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INNATE IMMUNE ACTIVATION POTENTIATES ALLOIMMUNE LUNG DISEASE INDEPENDENT OF CXCR3

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Abstract

Background—Pulmonary graft-versus-host disease (GVHD) after hematopoietic cell transplant (HCT) and allograft rejection after lung transplant are parallel immunologic processes that lead to significant morbidity and mortality. Our murine model of pulmonary GVHD after inhaled lipopolysaccharide (LPS) suggests that innate immune activation potentiates pulmonary transplant-related alloimmunity. We hypothesized that the CXCR3 receptor is necessary for development of LPS-induced pulmonary GVHD.

Methods—Recipient mice underwent allogeneic or syngeneic HCT followed by inhaled LPS. CXCR3 receptor inhibition was performed by using either CXCR3-knockout donors or systemic anti-CXCR3 antibody blockade. Pulmonary histopathology, cellular sub-populations, cytokine proteins and transcripts were analyzed.

Results—In comparison to lungs of LPS-unexposed and syngeneic controls, lungs of LPS-exposed allogeneic HCT mice demonstrated prominent lymphocytic perivascular and peribronchiolar infiltrates. This pathology was associated with increased CD4+ and CD8+ T cells as well as an increase in CXCR3 expression on T cells, a 2-fold upregulation of CXCR3 transcript and a 4-fold increase in its ligand CXCL10/IP10. CXCR3 inhibition using gene-knockout strategy or antibody blockade did not change the severity of pulmonary pathology (mean pathology score 6.5 for sufficient vs. 6.5 knockout, $p=1.00$; mean score 6.8 for antibody blockade vs. 7.4 control, $p=0.46$). CXCR3 inhibition did not prevent CD3 infiltration, nor prevent production of IL-12p40, nor significantly change other Th1, Th2, or Th17 cytokines in the lung.

Conclusions—In the setting of allogeneic HCT, innate immune activation by LPS potentiates pulmonary GVHD through CXCR3-independent mechanisms. Clinical strategies focused on inhibition of CXCR3 may prove insufficient to ameliorate transplant-related lung disease.

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Keywords

Pulmonary graft-versus-host disease; Lung rejection; CXCR3; Lipopolysaccharide; Innate immunity

INTRODUCTION

Pulmonary graft-versus-host disease (GVHD) after hematopoietic-cell transplantation (HCT) is becoming increasingly recognized, manifest acutely with interstitial pneumonitis and/or lymphocytic bronchiolitis (1,2) and chronically with airways fibrosis and airflow obstruction. A similar process affects recipients of human lung transplantation (3,4). Our laboratory has developed the novel hypothesis that activation of local pulmonary innate immunity critically regulates development of transplant-related lung disease. We have demonstrated that polymorphic variants in Toll-like receptor 4 (TLR4) regulate rates of allograft rejection in lung transplant recipients (5–7) and that inhaled LPS after murine allogeneic HCT initiates localized pulmonary GVHD (8). Similarly, polymorphic variation in LPS-binding proteins has been shown to influence airflow obstruction after human HCT (9).

The chemokine CXCL10/IP10, a ligand of the CXCR3 receptor, can be induced by LPS (10) and has been proposed as a potent chemoattractant for CXCR3+ T cells in multiple pulmonary diseases (11–14). CXCR3 blockade has been shown to reduce the severity of idiopathic pneumonia syndrome (15) and gastrointestinal GVHD (16) in murine HCT models as well as rejection in mouse models of heart (17), islet-cell (18), and tracheal transplant (19–21). However, more recent reports have questioned the dependence upon CXCR3 and demonstrated development of cardiac rejection despite CXCR3 deficiency (22,23). Given this controversy, we sought to evaluate the role of CXCR3 in our model of LPS-induced pulmonary GVHD.

MATERIALS AND METHODS

Mice

Experiments were approved by the Duke-Institutional-Animal-Care-and-Use-Committees. Male 8–10 week-old C57BL/6J(H2^b) and B10.BR-*H2^kH2-T18^a*/SgSnJ (B10.BR)(H2^k) mice were purchased from Jackson Laboratories (Bar Harbor, ME). CXCR3-knockout (CXCR3[−]) mice on C57BL/6 background (14,24) were obtained from the laboratory of Dr. Paul Noble. All animals were housed in a pathogen-free facility on LPS-free bedding (Shepherd Specialty Papers Inc., Kalamazoo, MI), with irradiated food (PicoLab Mouse Diet 20-5058, Purina Mills, Richmond, IN) and antibiotic water (Sulfamethoxazole/Trimethoprim 1.2/0.24mg/mL).

