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Impaired Humoral Immunity and Tolerance in K14-VEGFR-3-Ig Mice That Lack Dermal Lymphatic Drainage

Susan N. Thomas,* Joseph M. Rutkowski,∗ Miriella Pasquier,† Emma L. Kuan,† Kari Alitalo,‡ Gwendalyn J. Randolph,†,1 and Melody A. Swartz*‡,§

Lymphatic vessels transport interstitial fluid, soluble Ag, and immune cells from peripheral tissues to lymph nodes (LNs), yet the contribution of peripheral lymphatic drainage to adaptive immunity remains poorly understood. We examined immune responses to dermal vaccination and contact hypersensitivity (CHS) challenge in K14-VEGFR-3-Ig mice, which lack dermal lymphatic capillaries and experience markedly depressed transport of solutes and dendritic cells from the skin to draining LNs. In response to dermal immunization, K14-VEGFR-3-Ig mice produced lower Ab titers. In contrast, although delayed, T cell responses were robust after 21 d, including high levels of Ag-specific CD8+ T cells and production of IFN-γ, IL-4, and IL-10 upon restimulation. T cell-mediated CHS responses were strong in K14-VEGFR-3-Ig mice, but importantly, their ability to induce CHS tolerance in the skin was impaired. In addition, 1-y-old mice displayed multiple signs of autoimmunity. These data suggest that lymphatic drainage plays more important roles in regulating humoral immunity and peripheral tolerance than in effector T cell immunity. The Journal of Immunology, 2012, 189: 2181–2190.

It was suggested four decades ago that lymphatic drainage was involved in local immune tolerance (1, 2), yet since then, the role of lymph drainage (i.e., transport of soluble components from the periphery to lymph nodes (LNs)) in modulating adaptive immunity and tolerance has remained largely unexplored. There is a growing appreciation that tolerance to inhaled and ingested Ags is induced at the level of the draining LN (3) and that LN stromal cells mediate tolerance to self-Ag by endogenous expression on MHC molecules (4), but the importance of lymphatic drainage function, and soluble lymph delivery to the LN, is poorly understood.

Altered immunity has been reported in primary lymphedema, a congenital pathology of dysfunctional lymphatic drainage that commonly leaves edematous limbs prone to infections (5). Furthermore, lymphangiogenesis often occurs in areas of chronic inflammation such as in Crohn’s disease (6) and in LNs draining inflamed areas or after vaccination (7, 8), yet the function of an expanded lymphatic network in inflammation is unclear. In contrast, inflammatory lymphangiogenesis has been correlated with graft rejection in corneal, islet, and renal transplants (9–14), but it has also been shown to promote resolution of experimental inflammation (15) and in humans was correlated with long-term survival of renal transplants (16). In tumors, increased lymphatic drainage is correlated with tumor progression (17, 18), and tumor-associated lymphangiogenesis was recently shown to promote immune tolerance (19, 20). Thus, lymphatic drainage likely plays complex roles in modulating immunity.

Adaptive immune responses can be initiated by either peripheral dendritic cells (DCs) that pick up Ag in the periphery and migrate to the draining LNs or by LN-resident DCs that pick up soluble, lymph-borne Ag; both presumably educate T cells in the LN (21). In addition, lymph-borne Ag is taken up by subcapsular macrophages for delivery to LN follicle-resident B cells (22) or directly by the B cells themselves in the case of small Ags (23) to initiate humoral responses. Interestingly, cytokine responses by LN-resident DCs that take up soluble Ag differ from those of Ag-bearing DCs that travel to the LN after being activated in the periphery (24), suggesting the importance of lymph-borne Ag and therefore lymphatic drainage in fine-tuning the immune response.

In addition to facilitating adaptive immunity, the LN is an important site for the maintenance of self-tolerance (25). The stromal cells of the LN T cell zone form the structural network that guides lymphocyte trafficking (26) through secretion of the CCR7 ligands CCL21 and CCL19, which are essential to the positioning of CCR7+ regulatory T (TReg) cells, naïve T cells, and APCs in the LN paracortex (27). Without such positioning, as in the case of pli mice that lack CCR7 ligands (and subsequently have impaired trafficking of DCs and naïve T cells into the LN), Ag-specific effector T cell responses can be mounted in the spleen, leading to potent although delayed T cell immunity (28). However, self-
tolerance mechanisms fail in CCR7−/− mice, which develop generalized autoimmunity (29). This is because, in addition to CCR7 being required for the establishment of central tolerance within the thymus (29, 30), TReg cells require LN occupancy for their activation (25, 31–33). Furthermore, LN-resident stromal cells and lymphatic endothelium can present endogenous peripheral Ag for the deletion of self-reactive CD8+ T cells (26, 34, 35).

Thus, both APCs activated in the periphery, which then travel to the LN, as well as APCs activated in the LN from lymph-borne soluble Ag (including, importantly, B cells) contribute to the immune regulatory balance (25).

Hence, although the lymphatic system in immunity has largely been regarded in its cell transport roles, providing physical routes for immune cell trafficking from the periphery to the LN and chemokines for positioning these cells within the LN, the immune implications of fluid drainage by lymphatics from the periphery to the LN remain poorly understood. Unfortunately, mouse models of impaired lymphatic drainage require causative gene defects that affect immune responses. K14-VEGFR-3-Ig mice express soluble VEGFR-3-Ig via the keratin 14 promoter, which results in defective lymphatic growth that is restricted to the skin. As adults, they display a paucity of initial dermal lymphatic capillaries and decreased fluid clearance from the skin, and no other known physiological manifestations have been observed (36, 37); the mice survive to an advanced age (up to 2 y), the LNs are intact, and lymphatic vessels in other (nondermal) tissues appear normal (36). We examined the adaptive immune response to dermal versus peritoneal vaccination as well as acquired tolerance to dermal contact hypersensitivity (CHS) in these mice. Our findings support the hypothesis that local lymphatic drainage is critical for humoral immunity and acquired tolerance but less important for effector T cell immunity after vaccination.

