IL-12 overexpression in mice as a model for Sjögren lung disease

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McGrath-Morrow, Sharon, Beth Laube, Shey-Cherng Tzou, Cecilia Cho, Jeffrey Cleary, Hiroaki Kimura, Noel R. Rose, and Patrizio Caturegli. IL-12 overexpression in mice as a model for Sjögren lung disease. Am J Physiol Lung Cell Mol Physiol 291: L837–L846, 2006.—Interleukin-12 (IL-12), a Th1 proinflammatory cytokine, is reported to be increased in Sjögren syndrome. To evaluate the effects of local Th1/Th2 deregulation, we generated a transgenic mouse model that overexpresses IL-12 in the lungs. IL-12 transgenic mice developed bronchial and alveolar abnormalities strikingly similar to those found in the lungs of Sjögren patients. Pathologically, lung abnormalities began at ∼4 mo of age and were characterized by lymphocytic infiltrates around the bronchi, intraluminal periodic acid Schiff-positive debris, increased cell proliferation in the alveolar region, and increased interstitial and alveolar macrophages. Functionally, these abnormalities translated into decreased mucociliary clearance (P < 0.05 vs. wild-type littermates) and increased oxidative stress (P < 0.01). The pathological and functional abnormalities were accompanied by significant changes in lung natural killer (NK) cells. The number of NK cells was fourfold higher in IL-12 transgenic than wild-type lungs (20% of all lymphoid cells vs. 5%) during the first month of life. NK cells then decreased within a narrow window of time (from 30 to 50 days of age), reaching a nadir of ∼2% on day 50, and remained at these low levels thereafter. This new mouse model highlights the role of IL-12 in the initiation of Sjögren syndrome.

Sjögren syndrome is a systemic autoimmune disease that involves lacrimal glands (dry eyes), salivary glands (dry mouth), and numerous extraglandular sites such as lungs, kidneys, skin, and thyroid (18). In its primary form, Sjögren syndrome is defined as the presence of the above manifestations without additional connective tissue disease. In its secondary form, Sjögren syndrome associates with other rheumatic autoimmune diseases, mainly rheumatoid arthritis, systemic lupus erythematosus, or systemic sclerosis. Like most rheumatic diseases, Sjögren syndrome lacks a single distinguishing feature and therefore its diagnosis relies on a combination of clinical and laboratory findings. In particular, the revised American-European classification identifies the following six criteria: ocular symptoms, oral symptoms, ocular signs, lymphocytic infiltration of salivary glands on lower lip biopsy, objective evidence of salivary gland involvement, and antibodies to SSA and/or SSB ribonucleoprotein antigens. To establish the diagnosis of Sjögren syndrome, the patient must have either lymphocytic infiltration of salivary glands or SSA/SSB antibodies, plus any other three of the remaining criteria (54). Sjögren syndrome is more common in women (female-to-male ratio is 9:1), often occurring in the fifth or sixth decade of life, and has an estimated population prevalence of 0.5% (53). Therefore, on the basis of the Census 2000 estimate of the total US population (281,421,906), Sjögren syndrome affects ∼1.4 million Americans.

Lung abnormalities are frequent in patients with Sjögren syndrome (56). Davidson (13) found them in ∼25% of patients, but the frequency increases to 50% when thin-section chest computed tomography is used for diagnosis (51). Patients present with irritating dry cough (secondary to dryness of the tracheobronchial mucosa, tracheobronchial sicca) (11), dyspnea from small airway obstruction (39, 42), and increased bronchial responsiveness (20, 45). The clinical course is insidious and invariably requires the use of glucocorticoids. Historical changes in the lungs are numerous and are still awaiting a better classification (4), although all have lymphocytic infiltration in common. When the infiltrate is diffuse, interstitial lesions are described as lymphocytic interstitial pneumonia, usual interstitial pneumonia, and nonspecific interstitial pneumonia, all considered distinct pathological subsets. When the infiltrate becomes confluent, often with a lobar distribution, the terms “bronchiolitis obliterans organizing pneumonia” (BOOP), and more recently “cryptogenic organizing pneumonia” have been used (53). In addition to the above-described interstitial disease, other studies have also emphasized the involvement of the tracheobronchial airways (30) and an impaired mucociliary clearance (35). Patients with Sjögren syndrome can also develop tumors of the mucosa-associated lymphoid tissue present in the lungs (12). Finally, it is not unusual for patients with Sjögren syndrome to develop both autoimmune hypothyroidism and lung dysfunctions (18). Regarding this association, it is important to clarify that although nonautoimmune hypothyroidism can also be associated with lung abnormalities (55), these abnormalities are different from the inflammatory lung response seen in the autoimmune hypothyroidism associated with Sjögren syndrome (5).