Murine hematopoietic-cell transplantation

Donor mice (B10.BR, C57BL/6, or CXCR3[−]) were euthanized using CO₂. Tibia and femur bone marrow and splenocytes were isolated, filtered through 70µm filters (BD Biosciences, Franklin Lakes, NJ), counted, and resuspended in media containing 10% FBS (Hyclone, Logan, UT), 1% L-Glutamine (Sigma-Aldrich, St. Louis, MO) and 1% Penicillin/Streptomycin (Sigma-Aldrich). Recipient B10.BR mice were lethally irradiated using a Cesium irradiator (8Gy) and injected *via* retro-orbital route with 4×10⁶ bone marrow cells and 1×10⁶ splenocytes. Engraftment was evaluated 3 weeks post-transplantation using peripheral blood flow cytometry with anti-H2D^b-FITC (clone KH95) and anti-H2K^k-PE

(clone 36-7-5) (BD Biosciences). Animals with >95% donor-derived cells were used for subsequent experiments.

LPS exposures

Lyophilized LPS from *E. coli* 0111:B4 (Sigma-Aldrich) (25) was reconstituted and delivered to a 20L inhalation chamber using a constant-output six-jet atomizer 9306 (TSI Inc., Shoreview, MN) at 35psi, generating aerosol droplets with mean diameter of 0.5µm at a flow rate of 3.3L/min. Final LPS concentration was about 4.5ug/m³ (25,26). Mice received LPS for 2.5 hours/day, 5 days/week, for 2 weeks, starting 4 weeks post-HCT. Mice were euthanized 72 hours after last LPS exposure.

Anti-CXCR3 antibody treatment

Mice were injected intraperitoneally with 0.5mL of anti-murine CXCR3 antibody obtained from Dr. John Belperio, dosed as specified previously (19), or control goat-serum every-other-day for 2 weeks, starting the day before first LPS exposure.

Bronchoalveolar lavage (BAL) and analysis

After euthanasia, mouse trachea was cannulated and lungs were lavaged with 0.9% saline. BAL supernatant was analyzed using a CXCL10 ELISA kit (R&D systems, Minneapolis, MN) and mouse 23-plex cytokine assay (Bio-Rad Laboratories, Hercules, CA). IL-1β, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-12p40, IL-13, IL-17, GCSF, GMCSF, KC, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and TNFα levels were measured. IL-1α, IL-6, CCL11/eotaxin, and IFNγ were not detected. BAL cells underwent red-blood-cell lysis, counting, and flow cytometry (described below).

Lung tissue extraction and preservation

After pulmonary saline perfusion, the accessory (smallest) lobe of the right lung was preserved in RNAlater (Ambion/Applied Biosystems, Austin, TX). The remaining right lung was processed for flow cytometry. The left lung was gravity-inflated with and fixed in 10% formalin, then transferred into 70% EtOH after 24 hours.

Lung histology and immunohistochemistry

The left fixed lung was paraffin-embedded. 5µm sections were stained with hematoxylin-and-eosin (H&E). Pathological severity of lymphocytic lung inflammation was graded on a 9-point scale as described previously (8).

Sections were stained with rabbit anti-CD3 (Lab Vision Corp, Fremont, CA) (diluted 1:100) and anti-CD20 (Lab Vision Corp) (diluted 1:200). Rabbit Ig (diluted 1:60,000, Dako USA, Carpinteria, CA) was used as negative control. Primary antibody was detected with anti-rabbit horseradish-peroxidase (Dako USA).

RNA analysis

RNA was extracted (Ambion/Applied Biosystems): quantity was measured spectrophotometrically and quality was analyzed using Bio-RAD Experion chips (Bio-Rad Laboratories).

For realtime(RT)-PCR array, cDNA was reverse-transcribed using RT²First Strand Kit (SABiosciences, Frederick, MD). Transcripts were quantified in triplicate from 5ng cDNA using a Mouse-Inflammatory-Cytokines-and-Receptors RT-PCR Array PAMM-011 (SABiosciences); gene expression was normalized to housekeeping genes β-glucuronidase, hypoxanthine-phosphoribosyltransferase-1, Glyceraldehyde-3-phosphate-dehydrogenase,

and β -actin. Measured transcripts included markers of Th1 polarization (IL-12p40), Th2 (IL-4, IL-5, IL-5ra, IL-13), Th17 (IL-6ra, IL-6st, IL-17b, IL-21, IL-22, IL-23), as well as IL-10, IL-10ra, and IL-10rb. Data is expressed as fold-change with corresponding p-value, normalized to the control group.