Materials and Methods

Animals

K14-VEGFR-3-Ig mice are described earlier (36). Littermates lacking the transgene were used as wild-type (WT) controls. Mice were between ages of 8 and 20 wk, except in studies examining autoimmunity phenotypes in aged mice, which used mice at 12–18 mo. Notably, the mice were maintained on a specific chow diet that lacks any chicken-derived products (Diet 2918; Harlan Laboratories, Iningen, Switzerland). All procedures were approved by the Office Vétérinaire Cantonale Vaud (Switzerland).

Lymphatic uptake

The lymphatic uptake rate was determined as described previously (38). Briefly, mice were anesthetized (using an i.p. injection of 65 mg/kg ketamine and 13 mg/kg xylazine), and a 30-gauge needle catheter containing 0.9% NaCl with 2 mg/ml FITC–dextran 70 kDa (Invitrogen, Carlsbad, CA) was carefully placed intradermally (i.d.) into the tail tip. The catheter was attached to a low-pressure reservoir that permitted 5-cm stepwise changes from 40 to 60 cm H2O pressure. These infusion pressures allowed physiologic uptake into the lymphatic capillaries (when present) and postcapillary venules while minimizing potential swelling or tissue damage. By measuring the infusion flow rate together with the movement of the fluorescent dextran in the interstitial space (using a Leica MZ16 FA stereomicroscope) as functions of infusion pressure, we could estimate the hydraulic conductivity and relative clearance from the tissue. Calculations were made according to the theoretical framework outlined previously (38). Results (representing percent volume of injected solution cleared per time and pressure drop) were normalized relative to WT littermates.

Trypan blue drainage assay

Twenty microliters of 0.4% trypan blue (Invitrogen) was injected i.d. into the front and back legs and tail. Ten minutes postinjection, mice were sacrificed, and LNs were removed.

FITC Painting

The backs of mice were shaved and painted with 8% FITC (type I isomers; Sigma-Aldrich, St. Louis, MO) diluted in a 1:1 mixture of acetone and dibutylphthalate. After 4 d, mice were sacrificed, and draining LNs were analyzed for FITC+CD11c+ DCs by flow cytometry (FACSCanto; Becton Dickinson, San Diego, CA).

In vivo migration assay

Yellow-green polystyrene microspheres of 1 μm in diameter (Polysciences, Warrington, PA) diluted in PBS 1:20 were injected i.d. in each hind limb (40 μl/injection) and tail (20 μl) with 0.1 mg/ml chicken OVA (Profos, Marburg, Germany) in 1) PBS, 2) a 1:1 emulsion of PBS and alum (ThermoFisher, Rockford, IL), or 3) PBS with 25 μg Ultrapure 0111:B4 LPS (InvivoGen, Nunningen, Switzerland); solutions were injected i.d. using a 30-gauge needle. In select experiments, mice were immunized i.p. (100 μl).

Flow cytometry

All Abs used for flow cytometry were from eBioscience (San Diego, CA), unless otherwise stated. Streptavidin–Alexa 649 was used in conjunction with biotinylated Abs. Abs were prepared in HBSS/0.5% BSA and added to samples prior to incubation at 4˚C for 30 min in the dark. For detection of the OVA H-2Kb MHCI peptide SIINFEKL, samples were stained with PE-conjugated SIINFEKL tetramer (ProImmune, Oxford, UK) diluted 1:10 in HBSS/0.5% BSA for 10 min at room temperature in the dark. For intracellular staining of Langerin, surface-stained cells were fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) for 30 min at 4˚C, followed by goat anti-mouse Langerin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-goat PE (Jackson ImmunoResearch Laboratories, New Market, U.K.) staining. Both Abs were diluted in perm/wash buffer (BD Biosciences). For Foxp3 staining, cells were fixed/permeabilized using Foxp3 Fixation/Permeabilization kit (eBioscience) overnight at 4˚C and stained the following day in permeabilization buffer with anti-Foxp3. Data were acquired in a Dako CyAn flow cytometer (DakoCytomation, Glostrup, Denmark) with compensation using either calibration beads (BD Biosciences) or single-stained cells. Data analysis was performed using FlowJo software (version 8.8; Tree Star, Ashland, OR).