Interleukin-12 (IL-12) is a proinflammatory cytokine that has been found to be increased in Sjögren syndrome, in both the serum and the organs targeted by the autoimmune attack (27, 29, 38, 41). IL-12 is a heterodimer (p70) composed of a p40 subunit (expressed predominantly by macrophages, dendritic cells, and granulocytes) and a p35 subunit (expressed constitutively in numerous cell types); both subunits must be expressed by the same cell to produce a bioactive molecule. IL-12 binds to a specific plasma membrane receptor, mainly expressed on activated T, B, and natural killer (NK) lympho-

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cytes. The receptor is composed of a $\beta_1$-subunit, which binds p40, and a $\beta_2$-subunit, which binds p35. Ligation of the IL-12 receptor activates Tyk2 and Jak2 kinases, which, in turn, induce phosphorylation, dimerization, and nuclear translocation of signal transducer and activator of transcription-4 (3). The outcome is the transcriptional activation of numerous genes involved in prototypic Th1 responses, such as interferon-$\gamma$. The classic functions of IL-12 are to control parasitic infections and cancer cells by inducing an inflammatory response. However, uncontrolled IL-12 levels can induce a damaging lymphocyte and macrophage infiltration in many organs such as the lungs (10, 15). For this reason, we hypothesized that prolonged elevated levels of IL-12 could lead to many of the pathological changes observed in patients with Sjögren syndrome. We tested this hypothesis with a IL-12 transgenic mouse model previously developed in our laboratories (26).

**MATERIALS AND METHODS**

**Mouse population.** The study used IL-12 transgenic mice that express the full-length IL-12 heterodimer in the thyroid gland (26). These mice develop local features of thyroid disease (primary hypothyroidism and lymphocytic infiltration of the thyroid) but also produce enough IL-12 that IL-12 is increased in the serum (26) and can presumably exert effects at sites distant from the thyroid. IL-12 transgenic mice were bred to wild-type CBA mice (purchased from Jackson Laboratory, Bar Harbor, ME) for at least eight generations and maintained as heterozygous. A total of 187 mice, 123 transgenic mice between 41 and 689 days old and 64 age-matched controls, were analyzed in this study. In particular, 138 mice (98 transgenic and 40 control) were assessed for salivary, lacrimal, and lung histopathology; 13 mice (6 transgenic and 7 control) for lung IL-12 content, immunohistochemistry, immunofluorescence, and mucociliary clearance; and 36 mice (19 transgenic and 17 control) for lung NK cells. All experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set forth in National Institutes of Health guidelines, and approved by the Johns Hopkins University Animal Care and Use Committee.

