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ARTICLE

Pro-lymphangiogenic VEGFR-3 signaling modulates memory T cell responses in allergic airway inflammation

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In allergic airway inflammation, VEGFR-3-mediated lymphangiogenesis occurs in humans and mouse models, yet its immunological roles, particularly in adaptive immunity, are poorly understood. Here, we explored how pro-lymphangiogenic signaling affects the allergic response to house dust mite (HDM). In the acute inflammatory phase, the lungs of mice treated with blocking antibodies against VEGFR-3 (mF4-31C1) displayed less inflammation overall, with dramatically reduced innate and T-cell numbers and reduced inflammatory chemokine levels. However, when inflammation was allowed to resolve and memory recall was induced 2 months later, mice treated with mF4-31C1 as well as VEGF-C/-D knockout models showed exacerbated type 2 memory response to HDM, with increased Th2 cells, eosinophils, type 2 chemokines, and pathological inflammation scores. This was associated with lower CCL21 and decreased T_{Regs} in the lymph nodes. Together, our data imply that VEGFR-3 activation in allergic airways helps to both initiate the acute inflammatory response and regulate the adaptive (memory) response, possibly in part by shifting the T_{Reg}/Th2 balance. This introduces new immunomodulatory roles for pro-lymphangiogenic VEGFR-3 signaling in allergic airway inflammation and suggests that airway lymphatics may be a novel target for treating allergic responses.

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INTRODUCTION

Allergic asthma is a chronic inflammatory disease that is characterized by airway hyperresponsiveness, enhanced mucus production, type 2 immune cell infiltration, and smooth muscle contraction, all in response to inhalation of a specific allergen such as cat dander or house dust mite (HDM). Sensitization to the allergen involves the activation of type 2 $CD4^+$ (Th2) T cells and IgE-producing B cells, which in turn activate and recruit eosinophils, mast cells (in humans), and macrophages; other cellular modulators include Th17, Th9, and regulatory (T_{Reg}) $CD4^+$ T cells as well as type 2 innate lymphoid cells (ILC2).[1–3] Lymphatic vessels, which transport antigens, cytokines, and immune cells to the lymph nodes (LN), are critical to both the clearance of inflammatory cells and the induction and regulation of adaptive immune responses.[4,5] Lymphatic expansion or lymphangiogenesis, driven by vascular endothelial growth factor (VEGF) receptor 3 (VEGFR-3) signaling by its ligands VEGF-C and/or VEGF-D, occurs in allergic airway disease, particularly near bronchi and arteries,[6,7] although its pathological consequences are poorly understood.[8,9]

While lymphangiogenesis has been reported to occur in numerous types of chronic inflammatory diseases, including cancer, inflammatory bowel disease (IBD), chronic respiratory infection, and chronic skin inflammation, we still lack an integrated comprehension of how it alters adaptive immune responses in such diseases. In cancer, lymphangiogenesis plays complex roles in shaping the immune response,[4,5,10,11] both promoting immune suppression as well as, paradoxically, priming adaptive immune responses.[12] In mouse models of chronic inflammatory arthritis and IBD, blocking VEGFR-3 signaling was found to exacerbate inflammation,[13–15] but in Crohn's disease patients, excessively dilated and obstructed lymphatic vessels have been associated with lymph stasis, suggesting that blocking lymphangiogenesis should reduce inflammation.[16] In oxazolone-induced chronic skin inflammation, VEGFR-3 stimulation is protective, hampering the development of epidermal hyperplasia and accumulation of $CD8^+$ T cells.[17,18] In corneal, cardiac, and islet transplantation studies in mice, VEGFR-3 stimulation is promotes graft rejection.[19–21]

In non-allergy models of chronic lung inflammation, such as chronic respiratory infection of *Mycoplasma pulmonis*, lymphangiogenesis has been shown to play protective roles by promoting lymphatic drainage and resolution of inflammation.[22–24] However, those studies used inflammatory mediators that induce type 1 inflammation, such as mismatched transplantation[24] or bacterial infection.[22,23] Much less is known about lymphangiogenesis in chronic type 2 inflammatory diseases. Interestingly, Th2 cytokines, particularly IL-4 and IL-13, have been reported to inhibit lymphangiogenesis,[25,26] which appears inconsistent to reports of lymphangiogenesis in allergic airway disease.[6,7]

Here, we asked whether pro-lymphangiogenic signaling plays protective or pathogenic roles in allergic airway inflammation, focusing on the adaptive immune response with memory recall