Immunofluorescence

Frozen LNs were cryosectioned (8 μm) and subjected to standard immunofluorescence protocols using the following anti-mouse Abs: hamster anti-CD3e (1:100; BD Pharmingen, San Diego, CA), biotinylated rat anti-B220 (1:100; BD Biosciences), Alexa Fluor 647-conjugated CD11c (1:40; eBioscience), rat anti–ER-TR7 (1:50; Hycult Biotech, Uden, The Netherlands), rabbit anti-Vimentin (1:100; BD Biosciences), rat anti-para peripheral LN addressin (PNAd) (1:100; BioLegend, Uithoorn, The Netherlands), rabbit anti–Lyve-1 (1:250; Teco Medical, Neufahrn, Germany), rat anti-CD21/CD35 (1:400; eBioscience), rat anti-CXCL13 (1:10; R&D Systems, Minneapolis, MN), goat anti-gp38 (1:50; R&D Systems), rabbit anti-collagen I (1:50; Santa Cruz Biotechnology), and biotinylated rabbit anti-collagen IV (1:800; Abcam, Cambridge, U.K.); fluorescently conjugated secondary Abs and streptavidin were obtained from Invitrogen. Paraffin-embedded skin sections were cut into 8-μm-thick sections, blocked in goat serum, and stained with goat anti-mouse IgG–Alexa Fluor 647. Alternatively, ear tissue samples were whole-mount stained using standard techniques (39). Frozen sections (8 μm) of organs of healthy WT mice were first incubated in donkey serum and then stained with 1% sera from K14-VEGFR-3-Ig mice or age-matched WT littermates; after washing, sections were incubated with donkey anti-mouse IgG–AF647 (1:200). Sections were counterstained with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a LSM 510 or 710 confocal microscope (Carl Zeiss, Feldbach, Switzerland). Sections were scored in a blinded fashion.

Serum anti-OVA Ab titers

On day 21 postimmunization (p.i.) with a day 10 boost, mice were sacrificed, and their sera were analyzed by ELISA. Ninety-six-well plates (BD Biosciences) were coated overnight with 10 μg/ml OVA diluted in PBS. Plates were then blocked with 2.5% casein in PBS, and samples were added in blocking solution for 2 h, rinsed, and incubated with either HRP-conjugated anti-IgG, HRP-conjugated anti-IgGl, or biotinylated anti-IgGl.
ELISA (eBioscience), according to the manufacturer’s protocol.

From Life Technologies) and treated with 0.2 m

suspension culture plates (Greiner Bio-one, Frickenhausen, Germany) in

until analysis for IFN-

in the back legs and tail. Three or 6 d p.i., dermal-draining LNs (dLNs) and

liferated cells were normalized to the total CFSE+ cell counts on day 3 p.i.

estimated by summing the unproliferated (G0) cells and proliferating (G1–

transfer. The percentages of unproliferated and proliferated OT cells were

One hundred

penicillin/streptomycin; all from Life Technologies, Carlsbad, CA) with 0 or

m

media (RPMI 1640 medium with 10% FBS, 50 

m–dithiobis(2-nitrobenzoic acid) (DNTB) in aoo on day 0.

m

9

5,5

9

5

9

80˚C until IFN-γ, IL-10, and IL-4 cytokine production was analyzed by

ELISA (eBioscience), according to the manufacturer’s protocol.

Adoptive transfer experiments

OT-I and OT-II splenocytes were treated with ammonium chloride potas-

sium RBC lysis buffer and then positively selected for CD8+ cells (Ly-2

magnetic beads; Miltenyi Biotec, Bergisch Gladbach, Germany) or CD4+ cells (L3T4 beads; Miltenyi Biotec), respectively. Cells were labeled with 1 

m final concentration of CFSE (Invitrogen) prior to adoptive transfer of

× 10^6 OT-I cells or 2 × 10^6 OT-II cells in IMDM by tail vein injection

into naive mice. After 3 d, mice were immunized with OVA plus LPS i.d.

in the back legs and tail. Three or 6 d p.i., dermal-draining LNs (dLNs) and

spleens were harvested and processed to make single-cell suspensions.

Cells were analyzed by flow cytometry. Because K14-VEGFR-3-Ig mice

were on a C57BL/6 CD45.2 background, as were OT-I and OT-II mice, we

could only trace the OT-I and OT-II cells by CFSE labeling before adoptive

transfer. The percentages of unproliferated and proliferated OT cells were

estimated by summing the unproliferated (G0) cells and proliferating (G1–

G6) cells, respectively, and normalizing to the total numbers of parental

cells they arose from. For days 9 and 12 p.i., the frequencies of unpro-

liferated cells were normalized to the total CFSE+ cell counts on day 3 p.i.

Contact hypersensitivities

To establish contact sensitivity, 25 μl 0.5% dinitrofluorobenzene (DNFB)

in acetone with 20% olive oil (aoo) was painted onto the shaved backs of

mice. Five days later, one ear was challenged with 0.3% DNFB. Forty-

eight hours later, ear swelling was measured relative to the untreated ear.

In separate experiments to determine tolerance to DNFB-mediated CHS,

we first induced tolerance by treating the shaved stomachs of naive mice with

100 μl 1% 5,5'-dithiobis(2-nitrobenzoic acid) (DNTB) in aoo on day 0.

We then sensitized the mice on day 7 by treating their shaved backs with

25 μl 0.5% DNFB in aoo. Finally, mice were challenged on day 12 to
determine the extent of tolerance by treating one ear with 0.3% DNFB in

aoo. Forty-eight hours later, ear thickness was measured, and ear spec-

imens were harvested. Alternatively, resolution of ear swelling was deter-
m

mined by measuring ear thickness for 10–14 d postchallenge.

Histological analysis

Paraffin-embedded ears and frozen spleens were cut into 4- or 8-μm-thick

sections and H&E stained. Paraffin-embedded tail skin was cut into 8-μm-

thick sections and stained with Miller’s stain. Samples were imaged with a Zeiss (Feldbach, Switzerland) MRC camera.

Serum isotype titers and serum dsDNA titers

Serum isotype values were determined using an ELISA kit (BD Bio-

sciences), and dsDNA titers were determined by ELISA (VWR, Nyon, Switzerland), according to the manufacturer’s protocols.