**Lung content of IL-12 and other cytokines.** Lungs filter the majority of the cardiac output and contain abundant heparan sulfate proteoglycans, which are known to bind IL-12 (22). Lungs should therefore demonstrate increased IL-12 concentrations in response to increased serum IL-12 levels. To prove this prediction, proteins were extracted from the lungs of IL-12 transgenic and control mice as follows. The left lung lobes were removed and minced into small pieces (~5 mm in diameter) in 3 ml of phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (Sigma, St Louis, MO). Lung pieces were homogenized by ice-cold mechanical disruption with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 1000 g for 10 min and 4°C to remove undisrupted debris and nuclei. The supernatant was then ultraconcentrated (100,000 g for 1 h at 4°C) to separate the soluble cytosolic fraction from the pellet (formed by intracellular organelles). After the protein concentration was measured by bichinchoninic acid assay (Pierce Biotechnology, Rockford, IL), the cytosolic fraction was used immediately for cytokine array experiments (see below) or stored at −80°C until use. Lung cytosolic proteins were diluted 1:10 and incubated overnight onto a mouse cytokine array (RayBiotech, Norcross, GA), which allows the simultaneous detection of 31 molecules distributed as follows: 11 cytokines (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IFN-$\gamma$, and TNF-α), 11 chemokines [CCL2 [monocyte chemotactic protein (MCP)-1/JE], CCL3 [macrophage inflammatory protein (MIP)-1], CCL5 (RANTES), CCL11 (Eotaxin), CCL12 (MCP-5), CCL17 [thymus- and activation-regulated chemokine (TARC)], CCL19 (MIP-3), CCL21 (6Ckine), CCL27 (CTAK), CXCL1 (KC), and CXCL2 (MIP-2)]; 5 hematopoietic promoting molecules (IL-3, granulocyte colony-stimulating factor, granulocyte colony-stimulating factor, stem cell factor, and thrombopoietin); and 4 other molecules (leptin, tissue inhibitor of metalloproteinases 1, soluble TNF receptor 1, and vascular endothelial growth factor). After addition of biotinylated anti-cytokine antibody cocktail and horseradish peroxidase-conjugated streptavidin, a colorimetric signal was induced by addition of the detection buffer. The membrane was finally exposed to radiographic film (X-OMat AR; Kodak, Rochester, NY), and the signals were analyzed for gray scale intensity with the free software Image J (http://rsb.info.nih.gov/ij).

**TGF-β1.** Not present on the above-described cytokine array, was measured in isolation with a commercially available sandwich enzyme immunoassay (Quantikine; R&D, Minneapolis, MN).

**Salivary, lacrimal, and lung histopathology.** Lacrimal glands, salivary glands, and lungs were analyzed for the systemic effects of increased serum IL-12 concentrations.

Lacrimal and salivary glands were fixed overnight in Beckstead fixative, processed, and embedded in paraffin. Five-micrometer sections underwent hematoxylin and eosin staining. The extent of mononuclear cell infiltration was then graded with a five-category system, according to the system reported by Groom et al. (19). Briefly, a score of 0 represented a normal gland with no infiltration; 1 indicated the presence of 1–5 foci of mononuclear cells (≥ 20 cells per focus); 2 indicated >5 foci but no parenchymal destruction; 3 indicated multiple confluent foci with moderate degeneration of parenchymal tissue; and 4 indicated extensive infiltration of the gland with mononuclear cells and severe parenchymal destruction.

Lungs were infused through the trachea with a hot (60°C) 0.5% agarse solution, under 25 cmH$_2$O pressure, and then cooled on ice. Lungs were then fixed overnight in 10% buffered formalin, processed, and embedded in paraffin, cut into 5-μm sections, and then stained with hematoxylin and eosin for further analysis. The extent of mononuclear cell infiltration was graded with a four-category system that took into account the number and the size of the bronchus-associated lymphoid tissue (BALT), as well as the interstitial infiltration: 0 represented normal lungs with no infiltration; 1, 2, and 3 indicated mild, moderate, and severe infiltration, respectively.

For periodic acid Schiff (PAS) staining, hydrated sections were oxidized in 0.5% periodic acid solution for 5 min, rinsed in water, and incubated with Schiff reagent (Sigma) for 15 min. After a final 5-min wash, slides were counterstained with Mayer's hematoxylin solution for 1 min, dehydrated, and mounted with Permount.

**Lung immunohistochemistry.** Immunohistochemistry was used to detect CD4$^+$ or CD8$^+$ T cells, B220$^+$ B cells, MAC3$^+$ macrophages, nitrotyrosine, and phospho-Smad2 (pSmad2).