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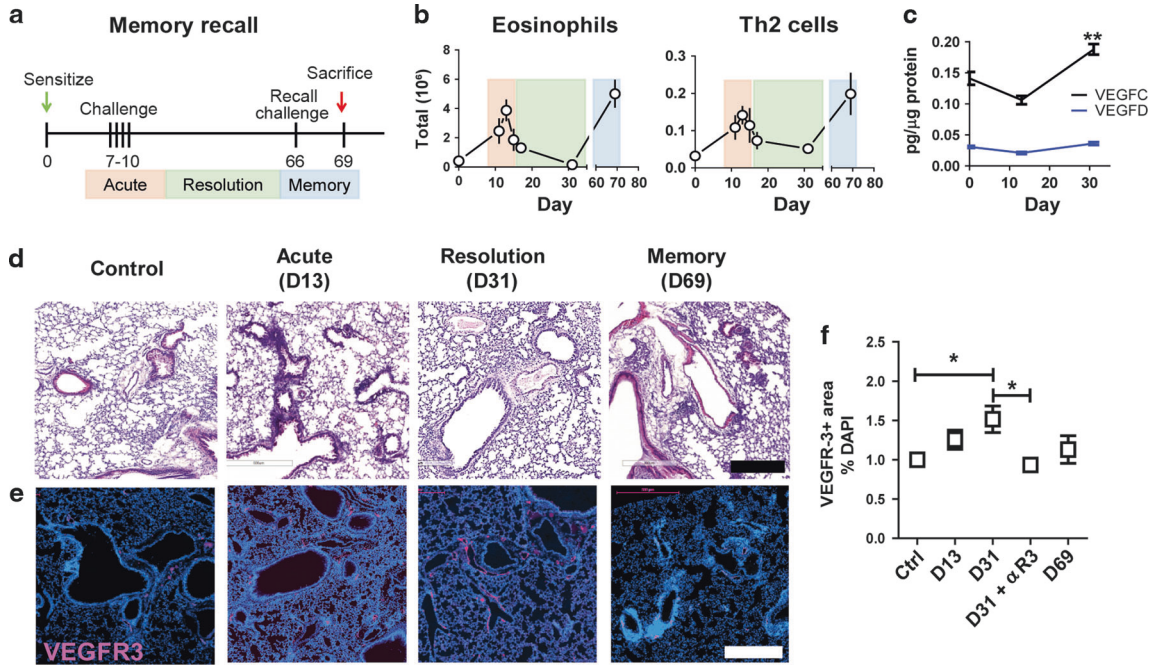

Fig. 1 HDM-mediated allergic airway inflammation induces lymphangiogenesis during resolution and lymphangiogenesis can be blocked using anti-VEGFR-3 antibody treatment. a Schedule of HDM-induced allergic airway inflammation in mice. On day 0, 100 µg HDM in 50 µl PBS was given intranasally on day 0, followed by four challenges of 25 µg each on days 7–10; after 8 weeks, 25 µg HDM was given as a memory recall on day 66, followed by sacrifice on day 69. **b** Numbers of eosinophils (Ly-6G-CD11c^{lo}Siglec-F^{+}, left) and Th2 (Foxp3^{-}Gata3^{hi}) CD4^{+} T cells (right) in the lung throughout the acute, resolution, and memory recall phases. **c** VEGF-C and VEGF-D levels in the lungs using ELISA. **d** H&E staining showing inflammation and **e** VEGFR-3 immunofluorescence showing lymphatic vessels, comparing control (PBS-challenged) with the three phases of HDM-induced inflammation. Scale bars, 500 µm. **f** Quantification of lymphatic area (VEGFR-3^{+} pixels/DAPI^{+} pixels) in the lungs at these time points, as well as at day 31 using anti-VEGFR-3 mF4-31C1 (αR3). Boxes represent median (central bar) with range from 25th to 75th percentile, and whiskers represent min to max value. Data are representative of $n \geq 4$ mice each in two to three experiments, and statistics (one-way ANOVA with Dunnett's multiple comparison test) were performed in GraphPad Prism. *$p < 0.05$.

challenges. Using both blocking antibodies against VEGFR-3 and transgenic models, we show that VEGFR-3 signaling facilitates the acute inflammatory response to HDM, but is protective with respect to memory challenges. This work highlights the complexity of pro-lymphangiogenic VEGFR-3 signaling in regulating both innate and adaptive immunity in allergic airway disease.

RESULTS

Lymphangiogenesis occurs late after the onset of allergic airway inflammation

To explore the roles of lymphangiogenesis and VEGFR-3 signaling in a murine model of HDM-mediated allergic airway inflammation, we developed a long-term model (Fig. 1a) that allowed us to study the acute inflammatory peak (day 13),[27] resolution (day 31), and memory recall response (day 69), as evidenced by the characteristic changes in eosinophilia and Th2 cells (Fig. 1b). Cell numbers were determined by flow cytometry (gating strategies in Supplementary Fig. 1A–C). In this model, we found that VEGF-C was significantly increased in the resolution phase at day 31 (Fig. 1c). Histopathological features of airway inflammation were seen at day 13 and after memory recall at day 69 (Fig. 1d). Lymphatic vessels, immunostained for VEGFR-3 (a common marker of lung lymphatics[22,23]), were increased in density at day 31 compared with PBS-treated mice (Fig. 1e, f), although LECs began to express the cell proliferation marker Ki-67 by day 11 (Supplementary Fig. 1C, D). We could prevent lymphatic vessel expansion with systemic administration of mF4-31C1 (Fig. 1f), a blocking antibody against mouse VEGFR-3 that prevents lymphangiogenesis without affecting existing lymphatics.[28]

Blocking VEGFR-3 signaling reduces overall CD45^{+} cells during acute allergic inflammation and modulates dLN cellular composition during acute inflammation and resolution

Having established that lymphangiogenesis occurs in the resolution phase, we sought to understand how blocking pro-lymphangiogenic VEGFR-3 signaling modulates the acute response and resolution in allergic airway inflammation. We blocked VEGFR-3 using mF4-31C1 throughout the entire experiment for the acute schedule and until day 21 for the resolution schedule (Fig. 2a). We found that VEGFR-3 blockade significantly decreased many inflammatory (CD45^{+}) cell populations present in lung tissues at day 13, including eosinophils, T cells, B cells, monocytes, and neutrophils (Fig. 2b, top, c), but not alveolar macrophages (Supplementary Fig. 2A). Blocking VEGFR-3 reduced these cell types similarly relative to the total CD45^{+} population (Fig. 2c, Supplementary Fig. 2A). During resolution at day 31, these were decreased below baseline levels in both the control and mF4-31C1-treated mice (Fig. 2b, bottom); however, relative to all CD45^{+} cells in the lung, both neutrophil and eosinophil populations were reduced more in mice treated with mF4-31C1 compared with controls upon postinflammatory cascade contraction (Fig. 2c). When we looked more specifically at the CD4^{+} T-cell subsets, we found that at day 13 in the lungs both T_{Reg} cells and naive CD4^{+} T cells (T_{naive}) were slightly decreased, though not significant, in mF4-31C1-treated mice. We also found that IL-4 and IL-10, but not IL-5, producing CD4+ T cells restimulated in vitro with HDM were decreased in lungs of mF4-31C1-treated mice (Supplementary Fig. 2B). At day 31, the lung subsets are unchanged (Fig. 2d).

