Experimental silicosis: a shift to a preferential IFN-γ-based Th1 response in thoracic lymph nodes

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Garn, Holger, Anke Friedetzky, Andrea Kirchner, Ruth Jäger, and Diethard Gemsa. Experimental silicosis: a shift to a preferential IFN-γ-based Th1 response in thoracic lymph nodes. Am J Physiol Lung Cell Mol Physiol 278: L1221–L1230, 2000.—In chronic silicosis, mechanisms leading to lymphocyte activation are still poorly understood, although it is well known that not only the lung but also the draining lymph nodes are affected. In the present study, we investigated T-cell activation by analysis of cytokine expression in the enlarged thoracic lymph nodes of rats 2 mo after an 8-day silica aerosol exposure. In the case of helper T cell (Th) type 1 cytokines, we found a significant increase in interferon (IFN)-γ mRNA expression, whereas interleukin (IL)-2 expression remained unchanged. In contrast, gene transcription for the Th2-type cytokines IL-4 and IL-10 was diminished. In addition, with use of an in vitro lymphocyte-macrophage coculture system, an enhanced IFN-γ and a reduced IL-10 release were shown with cells from silicotic animals. With regard to IFN-γ-inducing cytokines, we observed enhanced IL-12 mRNA levels in vivo, whereas IL-18 gene expression was slightly decreased. These data indicate that a persistent shift toward an IFN-γ-dominated type 1 (Th1/cytotoxic T cell type 1) T-cell reaction pattern occurred within the thoracic lymph nodes of silicotic animals. Thus a mutual activation of lymphocytes and macrophages may maintain the chronic inflammatory changes that characterize silicosis.

interferon-γ; chronic lung inflammation; particles; T lymphocytes; cytokines; interleukins; helper T cell type 1

SILICOSIS IS A CHRONIC FIBROTIC DISORDER of the pulmonary parenchyma caused by prolonged inhalation of crystalline particles (43). Whereas the contribution of macrophages to the pathogenesis of silicosis has been extensively studied over the last years (5), only little is known about the putative role that lymphocytes may play in the initiation and maintenance of the disease. However, clear evidence has accumulated indicating an involvement of lymphocytes in the progression of silicosis. Using an immunohistological examination of the lung, Kumar (23) observed a significant increase in total lymphocyte number after intratracheal instillation of silica particles. Kinetic analyses revealed that the influx of T cells preceded the occurrence of B lymphocytes. Furthermore, an increased number of lymphocytes expressing the α-chain of the interleukin (IL)-2 receptor (CD25) was reported (24). These results indicate the presence of an augmented proportion of activated lymphocytes in the total lymphocyte population of the lung.

It is known that inhaled silica particles not only affect the lung but also other organs, most importantly, the mediastinal lymph nodes and, to a lesser extent liver, spleen, kidneys, and thymus (43). It has been proposed that particle-laden macrophages leave the lung and enter the draining lymph nodes via lymphatic capillaries. Indeed, in a rat inhalation model of silicosis, Absher et al. (1) demonstrated the occurrence of silica particles within mediastinal lymph nodes, and Friedetzky et al. (13) showed that those particles were located within macrophages. Thus the appearance of silica-stimulated macrophages may represent the initial signal for the dramatic changes that subsequently occur within the draining lymph nodes. Friedetzky et al. (13) observed recently that, concomitant with the alterations in the lung, the affected thoracic lymph nodes increased tremendously in size and dramatic histological changes took place. One year after exposure, thoracic lymph nodes of silicotic animals displayed a 35-fold weight increase compared with the corresponding lymph nodes of control animals. Histologically, the normal lymph node structure had disappeared, and T- and B-cell areas including germinal centers were no longer detectable. Instead, granuloma-like structures consisting almost exclusively of macrophages, a portion of which contained silica particles, became the most prominent feature within these lymph nodes.

