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IL-22 is required for the induction of bronchus-associated lymphoid tissue in tolerant lung allografts

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Abstract

Long-term survival after lung transplantation remains profoundly limited by graft rejection. Recent work has shown that bronchus-associated lymphoid tissue (BALT), characterized by the development of peripheral nodal addressin (PNAd)-expressing high endothelial venules and enriched in B and Foxp3⁺ T cells, is important for the maintenance of allograft tolerance. Mechanisms underlying BALT induction in tolerant pulmonary allografts, however, remain poorly understood. Here, we show that the development of PNAd-expressing high endothelial venules within intragraft lymphoid follicles and the recruitment of B cells, but not Foxp3⁺ cells depends on IL-22. We identify graft-infiltrating gamma-delta ($\gamma\delta$) T cells and Type 3 innate lymphoid cells (ILC3s) as important producers of IL-22. Reconstitution of IL-22 at late time points through retransplantation into wildtype hosts mediates B cell recruitment into lymphoid follicles within the allograft, resulting in a significant increase in their size, but does not induce PNAd expression. Our work has identified cellular and molecular requirements for the induction of BALT in pulmonary allografts during tolerance induction and may provide a platform for the development of new therapies for lung transplant patients.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

1. Introduction

Bronchus-associated lymphoid tissue (BALT) is a pulmonary tertiary lymphoid organ (TLO) that plays a critical role in the inflammatory response to a range of lung pathology, including infection, autoimmunity, and allograft rejection (1-3). However, recent evidence has emerged that pulmonary TLOs are not synonymous with the generation of deleterious proinflammatory responses (4-7). To this end, the development of BALT has been associated with the downregulation of immune responses. Tumor-associated TLOs are one such example, which have been found in both human and mouse models of lung cancer (4). Pulmonary tumor-associated TLOs are enriched in Foxp3⁺ regulatory T cells, which when selectively depleted result in activation of dendritic cells as well as T lymphocytes and tumor regression. Similarly, we have found that BALT is induced in tolerant lung allografts that is enriched in regulatory Foxp3⁺ T cells (5). Our work has revealed that Foxp3⁺ T cells residing within BALT of tolerant lung allografts prevent antibody-mediated rejection by inhibiting the local activation of B cells (6).

Mechanisms leading to the induction of BALT, particularly that which is associated with downregulation of immune responses, remain poorly understood. BALT that develops in response to inflammatory stimuli, such as LPS administration or infection, principally depends on IL-17 production by T cells. Here, IL-17 stimulates lymphocyte-attracting chemokines, such as CXCL12 and CXCL13, resulting in B and T cell accumulation, and BALT formation; this process is independent of lymphotoxin signaling, distinguishing it from the mechanisms that mediate the formation of secondary lymphoid organs (SLO) (1, 8, 9). Other studies investigating the requirements for induction of TLOs in lungs and other tissues suggest that LTβ, IL-22, and IL-23, among other factors, may play a role in their development (10-12). The cell populations responsible for TLO induction also vary considerably among organ systems and include T_H17 cells, innate lymphoid cells (ILC), gamma-delta (γδ) T cells, and natural killer cells (8) (11, 13).

Here, we take advantage of our previously established orthotopic, vascularized mouse lung transplant model to show that the recruitment of B cells and the development of peripheral nodal addressin (PNAd)-expressing high endothelial venules (HEVs) in tolerant pulmonary allografts is dependent on IL-22 (14). We have found that graft-infiltrating γδ T cells and Type 3 ILCs (ILC3s) are important producers of IL-22 following lung transplantation. Remarkably, the formation of Foxp3⁺ cell aggregates within tolerant allografts does not depend on IL-22. Replantation experiments demonstrate that delayed restoration of IL-22 expression mediates B cell recruitment to the intragraft lymphoid follicles and increases their size, but does not result in the development of PNAd-expressing HEVs. Thus, our findings define a new pathway for the induction of BALT in tolerant lung allografts and suggest new therapeutic avenues.

2. Materials and methods

2.1 Mice

C57BL/6J (B6), Balb/cJ (BALB/c), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1⁺), B6.129P2-Tcrd^{tm1Mom}/J (TCR $\delta^{-/-}$), and B6 Foxp3-DTR (diphtheria toxin receptor) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). ROR γ t-cre AHR^{fl/fl} mice (referred to as AR mice) and IL-22-deficient mice were obtained from M. Colonna and S. Hultgren (Washington University, St. Louis, MO), respectively (15, 16).

2.2 Lung Transplantation

Left orthotopic vascularized lung transplants were performed as previously described (17). For select experiments mice were treated with costimulatory blockade consisting of MR1 (250 μ g intraperitoneally (i.p.)) and CTLA4- Ig (200 μ g i.p.) on days 0 and 2, respectively (Bio X Cell, West Lebanon, NH). For select experiments, lung recipients were additionally treated with anti-IL-23 antibody (20 μ g i.p., clone N71-1183; BD Biosciences, San Jose, CA) on days 0, 2, 4, 6 and then 3 times weekly thereafter (18). Lung retransplantations were performed as previously described (19). No additional immunosuppressants were given after retransplantation. The mice were sacrificed on day 7 or at least 30 days after primary transplantation or retransplantation as indicated. DT (List Biologic Laboratories, Campbell, CA) (1 μ g i.p.) was administered at the time of retransplantation (days 0, 1) to B6 mice receiving grafts that had initially been transplanted into IL-23-neutralized B6 Foxp3-DTR hosts. All procedures were approved by the Institutional Animal Care and Use Committee at Washington University.

2.3 Histology

Longitudinal sections of lung allografts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). For quantification of lymphoid follicles, two separate and non-adjacent sections were obtained from one paraffin-embedded tissue block. Cell aggregates with more than 100 cells were classified as lymphoid follicles. The number and area occupied by lymphoid follicles was quantified using Image J image processing and analysis software (National Institutes of Health, Bethesda, MD).

