Autoreactive T and B Cells Induce the Development of Bronchus-Associated Lymphoid Tissue in the Lung

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Rheumatoid arthritis–related interstitial lung disease (RA-ILD) is associated with significant morbidity and mortality. Studies in humans have found that the incidence of bronchus-associated lymphoid tissue (BALT) correlates with the severity of lung injury. However, the mechanisms underlying the development of BALT during systemic autoimmunity remain unknown. We have determined whether systemic autoimmunity in a murine model of autoimmune arthritis can promote the development of BALT by generating a novel murine model derived from K/BxN mice. Transgenic mice with the KRN T-cell receptor specific for the autoantigen, glucose-6-phosphate isomerase (GPI), were crossed with GPI-specific immunoglobulin heavy and light chain knock-in mice, producing mice with a majority of T and B cells specific for the same autoantigen. We found that 67% of these mice demonstrated lymphocytic infiltration in the lungs, localized to either the perivascular or peribronchial regions. Fifty percent of the mice with lymphocytic infiltration manifested lymphoid-like lesions resembling BALT, with distinct T and B cell follicles. The lungs from mice with lymphoid infiltrates had increased numbers of cytokine-producing T cells, including IL-17A⁺ T cells and increased major histocompatibility complex Class II expression on B cells. Interestingly, challenge with bleomycin failed to elicit a significant fibrotic response, compared with wild-type control mice. Our data suggest that systemic autoreactivity promotes ectopic lymphoid tissue development in the lung through the cooperation of autoreactive T and B cells. However, these BALT-like lesions may not be sufficient to promote fibrotic lung disease at steady state or after inflammatory challenge.

Keywords: autoimmunity; bronchus-associated lymphoid tissue; T cells; B cells

Rheumatoid arthritis (RA) is a common chronic, systemic autoimmune disorder characterized by inflammatory and erosive arthritis of the distal joints. Many patients with RA develop extraarticular disease, including lung, cardiac, and vascular

CLINICAL RELEVANCE

Rheumatoid arthritis–related interstitial lung disease is associated with significant morbidity and mortality, and bronchus-associated lymphoid tissue (BALT) was previously correlated with the severity of lung injury. However, the mechanisms underlying the development of BALT during systemic autoimmunity remain unknown. In this novel model, systemic autoreactivity promotes ectopic lymphoid tissue development in the lung through the cooperation of autoreactive T and B cells. The development of ectopic lymphoid aggregates was not sufficient to injure the lung tissue at steady state or after bleomycin lung injury. BALT formation may be a protective mechanism, rather than a mediator of lung injury.

disease. The lung can be a site of severe chronic inflammation, which can progress to endstage fibrotic interstitial lung disease and death (1). The pathophysiology of RA-related lung disease is poorly understood and difficult to treat. Data from human studies and murine models point to both a genetic predisposition with an association with major histocompatibility complex (MHC) Class II alleles and environmental exposures, with smoking as one of the major risk factors in the development of RA (2). The lung, with its constant exposure to the environment, has been implicated as the site of initiation for the autoimmune response, but no studies in animals have focused on dissecting the precise mechanisms involved (3). To develop therapies that are more specific and effective for lung disease, better models of RA-associated lung disease are needed in the laboratory.

Pathologists have long recognized that the lung pathology of patients with RA characteristically contains lymphocytic aggregates and areas of organized lymphoid tissue (4). Recently, Rangel-Moreno and colleagues reported on a correlation between inducible bronchus-associated lymphoid tissue (BALT) and the severity of lung destruction in patients with RA-related lung disease (5). Goronzy and Weyand also postulated that ectopic lymphoid tissue may propagate autoimmunity by providing regions of increased exposure of T cells to B cells, and the architecture of the arthritic synovium provides an ideal environment for the development of lymphoid tissue (6). These data suggest that ectopic lymphoid tissue plays a role in disease pathogenesis in the joints and in the lung.