The lymph node enlargement was due to an increase in cell number rather than an augmentation of extracellular connective tissue matrix (14). In accordance with the situation in the lung, the increase in T cells, especially of the CD4↑ phenotype, marginally preceded B-cell infiltration (13). Finally, all major lymph node cell populations contributed evenly to the enhancement of lymph node cell number, and no significant alterations between the relative portions of the different cell types were observed. Moreover, by cytofluorometric analysis with antibodies to the cell surface activation markers CD25 (IL-2 receptor α-chain) and CD54 (intercellular adhesion molecule-1) of lymph node cells obtained from animals 12 mo after silica exposure, we demonstrated an elevation in the relative number of activated T lymphocytes, especially the CD8↑ phenotype, and, to a lesser extent, CD4↑ T cells. In addition,
increased interferon (IFN)-γ mRNA expression was found at this late time point, whereas IL-2 and IL-4 mRNA levels remained unaltered (14).

Because these previous data represent the final stage of silicosis, the present study was designed to examine possible T-cell activation at an early time point (2 mo after silica exposure) by analyzing the cytokine gene expression and protein release of cells from affected lymph nodes compared with those from unaffected lymph nodes from the same animal or from healthy control animals. The aim was to examine whether a shift to either a helper T cell (Th) type 1-like or a Th2-like profile occurred that could further delineate the contribution of T cells to the development of the chronic state of silicosis.

**MATERIALS AND METHODS**

Animals. Silica exposure was carried out at the Votey Animal Inhalation Facility of the University of Vermont (Burlington, VT) with male Fischer 344 rats obtained from the National Cancer Institute (Bethesda, MD). The rats weighed ~165 g at this time. Subsequently, the animals were shipped to Germany by aircraft and housed at the animal facilities of the Institute of Immunology (University of Marburg, Marburg, Germany) where they were maintained in wire cages at a temperature of 18-22°C in a 12:12-h light-dark cycle. Food and water were given ad libitum. The animals appeared healthy during the time of the experiments and gained weight normally. Housing conditions and mineral exposure met National Institutes of Health and German guidelines.

Silica exposure. The animals were exposed in horizontal flow chambers for 6 h/day for 8 days to aerosols of α-cristobalite (C & E Mineral, King of Prussia, PA) as previously described in detail (17, 18, 28). Matched groups of rats were simultaneously exposed to the silica aerosol or to the carrier air (sham control). The aerosol was generated with a Wright dust feed apparatus and contained particles of respirable size; ~77% of the particles were <3.3 µm and 55% were <2.0 µm. The mean air concentration of silica particles was 9.28 mg/m³. Two months after exposure, the animals were killed by CO₂ intoxication. At this time point, the lungs of silica-exposed animals showed moderate changes typical of chronic silicosis in rodents (43). Lung and lymph node histology and characterization of lymph node cell populations have been previously described in detail (13). Normal histology was observed in the lungs and lymph nodes of control animals.

RNA preparation. Thoracic and cervical lymph node tissues of six control and six silica-exposed animals were shock-frozen in liquid nitrogen immediately after removal. Frozen tissues were homogenized with nitrogen-chilled pestles and mortars, and total RNA was prepared by use of the RNA clean preparation kit (Angewandte Gentechnologie Systeme, Heidelberg, Germany) according to the manufacturer’s guidelines. RNA yield was determined spectrophotometrically at 260 nm (40).

RT-PCR. First, mRNA from 2 µg of total RNA from each sample was reverse transcribed into cDNA by use of an oligo(dT)₃₀ primer (MWG Biotech, Ebersberg, Germany) and SuperScript II RT (Life Technologies, Gaithersburg, MD) as previously described (14).

For each of the first-strand cDNAs, the following PCRs were set up with specific primers for the sequences of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-γ, IL-2, IL-4, IL-10, IL-12 p40, and IL-18. Primer sequences were established with rat-specific gene sequences obtained from European Molecular Biological Laboratories gene bank (GAPDH, IL-12 p40, and IL-18) or kindly provided by Dr. A. Siegling (Institute of Immunology Charité, Berlin, Germany) (42) and were synthesized by MWG Biotech. Primer data are summarized in Table 1. PCRs were performed as heat-soaked PCRs (39): 1 µl of cDNA, 5 µl of 10× PCR buffer (Perkin Elmer, Germany), and 40.8 µl of double-distilled water were mixed in a 0.5-ml cup. After 20 min of incubation at 94°C (hot soak), 3.2 µl of a mix consisting of 1 µl of each sense and antisense primer (50 pmol/µl), 1 µl of deoxynucleotide triphosphates (40 mM), and 0.2 µl (1 U) of AmpliTaq DNA polymerase (Perkin-Elmer) were applied. The samples were subjected to 25–35 cycles (see Table 1) of denaturation (1 min at 94°C), 40 s of annealing (58-65°C; see Table 1), and extension at 72°C for 1 min with a TouchDown thermal cycler (Hybaid, Teddington, UK). PCR conditions were optimized for each primer pair to ensure that all products were obtained from the linear phase of the PCR.