Ex vivo B cell stimulation

Splenocytes were first treated with ammonium chloride potassium RBC

lysis buffer, and then splenocyte and dermal LN cell suspensions were

negatively selected for B cells (Ly-48, L3T4, and Ter-119 magnetic beads;

Miltenyi Biotec). B cells were plated at 5 × 10^3 cells/well in 96-well

suspension culture plates (Greiner Bio-one, Frickenhausen, Germany) in

IMDM with 10% FBS, 50 μM 2-ME, and 1% penicillin/streptomycin (all

from Life Technologies) and treated with 0.2 μg/ml Ultrapure LPS

(InvivoGen). After 24 h, supernatants were harvested and stored at ~80°C

until analysis for IFN-γ by ELISA (eBioscience). Cells were washed,
stained for B220 and B cell activation markers CD86 and IgM, and ana-
alyzed by flow cytometry.

Statistical analysis

Data are represented as the mean with the SEM. Statistics were calculated

using GraphPad Prism 5 (GraphPad, La Jolla, CA) software. Statistical

significance was defined as p < 0.05 following one-way ANOVA and post

hoc analysis. When normality tests failed, Kruskal–Wallis tests were

performed. For contingency analysis, Fisher’s exact test was used.

Results

Reduced lymphatic drainage and DC migration in K14-VEGFR-3-Ig mice

We first established the degree of impairment of lymphatic drainage

and transport of peripheral DCs to the dLNs in K14-VEGFR-3-Ig mice.

Consistent with their lack of dermal lymphatic capillaries (36), lymphatic drainage from the skin of K14-VEGFR-3-Ig mice was severely impaired, as seen qualitatively by the lack of trypan blue in the dLN 10 min after i.d. injection (Supplemental Fig. 1A) and quantitatively using a previously established tail model of quantitative microlymphangography (Fig. 1A) (40).

DC trafficking from the skin to the dLN was virtually abrogated in these mice, as demonstrated by a lack of FITC^CD11c^ and FITC^langerin^CD11c^ cells in dLNs 4 d after application of FITC-containing contact sensitizer to the epidermis (“FITC painting”; Fig. 1B, 1C). This abolishment of DC trafficking to skin-draining LNs was confirmed by the absence of bead^CD11c^ cells in dLNs 24 h after i.d. injection of 1-μm fluorescent microspheres (Fig. 1D), which can only reach the LN after being taken up by peripheral monocytes that differentiate into DCs (21).

Total frequencies of Lang^CD11c^ cells in dLNs were also se-

verely decreased (Fig. 1E). Importantly, dLNs of K14-VEGFR-3-

Ig mice had normal amounts of CCL21, a chemokine that attracts

DCs and naive T cells to the LN (Supplemental Fig. 1B), per

milligram of total LN protein, indicating that the impaired DC

trafficking was not due to decreased chemokine signaling in the

LN. Thus, the severely impaired lymphatic drainage and DC trafficking

from skin to dLNs in K14-VEGFR-3-Ig mice was presumably due to the lack of dermal lymphatic capillaries.

Altered LN organization in K14-VEGFR-3-Ig mice

We found disorganized B cell follicles in the dLNs but not mes-

enteric LNs (mLN) of K14-VEGFR-3-Ig mice (Fig. 1F); specifi-
cally, B cells were found scattered throughout the dLNs among

T cells and were less concentrated in follicles compared with those in WT mice. Similarly, the distributions but not amounts of

CCL21 and CXCL13, a chemokine that directs B cell positioning

within the LN, were abnormal in the dLNs of K14-VEGFR-3-Ig

mouse (Supplemental Fig. 1B-D). T cell zone fibroblastic reticular

cells and reticular fibers, which regulate T cell migration (41), also

appeared highly disorganized in dLN (Fig. 1G, Supplemental Fig. 2A) but not mLN (Supplemental Fig. 2A) of transgenic mice.

Finally, the lymphatic endothelial network and distribution of

follicular DCs within the dLNs, but not mLNs of transgenic mice

appeared very disorganized (Supplemental Fig. 2B) and contained

significantly smaller high endothelial venules (PNAd^) than in

WT dLNs (Fig. 1H, I, Supplemental Fig. 2C).

Interestingly, although naive dLNs of the transgenic mice were smaller (36) and less organized than their WT counterparts (Fig. 1F–H), they contained similar fractions (as percentage of CD45^ cells) of B cells (B220^) and T cells (CD3e^) (Fig. 2A). Fractions of DCs (CD11c^) and macrophages (F4/80^) were lower in dLNs but higher in mLNs of transgenic mice (Fig. 2A). However, higher

CD4/CD8 T cell ratios were found in both dLNs and mLNs as well as the spleens (Fig. 2B) of K14-VEGFR-3-Ig mice compared

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with WT mice. Interestingly, there were fewer TReg cells (Foxp3+, as the percentage of CD4+CD3ε+ cells) in the dLN and more in the mLN of transgenic mice compared with WT mice (Fig. 2C), suggesting that deficiencies in TReg cell homing to the dLN in K14-VEGFR-3-Ig mice is compensated by homing to other LNs.

The spleens and blood of transgenic mice contained higher frequencies of double-negative (CD4-CD8α-) CD3ε+ cells (Fig. 2B), which may negatively regulate immune responses (42). However, no significant differences were seen in systemic TReg cell frequencies other than a small increase in the adaptive TReg cell compartment (CD25+Foxp3+) (Fig. 2C). Another striking difference was the increased expression of B cell coreceptor CD19 by cells in the dLNs, but not mLNs or spleens, of transgenic mice (Fig. 2D). Because CD19 increases B cell sensitivity to Ag-specific stimulation, its increased expression could be associated with autoantibody production (43) and autoimmunity (44).