One of the murine models that has led to insights into RA is that of K/BxN arthritis, developed in 1996 by Kouskoff and colleagues (7). K/BxN mice spontaneously develop arthritis because of the expression of the transgene-encoded KRN T-cell receptor (TCR), which can recognize a self-peptide from

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glucose-6-phosphate isomerase (GPI), an enzyme involved in glycolysis (8), which is presented by the nonobese diabetic (NOD) MHC Class II allele A^{g7} (8). In this model, KRN T cells are activated and provide help to B cells specific for GPI (7–9). We have found that lymphocytic infiltrates develop in the lungs of K/BxN mice, but BALT-like tissue preferentially develops in mice that also carry Ig knock-in genes for high-affinity GPI-specific B cells (H-L-g7xKRN TCR, designated H-L-g7xK). These data suggest that ectopic lymphoid tissue in the lung develops from the cooperative activity of antigen-specific T and B cells. The presence of BALT-like lesions was not associated with interstitial lung pathology at baseline. Interestingly, after challenge with bleomycin, these mice were less prone to fibrosis than were wild-type animals. Thus, ectopic lymphoid tissue in the lung does not necessarily promote lung parenchymal injury, and may provide protection from inflammatory insults.

MATERIALS AND METHODS

Animals

K/BxN mice were generated by crossing B6.KRN TCR transgenic mice with NOD (A^{g7}) mice. To generate mice with a high-affinity B-cell receptor specific for GPI, mice previously generated with an anti-GPI Ig knock-in gene were crossed with C57BL/6 (B6).H2^{g7} congenic mice, which carry the MHC Class II A^{g7} allele required to present the GPI peptide to the autoreactive KRN TCR, and were then crossed with B6.KRN TCR transgenic mice (H-L-g7xK) (10). Wild-type control mice comprised either the first (F1) generation of C57BL/6 mice and NOD mice (BxN) or congenic B6-A^{g7} mice bred in-house or C57BL/6 mice, purchased from the National Cancer Institute (Frederick, MD). All mice were housed under specific pathogen-free conditions in the Animal Care Facility at the University of Chicago. All experimental murine protocols were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Lung Histology, Immunohistochemistry, and Tissue Digests

Lungs were fixed in 10% formalin, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin or Masson trichrome. For immunohistochemistry, after incubation in unmasking solution, histology slides were treated in 3% hydrogen peroxide and blocked, and anti-CD3 (PC630; Oncogene, San Diego, CA), anti-B220 (01122D; BD Biosciences, San Jose, CA), or biotinylated peanut agglutinin (PNA, catalogue number B-1075; Vector Laboratories, Inc., Burlingame, CA) was applied on tissue slides for a 1-hour incubation at room temperature in

a humidity chamber. After a PBS wash, slides were incubated with the Elite Vectastain ABC kit (PK-6100; Vector Laboratories, Inc.) Antigen-antibody binding was detected by the DAB Substrate Chromogen System (DAKO, Carpinteria, CA). Slides were briefly immersed in hematoxylin for counterstaining. For flow cytometry, the right upper and middle lobes of the lungs and the spleens were mechanically dissociated, and the lungs were further digested with collagenase I (Life Technologies, Grand Island, NY). After the lysis of red blood cells, single-cell suspensions were stained for flow cytometry.

Flow Cytometry

For cell-surface staining, cells from lung and spleen tissue were incubated with Fc block for 10 minutes, and then stained with antibodies to either CD4, CD3, CD19, MHC Class II, CD11c, CD11b, CD44, CD62L, V β 6 T cell receptor, or inducible costimulator (ICOS) (Ebioscience, San Diego, CA or BD Biosciences) in FACS buffer (PBS containing 2% BSA and 0.01% sodium azide). For intracellular cytokine staining, cells were incubated with phorbol 12-myristate 13-acetate (PMA) (20 ng/ml) and ionomycin (2 μ g/ml) for 5 hours, and with Brefeldin A (10 μ g/ml) for the final 4 hours of culture. Cells were stained with surface stains, fixed with 1% paraformaldehyde, and then permeabilized with 0.5% saponin buffer and stained intracellularly with anti-IL-17A, anti-IFN- γ , anti-TNF- α , or the respective isotype controls. Data were acquired on a LSRII (BD Biosciences) and analyzed with Flowjo software (Tree Star, Ashland, OR).

Bleomycin Lung Injury

Bleomycin (Cipla, Mumbai, India) at 1 U/kg was instilled intratracheally into anesthetized mice on Day 0. Mice were weighed on Day 0 and at time points after bleomycin instillation. Mice were killed on Day 21. Fibrosis was scored on trichrome-stained slides, as described elsewhere (11).