PCR products were separated by gel electrophoresis with 1.2% agarose gels (Life Technologies) with 1× Tris-acetate-EDTA as the running buffer (40) and were subsequently visualized by ethidium bromide staining. The gels were analyzed with a gel documentation and quantitation system (Vilbert-Lourmat, Marne la Vallée, France). Semiquantitative gel analysis was performed densitometrically with use of the system software Bio1D version 6.11d. Results are ex-

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GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL, interleukin.
pressed as arbitrary units that were calculated by integration of the intensity for each pixel over the spot area.

Northern blotting. Due to the presumably low copy numbers of the genes of interest within the total lymph node RNA, it was necessary to pool equal amounts of RNA from identical lymph node from three comparable animals to obtain sufficient signals. For each sample, 10 µg of total RNA (2 µg for GAPDH Northern blot) were denatured with a formamide-containing sample buffer and subsequently separated electrophoretically on 1% agarose-formaldehyde gels with a running buffer consisting of 20 mM 4-(N-morpholinol)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0 (40). RNAs were transferred to positively charged Biodyne Plus membranes (Pall Biosupport Division, Port Washington, NY) by capillary blotting with 10× saline-saline citrate as a blotting buffer. After drying and ultraviolet cross-linking, the membranes were hybridized with digoxigenin (Dig)-labeled antisense riboprobes in a solution containing 50% formamide at 68°C under continuous rotation in a hybridization oven (Biometra, Göttingen, Germany) as previously described (44). Thereafter, the membranes were carefully washed in 0.1× saline-saline citrate-0.1% sodium dodecyl sulfate at the same temperature, and the bound probes were visualized with the Dig nucleic acid detection kit (Boehringer Mannheim) and CDP-Star chemiluminescence substrate (Tropix, Bedford, MA). Semiquantitative analysis was performed as described in RT-PCR.

Generation of Dig-labeled riboprobes. Antisense riboprobes corresponding to rat GAPDH, IFN-γ, IL-2, IL-4, and IL-10 were generated by RT-PCR, cloning of the respective PCR products, and subsequent in vitro transcription. Briefly, 1 µg of total RNA from concanavalin A (Con A)-stimulated rat spleen cells was reverse transcribed into cDNA followed by PCR with the PCR conditions mentioned in RT-PCR to amplify the respective sequences. PCR products were cloned into the TA cloning site of the pCR II vector with the use of a TA cloning kit (Invitrogen, Leek, The Netherlands). Specificity and cloning direction were confirmed by sequencing. Dig-labeled RNA probes were generated with SP6 or T7 RNA polymerase in a Dig-RNA labeling kit (Boehringer Mannheim) with 1 µg of linearized vector as a template.

Preparation of lymphocyte suspensions. Thoracic, cervical, and mesenteric lymph nodes were removed aseptically and immediately transferred into cold (4°C) phosphate-buffered saline (PBS) without Ca2+ and Mg2+. The lymph nodes were dissected and passed through a 70-µm nylon cell strainer (Falcon, Becton Dickinson Labware) with a syringe plunger. The enlarged thoracic lymph nodes of silicotic rats delivered ample cells for study purposes. Thoracic lymph nodes of sham-exposed rats were too small to yield sufficient control cells. Therefore, the lymphocytes from the cervical and mesenteric lymph nodes of individual animals were pooled and served as control cells. The resulting cell suspensions were centrifuged at 300 g for 10 min at 4°C. The cell pellets were resuspended in culture medium (RPMI 1640 medium supplemented with 2 mM l-glutamate, 10 mM HEPES, 1 mM sodium pyruvate, 1× nonessential amino acids, 100 µM of penicillin, and 100 µg/ml of streptomycin; all purchased from Biochrom, Berlin, Germany) without fetal calf serum (FCS), filtered through a 70-µm nylon mesh to remove aggregates, and subsequently washed twice with the above-mentioned centrifugation conditions. Thereafter, the lymph node cells were resuspended in culture medium with 20% FCS and adjusted to a cell concentration of 2.5 × 10^6 cells/ml.