Poor humoral responses to dermal, but not peritoneal, vaccination in K14-VEGFR-3-Ig mice

Lymph flow from peripheral tissues provides Ag to draining LNs and transports APCs to LNs to initiate immune responses (21). To determine how adaptive immunity to dermal challenge was affected in K14-VEGFR-3-Ig mice, we compared immune responses to the model Ag OVA (confirmed endotoxin-free before injection) after i.d. or i.p. immunization with LPS (a Th1-promoting adjuvant) or i.d. immunization with alum (a Th2-promoting adjuvant). We note that mild responses to OVA plus saline in WT mice were expected because OVA is a foreign Ag, and the mice had been maintained on a chicken product-free chow diet.

On day 21 postimmunization (p.i.) with a day 10 boost, we found drastically reduced anti-OVA Ab titers in K14-VEGFR-3-Ig mice compared with WT controls with both adjuvants upon i.d. challenge (Fig. 3A–C). However, when immunized i.p., anti-OVA Ab titers in response to OVA plus LPS were normal (Fig. 3A–C). Poor humoral responses in response to i.d. challenge were not apparently due to deficiency in the functionality of the B cells, because B cells isolated from dLN and spleens of transgenic mice responded similarly to those from WT mice when stimulated ex vivo with LPS in terms of maturation (Fig. 3D) as well as IFN-γ and IgM production (Fig. 3E, 3F). Thus, our data suggest that the reduced Ab response to i.d. immunization seen in K14-VEGFR-3-Ig mice is likely due to deficiencies in TReg cell homing to the dLN.
Ig mice resulted from physiological differences in Ag transport to the dLN, rather than impaired B cell function.

K14-VEGFR-3-Ig mice show delayed but robust T cell responses to dermal vaccination

Despite the decreased Ag drainage and DC trafficking from the dermis to LNs of transgenic mice, however, their T cell responses 21 d after i.d. vaccination appeared surprisingly robust. Upon ex vivo OVA restimulation, splenocytes produced normal levels of IFN-γ, IL-4, and IL-10 (Fig. 4A–C), and both mouse strains developed similar frequencies of Ag-specific (SIINFEKL-MHC I pentamer+) CD8+ T cells 21 d p.i. (Fig. 4D).

To evaluate the kinetics and location of T cell priming in the K14-VEGFR-3-Ig mice, we performed i.d. immunization with OVA plus LPS after adoptive transfer of T cells from OT-I or OT-II transgenic mice, whose CD8+ and CD4+ T cells express restricted transgenic MHC class I and II molecules that bind OVA peptides, respectively. Indeed, we found that their expansion was delayed in K14-VEGFR-3-Ig mice relative to WT mice (Fig. 5, Supplemental Fig. 3A): although proliferation in the dLN of WT mice was seen 3 d p.i. for both OT-I and OT-II cells, in K14-VEGFR-3-Ig mice it was not observed until days 6 and 9 for OT-I and OT-II cells, respectively. Furthermore, OT-I proliferation appeared to occur both in the dLN as well as the spleen. In OVA-vaccinated mice without adoptive T cell transfer, at day 9 (i.e., the peak of the initial T cell response to vaccination), no differences were seen in IFN-γ or IL-10 production upon splenocyte restimulation in transgenic versus WT mice (Supplemental Fig. 3B). Interestingly, splenocytes from K14-VEGFR-3-Ig mice produced more of the Th2 cytokine IL-4 upon OVA restimulation with the Th1 adjuvant LPS at day 9 p.i. compared with those from WT mice (Supplemental Fig. 3B). Taken together, these data suggest that the spleen may partially compensate in a delayed fashion for the weakened LN functions of T cell priming in K14-VEGFR-3-Ig mice, which is consistent with previous reports in plt mice (in which T cell homing to the dLN is severely impaired) showing delayed but robust T cell responses after vaccination as a result of a shift in T cell priming from the dLN to the spleen (28).

FIGURE 2. Altered immune cell distributions in lymph nodes of K14-VEGFR-3-Ig mice. (A) Relative distributions of CD11c+ DCs, F4/80+ macrophages, B220+ B cells, and lymphocytes (CD3ε+) in the dermal LN (dLN), mesenteric LN (mLN), spleen, and blood. (B) Distributions of lymphocyte subtypes in each compartment as indicated show more CD4 and less CD8 T cells in transgenic (TG) versus WT mice. (C) Distributions of regulatory T cells in each compartment. (D) Normalized mean fluorescence intensity (MFI) of CD19 expression by cells in each compartment. n = 4–12. (A, B, and D) *p < 0.01, **p < 0.001. (C) *p < 0.05 for CD25- cells, **p < 0.01 for both CD25+ and CD25- cells.