2.4 Immunostaining

For immunohistochemistry, anti-mouse PNAd carbohydrate epitope (clone MECA-79; BD Biosciences, San Jose, CA), anti-Foxp3 monoclonal (clone FJK-16s; eBioscience, Waltham, MA), and anti-CCL21 polyclonal (R&D Systems, Minneapolis, MN) primary antibodies were used. For immunofluorescence, the following primary antibodies were used: anti-mouse CD45R (B220) (clone RA3-6B2, Thermo Fisher Scientific, Waltham, MA), anti-CD3e monoclonal antibody (clone SP7, Thermo Fisher Scientific), anti-CXCL13 polyclonal antibody (Thermo Fisher Scientific). Abundance of B220⁺ and CD3⁺ cells in lymphoid follicles was quantified using Image J and reported as B220 / CD3 ratios.

2.5 Flow cytometry

In order to obtain single-cell suspensions, lungs were digested with type I collagenase (Worthington Biochemical, Lakewood, NJ) and DNase I (Sigma-Aldrich, St. Louis, MO), red blood cell lysis was performed with ACK lysing buffer, and cells were passed through a 40 μ M wire mesh. For determination of IL-22 expression cell suspensions were stimulated overnight in complete-stimulation media using recombinant mouse IL-23 (50 ng/mL) (R&D Systems, Minneapolis, MN). Subsequently, cells were stimulated with PMA/ionomycin for 4 hours with Brefeldin present for the last 3 hours of stimulation as previously described (20). Surface and intracellular staining were performed with the following antibodies: anti-IL-22 (clone 1H8PWSR) (ThermoFisher/eBioscience, Waltham, MA), CD3e (clone 145-2C11, BD Biosciences and clone 17A2, Biolegend, San Diego, CA), CD4 (clone RM4-5, Biolegend), CD8 (clone 53-6.7, Biolegend), CD19 (clone 6D5, Biolegend), CD45.1 (clone A20, Biolegend), CD90.2 (clone 53-2.1, Biolegend), CD127 (clone A7R34, Biolegend), ROR γ t (clone Q31-378, BD Biosciences), TCR γ δ (clone GL3, BD Biosciences) and NKp46 (biotinylated polyclonal, R&D Systems). Biotin was detected with fluorochrome-conjugated streptavidin (BD Biosciences). Dead cells were excluded with Zombie Fixable Viability stain (Biolegend). Samples were acquired on an LSR Fortessa X-20 equipped with 5 lasers (355, 405, 488, 561, 640 nm; BD Biosciences) and analyzed using FlowJo Version 10.5 (TreeStar).

2.6 Quantitative PCR

Homogenized lung tissue was lysed with TRIzol (Thermo Fisher Scientific). Total RNA was isolated using QIAGEN RNeasy Mini Kit (QIAGEN), and quantitative PCR was performed as previously described (21). Primer sequences were as follows: IL-22, 5'-ATACATCGTCAACCGCACCTTTA -3' and 5'-AGCCGGACATCTGTGTTGTTA -3'; IL-23p19, 5'-GACCCACAAGGACTCAAGGAC -3' and 5'-ATGGGGCTATCAGGGAGTAGAG -3'; IL-17, 5'-TCAGCGTGTCCAAACACTGAG-3' and 5'-CGCCAAGGGAGTTAAAGACTT-3'; LTB, 5'-GATGACAGCAAACCGTCGTG-3' and 5'-CCTGGAAGCATTGGATCTCTG-3'

2.7 Statistics

Gene expression levels and continuous variables from histology specimens (i.e.: follicle number, B220/CD3 ratios) were compared between groups and presented in figures as mean \pm standard error of the mean. The nonparametric Mann-Whitney U test was used to determine statistical differences between groups. P values less than 0.05 were considered to be statistically significant, as indicated in the figure legends.

2.8 Data availability

The data that support the findings of this study are available from the corresponding author on request.

3. Results

3.1 Induction of BALT in tolerant lung grafts is dependent on IL-22

We have previously shown that peri-operative blockade of CD40-CD154 and B7-CD28 costimulatory pathways in recipient mice prevents acute graft rejection 7 days after lung transplantation (Figure 1A). In this model, allografts survive long-term, BALT is consistently induced by 30 days after transplantation and recipients develop donor-specific tolerance (Fig. 1B) (5). To determine the molecular cues responsible for BALT induction, we performed real-time PCR to quantify expression of cytokines and other factors which have been linked to TLO formation in other models. We found that expression levels of IL-22 (Fig. 1C) and IL-23 (Fig. 1D) were significantly elevated in tolerant lung allografts at both 7 and 30 days after transplantation compared to naïve lungs. The expression levels of LT β (Fig. 1E) and IL-17 (Fig. 1F), however, were not elevated in lung allografts compared to naïve lungs.

IL-23 signaling has been shown to trigger IL-22 expression in both humans and mice (22-24). Therefore, we next asked whether IL-22 and IL-23 are required for BALT induction in tolerant lung allografts. To examine this, we transplanted Balb/c lung grafts into IL-22-deficient B6 recipients (Fig. 1G) or wildtype B6 hosts that were treated with an IL-23-neutralizing antibody (Fig. 1H). We found that in both conditions allografts survived long term (>30 days), but developed significantly fewer lymphoid follicles when compared to wildtype conditions (Fig. 1I). When we transplanted Balb/c lungs into non-immunosuppressed B6 recipients grafts were acutely rejected irrespective of IL-22 expression in the recipient (Suppl. Fig. 1).