Statistical Analysis

All data are expressed as mean and SEM, unless otherwise noted. Statistical significance was determined by *t* tests using GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant at *P* < 0.05.

RESULTS

Mice with Autoimmune Arthritis Develop Lung Pathology

We hypothesized that mice with autoimmune arthritis would develop lymphoid lesions in the lungs, coincident with the development of arthritis. K/BxN mice with arthritis were evaluated

Figure 1. K/BxN mice develop perivascular and peribronchial lymphocytic infiltrates in the lungs. K/BxN mice were evaluated for lymphoid infiltration in the lung. (A) Representative lung with no infiltrates is shown (magnification, $\times 10$). (B) K/BxN lung exhibited multiple areas of lymphocytic infiltration around vessels (arrowheads) and airways (arrows) (magnification, $\times 4$). (C) Higher magnification of lung in B demonstrates peribronchial infiltrates around large airways (magnification, $\times 10$). (D) Higher magnification of infiltrates around small airways and vessels (magnification, $\times 10$).

Figure 2. H-L-g7xK mice develop lymphoid-like tissue. H-L-g7xK mice were evaluated for lymphoid infiltration in the lung. (A) Lymphoid-like lesions were found in the large airways of H-L-g7xK mice (magnification, $\times 10$). (B) Higher magnification was focused on bronchus-associated lymphoid tissue (BALT)-like area (magnification, $\times 20$). (C) Example of peribronchial and perivascular lymphocytic infiltration (magnification, $\times 10$). (D) Smaller airway with an example of peribronchial lymphocytic infiltration in an H-L-g7xK mouse (magnification, $\times 20$). (E) H-L-g7xK, K/BxN, and H-Lx-K lungs were scored for the presence of lymphoid-like tissue or lymphocytic peribronchial infiltrates according to the appearance of the histology, as already described.

for the presence of lymphoid tissue in the lung. Several mice exhibited no areas of lymphocytic infiltration (Figure 1). However, some had small lymphoid aggregates in the lungs, with larger areas of infiltration surrounding larger airways (Figure 1). The areas of lymphocyte aggregations were both peribronchial and perivascular. Most animals did not demonstrate extensive aggregations of lymphocytes that resembled organized lymphoid tissue (Figures 1 and 2). The GPI antigen is ubiquitous in the mouse, but may not be well presented by the bulk of B cells in the K/BxN mice (12). Antigen-specific B cells can present their cognate antigen very efficiently (13). To determine the influence of GPI-specific B cells, mice previously generated with an anti-GPI Ig knock-in gene were crossed with C57Bl/6 (B6).H2^{g7} congenic mice, which carry the MHC Class II A^{g7} allele required to present GPI peptide to the autoreactive KRN TCR (10). These mice were then crossed with B6.KRN TCR transgenic mice, to produce mice with an increased frequency of autoreactive T and B cells specific for the GPI autoantigen. These mice were designated H-L-g7xK.

Lung histology was evaluated in H-L-g7xK mice euthanized because of their severe arthritis, as determined by

scoring joint swelling. These mice had a mean ankle thickness of 3.98 mm, with a standard deviation of 0.49 and clinical scores of 10–12 (on a scale of 0–12, with 12 as most severe), according to the methods outlined by Monach and colleagues (14) and detailed elsewhere (9, 15). The mice ranged in age from 3–13 months. We found that approximately 67% of H-L-g7xK mice exhibited lymphocytic infiltration in the lungs, either perivascular or peribronchial (Figure 2E). Interestingly, half of the H-L-g7xK mice with lymphocytic infiltration had lymphoid-like lesions resembling lymphoid tissue, with more than five cell layers of predominantly T and B cells beneath the large airway mucosa of the lungs (Figure 2). In contrast, whereas most of the K/BxN mice had some lymphocytic infiltration, very few ($\sim 5\%$) demonstrated significant lymphoid aggregates in the lungs (Figure 2E). Mice with the anti-GPI Ig knock-in gene alone, crossed to KRN TCR mice on the B6 background (H-L-x-K), which do not develop arthritis, had no identifiable infiltrates (Figure 2E, and data not shown), suggesting that infiltrate formation is dependent on cognate interactions of T and B cells.