FIGURE 3. Minimal Ab responses to dermal vaccination but normal ex vivo B cell function in K14-VEGFR-3-Ig mice. (A–C) OVA-specific serum Abs 21 d after OVA immunization: total IgG (A), IgG2c (B), and IgG1 titers (C). *p < 0.05, **p < 0.01 using Mann–Whitney; n = 5–11. (D–F) B cells isolated from dLNs and spleens of K14-VEGFR-3-Ig and WT mice respond similarly to 24-h LPS stimulation ex vivo. Differences in CD86 mean fluorescence intensity (MFI) (D) and IFN-γ (E) or IgM secretion (stimulated cells less unstimulated cells) (F) as measured by ELISA. p Values by Mann–Whitney.
K14-VEGFR-3-Ig mice mount robust CHS reactions but fail to induce experimental CHS tolerance

To determine whether T cell responses to dermal immunization in K14-VEGFR-3-Ig mice would be recapitulated in another dermal immune challenge, we used a model of CHS. In this model, mice are sensitized to DNFB, and ear swelling occurs upon DNFB re-exposure as a result of T cell-mediated immune responses (45, 46). In dermal CHS experiments, ears of transgenic mice swelled more robustly than ears of WT mice for the first 4 d after DNFB challenge (Fig. 6A–C) but resolved on a similar timescale (Fig. 6C). These data support the concept of robust T cell immunity in transgenic mice.

In contrast, transgenic mice failed a well-established test of acquired tolerance to CHS. In WT mice, but not K14-VEGFR-3-Ig mice, pretreatment with DNTB (a tolerizing agent to DNFB) prevented DNFB-induced ear swelling (Fig. 6A, 6B, 6D). This suggests that transgenic mice are deficient in mechanisms of acquired tolerance against skin-encountered Ag and corroborates older studies in skin transplants that failed tolerance tests when lymphatic drainage was blocked (1, 2).

In support of the concept that lymphatic drainage to the LN is important in maintaining immune tolerance against peripheral Ag, we noted several hallmarks of autoimmunity in 1-y-old K14-VEGFR-3-Ig mice (Fig. 7). These included increased serum levels of IgG1, IgG3, and IgA but not IgG2b, IgM, and K or L chain Ab isotypes (indicating disruptions in B cell Ig class switching), the presence of megakaryocytes in the spleen [associated with increased plasma cell survival (47)], elevated serum titers of dsDNA, and Ab deposits in the skin as well as increased frequencies of mice with skin-reactive Abs in their serum (indicating autoimmune reactions against skin). Finally, in the skin of K14-VEGFR-3-Ig mice lacking Lyve-1+ lymphatic capillaries, we observed a decrease in CCL21 levels (Supplemental Fig. 4A), normal frequencies of skin-resident APCs (Supplemental Fig. 4B), and an increase in elastin (Supplemental Fig. 4C). Thus, although skin physiology appears altered in K14-VEGFR-3-Ig mice [consistent with earlier reports (25)], the normal densities of skin-resident APCs under steady-state conditions may suggest self-regulating mechanisms control APC density in skin, even when egress into lymphatics is impaired.

Discussion

LNs support the priming of adaptive immune responses as well as the maintenance of tolerance to autoantigens by orchestrating the interactions between APCs and T cells (25), whereas lymphatic vessels sustain Ag delivery to the LN through both APC trafficking and direct transport of peripheral lymph-soluble Ag (21). Our data in K14-VEGFR-3-Ig mice, which lack dermal lymphatic capillaries (36, 37) and thus have reduced transport of soluble Ag from the skin to the draining LNs (Fig. 1A), support the notion that lymphatic drainage from the periphery to the draining LN is critical for inducing humoral immunity and maintaining peripheral tolerance.

K14-VEGFR-3-Ig mice manifested profoundly impaired Ab responses to i.d. but not i.p. challenge with Ag (Fig. 3A–C), indicating that Ag delivery to the LN is critical for adaptive humoral immunity. This finding complements previous reports showing that lymph-borne Ag is delivered to LN-resident B cells either directly through the conduits in the case of smaller Ags (23) or by transfer from subcapsular macrophages for larger Ags (22). Humoral responses to dermal challenge in K14-VEGFR-3-Ig mice might also be impaired by the disorganized dLN architecture given recent reports of B cell priming and affinity maturation in response to s.c. challenge requiring the specialized topography of the LN (48), although B cell function appears robust in ectopic lymphoid structures that form in autoimmune-related disorders (49, 50). B cell function may also be impaired by the delay in CD4+ T cell priming, which plays a well-established role in mature B cell immune responses (51). Regardless of the mechanism, our data are consistent with the notion that lymph flow—which affects LN architecture, T cell priming, and Ag transport to the LN concurrently—plays an important role in humoral immunity.