For the hematopoietic markers (CD4, B220, and MAC3), 5-μm frozen sections were cut, mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), fixed in acetone, air-dried, and rehydrated in PBS. After block of endogenous peroxidase activity (5 min in 0.3% hydrogen peroxide) and antibody aspecific binding (1 h in 2% normal goat serum), sections were incubated overnight at 4°C in a humid chamber with a biotin-conjugated monoclonal antibody recognizing CD4 (clone H129.19; Pharmingen, San Diego, CA; 1:30 dilution), CD8 (Pharmingen 53-6.7; 1:30 dilution), B220 (Pharmingen RA3-6B2; 1:2,000 dilution), or MAC3 (Pharmingen 10-3-6; 1:2,000 dilution). Sections were washed in PBS and incubated with peroxidase-conjugated streptavidin (DakoCytomation, Carpinteria, CA) for 30 min at room temperature in the humid chamber. Staining was revealed by the addition of a diaminobenzidine substrate (Sigma). Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin (Polyscience, Bay Shore, NY), washed in running tap water, dehydrated through increasing concentrations of ethanol, and mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

For nitrotyrosine detection, sections were first incubated in citrate buffer for 20 min (antigen retrieval), washed, and blocked in 3% H$_2$O$_2$
for 15 min. The specific nitrotyrosine antibody (Ab7048–50; Abcam, Cambridge, MA; 1:1,000 dilution) was applied for 30 min and then washed with PBS. The secondary antibody, InnoGenex mouse-on-mouse Iso-IHC kit (HC-3119-06; InnoGenex, San Ramon, CA), was applied for 10 min at room temperature, washed, processed as per the Vectastain Universal Quick Kit (PK-8800, Burlingame, CA), developed with diaminobenzidine (no. K3468; DakoCytomation), counterstained, dehydrated, and mounted with Permount. The brown staining was quantified with Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and normalized to the lung perimeter.

For pSmad2 antibody detection, the primary antibody (no. 3101; Cell Signaling, Danvers, MA; 1:1,000 dilution) was applied for 1 h at room temperature. Secondary antibody, using Envision+ System HRP (no. K4010, DakoCytomation), was used for 1 h at room temperature and then processed as above.

**Lung immunofluorescence.** Immunofluorescence was used to detect proliferating cell nuclear antigen (PCNA) as follows. Five-micrometer deparaffinized, hydrated sections were rinsed and blocked with avidin-biotin for 10 min and washed with PBS. PCNA antibody (catalog no. SC-7907; Santa Cruz, Santa Cruz, CA; 1:50 dilution) was applied for 1 h at room temperature. Sections were washed, and a secondary antibody (goat anti-rabbit Alexa Fluor 488, no. A11001; Molecular Probes, Eugene, OR; 1:200 dilution) was applied for 30 min at room temperature and then processed as above.

**Mucociliary clearance.** Mucociliary clearance was measured in the lungs of transgenic mice with a noninvasive procedure that involved the aspiration of a gamma-emitting radioisotope that deposited in the airway mucus and gamma camera imaging of the lungs over time. A similar approach was used previously (29, 32, 33) to quantify mucus clearance and gamma camera imaging of the lungs over time. Aasia et al. (31) utilized a similar approach to quantify mucociliary clearance in the lungs of transgenic mice expressing a mutant form of the mucus-associated protein (MAP-18) (31). The mucociliary clearance assay was performed on 8- to 12-week-old male and female wild-type and transgenic mice (A/J mice) housed under standard laboratory conditions (12 h light:12 h dark) with food and water ad libitum. The lungs were stained, dehydrated, and mounted with Permount. The brown staining was quantified with Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and normalized to the lung perimeter.