Figure 3. Germinal centers are present in submucosal lymphoid aggregates in the lung. H-L-g7xK mice were euthanized at the time of development of severe arthritis, and were evaluated for the presence of lung pathology. *Top left:* Representative hematoxylin and eosin-stained section of lung lymphoid aggregate is similar to BALT at $\times 10$ magnification. *Top right:* CD3 staining (brown) of same lesion at $\times 20$ magnification. *Bottom left:* B220 staining (brown) of lesion. *Bottom right:* Peanut agglutinin (PNA) staining (brown) in B-cell follicles, as indicated by arrows, suggests germinal centers.

Figure 4. Lung infiltrates are associated with weight loss but not severity of arthritis. H-L-g7xK mice were evaluated for the presence of lung pathology, as described in Figure 2. Mice with BALT-like tissue or with any lymphocytic infiltration (LI), including peribronchial or perivascular lymphocytic infiltrates or more BALT-like areas, were compared with mice lacking lung pathology. (A) Clinical score. (B) Hind ankle thickness was measured before the mice were killed, and the right and left measurements were averaged. (C) Ages were compared between mice with and without lung infiltrates. (D) Weight. $**P < 0.01$; ns, no significance; ♀, female; ♂, male.

T-Cell and B-Cell Architecture in Lung Lesions Resembles Ectopic Lymphoid Tissue Formation

The submucosal lesions found in the lungs of the H-L-g7xK mice suggested the development of ectopic lymphoid tissue or BALT. Segregated T-cell and B-cell areas comprise one of the hallmarks of lymphoid tissue, and PNA is a marker for germinal center B cells (16). The lymphoid-like lesions had distinct areas with $CD3^+$ T cells and $B220^+$ B cells segregating into follicle-like structures. Within the B-cell areas were PNA-positive cells (Figure 3). A review of pathology determined the presence of germinal centers according to their characteristic light microscopic features. The presence of distinct follicle-like areas and germinal center B cells are consistent with ectopic lymphoid tissue development.

Lung Infiltrates Do Not Correlate with Severity of Arthritis or Age, but with Weight Loss

We had hypothesized that the severity of joint disease and lung abnormalities would be correlated. However, we found no correlation between the severity of arthritis and the presence of lung lymphoid aggregates (Figures 4A and 4B). We also found no correlation with age (Figure 4C). To evaluate whether the lung abnormalities may contribute to systemic disease, we determined whether weight correlated with lung pathology, and found a small but significant decrease in weight in male mice with lung infiltrates, compared with mice without any lung lesions (Figure 4D). These data suggest that lung lesions are

not likely to regulate the severity of arthritis directly, but may affect the overall health of the animals.

Mice with BALT Have Increased Numbers of CD4 Cells and Cytokine-Secreting T Cells

Because the H-L-g7xK mice exhibited increased lymphoid-like infiltrates compared with K/BxN mice, we postulated that differences would be evident in the frequency and phenotype of T and B cells in the lungs of these mice, compared with K/BxN and control mice. The T cells in the lungs, as well as in the spleens, of H-L-g7xK mice were $CD44^{Hi}CD62L^{Lo}$, and expressed high concentrations of ICOS compared with B6 control mice, and the majority expressed the clonotypic V β 6 chain (Figure E1 in the online supplement). T cells in the lungs of K/BxN mice showed a similar effector phenotype as the H-L-g7xK lung T cells, and also expressed high concentrations of ICOS (Figure E1). These data are consistent with the known phenotype of KRN T cells in mice that develop arthritis, and suggest that no other population of T cells has expanded in the lungs of H-L-g7xK mice (7). In a cohort of animals with lymphocytic infiltrates, we found that H-L-g7xK mice had an increased frequency and absolute number of $CD4^+$ T cells in the lungs, compared with K/BxN mice (Figure 5A). No significant difference was evident in comparison with wild-type mice. Despite the aggregation of B cells found on histology, no significant increase in the number or frequency of B cells occurred in the lungs of H-L-g7xK mice compared with K/BxN mice among those tested (Figure 5A). In contrast, a decrease in the frequency of B cells occurred in the lungs of H-L-g7xK mice compared with wild-type mice, although no differences were evident in terms of absolute number. Interestingly, the expression of MHC Class II was increased in B cells from the H-L-g7xK lungs compared with the K/BxN and wild-type mice, whereas MHC Class II expression on dendritic cells was not different (Figure 5B). A significant correlation was found between the severity of lymphocytic infiltrates and the level of MHC Class II expression according to mean fluorescence intensity (MFI) (Figures 5C and 5D). These data suggest that H-L-g7xK B cells may be more activated, and may predispose the animals to lymphoid-like aggregates in the lungs.