Besides transporting lymph-borne Ag, the lymphatics are highways for DC homing to LNs in both steady-state and inflamed conditions (21). Although naive K14-VEGFR-3-Ig mice showed normal APC frequencies in the skin (Supplemental Fig. 4B), and DC frequencies in dLNs were only slightly lower than those in control mice (Fig. 2), DC migration to the dLN was severely impaired when challenged with FITC painting or injection of 1-μm beads (Fig. 1B–E). However, it is important to note that the total levels of CCL21 were normal in the dLN (Supplemental Fig. 1B), even though CCL21+ lymphatic capillaries were mostly absent in the skin (Supplemental Fig. 4A). In contrast, high endothelial venules were smaller in transgenic mice (Fig. 1H, I, Supplemental Fig. 2C), and the T cell zone fibroblastic reticular cells and fibers were also disrupted (Supplemental Fig. 2A), which suggest that the homing of naive and TReg cells to the LN should also be impaired. Nevertheless, although the migration of APCs from the periphery to the draining LN in response to activating stimuli is widely considered critical for the initiation of Ag-specific T cell immunity, K14-VEGFR-3-Ig mice were able to mount robust (Fig. 4) but delayed (Fig. 5) T cell responses, as
exhibited by normal cytokine production upon splenocyte Ag restimulation after 21 d and normal frequencies of Ag-specific effector CD8 T cells with vaccination (Fig. 4), as well as robust ear swelling in response to dermal DNFB challenge (Fig. 6), a CHS reaction mediated by CD4+ and CD8+ T cells (45, 46). These findings are complementary to recent observations of delayed but strong CHS reactions to DNFB challenge in mice heterozygous for the expression of Kaposi’s sarcoma-associated herpesvirus latent-cycle gene k-cyclin under the control of the VEGFR-3 promoter (kCYC+/−) (52), which exhibit dermal edema and diminished DC trafficking from the skin to dLN (52, 53). Furthermore, delayed but robust T cell-mediated immune responses have also been observed in plt mice (28), another model of impaired DC trafficking to the LNs, although by a different mechanism (namely, the lack of CCR7 ligands that direct homing and positioning between APCs and naive T cells). Interestingly, splenectomized plt mice failed to mediate any T cell response to immunization (28), and efficient T cell immune responses were observed in alymphoplasia (aly/aly) mice (characterized by a complete lack of LNs and Peyer’s patches) reconstituted with functional T cells, although priming was supported in the spleen and liver (54). These data support our observations of T cell proliferation within the spleen of K14-VEGFR-3-Ig mice and indicate that lymphatic transport from the skin to the dLN after immunization is not essential for the initiation of CD8 T cell responses.

In dermal CHS experiments, ears of K14-VEGFR-3-Ig mice swelled robustly in response to dermal DNFB challenge (Fig. 6), indicating an efficient cutaneous immune reaction to DNFB mediated by Ag-specific T cells (45, 46) despite impaired transport from the skin to dLNs. Our observations contrast with the previously reported mechanism of VEGFR-3+ corneal DC-dependent, lymphatic-independent delayed-type hypersensitivity reactions to allografts (55). However, DCs outside of the cornea do not express VEGFR-3 (56); we therefore suppose that mechanisms of immune response in the cornea, which lack lymphatic vessels under non-inflamed conditions, are very different from those in the dermis. Ears of transgenic mice swelled more robustly compared with

FIGURE 5. T cell priming after immunization in K14-VEGFR-3-Ig mice is delayed. The number of OT-I (A, B) and OT-II (C, D) cells responding to immunization quantified as the total numbers of cells in each generation (G) (A, C) or as the percentage of unproliferated cells (determined by calculating the number of cells in each generation, normalized by the number of divisions, to determine the total) postimmunization with OVA + LPS in the spleen and dLNs (B, D). Initial OT-I and OT-II cell priming in WT (i, iii) and K14-VEGFR-3-Ig (ii, iv) mice, respectively. *p < 0.05, **p < 0.01 using two-way ANOVA; n = 3.
treated WT ears 5 d after sensitization with DNFB (Fig. 6C), which is consistent with responses to DNFB by kCYC+/− mice, presumably because of decreased inflammatory cytokine and chemokine expression in the dermis resulting in retention of cells and fluid within inflamed tissue (52). Although we cannot exclude differences in cytokine and chemokine expression in response to DNFB as causing the increased swelling, recent reports also implicate B cells in CHS, specifically with Ag-specific IgM-mediated CD8+ T cell recruitment (57) and inhibition of the elicitation phase by LN-resident B cells (58). The decreased ability of B cells (that normally serve inhibitory roles in CHS (57, 58)) to respond to i.d. immunization in the K14-VEGFR-3-Ig mice (Fig. 3), as well as the decreased egress of immune cells from the skin to the dLN (Fig. 1B–E), might therefore also contribute to enhanced swelling.

Stromal cells provide structure for DC migration within the T cell zone of the LN, supplying the framework for DC–T cell interactions important for adaptive immunity and tolerance. It has been suggested that afferent lymph flow is required for proper stromal organization within the LN (59). Furthermore, our laboratory previously used a tissue-engineered model of LN stroma to show that fluid flow is important for LN stromal cell organization in vitro and that CCL21 protein was localized in areas of higher fluid flow along fluid channels (60). The notion that fluid flow helps regulate stromal architecture is consistent with the disorganized architecture seen in the dLN (which presumably has less flow because of the lack of lymphatic drainage in the skin), but not mLN, of K14-VEGFR-3-Ig mice, including disorganized stromal cell distribution, minimal B cell follicle definition, and smaller or collapsed high endothelial venules (Fig. 1F–I, Supplemental Fig. 2), as has been reported within the dLN when afferent lymph flow is occluded (59). Surprisingly, the total concentration (but not distribution) of CCL21 in the dLNs appeared normal in transgenic mice, suggesting that autoregulatory mechanisms may exist to maintain LN CCL21 levels when lymph flow is decreased. Soluble VEGFR-3 in the dermis of transgenic mice or other cell types may also potentially contribute to architectural changes in the LN such as by disrupting lymphatic endothelial cell organization within

**FIGURE 6.** K14-VEGFR-3-Ig mouse ears swell in response to dermal CHS challenge but cannot be pretolerized. (A) Ear swelling 48 h after epicutaneous application of 0.3% DNFB 5 d after DNFB sensitization. Alternatively, tolerance to DNFB challenge, induced by epicutaneous DNTB treatment performed 7 d before DNFB sensitization, was seen in control mice but not in transgenic mice. (B) H&E-stained cross-sections of ears 48 h after challenge. Scale bar, 200 μm. *p < 0.05, **p < 0.01; n = 8–12. Resolution of ear swelling in DNFB challenge (C) and DNFB tolerance experiments (D).