3.2 $\gamma\delta$ T cells and ILC3s are important sources of IL-22 in tolerant lung allografts

Several cell populations have been found to produce IL-22 in response to IL-23 in both humans and mice. Pulmonary $\gamma\delta$ T cells are one such subset, which have largely been found to be proinflammatory and capable of producing IL-17 and IFN- γ in response to infection or sterile inflammation such as ischemia reperfusion injury (24-26). In addition, Type 3 ILCs (ILC3) have also been found to produce IL-22 and are implicated in the regulation of allergic lung disease (27, 28). An analysis of our transplants revealed that both $\gamma\delta$ T cells (Suppl. Fig. 2A) and ILC3s (Suppl. Fig. 2B, C) infiltrated lung allografts early during tolerance induction. To determine which cell type was the source of IL-22 in tolerant lung grafts, we harvested Balb/c lungs 7 days after transplantation into wildtype B6 recipients that had received peri-operative co-stimulatory blockade. Flow cytometric analysis revealed that virtually all IL-22⁺ cells also expressed RAR-related orphan receptor γ (ROR γ t). The majority of IL-22-expressing cells in tolerant lung grafts consisted of $\gamma\delta$ T cells (TCR $\gamma\delta$ ⁺CD3⁺) and ILC3s (TCR $\gamma\delta$ ⁻CD3⁻CD90⁺CD127⁺) (Figs. 2A, B). Other ROR γ t⁺IL-22⁺ cells included CD4⁺ T cells (TCR $\gamma\delta$ ⁻CD3⁺CD4⁺CD8⁻), non-T non-ILCs (TCR $\gamma\delta$ ⁻CD3⁻CD90⁻CD127⁻), and double-negative T cells that did not express CD4 or CD8 (TCR $\gamma\delta$ ⁻CD3⁺CD4⁻CD8⁻), which may include T cells that downregulated their co-receptors during stimulation.

To determine if $\gamma\delta$ T cells are required for BALT formation, we transplanted Balb/c lungs into B6 TCR $\delta^{-/-}$ hosts that were treated with peri-operative costimulatory blockade (29). Previous studies have employed conditional knockout mice in which aryl hydrocarbon receptor (AHR) deficiency is restricted to ROR γ t-expressing cells (AR mice) to examine the functional roles of ILC3s in inflammation (15). Therefore, to evaluate the role of ILC3s in the induction of BALT, we transplanted Balb/c allografts into AR recipients under peri-operative costimulatory blockade. Compared to wildtype recipients (Fig. 2C), $\gamma\delta$ T cell-deficient (Fig. 2D) and AR (Fig. 2E) recipients had substantially fewer lymphoid follicles per section (Fig. 2F), resembling IL-22 deficient (Fig. 1G) or IL-23 neutralized (Fig. 1H) hosts. Lymphoid follicles after transplantation into control AHR^{fl/fl} recipients resembled wildtype conditions (data not shown). To examine the size of induced BALT, the area occupied by lymphoid follicles per section was measured. We found that grafts transplanted into IL-22-deficient or IL-23-neutralized recipients had a lower mean area occupied by lymphoid follicles compared to wildtype hosts. Interestingly, while $\gamma\delta$ T cell-deficient and AR recipients developed fewer lymphoid follicles than wildtype recipients, the mean area occupied by lymphoid follicles per section was not significantly different (Fig. 2G).

3.3 IL-22 is required for PNAd expression and B cell, but not Foxp3⁺ cell, recruitment into lymphoid follicles

A hallmark of TLOs is the neogenesis of PNAd-expressing HEVs. PNAd facilitates the recruitment of lymphocytes into TLOs including BALT (30). Consistent with our previous report, tolerant lung grafts express abundant PNAd within BALT after transplantation into wildtype recipients (Fig. 3A). By contrast, we did not detect PNAd within the few, small lymphoid follicles that form within lung allografts after transplantation into IL-22-deficient (Fig. 3B) or IL-23-neutralized (Fig. 3C) recipients. PNAd staining was detected in lymphoid follicles in lung allografts after transplantation into AR hosts (Fig. 3D) or $\gamma\delta$ T cell-deficient (Fig. 3E), albeit less abundantly compared to wildtype recipients (Fig. 3A).

In accordance with our previous report, we observed that both B and T cells accumulated within the BALT of tolerant lung allografts after transplantation into wildtype recipients (Fig. 4A) (6). In stark contrast, while T cells were present within the small lymphoid follicles that formed in pulmonary grafts after transplantation into IL-22-deficient (Fig. 4B) or IL-23-neutralized hosts (Fig. 4C), very few B cells were detected resulting in significantly decreased B cell / T cell ratios in these mice (Fig. 4F). In AR or $\gamma\delta$ T cell-deficient recipients (Fig. 4D & E), B cell / T cell ratios were comparable to those seen in wildtype hosts (Fig. 4F). In models of respiratory viral infection, CXCL13 expression by pulmonary fibroblasts has been shown to result in B cell recruitment and ectopic germinal center formation (31). Immunostaining revealed CXCL13 expression in spindle shaped cells within lymphoid follicles that were induced in lung allografts after transplantation into wildtype (Suppl. Fig. 3A), AR mice (Suppl. Fig. 3B) or $\gamma\delta$ T cell-deficient recipients (Suppl. Fig. 3C), while staining was less pronounced after engraftment into IL-22-deficient (Suppl. Fig. 3D) or IL-23-neutralized (Suppl. Fig. 3E) recipients. CCL21 expression was comparable between grafts transplanted into wildtype and IL-22 deficient recipients (Suppl. Fig. 4).

Having recently demonstrated that Foxp3⁺ regulatory T cells that aggregate within BALT of lung allografts are critical in maintaining a tolerant state, we next assessed lymphoid follicles in our experimental groups for the presence of Foxp3⁺ cells (6). We observed that Foxp3⁺ cells accumulated within lymphoid follicles that were induced in pulmonary allografts after transplantation into wildtype (Fig. 4G), IL-22-deficient (Fig. 4H), IL-23-neutralized (Fig. 4I), AR (Fig. 4J) and $\gamma\delta$ T cell-deficient hosts (Fig. 4K) with no significant differences in their density between these groups (Fig. 4L).