One of the cytokines that was previously linked to BALT formation in the lung is IL-17A (17). Although both strains demonstrated an increase in the frequency of cytokine-producing cells in the lungs compared with wild-type mice, the H-L-g7xK mice exhibited the greatest numbers of IL-17A $^+$ CD4 $^+$ T cells in the lungs (Figures 6A and 6B). The H-L-g7xK mice also demonstrated increased TNF- α $^+$ CD4 $^+$ T cells and increased IFN- γ $^+$ CD4 $^+$ T cells, although the numbers of IFN- γ $^+$ CD4 $^+$ T cells were much lower than those of IL-17A $^+$ CD4 $^+$ T cells. The increase in cytokine-producing T cells in the arthritic strains compared with wild-type mice was also found in the spleen, but with no significant difference between the H-L-g7xK and K/BxN mice (Figure 6C and Figure E2 in the online supplement). These results suggest that H-L-g7xK animals have more activated T and B cells in the lungs, which may contribute to the development of BALT-like lesions.

Mice with Lymphocytic Infiltrates Are Protected from Bleomycin-Induced Fibrosis

Previous work found that the severity of lung-tissue damage in patients with RA-related lung disease correlated with the presence of BALT (5). In either strain of mice (K/BxN or H-L-g7xK), we did not find evidence of interstitial lung disease at baseline. We hypothesized that an inflammatory insult, such as bleomycin, may provoke greater injury in the lungs of mice with ectopic lymphoid tissue than in control mice because of the presence of activated T

Figure 5. Increased CD4⁺ T cells in lungs of mice prone to BALT-like infiltrates and increased major histocompatibility complex (MHC) Class II expression on B cells. The right upper and middle lobes of lungs from H-L-g7xK, K/BxN, and wild-type (WT) control mice (BxN or B6-Ag7) were digested and evaluated by flow cytometry. The remaining lung tissue was reserved for histology. (A) Percentages and absolute numbers of CD4⁺ T cells ($\times 10^3$) and CD19⁺ B cells ($\times 10^3$) in the lungs. (B) Expression of MHC Class II according to mean fluorescence intensity (MFI) was measured on dendritic cells (CD11b⁺CD11c⁺SSC_{lo}) and CD19⁺ B cells. (C) The extent of lymphocytic infiltrates was scored by a blinded observer on an arbitrary scale of 1–4. (D) A significant correlation was found between MHC Class II MFI and lymphocytic infiltration score (Pearson correlation, $r = 0.8127$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and B cells. To test this hypothesis, bleomycin was instilled intratracheally into control (BxN), K/BxN, and H-L-g7xK mice, and the mice were evaluated 21 days later. Remarkably, the K/BxN and H-L-g7xK mice showed attenuated weight loss compared with control mice after bleomycin instillation (Figure 7A). Furthermore, whereas interstitial cellular infiltrates were found in all animals, lung fibrosis on Day 21 after treatment was markedly diminished in both arthritic strains compared with control mice (Figures 7B and 7D). The decrease in fibrosis occurred although the mice exhibited lymphocytic infiltrates (Figure 7C). The data suggest that arthritic strains are protected from bleomycin injury, and that ectopic lymphoid tissue does not necessarily contribute to lung damage after bleomycin treatment.

DISCUSSION

We have found that mice with autoreactive T and B cells specific for the same autoantigen develop lung pathology that resembles the ectopic lymphoid lesions found in humans, and is consistent with BALT. These lesions were not as prominent or extensive in transgenic mice with only T cells specific for the autoantigen. The presence of lymphoid-like tissue did not correlate with the severity or incidence of arthritis or with the age of the mice, but did correlate with the expression of MHC Class II in B cells. Evidence of increased T-cell responses was observed in lungs with more T cells producing proinflammatory cytokines, especially IL-17A. The presence of BALT alone was not sufficient for interstitial lung disease at baseline or after challenge with bleomycin. Our data suggest that autoreactive T and B cells cooperate to induce BALT. Furthermore, augmented cognate T cell–B cell interactions may be sufficient to induce ectopic tissue formation in the lung, but do not necessarily predispose the mice to parenchymal lung pathology.

