and it is likely due to the increased number of donor lymphocytes transferred within the LN and to the trafficking of T cell through the lymphatic system. Although the clinical and immunological consequences of this observation seem to be limited in this acute rejection model, it must be noted that following transplantation, passenger donor T_{reg} can inhibit host adaptive immune responses and prolong allograft survival.[40] Moreover, donor T-cell chimerism is considered integral in the establishment of graft tolerance.[41] Therefore, the capacity of LN transfer to increase donor T-cell recirculation and mixed chimerism may be an important asset in the development of therapeutic protocols aimed to prolong graft survival and induce tolerance.

We did not observe any difference in TLO formation and frequencies of B cells within the graft in the 3 groups. TLO presence has been associated with both promotion of graft rejection and establishment of immunoregulation depending on the type of graft and the composition of these TLO.[7] Our data are in agreement with another report showing that TLO can be uniformly found in experimental and human VCA.[42] Notably, lymphangiogenesis inhibition using VEGFR3 blockade does not affect TLO formation and B-cell skin infiltration. This finding is also supported by a recent report revealing that an inflammatory state characterized by TLO formation is present, and even promoted, after the loss of lymphatic function,[43] and underlines the capacity of VCA and lung grafts to promote in situ alloresponse.

Interestingly, the presence of donor LN within the grafts was associated with an increased number of T_{reg} also of donor origin in the VCA skin, independently of VEGF-C (ie, D:LN$^+$ and VEGFR3 blocker groups). One potential explanation for this observation is that the preservation of the lymphoid tissue in grafts of the D:LN$^+$ group may help to maintain the skin-resident T_{reg} population. However, only 20% of the skin T_{reg} were of donor origin, suggesting that other mechanisms should be implicated. It has been shown in mice that LECs and LNSCs may induce antigen-specific tolerance and T_{reg} expansion.[15,16] Similar mechanisms have been described in humans.[44] Therefore, we speculate that the presence of donor LNSC in the D:LN$^+$ groups may induce T_{reg} formation by providing an ideal environment for T_{reg} expansion. Accordingly, in an LN transplantation model, it has been shown that major histocompatibility complex (MHC) II expression on LNSC is responsible for homeostatic maintenance of self-reactive T_{reg} and immune quiescence and is able to promote skin-graft survival.[45] More studies are warranted to verify this hypothesis and to evaluate whether LN transfer can be exploited for the generation of allospecific T_{reg}. In this direction, delivery of rapamycin and IL2 may be complemented by LN transfer to shift the LN microenvironment toward a regulatory function.[46-48]

This study has several limitations. First, we used an acute model of rejection without the use of any conditional immunosuppressive therapy. This has the advantage of avoiding any confounding effect of immunosuppressive drugs on the lymphangiogenic process (reviewed in Wong[5]). However, in this setting, surgical trauma is directly related to initiation of the rejection process. Follow-up studies for the characterization of the role of the lymphatics in VCA rejection should plan a conditioning regime that may amplify the effect of LN transfer without directly affecting the lymphatic system. Moreover, considering the emerging importance of chronic rejection in VCA,[49] the role of lymphatics in a chronic rejection model should be more closely investigated. Second, our tissue analysis was limited to the rejection point. This may mask some of the effects of LN transfer. Tissue analysis at earlier time points should be performed to better compare immunological parameters, also by analyzing immune cells composition, lymphangiogenesis, and microscopic histopathological signs of rejection in skin biopsies.

Overall, our results underline the potential of specifically targeting the LN component to influence VCA rejection, opening the way to new investigations in this direction. However, although significant, we showed that solely LN transfer has limited clinical and immunological effects. In our opinion. the administration of VEGF-C, ideally in a targeted and effective fashion, and the use of immunosuppressive regimens known to induce lymphangiogenesis represent the most promising approaches to implement lymphatic drainage and delay graft rejection in VCA. These 2 approaches may also be combined to increase their clinical potential.

ACKNOWLEDGMENTS

The authors thank Shruti Marwah for helping with the lymphangiogenesis scoring.