3.4 Reconstitution of IL-22 restores B cells recruitment to lymphoid follicles

We next wanted to examine whether reconstituting IL-22 at later time points was able to reconstitute the development of BALT within lung allografts. To accomplish this, we took advantage of a lung retransplantation model that was recently developed by our laboratory (5). We have previously reported that tolerant Balb/c lung allografts are not rejected when retransplanted into non-immunosuppressed allogeneic recipients demonstrating that immunoregulation is established locally within the graft (6). Therefore, we next transplanted Balb/c lungs into B6 IL-22-deficient recipients that were treated with peri-operative co-stimulatory blockade and retransplanted these grafts into non-immunosuppressed B6 wildtype mice at least 30 days after the initial transplantation. We examined these grafts 7 and 30 days after retransplantation. At both time points we noted a significant increase in the number of and area occupied by lymphoid follicles (Figs. 5A-D). Furthermore, following retransplantation into wildtype mice we observed that B cells aggregated in the lymphoid follicles resulting in significant increases in B cell / T cell ratios. (Figs. 5E-G). In addition, immunostaining revealed increased expression of CXCL13 within the lymphoid follicles after retransplantation (Suppl. Fig. 3F), which was comparable to our observations 30 days after transplantation of Balb/c lungs into wildtype B6 mice (Suppl. Fig. 3A). Furthermore, the density of Foxp3⁺ cells 30 days after re-transplantation was comparable to Balb/c lungs 30 days after engraftment into B6 IL-22-deficient hosts (Figs. 5H-I). Of note, however, we did not detect PNAd staining within lymphoid follicles at 30 days (Fig. 5J) after retransplantation. To examine whether Foxp3 cells are important in maintaining a tolerant state in conditions where IL-23/22 signaling is deficient and organized BALT does not form, we transplanted Balb/c lungs into B6 Foxp3-DTR mice that were treated with peri-operative costimulatory blockade and IL-23 neutralizing antibodies. At least 30 days later these tolerant lung grafts were retransplanted into non-immunosuppressed B6 wildtype mice that were treated with DT to deplete graft-resident Foxp3 cells, as previously described. Seven days after retransplantation all grafts were not ventilated and had histological features characteristic of antibody-mediated rejection, such as fibrinous deposits within the alveoli and hyaline membrane formation (Fig. 5K).

4. Discussion

Our study demonstrates that IL-22 plays an important role in the induction of BALT within tolerant lung allografts. Here, we have identified $\gamma\delta$ T cells and ILC3s as important producers of IL-22. We have shown that IL-22 is critical for the recruitment of B cells to lymphoid follicles as well as development of PNAd-expressing HEVs within these structures. Interestingly, Foxp3⁺ cells accumulate within the few, small lymphoid follicles

that form in pulmonary allografts when recipient mice lack IL-22, and recipient IL-22 expression is not required to induce tolerance after lung transplantation. Restoring IL-22 at late time points through retransplantation of lung allografts into wildtype hosts leads to a significant enlargement of the follicles with reconstitution of B cells, but does not result in the formation of PNA⁺ HEVs.

TLOs that are induced in transplanted grafts have been mostly associated with deleterious immune responses (32). For example, TLOs have been observed in cardiac allografts that have evidence of acute or chronic rejection (33). In aortic allografts TLOs were suggested to be the site of local generation of humoral immune responses that result in rejection (34). Some reports exist that BALT is associated with chronic lung allograft rejection (3). By contrast, we and others have observed intra-graft Foxp3-rich TLOs that play an important role in the maintenance of a tolerant state (5, 6, 35). For example, the development of such structures is associated with improved graft function within murine renal allografts (35). While we have previously reported that BALT is not induced in acutely rejected lungs, our laboratory has shown that Foxp3-rich BALT is induced in tolerant murine pulmonary allografts (5, 36). Perhaps more importantly, we have recently reported that depletion of Foxp3⁺ cells from tolerant lung allografts results in activation of B cells by T cells within the graft and triggers antibody-mediated rejection (6). Foxp3-rich TLOs have also been recently described in tumor models where they suppress immune responses that are directed against the tumor (4). At the present time it remains unknown why tertiary lymphoid organs promote deleterious immune responses in some grafts while they apparently protect others from rejection. We speculate that the inflammatory state at the time when tertiary lymphoid organs are induced determines their cellular composition. To this end, their induction in a tolerogenic setting, such as following treatment with co-stimulatory blockade in our experiments, may favor the enrichment of regulatory immune cells within these structures. Future studies will need to examine whether inducing cellular and soluble mediators play a role in determining whether “pro-inflammatory” or “tolerogenic” tertiary lymphoid organs form within allografts.

Only a few studies have addressed the cellular and molecular requirements that mediate the induction of TLOs after transplantation. It has been shown in skin allografts that overexpression of lymphotoxin- α results in the neogenesis of lymphoid follicles that harbor PNA⁺ vessels (37). Similarly, lymphotoxin β receptor signaling has been shown to promote the development of TLOs in other disease models, such as atherosclerosis or autoimmunity (10, 38). In cardiac allografts inhibiting lymphotoxin β receptor signaling abrogates the development of B cell-enriched TLOs and attenuates humoral immune responses (39). Graft-infiltrating T_H17 cells have also been implicated in promoting lymphoid neogenesis in a study examining chronically rejected human renal allografts (40); this observation is somewhat reminiscent of reports in murine models where the induction of TLOs also depends on IL-17. For example, the development of BALT following intranasal administration of LPS to neonatal mice required IL-17 production by T cells (8). Interestingly, in this study BALT was induced in ROR γ t- or Id2-deficient mice indicating that lymphoid tissue inducer cells did not play an important role in this process.

Our study represents the first study to show the role of IL-22 in TLO formation after organ transplantation. Our findings extend the observations of few reports that have implicated IL-22 in the development of lymphoid follicles at peripheral sites. In a virus-induced model of murine salivary gland inflammation, TLO induction depends on IL-22 (11). Similar to this study, we identified $\gamma\delta$ T cells and ILC3s as the major producers of IL-22 in tolerant lung allografts (11). These cells play an important role in BALT induction in our model as evidenced by the reduced number of lymphoid follicles after engraftment into $\gamma\delta$ T cell-deficient or AR mice. However, compensatory mechanisms of IL-22 production appear to exist in the absence of $\gamma\delta$ T cells or ILC3s, as evidenced by B cell recruitment and the development of PNAd-expressing HEVs in TCR $\delta^{-/-}$ hosts or AR recipients. As we did not observe increases in the expression of IL-17 transcripts in tolerant lung allografts, we did not formally examine a role for IL-17 in the induction of BALT in the current study. Our experiments indicate that, in the absence of IL-22, other cytokines such as IL-17 cannot mediate the induction of organized BALT in tolerant lung allografts.