To obtain alveolar macrophages, the lungs of animals were lavaged with 100 ml of prewarmed (37°C) PBS without Ca2+ and Mg2+. The resulting cell suspensions were washed twice with PBS. Subsequently, mononuclear cells were prepared by density gradient centrifugation with Lympholyte-Rat (Cedarlane, Hornby, ON) followed by two washing steps, the first in PBS and the second in culture medium without FCS. After resuspension in FCS-free culture medium, the cells were adjusted to 2.5 × 10^6 cells/ml. Purity of the alveolar macrophages was >98% and as shown in a previous study (28), ~30% of alveolar macrophages from silicotic animals contained detectable silica particles.

One hundred microliters of the alveolar macrophage suspensions were added to each well of 96-well cell culture plates (Corning Costar, Cambridge, MA) and incubated at 37°C in a humid atmosphere containing 5% CO2. Two hours later, 100 µl of the different lymph node cell suspensions were added, resulting in a macrophage-to-lymphocyte ratio of 1:10. For the control cells, similar numbers of lymphocytes and macrophages were cultured separately. Finally, 1 µg/ml of indomethacin (Sigma-Aldrich, Desehnten, Germany) to block production of suppressive prostaglandin E2 (15) and 2.5 µg/ml of Con A (Sigma-Aldrich) dissolved in culture medium with 10% FCS were added, yielding a total volume of 25 µl/well. The final FCS concentration for all cultures was 10%. Cultures were incubated under the above-mentioned conditions, and 24 or 48 h later, the culture supernatants were removed and stored at −80°C until cytokine analysis.

Cytokine quantitation by ELISA. Culture supernatants were tested for cytokine content with ELISAs specific for rat IFN-γ, IL-2, IL-4, and IL-10. Matched antibody pairs with a biotinylated monoclonal detection antibody and recombinant standard proteins were purchased from either Biosource (IFN-γ; Camarillo, CA) or PharMingen (IL-2, IL-4, and IL-10; San Diego, CA). Sandwich ELISAs were set up according to standard procedures. Briefly, Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with 50 µl/well of the relevant capture antibody diluted to a predetermined concentration and incubated overnight at 4°C. All subsequent steps were performed at room temperature. After three washes with PBS-5% Tween 20, the nonspecific binding sites were blocked by the addition of 250 µl of blocking buffer (1% bovine serum albumin in PBS) to each well and 20 min of incubation. The plates were washed three times, and 25 µl of sample buffer (0.5% Tween 20 in blocking buffer) were added followed by 25 µl of sample or standard (diluted in culture medium with 10% FCS). Four hours later, the plates were washed three times, and 50 µl of the appropriately diluted biotinylated detection antibody were added per well. After incubation for 1 h and 4 washing steps, 100 µl of horseradish peroxidase conjugated streptavidin/well (1:10,000 diluted in sample buffer; Boehringer Mannheim) were applied and incubated for 30 min. After four washes, 100 µl of substrate solution, 1 mg/ml of o-phenylenediamine (Sigma-Aldrich) in substrate buffer (0.05 M phosphate-citrate buffer, pH 5.0 with 0.5 µl/ml of 30% H2O2), were applied. The reaction was stopped 15–30 min later by the addition of 25 µl of 2.5% sulfuric acid. Absorption was read with microplate reader MR7000 (DynaTech Laboratories, Denkendorf, Germany) at 570 nm with a 630-nm reference filter.

Statistics. All data were calculated and are expressed as means ± SD. After analysis of the sample values for Gaussian distribution, determination of significance was carried out with Student’s t-test or the paired t-test.

RESULTS

Th1/Th2 cytokine gene expression in lymph nodes of silicotic animals. As previously shown in rats 12 mo after silica exposure, the enormous enlargement of two
lung-draining lymph nodes coincided with an apparent T-lymphocyte stimulation (14). The present investigation was designed to examine these lymph nodes earlier, 2 mo after silica exposure, and to study in detail Th1 and Th2 cytokine gene expression pattern by RT-PCR and Northern blot analysis.