REFERENCES

1. Thomas SN, Rohner NA, Edwards EE. Implications of lymphatic transport to lymph nodes in immunity and immunotherapy. *Annu Rev Biomed Eng*. 2016;18:207–233.
2. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest*. 2014;124:943–952.
3. Shields JD. Lymphatics: at the interface of immunity, tolerance, and tumor metastasis. *Microcirculation*. 2011;18:517–531.
4. Sáinz-Jaspeado M, Claesson-Welsh L. Cytokines regulating lymphangiogenesis. *Curr Opin Immunol*. 2018;53:58–63.
5. Wong BW. Lymphatic vessels in solid organ transplantation and immunobiology. *Am J Transplant*. 2020;20:1992–2000.
6. Iwami D, Brinkman CC, Bromberg JS. Vascular endothelial growth factor C/vascular endothelial growth factor receptor 3 signaling regulates chemokine gradients and lymphocyte migration from tissues to lymphatics. *Transplantation*. 2015;99:668–677.
7. Hsiao HM, Li W, Gelman AE, et al. The role of lymphoid neogenesis in allografts. *Am J Transplant*. 2016;16:1079–1085.
8. Simon T, Li L, Wagner C, et al. Differential regulation of T-cell immunity and tolerance by stromal laminin expressed in the lymph node. *Transplantation*. 2019;103:2075–2089.
9. Dietrich T, Bock F, Yuen D, et al. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation. *J Immunol*. 2010;184:535–539.
10. Yin N, Zhang N, Xu J, et al. Targeting lymphangiogenesis after islet transplantation prolongs islet allograft survival. *Transplantation*. 2011;92:25–30.
11. Dashkevich A, Raissadati A, Syrjälä SO, et al. Ischemia-reperfusion injury enhances lymphatic endothelial VEGFR3 and rejection in cardiac allografts. *Am J Transplant*. 2016;16:1160–1172.
12. Cui Y, Liu K, Monzon-Medina ME, et al. Therapeutic lymphangiogenesis ameliorates established acute lung allograft rejection. *J Clin Invest*. 2015;125:4255–4268.
13. Schwager S, Renner S, Hemmerle T, et al. Antibody-mediated delivery of VEGF-C potently reduces chronic skin inflammation. *JCI Insight*. 2018;3:e124850.
14. Fletcher AL, Malhotra D, Turley SJ. Lymph node stroma broaden the peripheral tolerance paradigm. *Trends Immunol*. 2011;32:12–18.
15. Hirosue S, Dubrot J. Modes of antigen presentation by lymph node stromal cells and their immunological implications. *Front Immunol*. 2015;6:446.
16. Rouhani SJ, Eccles JD, Riccardi P, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun*. 2015;6:6771.
17. Dieterich LC, Detmar M. Tumor lymphangiogenesis and new drug development. *Adv Drug Deliv Rev*. 2016;99(Pt B):148–160.
18. Nakayama Y, Brinkman CC, Bromberg JS. Murine fibroblastic reticular cells from lymph node interact with CD4+ T cells through CD40-CD40L. *Transplantation*. 2015;99:1561–1567.
19. Shores JT, Brandacher G, Lee WP. Hand and upper extremity transplantation: an update of outcomes in the worldwide experience. *Plast Reconstr Surg*. 2015;135:351e–360e.
20. Schneeberger S, Morelon E, Landin L; ESOT CTA Committee. Vascularized composite allotransplantation: a member of the transplant family? *Transplantation*. 2012;93:1088–1091.
21. Leonard DA, Cetrulo CL Jr, McGrouther DA, et al. Induction of tolerance of vascularized composite allografts. *Transplantation*. 2013;95:403–409.