While in the lungs no significant changes in CD4^{+} T-cell subsets were found, CD4^{+} T-cell subsets in the dLN are significantly

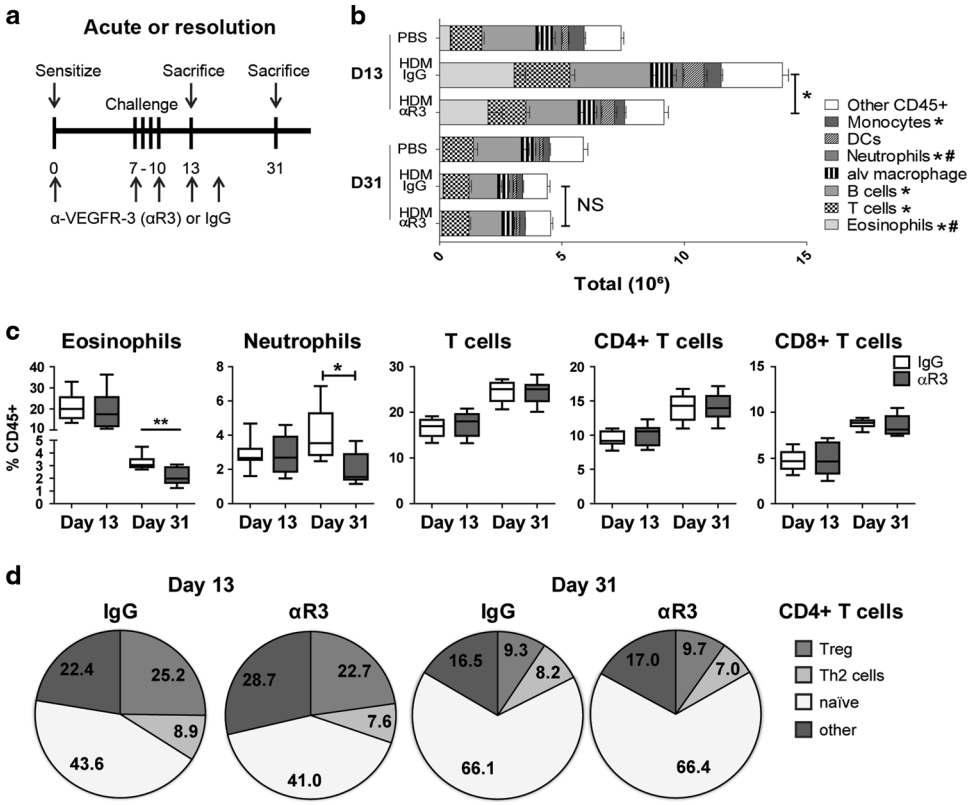

Fig. 2 VEGFR-3 blocking dampens acute HDM-mediated inflammation in murine lungs and modulates dLN composition in the acute phase and during resolution. a Schedule of HDM-induced allergic airway inflammation in mice. Mice were challenged with 100 µg HDM on day 0, followed by four consecutive challenges of 25 µg of HDM on days 7–10. Pro-lymphangiogenic signaling was blocked using mF4-31C1 and mice were sacrificed to assess the level of inflammation either at day 13 (inflammatory peak) or at day 31 (final dose of mF4-31C1 given on day 21). **b** Total numbers of overall CD45$^+$ cells at day 13 and 31, with subsets including monocytes, neutrophils, B cells, alveolar macrophages, DCs, T cells, and eosinophils in the lungs. ($p < 0.05$: *—day 13, #—day 31). **c** Eosinophils, neutrophils, T cells, CD4$^+$ T cells, and CD8+ T cells as %CD45$^+$ cells at day 13 and 31. **d** CD4$^+$ T-cell subsets in lungs at day 13 and day 31, including T_{Reg}, Th2, and T_{naive} cells as %CD4+ cells. All cell numbers were assessed via flow cytometry. Boxes represent median (central bar) with range from 25th to 75th percentile, and whiskers represent min to max value. Data are representative of $n \geq 4$ mice and two to three experiments, and statistics (two-tailed Student's t test) were performed in GraphPad Prism. * or $^{\#}p < 0.05$, and $^{+}0.05 < p < 0.1$.

affected by mF4-31C1 treatment at both time points: CD4$^+$ T_{naive} cells are increased, while T_{Rreg} cells are decreased (Fig. 3a). Th2 cells are only decreased at day 13 (Fig. 3a), consistent with overall decreased inflammation in the lungs upon mF4-31C1 treatment, but are unchanged at day 31 (Fig. 3a). Naive T cells are known to express CCR7, the receptor for the chemokine CCL21. It has been established that lymphatics, particularly during inflammation and in response to pro-lymphangiogenic VEGF-C, produce CCL21.[4,5,29] CCL21 is present at steady state in both lungs and dLNs (lungGENS database).[30–32] Since VEGFR-3 signaling induces CCL21 upregulation in LECs and we observed changes in the CCR7$^+$ T-cell compartment, we sought to analyze CCL21 levels in the lungs upon VEGFR-3 blocking. At day 13, mF4-31C1 treatment decreased CCL21 in the lungs, quantified by immunofluorescence and ELISA (Fig. 3b, c). In the dLNs, however, CCL21 levels did not decrease with mF4-31C1 treatment at day 13 (Fig. 3c). In our experiments, we observed proportional decreases of several innate cells along with T cells, including neutrophils and monocytes (both were decreased in total number upon mF4-31C1 treatment), which are not known to express CCR7. We therefore sought to assess changes in other chemokines due to mF4-31C1 treatment. Using a multiplex kit (Luminex), we found that several other chemokines, including CXCL2, CCL5, CCL7, CXCL10, CCL11, CCL12, CCL2, CXCL13, CCL3, and CXCL12, are also decreased by mF4-31C1 treatment (Fig. 3d).