**RESULTS**

**IL-12 content is increased in lungs of transgenic mice.** We extracted proteins from the lungs of six IL-12 transgenic and seven control mice and tested them on a cytokine array to measure the levels of IL-12 p70 and other inflammatory cytokines. Compared with wild-type lungs (Fig. 1A), IL-12 transgenic lungs (Fig. 1B) contained significantly greater levels of IL-12 (P < 0.0001 by Wilcoxon rank sum test; Fig. 1C). MIP-2, another inflammatory marker, was also detected in IL-12 transgenic lungs (Fig. 1B), but not in wild-type lungs. The other cytokines present on the array were not detected in either wild-type or transgenic lungs. These findings indicate...
of infiltrating lymphocytes could be seen in the salivary and lacrimal glands of wild-type controls, but they never surpassed grade 2 (Fig. 2, B and C). Gland pathology was more severe and prevalent in female mice, reflecting the sex bias typical of Sjögren syndrome. In particular, females had scores that were on average 0.97 higher than males (95% CI: 0.26–1.67; \( P = 0.007 \)).

In lungs, lymphocytes were predominantly localized around large bronchial and pulmonary vessels, yielding a striking expansion of the BALT (Fig. 3: compare transgenic lungs in B to wild-type lungs in A). Lymphocytes populating the BALT were mainly B220-positive B lymphocytes (Fig. 3C and Table 1), followed by CD4-positive T lymphocytes (Fig. 3D and Table 1), similar to what was observed in salivary and lacrimal glands. The lung mononuclear cell infiltration was significantly more severe in IL-12 transgenic than in wild-type littermates (Fig. 2D). In particular, going from the wild-type to the IL-12 transgenic genotype, the lung score increased 3.01 (95% CI: 2.16–3.93; \( P < 0.0001 \)). The lung score worsened with age: lung alterations began at \( \approx 2 \) mo of age, increased in prevalence and severity by 4 mo of age, and reached 100% prevalence by 6 mo of age. For every 100-day increase in age, the score increased 0.21 (95% CI: 0.02–0.42; \( P = 0.048 \)), with genotype and sex held constant. Male and female transgenic mice developed similar lung lesions.

Macrophages are increased in IL-12 transgenic lungs. IL-12 transgenic lungs also contained numerous MAC3-positive macrophages scattered throughout the interstitium and the alveolar spaces (Fig. 4, A and B). In some areas, macrophages and epithelial cells clustered, remotely resembling granulomas (Fig. 4, B and C). Macrophages were also found in close approximation to areas of BOOP (Fig. 4D). These changes were not seen in lungs from wild-type controls (Fig. 4E).

Cell proliferation and PAS staining are increased in IL-12 transgenic lungs. Because IL-12 transgenic lungs had many features consistent with an inflammatory process, we evaluated cell proliferation with PCNA immunofluorescent staining. PCNA staining (not shown) demonstrated that proliferating cells were significantly more numerous in IL-12 transgenic than wild-type lungs (\( P < 0.02 \)).

As mice aged, the number of inflammatory cells in the lungs increased. Several older mice showed increased PAS staining in the large and small airways (Fig. 4C). This PAS-positive debris was acellular, suggesting an increase in mucin debris from IL-12 transgenic airways. No PAS-positive material was found in the airways of any wild-type lungs examined (Fig. 4E). Some of the IL-12 transgenic lungs also showed cells that pealed off in layers into the airways and resembled bronchial epithelial cells (Fig. 4F). The findings of PAS-positive acellular debris and increased cells in the airways prompted us to study mucociliary clearance.

Mucociliary clearance is decreased in IL-12 transgenic mice. Mucociliary clearance was measured in six IL-12 transgenic females (141 ± 14 days old) and seven wild-type females (113 ± 48 days old). Representative images of a pair of wild-type (A and C) and transgenic (B and D) mouse lungs are shown in Fig. 5. Six hours after the aspiration of the radioactive tracer, wild-type lungs (Fig. 5C) retained only 82.2% of the radioactivity deposited at time 0 (Fig. 5A), yielding a robust mucociliary clearance of 17.8%. In contrast,
IL-12 transgenic lungs at 6 h (Fig. 5D) retained 94.5% of the time 0 radioactivity (Fig. 5B), yielding a mucociliary clearance of only 5.5%. Analysis of the entire data set (Fig. 5E) showed that mucociliary clearance was significantly decreased in IL-12 transgenic mice ($P = 0.05$). One wild-type mouse (Fig. 5E) was unusual with respect to the other wild-type animals because it showed no clearance at all. When the outlier was excluded from the analysis, a $P$ value of 0.015 was obtained. Overall, these data suggest that the presence of IL-12 in the lungs leads to a significant decrease in mucociliary clearance, a finding similar to that described in patients with Sjögren syndrome (35).