The presence of BALT has long been recognized in patients with RA-related lung disease, and was recently correlated with the severity of tissue damage (4, 5). BALT is not constitutive in either mice or humans but can develop postnatally, and this has been termed inducible BALT (iBALT) (18, 19). The development of BALT in human lungs was previously thought to be a response to microbial stimuli or a consequence of inflammation; BALT can be induced in mice by viral infections or other microbial stimuli (19–21). Our data suggest that B cells and an increased frequency of antigen-specific B cells, as in H-L-g7xK mice, can be inducers of ectopic lymphoid tissue. The B cells may provide the necessary chemokines and cytokines for initiating ectopic lymphoid tissue formation (22). Surprisingly, the overall numbers of B cells were not increased in the lungs of these mice. This may be attributable to the patchy nature of the disease and the localization of the largest infiltrates to the large airways. The large airways may not have been included in the lung digests. By isolating a portion of the lung for flow cytometry and using the remaining tissue for histology, we may be missing the areas with the most significant infiltrates.

Our data are in agreement with previous work involving a murine model of multiple sclerosis. In that study, the authors also found that transgenic mice with B and T cells specific for myelin-derived antigens exhibited ectopic lymphoid tissue formation in the spinal cord, as well as increased T-cell and B-cell activity (23). The antigen-specific B cells in H-L-g7xK mice may more efficiently activate T cells, given their high-affinity B-cell receptor and increased expression of MHC Class II. In support of this hypothesis, we found a significant correlation between the level of MHC Class II expression and the severity of lymphoid infiltrates. Dendritic cells have also been found to be required for iBALT formation in response to viral infection (24, 25). However, we did not find a significant difference in the level of MHC

Figure 6. Increased IL-17A-producing, TNF- α -producing, and IFN- γ -producing T cells in the lungs of mice with BALT-like lesions. (A) Representative dot plots from intracellular cytokine staining of lungs are shown for WT (BxN), K/BxN (K/B), and H-L-g7xK mice. (B) Percentages and absolute numbers of IL-17A-producing, TNF- α -producing, and IFN- γ -producing CD4⁺ T cells in the lungs. (C) Absolute numbers of cytokine-producing CD4⁺ T cells found in the spleens. *Significant compared with wild-type, unless noted otherwise on the graph; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Class II MFI on dendritic cells. Other alterations in dendritic cells are possible, and more detailed studies on dendritic cells may be revealing. Further studies are also needed to understand the mechanisms by which autoantigen-specific B cells may promote ectopic tissue formation in the lung.

IL-17A was previously found to play a role in germinal center formation in lymphoid tissue and in BALT formation in the lungs (17, 26). Given the increase in numbers of CD4⁺IL-17A⁺ T cells in the lungs of H-L-g7xK mice, IL-17A may be one of the main drivers of the lymphoid infiltrates in these mice. In another murine model of autoimmune arthritis, IL-17A was found to promote spontaneous germinal center formation through effects on B-cell responses (26). Interestingly, a recent report on humans with B-cell immunodeficiency found defects in the circulating Th17 cells in these individuals, and suggested that B cells play a role in the homeostasis of Th17 cells (27). All of the IL-17A⁺ cells were also TNF- α ⁺, constituting a phenotype that has been suggested to be more pathogenic in the brain (28). However, we did not find evidence of direct injury to lung tissue. TNF- α has also been implicated in the development of BALT, and is known to play a role in lymph node homeostasis (29, 30). The increase in CD4⁺ T cells producing IFN- γ and TNF- α may also be attributable to the increased antigen-presenting function of the B cells in H-L-g7xK mice. The differences were not limited to the lung, because both strains of arthritic mice demonstrate increased IL-17A-producing and IFN- γ -producing T cells in the spleens, compared with wild-type mice. This is consistent with previous studies in K/BxN mice (31). However,

only the H-L-g7xK mice showed an increase in the numbers of cytokine-producing cells in the lungs, suggesting that B cells in these mice may affect the distribution of these effector T cells or promote their differentiation in the lung. A positive feedback loop may occur, by which activated B cells in the lungs regulate the accumulation or differentiation of Th17 cells, and IL-17A and possibly TNF- α in turn promote ectopic lymphoid tissue formation.