**FIGURE 7.** One-year-old K14-VEGFR-3-Ig mice display autoimmune phenotypes. (A) Increased IgG1, IgG3, and IgA mAb isotypes in serum of K14-VEGFR-3-Ig mice. (B and C) Increased frequencies of spleen-resident megakaryocytes (black arrowheads) by H&E staining. Scale bar, 50 μm. (D) Increased serum titers of dsDNA-reactive Ig. (E) Ab deposition (red) in the skin. (F and G) Frequencies of mice with serum reactive toward indicated mouse tissues. Scale bars, 50 μm (E), 100 μm (F). n = 5–12; *p < 0.05, **p < 0.01 by Mann–Whitney, #p < 0.05 by Fisher’s exact test.
dLNs. Regardless of the mechanism, it is likely that the disorganized LN stroma and B cell follicles in transgenic mice contribute to their impaired immune responses to dermal vaccination.

Our findings of autoimmune outcomes (Fig. 7) as well as the failure to induce CHS tolerance to DNPB with DNTB pretreatment (Fig. 6) in K14-VEGFR-3-Ig mice corroborate 40-year-old reports (1, 2) that propose lymphatic drainage to a sentinel LN is essential to acquired peripheral tolerance. Those studies used a model of skin transplant where the blood, but not lymphatic, vasculature was connected to the surrounding tissue, and CHS tolerance could not be induced. Our current model is not complicated by the factor of tissue damage and inflammation, which could also affect local immunity, present in the past model. Unfortunately, those older studies were not followed up since the mid-1970s, even though there is an emerging appreciation for correct LN function and organization in peripheral tolerance, particularly with regards to the positioning of B cells and trafficking of T cells (25). Autoimmune phenotypes have been observed in mice lacking either CCR7 or CXCR5 (29, 61), the receptors for CCL21 and CXCL13 important in the segmentation of the T and B cell zones of the LN, respectively. Although those reports highlighted the need for proper B cell and TReg cell positioning in the LN, which are guided by CXCL13 and CCL21, our findings suggest that lymphatic drainage may help guide the distribution and segmentation of these cytokines, because their distributions but not total amounts were abnormal in the dLNs of K14-VEGFR-3-Ig mice (Supplemental Fig. 1B). Moreover, CCL21 expression in the skin of transgenic mice appeared drastically reduced, mostly because their main source in the skin—lymphatic capillaries—were largely absent (Supplemental Fig. 4A). Thus, the lack of lymphatic drainage from skin to the dLN could promote autoimmunity in multiple ways. However, although we see no evidence of impaired lymphatic drainage in other tissues of adult K14-VEGFR-3-Ig mice, altered lymphatics in organs other than the skin during development as characterized by Makinen and colleagues (36) may confound a strict interpretation of the observations of autoimmune phenotypes as being solely the result of impaired dermal lymphatic drainage.

In addition to the decreased availability of peripheral Ag, decreased DC trafficking, and disorganization of the dLN, the immune cell distributions within the dLN may contribute to the development of autoimmune reactions seen in 1-y-old K14-VEGFR-3-Ig mice (Fig. 7). For example, K14-VEGFR-3-Ig mice demonstrated higher systemic CD4/CD8 T cell ratios (Fig. 2B) but only local disturbances (in the LNs) Foxp3+ T cell distributions (Fig. 2C); this is likely a result of disruptions in TReg cell homeostatic migration to the dLNs via the skin draining lymphatics (62). CD19, which is expressed by B cells, complexes with the AgR of B cells to increase sensitivity to Ag-specific stimulation. Consequently, increased expression of CD19 is associated with autoimmunity (44), and overexpression of CD19 causes increased autoantibody production (43). Taken together with the disrupted stromal cell organization and decreased frequencies of TReg cells, the increased CD19 expression by cells in the LNs of K14-VEGFR-3-Ig mice (Fig. 2D) suggests that the B cell regulatory balance normally maintained by the LN (25) might be dysregulated in the transgenic mice.

In addition, there is increasing evidence linking skin fibrosis to aberrant B cell signaling and autoimmunity. TSK+/+ mice, which are heterozygous for a mutation in fibrillin-1 that results in cutaneous hyperplasia, exhibit skin sclerosis and autoimmunity as well as chronic B cell activation as a result of augmented CD19 signaling (43). Knockdown of CD19 in TSK+ mice reduces hypodermal thickness and skin collagen deposition, suggesting CD19 involvement in regulating skin pathology. IL-4 deficiency also rescues TSK+/+ mice from skin fibrosis and autoimmune body production (63). K14-VEGFR-3-Ig mice exhibit increased skin elastin deposition (Supplemental Fig. 4C). The correlating impairments in LN-resident B cell function, increased CD19 expression, and the heightened IL-4 production following LPS immunization in K14-VEGFR-3-Ig mice suggest a strong coupling between CD19-mediated B cell autoimmunity and skin fibrosis with impaired dermal lymphatic drainage.

In conclusion, this study demonstrates the pivotal role of dermal lymphatic drainage in the organization and function of B cells resident in the draining LN, efficient Ab responses to dermal vaccination, and postdevelopmental tolerance induction against Ag encountered in the skin. In contrast, T cell responses to dermal challenge appeared to be only delayed by impaired lymphatic flow, with the exception of possibly skewing responses toward a Th2 phenotype. These findings implicate lymphatic drainage as critical to regulating humoral immunity and peripheral tolerance.

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References