Blocking VEGFR-3 signaling exacerbates the memory response to HDM

We sought to assess changes in the memory response when performing mF4-31C1 treatment during the first 21 days of the allergic response (Fig. 4a) and rechallenging with HDM after several weeks. We found that overall lung inflammation, as indicated by CD45$^+$ cell numbers (Fig. 4b), including total T cells and CD4+ T cells (Supplementary Fig. 3A), as well as histologically (Fig. 4c), were not enhanced in mice receiving mF4-31C1 treatment. However, we found an increase in eosinophils (Fig. 4d, Supplementary Fig. 3B), CD4$^+$ Th2s (Fig. 4d), and CD4$^+$ T_{EM} (Supplementary Fig. 3C), but not T_{Reg} cells (Fig. 4d) or serum IgE levels (Supplementary Fig. 3D). Furthermore, both the ratios of CD4$^+$ T_{EM} to T_{CM} and Th2 to T_{Reg} cells are increased in mF4-31C1 vs. isotype antibody (IgG) treated mice (Fig. 4e). In addition, in vitro restimulation with PMA and ionomycin clearly showed enhanced levels of IL-4$^+$, IL-13$^+$, and IL-4$^+$ IL-13$^+$ double positive CD4$^+$ T cells (Fig. 4f, Supplementary Fig. 3E), but not IFNγ$^+$ CD4$^+$ T cells in mF4-31C1-treated mice. Overall type 2 cytokine producing cells made up ~50% of CD4$^+$ T cells in mF4-31C1-treated mice, compared with ~30% in mice receiving isotype antibody treatment (Fig. 4f). HDM specific restimulation of T cells showed increased IL-5 and IL-13, though not significant ($0.05 < p < 0.1$), but not IFNγ (Fig. 4g).