The penetrance of ectopic lymphoid tissue was variable and did not correlate with arthritis, and the majority of extensive lymphoid aggregates were located in the large airways. The lung has been proposed as the site of initiation of autoantigen sensitization in humans (3). Smokers have been found to have citrullinated proteins in their lungs, and anti-citrullinated protein antibodies comprise a very sensitive marker for human RA (32). The reason for the lungs of many H-L-g7xK mice, but not others, to contain lymphoid-like lesions may involve environmental microbial stimulation augmenting the activation of B cells. B cells in K/BxN mice have been found to be better T-cell activators when microbial stimuli are present (12). The lung may provide a unique environment for the promotion of tertiary lymphoid structures through the activation of B cells, and the increased frequency of antigen-specific B cells may provide an augmented feedback loop to perpetuate iBALT. We also did not find a correlation between the severity of arthritis and the age of mice with lung pathology. These data suggest that the lung may be a reservoir for cells inducing arthritis, but not a critical mediator of arthritis pathology. This conclusion is supported by a study of

Figure 7. H-L-g7xK and K/BxN mice are protected from bleomycin lung injury. (A) Weight loss (%) was compared with initial weight after bleomycin was instilled intratracheally on Day 0 ($n = 5$ mice per group). (B) Fibrosis was scored by a blinded observer according to the technique described in MATERIALS AND METHODS. (C) The extent of lymphocytic infiltrates was scored as described in Figure 5C. (D) Masson trichrome-stained (Tri) and hematoxylin and eosin-stained (H&E) lungs from untreated and bleomycin-treated (Bleo) mice on Day 21. Arrows represent areas scored as fibrosis. Magnification, $\times 4$.

another autoimmune arthritis model that found lung injury alone was not a sufficient stimulus to promote arthritis in susceptible mice, whereas an injection of the fungal β -glucan, zymosan, was sufficient (33, 34).

Although RA-related lung disease occurs in older adults, we did not find a correlation with age. However, we did not investigate mice much beyond 1 year of age, and therefore aging may still affect the severity of lesions. Interestingly, we saw a difference in weights among male mice with lymphoid infiltrates in the lung. Although their arthritis was not more severe, this may suggest that systemic inflammation in mice induces weight loss. In a subset of mice examined, we did not find evidence of gut, spleen, or liver pathology to suggest that other foci of pathology may be contributing to weight loss. The variation in penetrance is similar to findings in humans with RA, because not all patients develop lung disease (1).

Although we found that H-L-g7xK mice had extensive lymphoid tissue surrounding the airways, we did not find evidence of parenchymal lung pathology, such as interstitial fibrosis, pneumonitis, or organizing pneumonia. Because previous work suggested that BALT may play a role in the pathology of fibrotic lung disease in humans (5), we determined the effect of an inflammatory insult, using bleomycin on the lungs of H-L-g7xK and K/BxN mice compared with wild-type control mice. In contrast to expectations, we found that both strains of arthritic mice were resistant to weight loss and the fibrosis induced by bleomycin. These data suggest that BALT-like lesions alone are not sufficient to promote inflammatory lung damage and fibrosis. We had also postulated that the increased presence of $CD4^+$ IL-17A $^+$ T cells in arthritic mice may contribute to increased fibrosis in the lungs of these mice, because IL-17A was found to be a mediator of fibrosis in response to bleomycin (35). The lack of effect

in the transgenic mice may be attributable to the antigen specificity of the T cells in the lungs, because both strains carry the KRN TCR transgene, and the majority of T cells in the lungs expressed the clonotypic V β 6 chain, suggesting that they express the KRN TCR (Figure E1, and data not shown). The exact antigens associated with fibrotic lung disease in patients with RA remain unknown, and although anti-GPI antibodies have been found in human RA, their exact role is not clear (36). Previous work also found that even in the absence of T cells, bleomycin can induce fibrosis (37). The IL-17A that promotes fibrosis in the bleomycin model may not be derived from $CD4^+$ T cells. Alternately, the NOD background may modulate fibrosis. However, the control mice used were B6xNOD F1, and the K/BxN mice were B6.KRN transgene X NOD F1. The H-L-g7xK mice have even less of the NOD background, because they are B6.H/L knock-in crossed with C57BL/6 (B6).H2 g7 congenic mice, which carry the MHC Class II A g7 allele required to present GPI peptide to the autoreactive KRN TCR. Thus the NOD background seems unlikely to explain differences in the fibrotic response to bleomycin.