In the first set of experiments, we examined the specific cytokine mRNA content by semiquantitative RT-PCR, which was the only available method that allowed the individual detection of mRNA expression in the particularly small thoracic lymph nodes of sham-exposed control rats. We compared cytokine gene transcription in the thoracic and cervical lymph nodes of six silicotic and six control animals. As shown in Fig. 1, a significantly higher mRNA expression for the Th1 cytokine IFN-γ was found in the thoracic lymph nodes of silicotic animals compared with that in the cervical lymph nodes of silicotic animals or both types of lymph nodes of control rats (Fig. 1A). Interestingly, the level of gene transcription of IL-2, the second important Th1/cytotoxic T cell (Tc) type 1-derived cytokine, remained unaltered (Fig. 1B). In contrast, the mRNAs for IL-4 and IL-10, which are dominantly expressed by Th2/Tc2 cells, were found to be significantly less expressed in the thoracic lymph nodes of silicotic animals (Fig. 1, C and D).

In the second series of experiments, Northern blot analyses were performed to confirm the semiquantitative RT-PCR data on cytokine gene transcription. Due to the limited availability of total RNA from the lymph nodes of control animals and the weak expression of some genes of interest, we had to pool RNA from the lymph nodes of three individual animals to obtain detectable signals. When comparing cytokine gene expression in the enlarged thoracic lymph nodes of silicotic animals with that in pooled cervical and mesenteric lymph nodes from either silicotic or healthy rats, we could entirely confirm the results of the RT-PCR experiments. We found increased mRNA levels for IFN-γ, no changes for IL-2, and decreased levels of IL-10 (Fig. 2, A–C). IL-4 mRNA was not detectable, even with 10 µg total RNA/lane (data not shown). This is in accordance with the RT-PCR results, where 35 PCR cycles were necessary to obtain just-visible bands of IL-4-specific PCR products. Thus cytokine mRNA results obtained by RT-PCR corresponded well with Northern blot data.

Cytokine release from in vitro stimulated cells. To establish that enhanced cytokine gene expression resulted in increased production and release of cytokines, we set up in vitro cultures with cells from enlarged

Fig. 1. Expression of interferon (IFN)–γ (A), interleukin (IL)-2 (B), IL-4 (C), IL-10 (D), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; E) in total lymph node RNAs obtained from thoracic and cervical lymph nodes of silica-exposed and control rats. Bottom: gene-specific PCR products in ethidium bromide-stained agarose gels of 6 individual animals/group. Top: corresponding densitometric analyses. Values are means ± SE. *Significant difference from all other groups, \( P < 0.01 \).

A

B

C

D

E
trophic lymph nodes from silicotic animals and compared them with pooled cells from cervical and mesenteric lymph nodes. The lymphocytes were cultured alone or in the presence of alveolar macrophages at a 10:1 ratio with and without the addition of Con A as a mitogenic stimulus. Lymphocytes and alveolar macrophages were obtained from silicotic and control rats, and various combinations were tested with Con A at an optimal (2.5 µg/ml) concentration.

When the lymphocytes and macrophages were cultured alone or in combination for 24 or 48 h in the absence of Con A, no measurable amount of cytokines was released into the culture medium (data not shown). The addition of Con A to the lymph node cells induced the release of moderate amounts of IFN-γ, IL-2, and IL-10 (Figs. 3–5, respectively). Even under those conditions, it became apparent that IFN-γ secretion from the lymphocytes of thoracic silicotic lymph nodes was higher and IL-10 production was lower than that from the other two types of lymph node preparations, whereas IL-2 release remained unchanged.

This pattern of cytokine release was clearly enhanced and, with respect to individual cytokines, accen-
tuated when alveolar macrophages were added to the lymphocyte cultures. In general, alveolar macrophages from silicotic rats were more potent in assisting cytokine release from lymphocytes of all different lymph node preparations. This enhancing effect was most pronounced when alveolar macrophages as well as lymphocytes from the thoracic lymph nodes of silicotic rats were combined (Figs. 3A, 4A and 5A). The consistent finding was always that lymphocytes from silicotic thoracic lymph nodes released higher amounts of IFN-γ (Fig. 3A) than lymphocytes from nonaffected cervical and mesenteric lymph nodes of silicotic or sham-exposed rats (Fig. 3, B and C). In accordance with the results of the experiments on mRNA expression, there was also no significant difference in IL-2 production between the three lymph node cell populations (Fig. 4). When IFN-γ release was assayed at 48 h of incubation, the marked differences between the groups disappeared with a Con A dose of 2.5 µg/ml but were still present with a Con A dose of 0.5 µg/ml (data not shown).