For additional transcript analysis, cDNA was transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). 40ng of cDNA was used for RT-PCR (in triplicate) using Taqman probe-and-primer combinations for FOXP3 (Mm00475162_m1), CD3 ϵ (Mm0599683_m1), and the endogenous β -actin (4352933) (Applied Biosystems). Ct values were determined using ABI 7500 RealTime PCR System with SDS software 1.3.1. Change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to β -actin expression, and expressed as fold-change compared to the control group.

Lung flow cytometry

Lung was homogenized, digested with collagenase-A (Roche Diagnostics, Mannheim, Germany) and DNase-I (Sigma-Aldrich), filtered through a 70 μ m filter (BD Biosciences), red-cell-lysed, washed, and resuspended in PBS with 3% FBS, 0.05% sodium azide (VWR International, West Chester, PA), and 10mM EDTA. Live cells were counted using 0.4% Trypan-Blue (Sigma-Aldrich) dead-cell exclusion. Cells were blocked using 5% normal-mouse-serum, 5% normal-rat-serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and 1% F $_c$ -receptor-block (anti-mouse CD16/32) (eBioscience, San Diego, CA), and then stained with anti-mouse antibodies for 30 minutes using anti-CD3-FITC, anti-CD49b-PE (clone DX5), anti-CD4-PE-Cy7, anti-CD11b-APC, anti-CD8-APC-Cy7, anti-CD19-PE-Cy5, anti-CD3-PE-Cy7 (eBioscience), and anti-CXCR3-PE (R&D systems). Fluorescence was measured using BD FACSCantoII flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star Inc., Ashland, OR): a singlet gate was used to exclude cell aggregates, followed by an all-cell gate to exclude small debris and dead cells. Cell percentages are expressed as percentage of all cells and converted to absolute numbers by multiplying by live-cell counts.

Statistical analysis

Data are expressed as means $\pm$ SEM. Between-group comparisons were performed to specifically determine if LPS-treated allogeneic HCT mice were significantly different from allogeneic untreated or syngeneic treated mice and whether CXCR3 inhibition (*via* knockout or antibody strategy) made a significant difference compared to control. Comparisons were performed using a two-tailed Student's t-test. P-values of ≤ 0.05 were considered significant.

RESULTS

Inhaled LPS potentiates pulmonary GVHD

After LPS exposure, B10.BR recipient mice that had undergone allogeneic HCT (AlloLPS mice) had significantly increased lymphocytic inflammation in their lung tissue with perivascular and peribronchiolar infiltrates, seen to a much lower extent in allogeneic HCT mice not exposed to LPS (AlloNoLPS) or syngeneic mice exposed to LPS (SynLPS) (figure 1). Flow cytometry confirmed a predominance of CD3 $^+$, CD4 $^+$, and CD8 $^+$ T cells in the lung tissue of AlloLPS compared to AlloNoLPS and SynLPS mice (figure 2).

CXCR3 and CXCL10 are upregulated in LPS-induced pulmonary GVHD

In allogeneic mice, LPS increased CXCR3 expression on CD3 $^+$ T cells (figure 3A). Transcripts were significantly upregulated in AlloLPS as compared to SynLPS mice for

CXCR3 and CXCL10 (figure 3B), but not for CXCL9 and CXCL11. Confirmatory analysis showed elevated CXCL10 protein levels in BAL of AlloLPS as compared to controls (figure 3C).

LPS-induced pulmonary GVHD occurred independent of CXCR3

Lymphocyte CXCR3-deficiency in AlloLPS mice did not decrease pulmonary GVHD. In fact, mice reconstituted with CXCR3-deficient (CXCR3⁻) or CXCR3-sufficient cells did not differ in their lymphocytic inflammation on pathology (figure 4A) or in pulmonary T cells by flow cytometry (figure 4B,C). Furthermore, the pattern of T cell infiltration around blood vessels and airways was the same in the two groups (figure 4D–G). Since >95% of allogeneically-transplanted lung lymphocytes in this model are donor-derived (data not shown) and CXCR3 expression is mostly limited to lymphocytes (27), we studied CXCR3-deficiency using knockout donors. However, a role for CXCR3 on non-lymphocyte recipient cells cannot be entirely ruled-out: antibody blockade was performed to address this. AlloLPS mice treated with anti-CXCR3 antibody developed the same amount of lymphocytic inflammation as mice injected with control serum (figure 4H), with similar percentages and absolute counts of lung T cells (figure 4I,J).