Interestingly, the K/BxN and H-L-g7xK mice that received bleomycin did have lymphocytic infiltrates in their lungs. We do not know whether the infiltrates were increased by the bleomycin or preceded the bleomycin. These data suggest that lymphocytic infiltrates may be protective. The aggregation of T and B cells in the lungs may provide adaptive defense mechanisms through the recruitment of specific T and B cells capable of modulating the deleterious effects of inflammatory insults. In the case of influenza infection, iBALT was found to be sufficient for survival because of the priming of influenza-specific T and B cells in the lungs (19). In agreement with our findings, C-C chemokine receptor type 7 knockout mice (CCR7 $^{-/-}$), which have increased BALT formation, also have a diminished response to bleomycin-

induced lung fibrosis (38). The increased BALT formation and the protection from bleomycin are associated with defects in T regulatory cell trafficking, which underlies the postnatal development of BALT in CCR7^{-/-} mice (38, 39). In arthritic mice, the ectopic lymphocytic infiltrates may harbor regulatory T cells capable of modulating the immune response to bleomycin. Although we found a protective effect with bleomycin, we cannot rule out that other inflammatory insults, from other viruses or bacteria, may lead to a different response in the presence of BALT/iBALT that is harmful to lung tissue. In clinical lung samples from subjects with RA-related lung disease, the presence of iBALT correlated with increased collagen deposition, the citrullination of proteins, and cytokine production, suggesting that iBALT contributed to the fibrotic lesions (5). In mice with a full repertoire of T and B cells, autoreactivity to lung antigens such as citrullinated proteins may promote lung damage and fibrosis. More studies are needed to understand the mechanisms by which these transgenic mice modulate their response to bleomycin and other inflammatory triggers.

Our findings are in contrast to two other models of autoimmune arthritis that reported an association with autoimmune arthritis-related inflammation and fibrotic lung disease (34, 40, 41). These models promoted autoimmune arthritis via different mechanisms, one through altering T-cell signaling with a mutation in zeta chain-associated protein kinase 70 (ZAP-70) (SKG mice), and the other via aberrant MHC Class II expression. Unlike our model, neither of these models was dominated by the T-cell response to one specific autoantigen. The lung disease found in those studies may have been promoted by T cells specifically reactive to lung antigens. In the case of aberrant MHC Class II expression, collagen II immunization is required to provoke disease, and may be the driver of lung fibrosis. In addition, in the SKG mouse, CD8 T-cell clones appear capable of promoting lung inflammation and fibrosis (42). In our model, the lack of a robust CD8 T-cell response may underlie the absence of parenchymal damage or fibrosis.

Taken together, our data suggest that the development of ectopic lymphoid aggregates is not sufficient to injure lung tissue at steady state or after bleomycin injury. Other inflammatory insults, such as viral or bacterial infections or environmental insults (e.g., smoking), may lead to different results. BALT/iBALT may be detrimental in these circumstances and in mice with a full repertoire of T and B cells. Other forms of RA-related lung disease, such as follicular, constrictive, or obliterative bronchiolitis, may develop in mice with BALT/iBALT in response to these different inflammatory stressors. Our data also show that the severity of arthritis does not correlate with lymphocytic infiltration of the lung. This lack of correlation is similar to findings in humans, and suggests that independent triggers exist for joint and lung disease.

In conclusion, the frequency of autoreactive B cells and their cognate interactions with antigen-specific T cells promoted BALT-like tissue in arthritis-prone mice. This novel model will be useful for dissecting the mechanisms involved in BALT formation and the role of ectopic lymphoid tissue in modulating inflammatory lung injury and promoting RA-related lung disease.

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