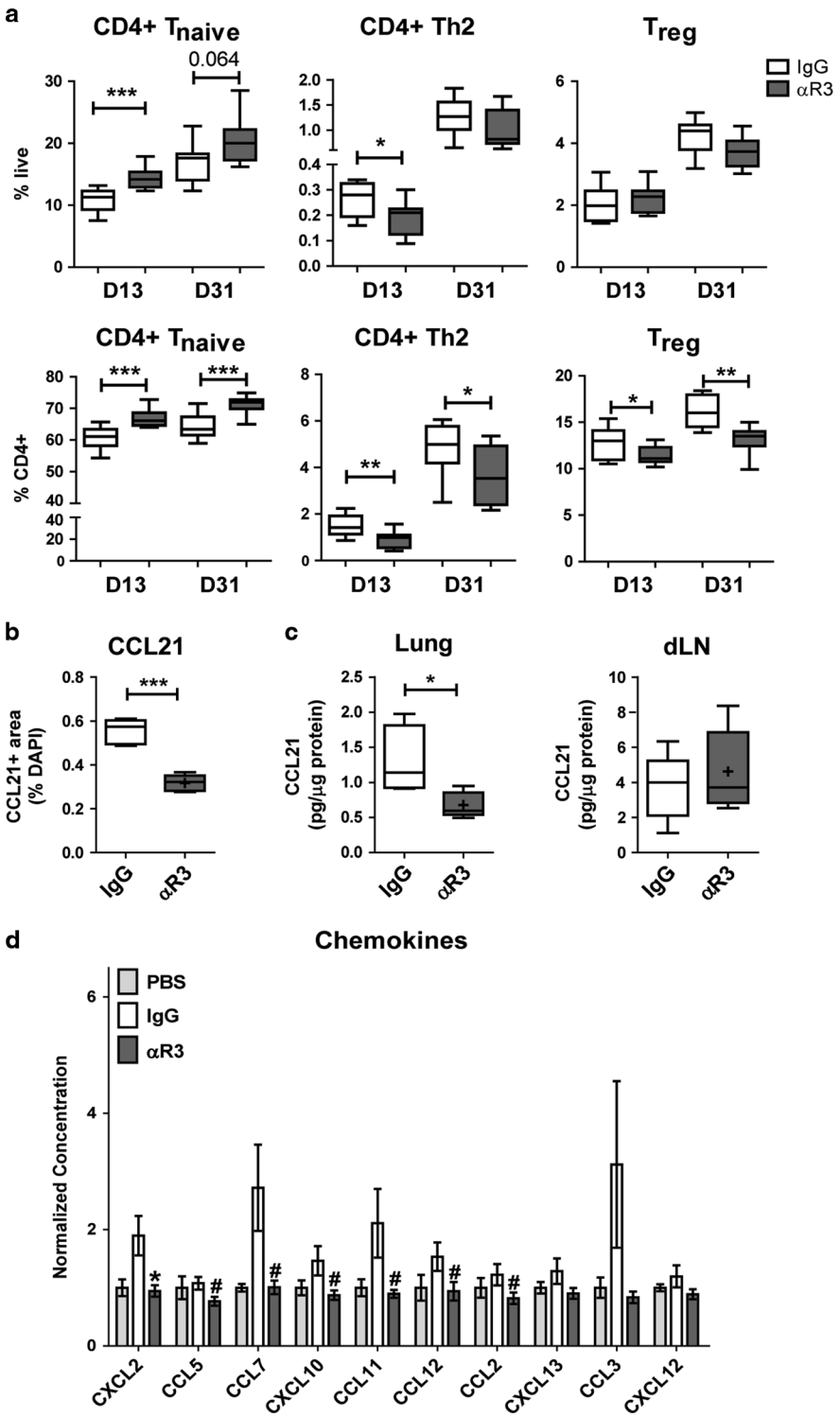

Fig. 3 VEGFR-3 blocking treatment increases CD4+ T_{naive} in the dLN during acute inflammation and resolution, and decreases CCL21 and other chemokines in the lungs during acute inflammation. Mice were challenged as described in Fig. 2. $CD4^+$ T-cell subsets including $CD4^+$ T_{naive}, T_{Reg}, and Th2 cells in the dLN at **a** day 13 and day 31. Levels of the CCR7 chemokine, CCL21, in lungs and dLN as determined by **b** immunofluorescence staining, possible since CCL21 both sticks to extracellular matrix and cytoplasm, (scale bar indicates 400 μm) and **c** ELISA. **d** Luminex assay on lung lysates to assess chemokine levels including CXCL2, CCL5, CCL7, CXCL10, CCL11, CCL12, CCL2, CXCL13, CCL3, and CXCL12 at day 13. Boxes represent median (central bar) with range from 25th to 75th percentile, and whiskers represent min to max value. Data are representative of $n \geq 4$ inflamed mice and statistics (two-tailed Student's t test) were performed in GraphPad Prism. $^{\#}0.05 < p \leq 0.15$, $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p < 0.001$.

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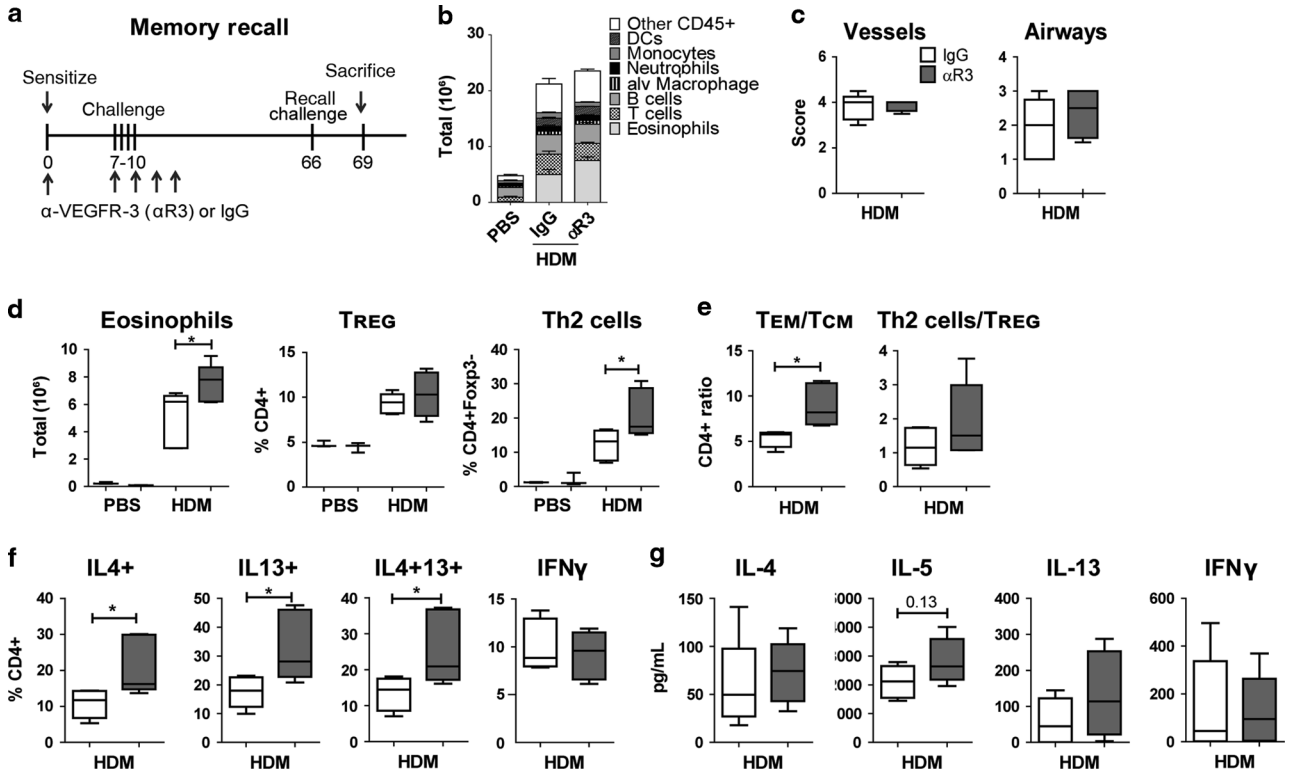