Absence of CXCR3, but not anti-CXCR3 antibody blockade, slightly alters the recruited lymphocyte population in LPS-induced pulmonary GVHD

There was a significant decrease in the percentage of CD8⁺ T cells and an increase in the absolute numbers of B cells in lungs of AlloLPS mice transplanted with CXCR3⁻ compared to mice transplanted with CXCR3-sufficient donors (figure 5A,B). Similar recruitment of CD4⁺ T, NKT, and NK cells were seen in the two groups (figure 5A,B). The B cell difference was not apparent when evaluating CD20⁺ cells by immunohistochemistry with similar perivascular and peribronchial distribution (figure 5C–F). AlloLPS mice treated with anti-CXCR3 antibody or control had similar percentages and numbers of pulmonary CD4⁺ T, CD8⁺ T, NKT, NK, and B cells (figure 5G,H).

Role of alternative mechanisms accounting for lymphocyte recruitment during CXCR3 deficiency or blockade

In order to better understand the pulmonary lymphocyte recruitment in AlloLPS mice in the absence of CXCR3, we compared levels of 23 cytokines in the BAL of AlloLPS mice transplanted with CXCR3⁻ or CXCR3-sufficient donors (supplemental figure) as well as in the BAL of AlloLPS mice treated with anti-CXCR3 antibody or control (data not shown). Furthermore, cytokine, cytokine receptor, and related protein transcripts were measured in lungs of AlloLPS mice transplanted with CXCR3⁻ or CXCR3-sufficient donors (data not shown). No specific cytokine pathway upregulation was found in CXCR3-deficiency or blockade as compared to controls, with similar markers of Th1, Th2, Th17, and regulatory T cell polarization. Of note, persistent high levels of IL12p40 were identified in the CXCR3-deficient group, indicating an unperturbed strong Th1 response.

DISCUSSION

After murine allogeneic HCT, inhaled LPS leads to pulmonary T cell recruitment with perivascular and peribronchiolar inflammation, resembling human pulmonary GVHD and lung allograft rejection. These findings are consistent with reports of T cell predominance in human pulmonary GVHD (15) and human and mouse lung transplant rejection (28–30). We hypothesized that CXCL10 facilitates recruitment of CXCR3⁺ T cells into the lung and that interrupting this pathway will lead to decreased pulmonary GVHD. However, despite significant upregulation of CXCL10 and CXCR3, inhibition of the CXCR3 pathway, using two independent strategies, did not prevent lymphocytic lung inflammation. Analysis of

pulmonary lymphocyte subsets in the absence of CXCR3 revealed a reduced percentage of pulmonary CD8+ T cells, contrasting with other studies showing CXCR3-mediated CD4 cell trafficking (31). CXCR3 deficiency also led to a mild increase in B cell numbers, consistent with TLR4-mediated B cell activation published in prior studies (32). No differences in lymphocyte subsets were identified between CXCR3-antibody and control mice, perhaps due to the presence of CXCR3 signaling during the first 4 weeks post-transplant. While both strategies led to the same overall conclusion, CD3, CD4 and CD8 cells were reduced in the antibody approach as compared to the knockout experiments, possibly reflecting non-specific effects of serum on T cell trafficking. Most surprisingly, interruption of the CXCR3 pathway did not change markers of Th1, Th2, Th17, or regulatory T cell differentiation. Our findings are consistent with two recent studies in murine cardiac transplantation where CXCR3 blockade also failed to prevent acute cardiac rejection (22,23), suggesting that acute rejection in the setting of several clinically-relevant allotransplant models is independent of CXCR3.