We have uncovered that IL-22 is critical for B cell recruitment to the lymphoid follicles as well as for the development of PNAd⁺ vessels, but not for the expression of CCL21. Similarly, in a murine model of virus-induced autoimmune inflammation of salivary glands, expression of IL-22 is critical for the local induction of the B cell chemokines CXCL12 and CXCL13 (11). Consequently, B cell accumulation in TLOs that formed within salivary glands was impaired in the absence of IL-22, which resulted in reductions in autoantibody production. Numerous studies have shown that IL-23, a proinflammatory cytokine that can be produced by dendritic cells, drives the synthesis of IL-22 (41, 42). Therefore, it is not surprising that neutralization of IL-23 and absence of IL-22 in recipient mice yielded similar phenotypes. To this end, a previous study investigating *M. tuberculosis*-induced BALT demonstrated that IL-23 played an important role in the expression of B cell chemokines and B cell aggregation (12).

It is the prevailing view that lymphocyte homing into BALT and other TLOs depends on HEVs, where PNAd is required for T and B cell extravasation (30). We have found that IL-22 is required for the development of PNAd-expressing vessels within lymphoid aggregates in tolerant lung allografts. Interestingly, late reconstitution of IL-22 through retransplantation of IL-22-deficient grafts into wildtype hosts triggers B cell recruitment, but fails to induce PNAd⁺ vessels. Previous studies have suggested that lymphotoxin signaling may be critical for the development of PNAd⁺ vessels during induction of TLOs, and recent reports have suggested that during intestinal inflammation lymphotoxin β receptor signaling in dendritic cells triggers the production of IL-23, which in turn induces the production of IL-22 by ROR γ t⁺ cells (42-44). Thus, it is possible that in certain inflammatory models IL-22 can play a role in the development of PNAd⁺ vessels downstream of lymphotoxin signaling. Other signals such as activation of pattern recognition receptors and signaling via MyD88 can trigger the production of IL-23 (45). Future studies need to determine which signals result in the increase in IL-23 expression in lung allografts during tolerance induction.

We have recently shown that graft-resident Foxp3⁺ cells are critical for maintenance of tolerance after pulmonary transplantation (6). Interestingly, in this study we found that

transplantation into IL-22-deficient or IL-23-neutralized recipients did not affect Foxp3⁺ cell accumulation in lung allografts. In fact, their density was comparable to our findings in wildtype recipients, providing an explanation for the survival of grafts after transplantation into IL-22-deficient or IL-23-neutralized mice. Although the Foxp3⁺ cells do not accumulate within organized BALT when recipients lack IL-22/IL-23 signaling, our retransplant experiments show that the tolerance of such lungs depends on the presence of Foxp3⁺ cells as their selective depletion from the graft triggers rejection. These observations extend the notion that immune circuits that are established locally within the lung graft play an important role in the regulation of tolerance. Lung grafts are unique as they are constantly exposed to the external environment. Since external stimuli are known to impact the function of pulmonary immune cells including impairment of regulatory T cell function, our findings may provide a mechanistic link between exposure to respiratory pathogens as well as air pollutants and lung allograft rejection (46-49). Notably, Foxp3⁺ cells accumulated in lung allografts that were transplanted into IL-22-deficient or IL-23-neutralized mice despite the absence of PNAd⁺-expressing HEVs suggesting that they can home to BALT via alternative routes. Accordingly, a study of nasal-associated lymphoid tissue (NALT), a form of mucosal TLO, in mice that lack PNAd expression revealed that while B cells and CD4⁺CD25⁻ T cells have less accumulation within NALT, regulatory T cell (CD4⁺CD25⁺) accumulation is unaffected (50). Our findings in IL-22-deficient or IL-23-neutralized lung grafts are reminiscent of Foxp3-rich organized lymphoid structures that have been observed in stable kidney allografts, which also lack PNAd expression (51). Furthermore, a recent study has revealed that two distinct routes exist for lymphocyte homing into BALT - one from the blood via PNAd-expressing HEVs and one via the lung parenchyma that is independent of HEVs (52).