Fig. 4 Blocking pro-lymphangiogenic signaling using the VEGFR-3 blocking antibody mF4-31C1 induces an exacerbated memory response to HDM. a Schedule of HDM-induced allergic airway inflammation in mice (as described previously) including anti-VEGFR-3 blocking treatment using mF4-31C1. **b** Overall $CD45^+$ inflammatory cell numbers. **c** Blinded inflammation score performed by a pathologist: perivascular inflammation and peribronchiolar inflammation was scored from 0 to 5 on an ordinal scale, with 0 indicating normal structures with no pathologic inflammatory cells, 1 indicating few scattered inflammatory cells, 2 indicating a circumferential inflammatory band 1 cell thick, 3 indicating a circumferential inflammatory band 2–4 cells thick, 4 indicating a circumferential inflammatory band >4 cells thick, and 5 indicating the presence of nodular lymphoid follicular hyperplasia. **d** Eosinophil, Th2 cell, and T_{Reg} cell levels as assessed by flow cytometry. **e** $CD4^+$ T_{EM} to T_{CM} and Th2 to T_{Reg} ratios. **f** IL-4^+ and IL-13^+ $CD4^+$ T cells levels after in vitro restimulation with PMA and ionomycin. **g** ELISAs for IL-4, -5, and -13 on HDM specific in vitro restimulated single cell lung lysate samples (48 h). Boxes represent median (central bar) with range from 25th to 75th percentile, and whiskers represent min to max value. Data are representative of $n \geq 4$ inflamed mice ($n \geq 3$ for PBS-treated mice) and two to three experiments, and statistics (two-tailed Student's t test, comparing only HDM treated groups) were performed in GraphPad Prism. *$p < 0.05$, **$p \leq 0.01$.

Mice lacking VEGF-C and VEGF-D show exacerbated memory response to HDM

To understand if the exacerbated memory response is dependent on the absence of one of, or both, the pro-lymphangiogenic growth factors (i.e., VEGF-C and/or VEGF-D), we compared the memory response induced by HDM in wild-type ("WT") mice with that in mice lacking either VEGF-C (*Rosa26*$^{Cre\text{-}ERT2}$;*Vegfc*$^{flox/flox}$, herein referred to as "VEGF-C KO" or "VC KO" in figures), VEGF-D (*Vegfd*$^{-/-}$, herein referred to as "VEGF-D KO" or "VD KO" in figures), or both (*Rosa26*$^{Cre\text{-}ERT2}$;*Vegfc*$^{flox/flox}$;*Vegfd*$^{-/-}$, herein referred to as "double KO") that have been previously described.[33,34] We induced *Vegf-c* deletion prior to HDM challenge in our established memory schedule (Fig. 5a), and then assessed the histopathology of the lungs (Fig. 5b). After blinded scoring, we found that inflammation surrounding both vessels and airways was enhanced from 3 ± 1 around vessels and 2 ± 1 around airways in WT mice to 5 ± 1 (vessels) and 4 ± 1 (airways) in double KO mice (Fig. 5c, scoring criteria can be found in the Supplementary Methods). It also appears that lack of VEGF-D alone results in these pathological changes (Fig. 5c).

We next sought to assess differential inflammatory cell infiltration into the lungs in these mice (Fig. 5d–g). With HDM treatment, lungs of double KO mice contained slightly increased eosinophils (though not significant, Fig. 5d), increased $CD4^+$ T_{EM}/T_{CM} ratios (Fig. 5e), increased Th2 $CD4^+$ T cells (Fig. 5f), and higher ratios of Th2/T_{Reg} cells (Fig. 5g). No inflammation was induced by removing VEGF-C and/or VEGF-D in control PBS-treated mice (Fig. 5d, f). When we examined the functionality of these cells by restimulating in vitro with PMA and ionomycin, we found a 2.8-fold increase in both IL-5^+ and IL-13^+, but not in IFNγ$^+$ $CD4^+$ T cells (Fig. 5h, i). This effect appeared to be antigen specific, as HDM restimulation of lung single cell suspension resulted in enhanced IL-4, IL-5, and IL-13 but not IFNγ levels produced by lung cells (Fig. 5j).

DISCUSSION

In our presented work, we have found that pro-lymphangiogenic VEGFR-3 signaling modulates allergic airway inflammation. During acute inflammation, VEGFR-3 blocking decreased immune cell recruitment into the lungs, though more naive T cells were found in the dLN. In contrast, the memory response to allergen is significantly exacerbated by a lack of VEGFR-3 signaling (either by mF4-31C1 treatment or in mice lacking VEGF-C and/or VEGF-D). We found that the exacerbated memory response is dependent on the absence of both VEGF-C and VEGF-D, suggesting that these two growth factors may compensate for each other in allergic airway inflammation. We hypothesize that the changes in the memory response may at least in part be due to the decrease of CCL21 levels in the lungs (but not in the dLN) at day 13 that cause accumulation of naive T cells in the dLN, leading to more priming of the increased naive T cells. Increased T-cell priming could ultimately lead to the exacerbated memory response we find at