There are several potential explanations for why our results differ from previous studies, which found a role for CXCR3 blockade in the prevention of transplant-related lung diseases. First, unlike previous studies, our model involves repeated exposures to LPS, a common environmental innate stimulus. Thus, although CXCR3 blockade might prevent inflammation in the setting of an isolated alloresponse (15,18,20,21), the addition of environmental stimuli likely leads to potentiation of a strong Th1 response that overshadows CXCR3-dependent pathways. Additional Th1 cytokines, such as IL-23 and IL-12p80, or other compensatory pathways, including antibody-mediated mechanisms (33), not assessed in these studies, represent potential alternative mechanisms of disease worthy of further study. Second, the level of MHC mismatch may affect the strength of the alloimmune inflammation. We focused on a model involving both class I and class II MHC mismatch because many human allogeneic HCT and all lung transplants are done across major HLA mismatches. CXCR3 blockade reduced the severity of alloimmune disease in published models of HCT across single or minor MHC mismatches (15,16). Therefore, further study of CXCR3 blockade in transplant models involving a spectrum of allomismatch or immunosuppression would be useful to determine if the contribution of CXCR3 varies with the strength of alloimmune stimulation. Third, while CXCR3 blockade has been shown to prevent obliterative disease in the setting of tracheal transplantation (19,20), our model replicates features of acute post-transplant lymphocytic lung inflammation rather than chronic fibrosis.

In summary, we have shown that innate immune activation with LPS locally potentiates murine pulmonary GVHD with marked elevations of pulmonary CXCR3 and CXCL10 and a strong Th1 response with prominent production of IL-12p40. However, blocking the CXCR3 receptor through genetic deletion or antibody inhibition does not alter the overall severity of LPS-induced pulmonary GVHD or prevent development of strong Th1 response, which appears to promote disease, independent of CXCR3. Given the almost constant burden of innate immune activation in the lungs of human hematopoietic-cell and lung transplant recipients, our results suggest that CXCR3 blockade alone may not be a sufficient strategy to prevent human transplant-related lung disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HCT	hematopoietic-cell transplant
GVHD	graft-versus-host disease
LPS	lipopolysaccharide
CXCR3	CXC receptor 3
TLR4	Toll-like receptor 4
BAL	bronchoalveolar lavage
CXCR3⁻	CXCR3-deficient mice
FBS	fetal bovine serum
H&E	hematoxylin and eosin
WT	wild-type
AlloLPS	allogeneically HCT-transplanted mice exposed to LPS
AlloNoLPS	allogeneically HCT-transplanted mice not exposed to LPS
SynLPS	syngeneically HCT-transplanted mice exposed to LPS
SynNoLPS	syngeneically HCT-transplanted mice not exposed to LPS
MHC	major histocompatibility complex

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Figure 1. Pulmonary GVHD in allogeneic HCT mice after inhaled LPS exposure

Allogeneically-reconstituted HCT mice were exposed vs. not exposed to 2 weeks of daily inhaled LPS and sacrificed 72 hours after last exposure. Lung pathology was evaluated and pathology grades were determined in a blinded fashion using a semi-quantitative scoring system based on the thickness of perivascular and peribronchiolar inflammation as well as the overall percentage of lung involved. (A) Lymphocytic perivascular and peribronchiolar inflammation was most prominent in the allogeneic HCT mice exposed to LPS ($n = 4$) with significantly less pathology in allogeneic HCT mice not exposed to LPS ($n = 5$) ($p = 0.04$) and syngeneic HCT mice exposed to LPS ($n = 4$) ($p = 0.01$) (data replicated in 4 independent experiments). (B–E) Representative histology sections are shown for all groups (H&E stain, 200 $\times$).

Figure 2. T cell recruitment in LPS-induced pulmonary GVHD

After HCT and 2 weeks of daily LPS exposures, digested lung tissue cells were analyzed by flow cytometry. (A) CD3+, (B) CD4+, and (C) CD8+ T cells, expressed as a percentage of all cells, were increased in allogeneic mice exposed to LPS (n = 3) as compared to allogeneic mice not exposed to LPS (n = 3) and syngeneic controls exposed to LPS (n = 3). Absolute numbers of (D) CD3+, (E) CD4+, and (F) CD8+ T cells, obtained by multiplying the percentage of all cells by the absolute number of live lung cells per mouse, generally parallel the percentages, trending towards an increase in allogeneic mice exposed to LPS. (* = p-value less than 0.05, ** = p-value less than 0.005, *** = p-value less than 0.0005) (data replicated in 2 independent experiments). (G) Representative flow cytometric plots show CD4+ and CD8+ T cell populations within small cells for each experimental group.