Fig. 2. Salivary gland histopathology and salivary, lacrimal, and lung histopathology scores. A: foci of mononuclear infiltration in salivary glands of IL-12 transgenic mice. B–D: salivary, lacrimal, and lung histopathology scores, respectively, in entire cohort of 98 IL-12 transgenic and 40 wild-type mice.

Fig. 3. Lung histopathology. IL-12 transgenic lungs were characterized by a marked infiltration of mononuclear cells and expansion of the bronchial-associated lymphoid tissue (B). Lymphocytes were predominantly B220$^+$ B cells (C) and CD4$^+$ T cells (D). A wild-type lung is shown for comparison in A.
Oxidative stress and TGF-β signaling are increased in IL-12 transgenic lungs. Given the increase in inflammatory cells in IL-12 transgenic lungs, we quantified oxidative stress in the lungs with an antibody directed against nitrotyrosine. IL-12 transgenic lungs (Fig. 6B) showed significantly greater nitrotyrosine staining than control lungs (Fig. 6A) (P < 0.03), suggesting a link between the development of an inflammatory phenotype and the increase in oxidative stress.

Increased oxidative stress and inflammation are described in autoimmune diseases, along with a compensatory increase in TGF-β signaling (2). We therefore evaluated the TGF-β signaling pathway with an antibody directed against pSmad2, a key cytoplasmic protein necessary for mediating intracellular signaling of TGF-β (34). IL-12 transgenic lungs (Fig. 6D) showed significantly greater pSmad2 staining than wild-type lungs (Fig. 6C) (P < 0.03), present both in the inflammatory cells and in the interstitial cells of the alveolar septum (inset in Fig. 6D). We also measured the overall TGF-β1 content in whole lung homogenates from five IL-12 transgenic and five wild-type control mice. IL-12 transgenic lungs contained higher TGF-β1 levels (5.1 ± 0.7 vs. 3.4 ± 0.4 pg/ml), although the difference did not reach statistical significance (P = 0.11 by Wilcoxon rank sum test), probably because of the focal nature of the lung infiltrate.

Lung NK cells initially increased and then decreased in IL-12 transgenic mice. Lung NK cells were studied in 19 IL-12 transgenic mice, ranging in age from 15 to 381 days, and in 17 age-matched controls. In wild-type lungs, NK cells represented 5% of the total lymphoid population in the first month of life, increased to 12% during the second month of life, and then remained stable at this level thereafter (Fig. 7A). These data are in keeping with the previously reported kinetics of rodent NK cells in the spleen (23).

Table 1. Distribution of main lymphoid populations present in lungs of IL-12 transgenic and wild-type littermates

<table>
<thead>
<tr>
<th>B220</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4 CD25 CTLA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>%</td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>Wild type</td>
<td>1,928 ± 317</td>
<td>41 ± 4.6</td>
<td>2,036 ± 444</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>IL-12 Tg</td>
<td>2,907 ± 728</td>
<td>41 ± 4.9</td>
<td>6,066 ± 3,773</td>
<td>44 ± 4</td>
</tr>
</tbody>
</table>

Values represent means ± SE of B and T lymphocytes, expressed as absolute number and as % of the lymphoid gate [natural killer (NK) cells are presented separately in Fig. 7]. Transgenic (Tg) mice always showed a greater number of B and T lymphocytes, although differences did not reach statistical significance. IL-12, interleukin-12.
In contrast, NK cells showed a striking biphasic kinetic in IL-12 transgenic lungs (Fig. 7A). NK cells were fourfold higher than in wild-type littermates (20% vs. 5%) during the first month of life. NK cells then decreased during a narrow window of time (from 30 to 45 days of age), reaching a nadir of ~2% around day 45 and remaining at this low level throughout their life. Lung NK cell changes were also present when data were analyzed as absolute numbers rather than percentages (data not shown). Regression analysis showed that lung NK cells in IL-12 transgenic mice were significantly different from those in wild-type controls at all time points examined (P < 0.0001).