Just the opposite to the release of IFN-γ was found for IL-10, which became detectable in 48-h but not in 24-h culture supernatants and which showed a markedly suppressed release of lymphocytes from silicotic thoracic lymph nodes (Fig. 5A). It was also noted that the addition of macrophages only moderately enhanced IL-10 secretion from silicotic lymph node lymphocytes compared with the other two types of lymphocyte preparations (Fig. 5, B and C). The other important Th2 cytokine, IL-4, could not be detected in the culture supernatants either at 24 or 48 h after in vitro stimulation of the lymph node cells.

In conclusion, it became apparent that lymphocytes from the enlarged silicotic thoracic lymph nodes produced higher levels of IFN-γ, whereas IL-10 release

![Fig. 4. Release of IL-2 into the culture supernatants from primary cultures of lymphocytes derived from thoracic lymph nodes of silicotic rats (A) and control (cervical plus mesenteric) lymph nodes of silica-exposed (B) and control (C) rats. Lymphocytes (1 × 10^6 cells/ml) were cultured alone or together with alveolar macrophages (10:1) in presence of 2.5 µg/ml of Con A and 1 µg/ml of indomethacin for 24 h. IL-2 was quantitated by ELISA. Values are means ± SD of 1 of 4 experiments, each performed in triplicate. All differences are significant at P < 0.05.](image1)

![Fig. 5. Release of IL-10 in culture supernatants from primary cultures of lymphocytes derived from thoracic lymph nodes of silicotic rats (A) and control (cervical plus mesenteric) lymph nodes of silica-exposed (B) and control (C) rats. Lymphocytes (1 × 10^6 cells/ml) were cultured alone or together with alveolar macrophages (10:1) in presence of 2.5 µg/ml of Con A and 1 µg/ml of indomethacin for 48 h. IL-10 was quantitated by ELISA. Values are means ± SD of 1 of 4 experiments, each performed in triplicate. All differences are significant at P < 0.05 except those marked not significant (ns).](image2)
was significantly reduced. This central finding is summarized in Fig. 6.

IL-12 and IL-18 gene expression in lymph nodes of silicotic animals. To investigate possible mechanisms for the specific increase in IFN-γ gene expression and subsequent protein release within the enlarged thoracic lymph nodes of silicotic rats, we examined the level of gene transcription for the two major IFN-γ-inducing cytokines IL-12 and IL-18 by semiquantitative RT-PCR (Fig. 7). Interestingly, the gene expressions of these two cytokines were regulated in quite opposite directions. Whereas for IL-12, a significant increase in mRNA amounts could be detected (Fig. 7A), IL-18 expression seemed to be less pronounced within the silicotic thoracic lymph nodes compared with that in control lymph nodes (Fig. 7B). Unfortunately, no antibodies against these rat cytokines are as yet available to detect actual IL-12 and IL-18 release.

DISCUSSION

Although it is known from patients as well as from experimental animal data that in silicosis the lung-draining thoracic lymph nodes are also affected, the underlying mechanisms and the possible involvement of lymphocytes in the pathogenesis of the disease are still poorly understood. At present, the available data remain controversial, which indicates that an in-depth investigation of the role of lymphocytes in silicosis and related lung diseases is urgently required. Kumar et al. (24) observed an activation of lymphocytes in the pulmonary response to silica, and Li et al. (26) supposed that lymphocytes contribute to the regulation of macrophage activities. This concept was further supported by data from Suzuki et al. (46), who showed that athymic mice developed less severe silica-induced interstitial pneumonitis and inflammatory responses than euthymic mice. In contrast, in the rather artificial model of quartz injection into the footpads and subsequent analysis of the popliteal lymph node reaction, Weirich et al. (50) ignored a possible participation of T lymphocytes in the development of silicotic nodules within the lymph nodes. Also, Corsini et al. (3) described a protective role for T lymphocytes in asbestos-induced pulmonary inflammation and collagen deposition in mice.