22. Madariaga MLL, Shanmugarajah K, Michel SG, et al. Immunomodulatory strategies directed toward tolerance of vascularized composite allografts. *Transplantation*. 2015;99:1590–1597.
23. Thaunat O, Badet L, El-Jaafari A, et al. Composite tissue allograft extends a helping hand to transplant immunologists. *Am J Transplant*. 2006;6:2238–2242.
24. Lese I, Leclère FM, Gayanayake T, et al. Regional lymphatic inclusion in orthotopic hindlimb transplantation: establishment and assessment of feasibility in a rodent model. *Transplant Direct*. 2020;6:e592.
25. Roy SF, Krishnan V, Trinh VQ, et al. Lymphocytic vasculitis associated with mild rejection in a vascularized composite allograft recipient: a clinicopathological study. *Transplantation*. 2020;104:e208–e213.
26. Kirkin V, Mazitschek R, Krishnan J, et al. Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur J Biochem*. 2001;268:5530–5540.
27. Ruddle NH. High endothelial venules and lymphatic vessels in tertiary lymphoid organs: characteristics, functions, and regulation. *Front Immunol*. 2016;7:491.
28. Buretta KJ, Brat GA, Christensen JM, et al. Near-infrared lymphography as a minimally invasive modality for imaging lymphatic reconstitution in a rat orthotopic hind limb transplantation model. *Transpl Int*. 2013;26:928–937.
29. Pedersen MS, Müller M, Rülicke T, et al. Lymphangiogenesis in a mouse model of renal transplant rejection extends life span of the recipients. *Kidney Int*. 2020;97:89–94.
30. Maltzman JS, Reed HO, Kahn ML. HA-ving lymphatics improves lung transplantation. *J Clin Invest*. 2015;125:3999–4001.
31. Schwager S, Detmar M. Inflammation and lymphatic function. *Front Immunol*. 2019;10:308.
32. Mundinger GS, Narushima M, Hui-Chou HG, et al. Infrared fluorescence imaging of lymphatic regeneration in nonhuman primate facial vascularized composite allografts. *Ann Plast Surg*. 2012;68:314–319.
33. Farias-Cisneros E, Chilton PM, Palazzo MD, et al. Infrared imaging of lymphatic function in the upper extremity of normal controls and hand transplant recipients via subcutaneous indocyanine green injection. *SAGE Open Med*. 2019;7:2050312119862670.
34. Gardenier JC, Kataru RP, Hespe GE, et al. Topical tacrolimus for the treatment of secondary lymphedema. *Nat Commun*. 2017;8:14345.
35. Taddeo A, Tsai C, Vögelin E, et al. Novel targeted drug delivery systems to minimize systemic immunosuppression in vascularized composite allotransplantation. *Curr Opin Organ Transplant*. 2018;23:568–576.
36. Gajanayake T, Olariu R, Leclère FM, et al. A single localized dose of enzyme-responsive hydrogel improves long-term survival of a vascularized composite allograft. *Sci Transl Med*. 2014;6:249ra110.
37. Dzhonova DV, Olariu R, Leckenby J, et al. Local injections of tacrolimus-loaded hydrogel reduce systemic immunosuppression-related toxicity in vascularized composite allotransplantation. *Transplantation*. 2018;102:1684–1694.
38. Olariu R, Denoyelle J, Leclère FM, et al. Intra-graft injection of tacrolimus promotes survival of vascularized composite allotransplantation. *J Surg Res*. 2017;218:49–57.
39. Dzhonova D, Olariu R, Leckenby J, et al. Local release of tacrolimus from hydrogel-based drug delivery system is controlled by inflammatory enzymes in vivo and can be monitored non-invasively using in vivo imaging. *PLoS One*. 2018;13:e0203409.
40. Harper IG, Gjorgjimajkoska O, Siu JHY, et al. Prolongation of allograft survival by passenger donor regulatory T cells. *Am J Transplant*. 2019;19:1371–1379.
41. Leonard DA, Kurtz JM, Cetrulo CL. Mixed chimerism for tolerance induction of vascularized composite allografts. In: Brandacher G, ed. *The Science of Reconstructive Transplantation*. Humana Press, New York, NY; 2015:203–225.
42. Hautz T, Zelger BG, Nasr IW, et al. Lymphoid neogenesis in skin of human hand, nonhuman primate, and rat vascularized composite allografts. *Transpl Int*. 2014;27:966–976.
43. Reed HO, Wang L, Sonett J, et al. Lymphatic impairment leads to pulmonary tertiary lymphoid organ formation and alveolar damage. *J Clin Invest*. 2019;129:2514–2526.
44. Nörder M, Gutierrez MG, Zicari S, et al. Lymph node-derived lymphatic endothelial cells express functional costimulatory molecules and impair dendritic cell-induced allogenic T-cell proliferation. *FASEB J*. 2012;26:2835–2846.
45. Baptista AP, Roozendaal R, Reijmers RM, et al. Lymph node stromal cells constrain immunity via MHC class II self-antigen presentation. *Elife*. 2014. 3:e04433.
46. Tostanoski LH, Chiu YC, Gammon JM, et al. Reprogramming the local lymph node microenvironment promotes tolerance that is systemic and antigen specific. *Cell Rep*. 2016;16:2940–2952.
47. Fisher JD, Balmert SC, Zhang W, et al. Treg-inducing microparticles promote donor-specific tolerance in experimental vascularized composite allotransplantation. *Proc Natl Acad Sci U S A*. 2019;116:25784–25789.
48. Sutter D, Dzhonova DV, Prost JC, et al. Delivery of rapamycin using in situ forming implants promotes immunoregulation and vascularized composite allograft survival. *Sci Rep*. 2019;9:9269.
49. Kanitakis J, Petruzzo P, Badet L, et al. Chronic rejection in human vascularized composite allotransplantation (hand and face recipients): an update. *Transplantation*. 2016;100:2053–2061.