A representative flow cytometry dot plot from a pair of 4-mo-old mice is also shown in Fig. 7, B and C; the top left quadrants identify the NK cell population, which is CD3 negative and DX5 positive. At this age, NK cells in IL-12 transgenic lungs (Fig. 7B) were sevenfold lower than in wild-type lungs (Fig. 7A) (2.5% vs. 14.3%). These data suggest that the decrease in NK cell population may be an important determinant of the lung phenotype.

**DISCUSSION**

This study shows that increased IL-12 content in murine lungs induces a phenotype similar to that observed in Sjögren syndrome. The lung phenotype was mainly characterized by a marked mononuclear infiltration (lymphocytes and macrophages). With age, lungs developed striking expansion of BALT, areas of BOOP in the peripheral airways, evidence of increased oxidative stress and TGF-β signaling, decreased mucociliary clearance, and derangement of the normal lung NK cell population. The mononuclear infiltration and the BALT expansion strongly indicate an autoimmune pathogenesis and are reminiscent of the findings observed in a well-characterized mouse model of autoimmunity: the lupus-prone MRL-Fas<sup>−/−</sup> mouse (48, 52). Similar to our IL-12 transgenic mice, MRL-Fas<sup>−/−</sup> mice also develop mononuclear infiltrates in lungs and salivary and lacrimal glands. Interestingly, when MRL-Fas<sup>−/−</sup> mice are crossed onto a IL-12 p40-knockout background, they show an improvement in lung and salivary and lacrimal gland histopathology (25), emphasizing the role of IL-12 in the disease pathogenesis. Our findings also suggest...
that the bronchial airways may be targeted for inflammation in Sjögren syndrome, and this notion is supported by the observation that mononuclear peribronchial infiltrates are seen on transbronchial biopsy of Sjögren patients (42).

The impaired mucociliary clearance observed in the IL-12 transgenic mice may be explained by at least three possible mechanisms. First, IL-12 transgenic lungs contained PAS-positive, acellular debris that partially occluded the airways and may have slowed mucociliary clearance by mechanical obstruction. Interestingly, glycoprotein-containing substances, including mucins, stain positive for PAS. This suggests that the PAS-positive material in the IL-12 transgenic mouse may have been material that was associated with mucin, a component of mucus. Second, IL-12 transgenic lungs also contained intraluminal cellular debris, resembling denuded airway epithelial cells. The sloughing of airway epithelial cells is likely secondary to the increased levels of oxidative stress, with generation of free oxygen species by the infiltrating macrophages. In keeping with these findings, Kay and colleagues (24) showed that increased oxidative stress enhances the loss of cilia in a rat tracheal culture system. Finally, cytokines such as IL-8 (1) and IL-13 (31) can directly inhibit the beat frequency of ciliated respiratory epithelial cells, a mechanism that can similarly be proposed for IL-12. Thus the combination of acellular and cellular debris and altered cytokine milieu in IL-12 transgenic airways contributed to delay the mucociliary clearance of the tracheobronchial tree, a finding also present in patients affected by Sjögren syndrome (35).