Garn et al. (14) recently demonstrated that the enlargement of thoracic lymph nodes after silica exposure was mainly caused by an influx of mononuclear cells. Initial lymph node changes could be observed as early as 2 wk after particle exposure (13). Even 1 yr after silica exposure, an increased number of activated T lymphocytes could be detected with flow cytometric analysis by the expression of surface activation markers (14). The present study was designed to examine in detail the cytokine profile within the enlarged thoracic lymph nodes 8 wk after silica exposure. Our main interest was focused on the expression of cytokines that may drive an immune response in either the Th1 or a Th2 direction.

The most important finding of the present study was the differential regulation of IFN-γ and IL-10 within the affected thoracic lymph nodes of silicotic animals. In the case of IFN-γ, an increase in gene expression could be demonstrated by Northern blot analysis and semiquantitative RT-PCR (Figs. 1 and 2). In addition, further evidence for an important role for IFN-γ was obtained by in vitro stimulation experiments. Lymph node cells from the enlarged silicotic lymph nodes released significantly higher amounts of IFN-γ into the culture medium compared with similar cells obtained from unaffected lymph nodes from either control or silica-exposed animals (Fig. 3). Opposite results were observed for IL-10 and IL-4, which showed reduced mRNA amounts (Figs. 1 and 2), and in the case of IL-10, a decrease in protein release after in vitro stimulation could be demonstrated (Fig. 4). These data indicate that in silicosis those lymphocytes that produce the Th1 cytokine IFN-γ were preferentially stimulated, whereas the number or activity of Th2 cytokine-
producing cells seemed to be diminished. IFN-γ itself is known to efficiently suppress a Th2/Tc2 response by inhibiting the transcription and release of IL-4 and IL-10 (2, 29, 32, 35), the first of which is considered to be necessary for the onset of a Th2 response (25, 49). Thus IFN-γ may be responsible for the downregulation of IL-4 and IL-10.

Because of its inhibitory effects on inflammatory responses, the decreased expression of IL-10 is of special interest. IL-10 is known to inhibit several functions of macrophages such as synthesis of proinflammatory cytokines, release of oxygen radicals and nitric oxide, and antigen-presenting capacity (4, 9). In addition, IL-10 may reduce the production of Th1/Th2-derived cytokines (12, 29) but, on the other hand, may stimulate B-cell proliferation (8, 38). Thus IL-10 is clearly involved in the shift of an immune reaction toward a Th2/Tc2 response. Using a quartz instillation model in mice, Huaux et al. (20) showed increased levels of IL-10 mRNA and protein within the lung tissue and in the cells of bronchoalveolar lavage fluid 24 h after instillation of particles. These data indicate a regulatory role for IL-10 in the early inflammatory response that was further confirmed by Driscoll et al. (10), who demonstrated an attenuating effect of exogenously applied IL-10, whereas the application of an anti-IL-10 antisemum increased the inflammatory reaction. Beneficial effects of IL-10 have also been described in other inflammatory lung diseases such as adult respiratory distress syndrome (27) and acute lung injury caused by acute necrotizing pancreatitis (34). Compared with the results by Huaux et al. (20) and Driscoll et al. (10), our data clearly indicate a differential expression of IL-10 in the immediate inflammatory response in the lung and the chronic state of the disease when studied in the lung-draining lymph nodes. However, it cannot be excluded that a principally different IL-10 expression pattern may occur in different tissue compartments and at different time periods during the development of silicosis. On the basis of our results, we postulate that the decreased expression of IL-10 at later time points after silica exposure is a crucial point for the development of the chronic state of silicosis. Whether differences in IL-10 expression in the lung and lymph nodes are caused by a differential regulation in a distinctive cell population or are rather due to an involvement of different cells (Th2/Tc2 lymphocytes, macrophages, epithelial cells) has to be investigated in the future. The extremely weak mRNA expression for IL-4 and, subsequently, the lack of IL-4 in culture supernatants of in vitro stimulated cells may be due to the animal model employed here because our data are in agreement with results by Odinot et al. (31), who observed in an infection model almost no IL-4 expression in spleen and lymph nodes when using Fischer 344 rats compared with Lewis or Norway rats.