Figure 3. Upregulation of CXCR3 receptor and its ligand CXCL10 in LPS-induced pulmonary GVHD

After HCT and 2 weeks of LPS exposures, lung tissue was analyzed by flow cytometry and RT-PCR and BAL was analyzed using a CXCL10 ELISA. (A) In allogeneic mice (n = 4/group), LPS increases the absolute number of CD3+CXCR3+ T cells (p = 0.01) as well as the CD3+CXCR3+ percentage among small cells (representative flow cytometric plots are shown, along with the negative IgG control) (data replicated in 2 independent experiments). (B) Lung tissue of allogeneic HCT mice exposed to LPS (n = 3), as compared to syngeneic mice exposed to LPS (n = 3), had significantly increased transcripts for CXCR3 (2.1-fold, p = 0.01) and CXCL10 (6.2-fold, p = 0.04). (C) Confirmatory analysis showed elevated CXCL10 protein levels in BAL fluid of allogeneic mice exposed to LPS (n = 3) as compared to allogeneic mice not exposed to LPS (n = 4) (p = 0.008) and syngeneic mice exposed to LPS (n = 4) (p = 0.02) (data replicated in 4 independent experiments).

Figure 4. CXCR3 deficiency and antibody-blockade do not prevent LPS-induced pulmonary GVHD

(A–G) Allogeneic HCT mice were reconstituted with CXCR3-deficient (CXCR3⁻) or sufficient wild-type (WT) hematopoietic cells and subsequently exposed to daily LPS for 2 weeks. (A) CXCR3⁻ (n = 12) and WT-reconstituted (n = 4) AlloLPS mice exhibited the same amount of pulmonary GVHD as reflected in the pathology grade (p = 1.00). (B, C) CXCR3⁻ (n = 3) and WT-reconstituted (n = 3) AlloLPS mice demonstrated similar T cell percentages (p = 0.10) and absolute numbers (p = 0.33) in the lung tissue by flow cytometry. (D–G) Representative images of lung tissue immunohistochemistry for CD3⁺ cells are shown, demonstrating similar perivascular and peribronchial T cell infiltration in (D) WT and (E) CXCR3⁻-reconstituted AlloLPS mice, as well as a (F) mouse lymph node positive control and (G) AlloLPS lung stained with isotype control. (H–J) Allogeneic HCT LPS-exposed mice were treated with anti-murine CXCR3 antibody (Ab) (n = 5) or control serum (control) (n = 5). (H) Both groups exhibited the same amount of pulmonary GVHD as reflected in the pathology grade (p = 0.46). (I, J) AlloLPS mice treated with anti-murine CXCR3 Ab (n = 4) and control serum (n = 4) demonstrated similar T cell percentages (p = 0.54) and absolute numbers (p = 0.24) in the lung tissue by flow cytometry (data replicated in 2 independent experiments).

Figure 5. Effect of CXCR3 deficiency and antibody-blockade on pulmonary lymphocyte subsets in LPS-induced pulmonary GVHD

(A–F) Allogeneic HCT mice were reconstituted with CXCR3-deficient (CXCR3^{-/-}) (n = 3) or sufficient (WT) (n = 3) hematopoietic cells and subsequently exposed to daily LPS for 2 weeks. Digested lung tissue was analyzed by flow cytometry. (A) Compared to WT controls, allogeneic HCT mice reconstituted with CXCR3^{-/-} cells and exposed to LPS exhibited a significant decrease in lung CD8⁺ T cell percentage (p = 0.01) with no change in CD4⁺ T, NKT, NK, and B cell percentages. (B) Analysis of absolute pulmonary cell counts showed that CXCR3^{-/-} AlloLPS mice had similar numbers of CD4⁺ T, CD8⁺ T, NKT, and NK cells but increased B cells (p = 0.005). (C–F) Representative images of lung tissue immunohistochemistry for CD20⁺ cells are shown, demonstrating (C) similar B cell infiltration near blood vessels and bronchi in WT and (D) CXCR3^{-/-} AlloLPS mice, as well as (E) a mouse lymph node positive control and (F) an AlloLPS lung stained with isotype control. (G, H) AlloLPS mice treated with anti-murine CXCR3 Ab (n = 4) and control serum (n = 4) demonstrated similar CD4⁺ T, CD8⁺ T, NKT, NK, and B cell percentages and absolute numbers by flow cytometry (data replicated in 2 independent experiments).