An increase in TGF-β signaling in IL-12 transgenic lungs was reflected by increased expression of pSmad2, a cytoplasmic protein that becomes phosphorylated after ligation of the TGF-β receptor 1. During TGF-β signaling, pSmad2/Smad3 forms a complex with Smad4 that translocates into the nucleus and increases transcription of TGF-β-responsive genes (34). TGF-β is considered an immunosuppressive cytokine, involved in lymphocyte homeostasis and self-tolerance (6). In the absence of TGF-β, mice develop multiorgan inflammation/autoimmunity and die ~20 days after birth because of organ failure (49). Removal of B cells, CD4+ T cells, or CD8+ T cells alone does not improve longevity or inflammation, whereas removal of all T cells does improve both (7). Induction of TGF-β in our autoimmune-type IL-12 transgenic model may have occurred as a compensatory mechanism, in response to the increased inflammation and oxidative stress, as a mechanism to suppress the autoimmune phenotype. Supporting this notion is a study that found that lupus-prone MRL-lpr/lpr mice had decreased inflammation, decreased total IgG, and increased survival when given monthly intramuscular injections of a TGF-β cDNA expression vector (46). Another study using mice that develop Sjögren-like salivary gland infiltrates found that calorie restriction in the older mice decreased salivary gland inflammation and that this correlated with an increase in TGF-β and a decrease in inflammatory cytokines (9). Alternatively, increased in pSmad2 in our model may reflect the increased inflammatory state of the IL-12 transgenic lung. In fact, the lungs of these mice have an increased number of infiltrating macrophages, which are known to produce TGF-β. The increased TGF-β signaling under conditions of inflammation can induce extracellular matrix protein production and lead to impaired healing and fibrosis (8).

The derangement of the normal lung NK cell population in this mouse model may provide additional insights into the pathogenic mechanisms underlying Sjögren syndrome. NK cells are a subset of lymphocytes characterized morphologically by large cytoplasmic granules and genetically by the lack of rearrangement in the gene segments forming B-cell or T-cell receptors. NK cells have been typically associated with innate immune responses, controlling intracellular pathogens (viruses, bacteria, and parasites) and tumors (21), but more recently they have also been implicated in adaptive immune responses (14). NK cells can in fact kill dendritic cells, limiting their antigen presentation capacity and overt immune reactions (16, 44).

In our model, NK cells showed a bimodal kinetics. They first significantly increased during the first month of life; then, between days 30 and 45, they dropped precipitously, remaining significantly lower than controls thereafter. Interestingly, the decline in NK cells preceded the appearance of the lung histological and functional lesions, suggesting a possible causal relationship. The initial increase in NK cells is in line with the well-established effects of IL-12. IL-12 was in fact first identified as a factor capable of enhancing NK cell activity and named “NK cell stimulatory factor” (28). The subsequent decrease in NK cells can also be explained as a direct effect of IL-12. It is known that prolonged exposure of lymphocytes (T, B, and NK) to IL-12 decreases their number and activity. For
example, prolonged IL-12 treatment decreases the number of NK cells in the lungs of mice infected with *Mycobacterium tuberculosis* (40). Other factors, such as the observed increase in TGF-β signaling, may contribute to the decline in NK cells. TGF-β is a potent inhibitor of NK cell functions, capable of attenuating both NK cytolytic activity and IFN-γ production (47).

Little is known about NK cells in Sjögren syndrome, but most studies have shown that in peripheral blood of Sjögren patients the number and/or activity of NK cells are decreased. Minato et al. (36) studied 13 patients with primary Sjögren syndrome and found a significantly depressed NK activity against measles-infected HeLa cells. Similar findings were reported by Miyasaka et al. (37) in 27 patients, by Pedersen et al. (43) in 21 patients, and by Struyf et al. (50) in 16 patients. Our mouse model provides evidence that a decrease in NK cells is associated with a Sjögren phenotype. In addition, the model shows that disease pathogenesis is initiated by prolonged exposure to increased IL-12 concentrations.

Together, these data suggest that IL-12 is necessary for the development of autoimmune pathology in specific organs. These organs include the lungs and salivary and lacrimal glands, organs that are specifically targeted in Sjögren disease. Our mouse model of IL-12 overexpression is novel and is the first to directly link overproduction of the Th1 cytokine IL-12 with autoimmune changes in the lung. This IL-12 transgenic model is also important in that it demonstrates several histological and functional characteristics that are found in Sjögren lung disease. Furthermore, the lung phenotype in this model progresses with age, which could allow for future testing of early interventions that might be useful in preventing, or halting, the progression of Sjögren disease.

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