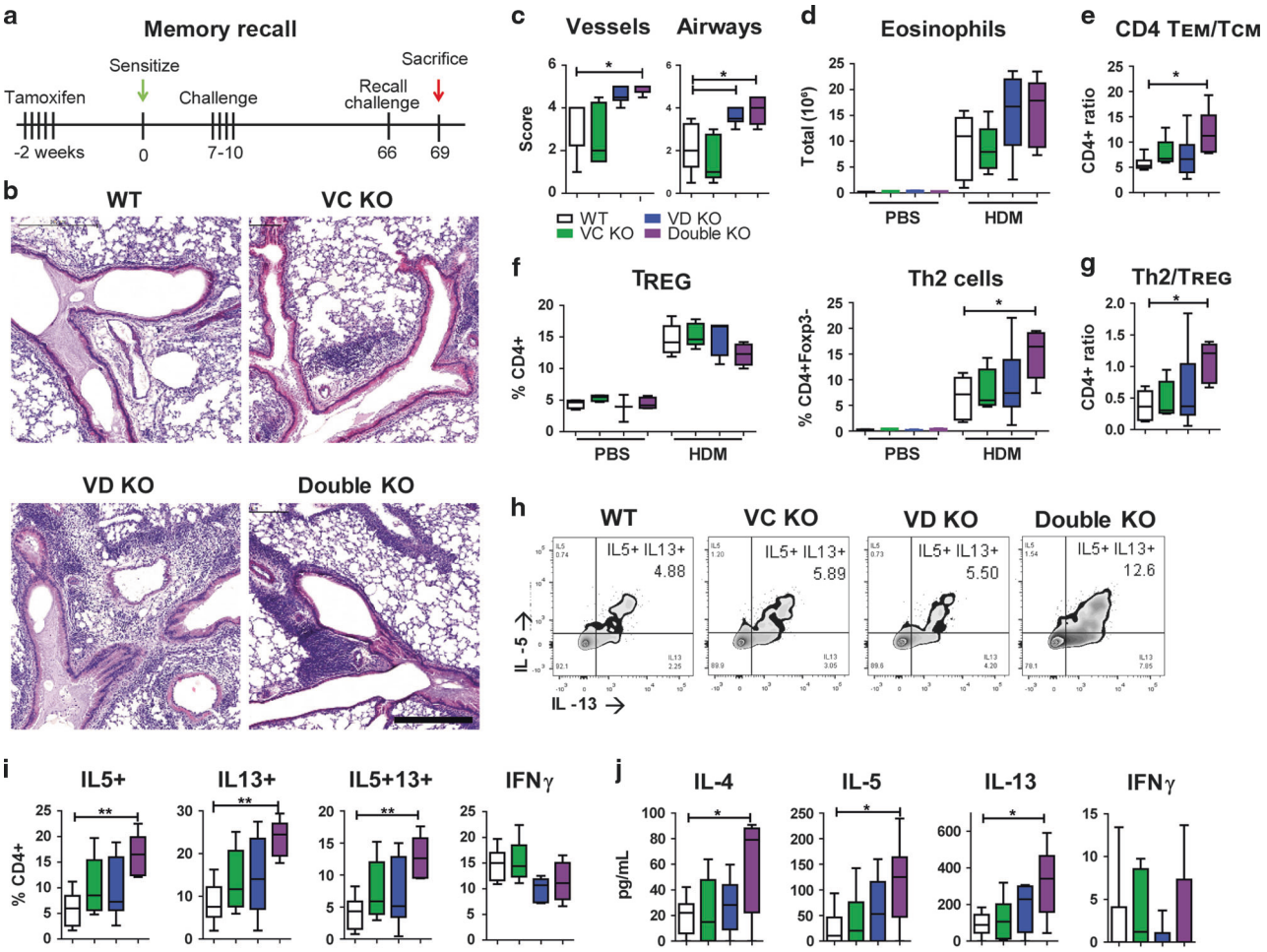

Fig. 5 The exacerbated memory response is dependent on absence of both VEGF-C and VEGF-D. a Schedule of HDM-induced allergic airway inflammation in mice including treatment of all mice with tamoxifen more than 2 weeks prior to inducing allergic inflammation. Allergic inflammation was induced in wild type ('WT') mice and mice lacking either VEGF-C (*Rosa26*$^{Cre\text{-}ERT2}$;*Vegfc*$^{flox/flox}$, here referred to as "VC KO"), VEGF-D (*Vegfd*$^{-/-}$, here referred to as "VD KO"), or both (*Rosa26*$^{Cre\text{-}ERT2}$;*Vegfc*$^{flox/flox}$;*Vegfd*$^{-/-}$, here referred to as "double KO") that have been previously described.[33,34] **b** H&E staining showing inflammation (scale bar indicates 500 µm), and **c** blinded inflammation scores. Flow cytometric analysis of immune cell infiltration into the lungs including **d** eosinophils, **e** CD4+ T_{EM}/T_{CM} ratios, **f** T_{Reg} cells, and CD4+ Th2 cells, and **g** Th2/T_{Reg} ratios. **h–i** IL-5$^+$, IL-13$^+$, IFNγ$^+$, and IL5$^+$13$^+$ double producing CD4+ T cells after in vitro restimulation with PMA and ionomycin on lung single cell suspensions quantified via flow cytometry. **j** IL-4, IL-5, and IL-13 levels after 48 h in vitro restimulation with HDM on lung single cell suspensions to assess antigen specificity of the Th2 cells in the lungs. Boxes represent median (central bar) with range from 25th to 75th percentile, and whiskers represent min to max value. Data are representative of $n = 4$ mice for PBS treated and $n = 6$ mice for inflamed mice ($n = 1$ experiment for mice lacking VC/VD), and statistics (one-way ANOVA with Dunnett's multiple comparison mice lacking VEGF-C and/or VEGF-D). Tests were performed in GraphPad Prism. *$p < 0.05$, **$p \leq 0.01$.

D69. We suspect that effects of blocking pro-lymphangiogenic VEGFR-3 signaling were both due to altering lung lymphatics, and by affecting immune cells such as macrophages that may also respond to VEGFR-3 stimulation. Taken together, our work is the first study to describe critical roles of pro-lymphangiogenic signaling in modulating immunity during allergic airway inflammation, leading to enhanced acute inflammation but an ameliorated memory response later on.

Existing studies on allergic inflammation and lymphangiogenesis generally focused on whether or not lymphangiogenesis occurs, and have led to conflicting findings.[6,9,25,35,36] Our findings corroborate studies showing lymphangiogenesis in an HDM model of respiratory allergies in rats,[6] and studies by Kretschmer et al. demonstrating that HDM challenge leads to lymphangiogenesis and T-cell infiltration in murine lungs.[37] Other studies have also demonstrated that lymphangiogenesis occurs more broadly in chronic airway inflammation, particularly during diseases such as lymphangioleiomyomatosis, and in certain regions within lungs of idiopathic pulmonary fibrosis patients.[7,22,23,37–40] Interestingly, fatal asthma in humans, where fibrosis has occurred in much of the lung, is associated with fewer lymphatic vessels in the lungs.[9,25,35] However, a thorough study of how lymphatic vessels are altered during disease progression in human asthmatic patients has yet to be performed, and existing studies in mice and rats likely are more representative of earlier time points than fatal asthma.