In conclusion, our findings provide a novel pathway for the induction of TLOs in allografts. We have shown that IL-22 is required for B cell accumulation and development of PNAd-expressing HEVs within BALT that is induced in tolerant lung transplants. We further show that reconstitution of IL-22 at late time points promotes B cell recruitment and enlargement of the lymphoid follicles, but does not result in the development of PNAd-expressing HEVs (6). The formation of Foxp3 aggregates in lung allografts that we have shown to be critical for maintaining a tolerant state, does not depend on IL-22 or PNAd-expressing HEVs. Given that chronic rejection is the leading cause of late-term morbidity and mortality in lung transplant patients, novel therapies that induce tolerance are critically needed (53). Collectively, our findings provide new insights into immunological events that contribute to tolerance after lung transplantation, and these findings need to be taken into consideration when developing new therapies. Our work raises the possibility that outcomes after lung transplantation may be improved by pharmacological or gene therapy interventions that facilitate the induction of Foxp3-rich aggregates in pulmonary grafts. To this end, ex vivo lung perfusion provides a platform to treat lung allografts prior to transplantation (54).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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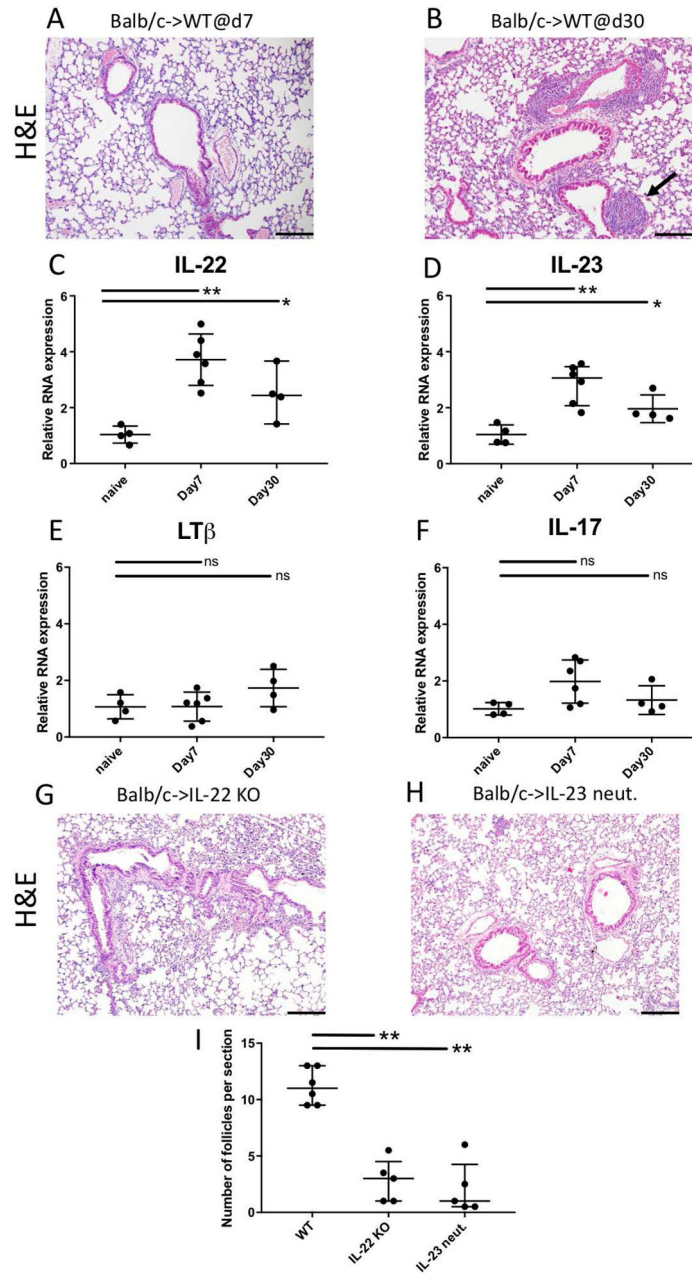


Figure 1: BALT induction in tolerant lung allografts requires IL-22 and IL-23 expression. Histological appearance of Balb/c lung allografts (A) 7 and (B) 30 days after transplantation into B6 wildtype (WT) mice that received WT peri-operative costimulatory blockade, with arrow depicting BALT at 30 days. Relative RNA expression of (C) IL-22, (D) IL-23, (E) LTβ, and (F) IL-17 from whole lung lysates of naïve Balb/c lungs and transplanted lungs at days 7 and 30. Appearance of lung allografts 30 days after transplantation into (G) IL-22 deficient hosts or (H) wildtype recipients receiving IL-23 neutralization. Recipients were treated with peri-operative costimulatory blockade. Scale bars: 100μM. (I) Quantification of the number of

lymphoid follicles per section 30 days after transplantation into various recipients. n = 4 for all conditions. * p<0.05; **p<0.01; ns=not significant.

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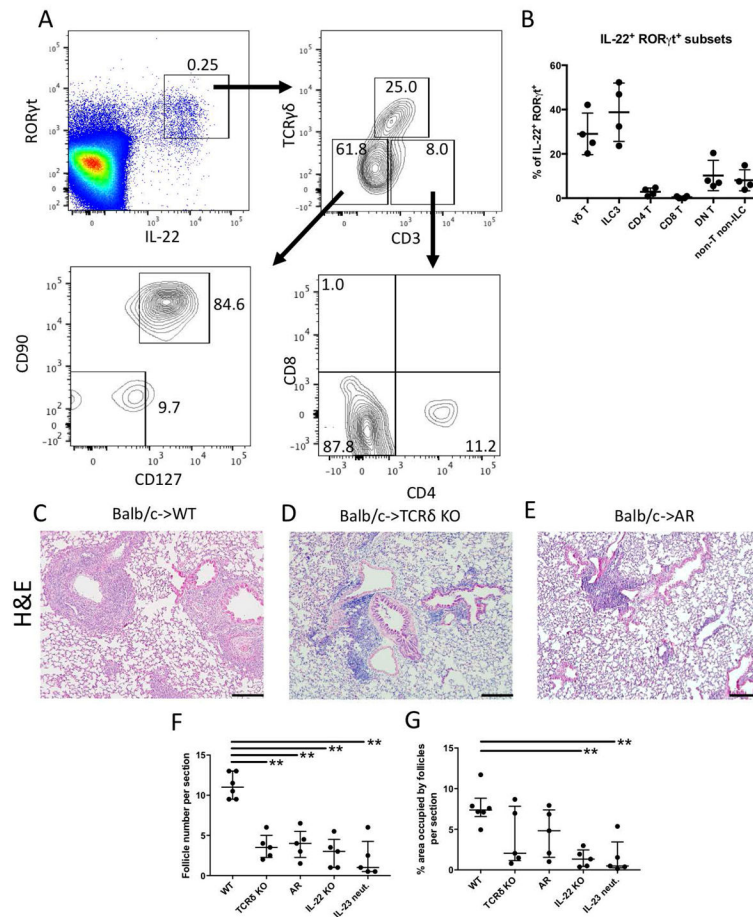


Figure 2:

Graft-infiltrating $\gamma\delta$ T cells and ILC3s are important sources of IL-22 after lung transplantation. (A) Flow cytometric characterization of IL-22⁺ cell types in tolerant Balb/c \rightarrow B6 CD45.1 lung grafts reveals $\gamma\delta$ T cells (ROR γ t⁺CD3⁺TCR $\gamma\delta$ ⁺) and ILC3s (ROR γ t⁺CD3⁻TCR $\gamma\delta$ ⁻CD127⁺CD90⁺) as main producers. Other IL-22⁺ cells include CD4⁺ T cells (ROR γ t⁺CD3⁺TCR $\gamma\delta$ ⁻CD4⁺CD8⁻), double-negative (DN) T cells (ROR γ t⁺CD3⁺TCR $\gamma\delta$ ⁻CD4⁻CD8⁻), CD8⁺ T cells (ROR γ t⁺CD3⁺TCR $\gamma\delta$ ⁻CD4⁻CD8⁺) and non-T non-ILCs (ROR γ t⁺CD3⁻TCR $\gamma\delta$ ⁻CD127⁻CD90⁻). Plots are gated on live single CD45.1⁺ cells. (B) Quantification of ROR γ t⁺IL-22⁺ cell subpopulations in tolerant lung grafts based on the gating plots described above. Histological appearance of lung allografts 30 days after transplantation into (C) wildtype (WT), (D) $\gamma\delta$ T cell-deficient (TCR δ KO) recipients and (E) AR mice (ROR γ t-cre Ahr^{fl/fl}) that received peri-operative co-stimulatory blockade. Quantification of the (F) number of lymphoid follicles and (G) percentage of total area occupied by lymphoid follicles per section 30 days after transplantation into various recipients. n = 4 for all conditions. **p<0.01. IL-22 KO: IL-22-deficient; IL-23 neut: IL-23 neutralization. Scale bars: 100 μ m.

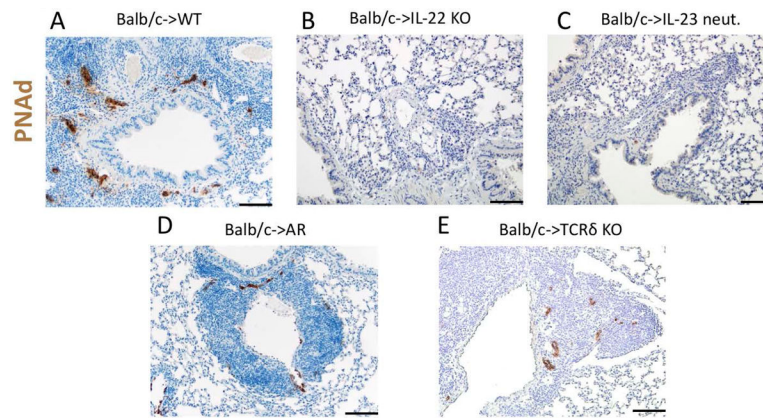


Figure 3: Expression of peripheral node addressin in lymphoid follicles is dependent on IL-22. Histological appearance and representative staining for PNAd (brown) in Balb/c lung allografts 30 days after transplantation into (A) wildtype (WT), (B) IL-22-deficient, (C) IL-23-neutralized, (D) AR ($ROR\gamma t\text{-cre Ahr}^{fl/fl}$) mice, and (E) $\gamma\delta$ T cell-deficient ($TCR\delta^{-/-}$) recipients. Recipient mice were treated with peri-operative costimulatory blockade. Scale bars: 50 μ m.

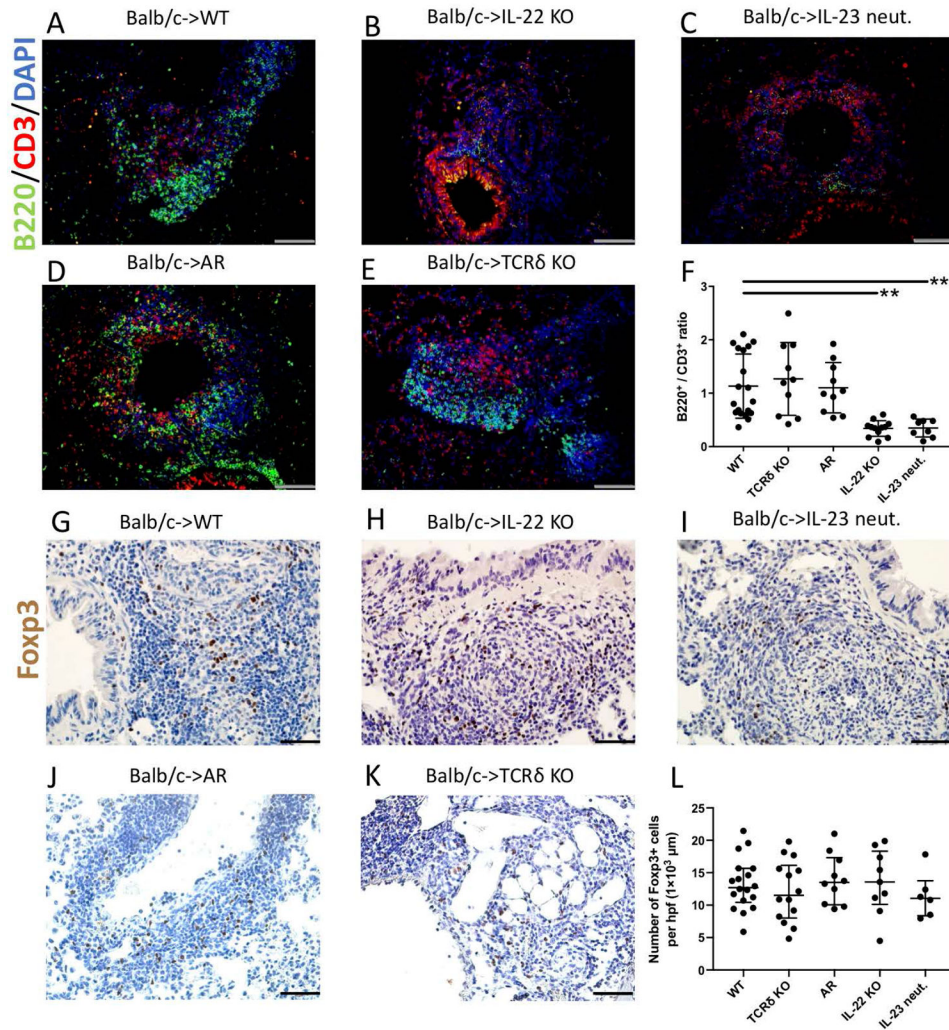


Figure 4:

IL-22 is required for B cell, but not Foxp3⁺ cell recruitment into lymphoid follicles. Immunofluorescent staining of B cells (green), T cells (red), and DAPI (blue) in Balb/c lung allografts 30 days after transplantation into (A) wildtype (WT), (B) IL-22-deficient, (C) IL-23-neutralized, (D) AR mice (RORγt-cre Ahr^{fl/fl}) and (E) γδ T cell-deficient (TCRδ^{-/-}) recipients. Scale bars: 100 μm (F) Quantification of B220 / CD3⁺ positive cell ratio per lymphoid follicle 30 days after transplantation into the indicated recipients. Each dot represents one lymphoid follicle (n = 2 mice per group). Immunostaining for Foxp3 (brown) in Balb/c lung allografts 30 days after transplantation into (G) wildtype, (H) IL-22 deficient, (I) IL-23 neutralized, (J) AR mice, and (K) γδ T cell-deficient recipients. (L) Quantification of Foxp3 cells per lymphoid follicle 30 days after transplantation of Balb/c lungs into the indicated recipients. Each dot represents one lymphoid follicle (n = 2 mice per group). Recipients were treated with peri-operative co-stimulatory blockade for all conditions. Scale bars: 50 μm.

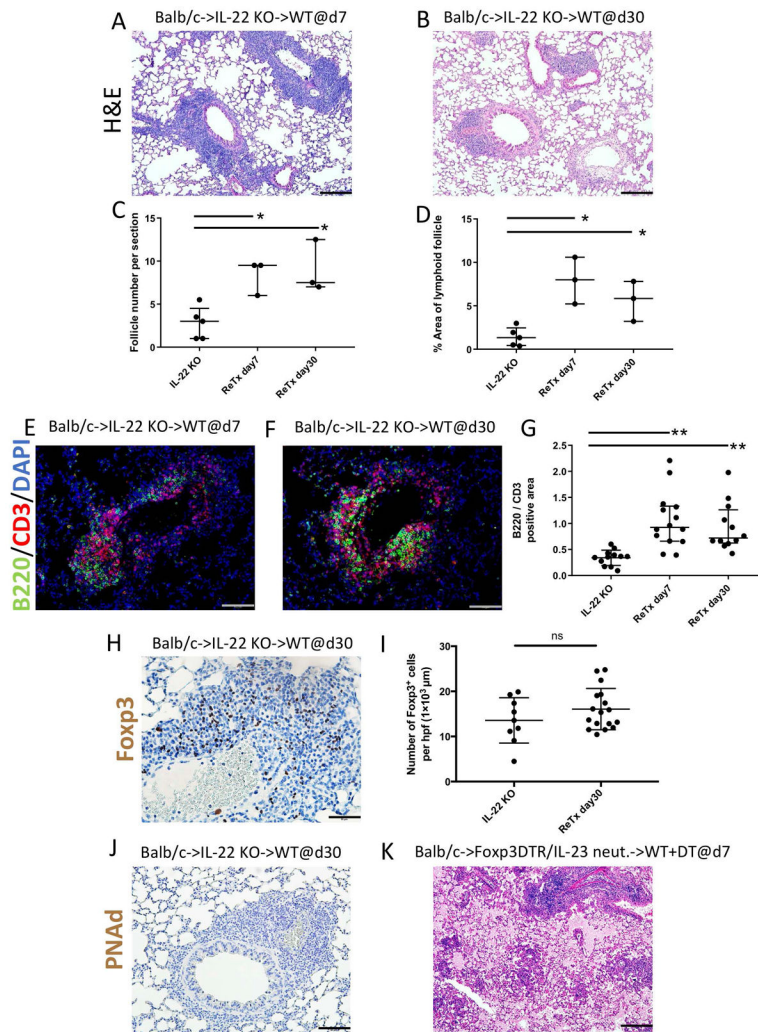


Figure 5:

Late reconstitution of IL-22 expression mediates B cell recruitment to lymphoid follicles. Histological appearance of Balb/c lung grafts initially transplanted into IL-22 deficient recipients that received peri-operative costimulatory blockade and then retransplanted into non-immunosuppressed wildtype secondary recipients 30 days later. Images show lung allografts harvested (A) 7 and (B) 30 days after retransplantation. $n=3$. Scale bars = 100 μm . Quantification of lymphoid follicle (C) number and (D) percent of total area per section in IL-22-deficient recipients 30 days after primary transplantation, as well as 7 and 30 days after retransplantation into wildtype secondary hosts. Histological appearance of B cell (green), T cell (red), and DAPI (blue) immunofluorescent staining from Balb/c lung grafts (E) 7 and (F) 30 days after retransplantation (scale bars = 50 μm) and (G) quantification of B220 / CD3 cell ratio per lymphoid follicle among the various conditions. Each dot represents one lymphoid follicle ($n=2$ mice per group). Immunostaining for Foxp3 (brown) (H) 30 days after retransplantation and (I) quantification of Foxp3 cells per lymphoid follicle at 30 days among various conditions. Scale bars = 50 μm . Each dot represents one lymphoid follicle ($n=2$ mice per group). Immunostaining for PNAd (brown) (J) 30 days after retransplantation. Scale bars = 50 μm . (K) Histological appearance of Balb/c lung grafts

initially transplanted into IL-23 neutralized B6 Foxp3-DTR recipients that received peri-operative costimulatory blockade and then retransplanted into non-immunosuppressed wildtype B6 recipients 30 days later that received DT at the time of retransplantation. Images are shown 7 days after retransplantation (H&E) (n=4). Scale bars =100 μ m. ns= not significant; *p<0.05; **p<0.01.

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