The fact that no changes in IL-2 gene transcription or protein release after in vitro stimulation of the lymph node cells from silicotic lymph nodes were detected raises the question of which cell populations were responsible for the increased IFN-γ release. Generally, IFN-γ may be produced by CD4+ Th1-lymphocytes, CD8+ Tc1 lymphocytes, and natural killer cells (11). Because these cell types may also produce IL-2 (30), special signaling mechanisms inducing a selective IFN-γ gene expression have to be proposed. To further address this question, we examined mRNA expression for the cytokines IL-12 and IL-18, both of which are strong inducers of IFN-γ (33). Interestingly, we found increased levels of IL-12 mRNA, whereas IL-18 gene transcription was even slightly diminished (Fig. 7).

IL-12 is known to preferentially regulate the proliferation of Th1 T-cell clones (22, 47), which subsequently produce IFN-γ. In contrast, IL-18 acts mainly as an amplifier of IFN-γ production, for example, in IL-12-activated Th1 lymphocytes (33). Thus an increase in IL-12 release by activated macrophages within the affected thoracic lymph nodes could be responsible for activation of IFN-γ-producing Th1 cells.

In addition, the stimulatory role of alveolar macrophages for the activation of T lymphocytes is of particular interest. In contrast to previous findings (37) describing an inhibitory influence of alveolar macrophages on T-cell functions (19), our results show significant stimu-
lating effects of alveolar macrophages, independent of whether they are derived from silicotic or normal animals, at least in the release of the cytokines IFN-γ, IL-2, and IL-10 by mitogen-stimulated T lymphocytes (Figs. 3–5). One reason for this discrepancy might be that we introduced indomethacin into the cell cultures to abolish the inhibitory effects caused by the release of prostaglandin E₂ by macrophages (15, 41). Because alveolar macrophages from silicotic animals are more potent in aiding cytokine production by lymphocytes, it seems likely that activated macrophages exert their stimulatory effects by the release of cytokines. However, we could not detect any IL-1 in the culture supernatants (data not shown), which indicates a minor role for proinflammatory cytokines at this stage of the disease. Instead, regulatory cytokines such as IL-12 may be responsible candidates for the apparent lymphocyte stimulation. In addition to cytokine-mediated mechanisms, specific physical cell-cell interactions, i.e., by costimulatory cell surface molecules and/or adhesion molecules, have to be taken into account. In vivo, macrophage properties may change when activated lung macrophages leave the lung and migrate to the lymph nodes. Because alveolar macrophages have been described to have poor accessory activity (36) and suppressive properties with respect to T-cell activation (45), it is feasible that they will become more activated when they leave the lung and enter the lymph nodes. Additional experiments are in progress to further analyze the properties of lymph node macrophages and to investigate the mechanisms of in situ and in vitro macrophage-lymphocyte interactions.

In conclusion, activated alveolar macrophages appear to play an important role in the initiation of pathological changes within the lung as well as in the lung-draining lymph nodes during silicosis. Activated macrophages enter these thoracic lymph nodes (16) where they most likely contribute to the observed lymphocyte stimulation. The initiating mechanisms of lymphocyte activation still remain unresolved. An antigenic structure has not been described on silica particles, which suggests that other mechanisms such as oligodendral or polycytoplasmic T-cell activation by macrophage-derived cytokines, e.g., IL-12, may have taken place (48). Once activated, T lymphocytes produce IFN-γ, which leads to an amplifying mechanism driven by the mutual stimulation of macrophages and lymphocytes within the affected thoracic lymph nodes that has been initiated and, in an unknown way, maintained by silica particles. This continuous process may be responsible for the chronic changes within the lymph nodes of silicotic animals and patients. Furthermore, activated lymphocytes are able to leave the lymph nodes via the efferent lymph and may enter the lung. Indeed, increased lymphocyte numbers (23) with an augmented portion of activated (24) and IFN-γ-producing (6) lymphocytes have been described in the lungs of silicotic rodents. Within the lung, those lymphocytes may sustain the macrophage infiltrate (21) and contribute to macrophage activation via release of IFN-γ. Thus IFN-γ may be the postulated lymphocyte-derived cytokine stimulating the alveolar macrophage-secreted fibroblast mitogenic activity (26). This view is supported by most recent observations of Davis et al. (7), who showed significantly reduced lung fibrosis in IFN-γ knockout mice. The presence of IFN-γ-activated macrophages and macrophage-derived mediators appear to form the basis for continuous fibrotic and inflammatory processes and subsequent pathological changes in the lung.

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REFERENCES