The CCL21/CCR7 axis is a well-established mechanism by which lymphatics modulate immunity, and CCL21 production can be induced in LECs with pro-lymphangiogenic VEGF-C. In the dLNs, lymphatics are one of the major sources of CCL21, which attracts CCR7$^+$ T cells and DCs.[29,41] Previous studies by Baluk et al. demonstrated that CCL21 is increased in mouse lung lymphatics during chronic bacterial infections.[23] Here, we have found that CCL21 is reduced in the lungs during acute inflammation when pro-lymphangiogenic signaling is blocked. This axis has also been studied in allergic airway inflammation using *Ccr7*$^{-/-}$ knockout mice, or mice lacking CCL21/CCL19 expression in secondary lymphoid organs (*plt*/*plt* mice).[30,31] Kawakami et al. showed that

Ccr7$^{-/-}$ KO mice have an exacerbated response to HDM, with increased eosinophilia, IL-13 levels in the lungs, and airway response to acetylcholine.[30] In contrast, Ploix et al. demonstrated that *plt/plt* mice did not appear to have significantly changed responses to an OVA/alum based model of allergic airway inflammation.[31] Another study by Xu et al. with *plt/plt* mice appears to contradict this, clearly demonstrating greater eosinophilia and lymphocyte numbers in the bronchoalveolar lavage fluid (BAL), inflammation score of lung histology, type 2 cytokines (as detected by RNA), and IgE production in *plt/plt* mice compared with controls.[42] A third study by Yamashita et al. suggests that early on, airway inflammation was reduced in *plt/plt* mice, as indicated by airway response to acetylcholine and overall lymphocytes and cell numbers in BAL.[43] However, after cessation of antigen dosing, inflammation appears to resolve less in *plt/plt* mice compared with WT controls, reconciling the earlier studies.[43] Similarly, our data demonstrate that overall inflammatory cell numbers are decreased early on, though allergic inflammation is still present, and a robust memory response is formed. Differences between our results and those from *plt/plt* mice likely stem from the fact that in *plt/plt* mice, CCL21 is still produced at low levels in LECs, which may have been increased during inflammation, a possibility that was not considered in the discussed studies. In addition, both CCL21 and CCL19 are abrogated entirely from the dLNs in *plt/plt* mice, whereas our studies likely affected mostly the LEC population. This is substantiated by previous studies showing that VEGFR-3 blocking abrogates CCL21 production mainly in LECs, but may not modulate CCL21 production in other cell types.[20,44,45] In addition, we found that VEGFR-3 blocking not only modulated CCL21, but also decreased other chemokines involved in recruitment of immune cells including T cells, eosinophils, neutrophils, and basophils, which could explain changes in non-CCR7^{+} inflammatory cells we have observed. Furthermore, other cells, including macrophages and ILCs, have been shown to express VEGFR-3 and produce chemokines, and thus may be responsible for some of the changes in chemokines and thus inflammatory cells we have observed (see ImmGen database[46–49]). It is therefore likely that VEGFR-3 blocking modulates other chemokine secretion by other immune cells during the allergic response.

LECs have been shown to produce several cytokines. This includes IL-7 in response to viral infection and IL-15 in response to lipopolysaccharide induced inflammation.[50,51] IL-7 and IL-15 production suggests a role for lymphatics in the maintenance of T-cell homeostasis (by IL-7)[52] and memory, as well as homeostasis of NK cells and NK T cells (by IL-15).[53] Interestingly, in a model of allergic airway inflammation that specifically induces bronchus-associated lymphoid tissues by adoptive transfer of activated Th2 cells from DO11.10 OVA-specific Tg mice, Thy1^{+} LECs were shown to produce IL-7.[36] The authors postulate that this enhances local survival of the memory Th2 population in the lungs, suggesting a critical role for LECs in immune memory maintenance.[36] Interestingly, we have also seen that chronic allergic airway inflammation enhances IL-7 production in lung LECs (data not shown), and studies on whether VEGFR-3 signaling is critical in inducing memory T-cell-maintaining phenotype in LECs during chronic HDM-mediated allergic inflammation are currently ongoing.

In summary, we have found that blocking pro-lymphangiogenic signaling exacerbates allergic memory responses. This is likely in part due to an increase in naive T cells in the dLN, and decrease in chemokine levels in the lungs. Therefore, our studies provide new evidence that blocking pro-lymphangiogenic signaling modulates the allergic response by first reducing inflammatory cells in the acute phase and then exacerbating memory responses.

METHODS

We induced HDM-mediated allergic airway inflammation in mice. To test the effects of pro-lymphangiogenic signaling, mice were treated with VEGFR-3 antibody (mF4-31C1) blockade and lung and lymph node inflammatory cell infiltration and lymphatics were assessed using flow cytometry, in vitro restimulation, fluorescence immunostaining, and histology, and cytokine and chemokine levels were assessed using ELISA and multiplex assays.[54–59] More extensive methods can be found in the Supplementary Materials.

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AUTHOR CONTRIBUTIONS

K.M. designed, performed, and analyzed experiments and wrote the manuscript. C.L.H., D.F.C., and L.P. aided in experimental design, analysis, data interpretation, and manuscript preparation. D.B.C. performed histological analysis, and J.E.G.M., H.N., and R.G. aided in experiments and data collection. K.A., A.I.S., and M.A.S. helped design experiments, interpreted data, and edited the manuscript.

ADDITIONAL INFORMATION

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