

CD4 T Lymphocyte-mediated Lung Disease

Steady State between Pathological and Tolerogenic Immune Reactions

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Although considerable evidence indicates a role for CD4⁺ T lymphocytes (T cells) in airway inflammation, little data exist regarding the mechanisms underlying the induction and regulation of CD4⁺ T cell reactivity to lung-specific antigens. To dissect the immunologic and molecular mechanisms of CD4⁺ T cell dysregulation, reactivity to a self-antigen expressed in the lung of mice bearing a major histocompatibility complex class-II-restricted T cell receptor specific for this antigen was studied. Transgenic mice developed a progressive interstitial pneumonitis characterized by massive lymphocytic and plasmacytic infiltration of interalveolar septa, a clinical picture closely resembling some of the interstitial lung diseases. Pulmonary inflammation reached a plateau state in older mice with prominent formation of lymphoid follicles but reduced interstitial infiltration. Extensive immunologic characterization of self-reactive CD4⁺ T cells isolated from the inflamed lung suggested the induction of regulatory T cells in the site of inflammation. Moreover, inflammation was accompanied by broad changes in the gene expression pattern toward a profile partially resembling that of activated, but strikingly, also that of regulatory CD4⁺ T cells. Together our data provide important insights into functional and molecular alterations being associated with the induction and/or regulation of T cell-mediated pulmonary inflammation.

Keywords: airway inflammation; mucosal self-antigen; peripheral tolerance; transgenic mice

A significant number of lung diseases are presumed to be T cell-mediated based in part on the observation of T cell accumulation in sites of disease activity. Several of these disorders are notably characterized by the preferential accumulation of CD8⁺ T cells in the alveolar space of the interstitium (1–3). Moreover, the participation of CD4⁺ T cells in interstitial lung disease (ILD) has been suggested. ILD represents a large class of heterogeneous disorders involving the lung parenchyma, i.e., the alveolar epithelium, the interstitial connective tissue, vasculature, and lymphatic tissue. Sarcoidosis, idiopathic interstitial pneumonias, autoimmune connective tissue diseases, and pulmonary hemorrhage syndromes represent some of the major categories of ILD. Sarcoidosis, for example, appears to be associated with an exaggerated cellular immune response to an unknown antigen, and CD4⁺ Th1 lymphocytes are important effectors of pulmonary injury in this disease (4). A different ratio of Th1/Th2 lympho-

cytes has been reported for the interstitial lung compartment and the bronchoalveolar space (5). Undoubtedly lymphocytes play a central role in sarcoidosis (6). However, the different aspects influencing the balance of lymphocyte immigration, local proliferation, and apoptosis, and thereby the numbers of lymphocytes in the lung under disease conditions, are only partially understood in interstitial lung disease (7). In addition to ILD, the involvement of T cells has also been suggested in other pulmonary disorders, such as chronic obstructive pulmonary disease and asthma (8, 9). In these, it is postulated that cigarette smoke or allergen-induced immune responses can, under certain conditions, progress to T cell-mediated autoimmune disease. However, the mechanisms regulating CD4⁺ T cell responses directed to lung specific proteins and the regulation of their effector activity are not well characterized. As lymphocyte reactions in the lung compartments are mostly not reflected by the situation of lymphocytes in the blood, the lung has to be studied in more detail (10).

To address the role of self-reactive CD4⁺ T cells in the progression and resolution of autoimmune-mediated inflammatory lung disease, and to characterize the cellular and molecular mechanisms involved in disease pathogenesis, we generated surfactant protein C (SPC)-hemagglutinin (HA) transgenic mice expressing influenza strain A/PR8/34 HA under control of the SPC promoter specifically in type II alveolar epithelial cells. To establish an autoimmune environment, these mice were crossed to mice expressing a transgenic T cell receptor specific for a major histocompatibility complex class II restricted HA-derived peptide. We show here that the concomitant presence of a lung-specific self-antigen and self-reactive CD4⁺ T cells is sufficient to initiate mucosal inflammation progressing to severe autoimmune interstitial pneumonia. We show that under these autoimmune conditions peripheral tolerance mechanisms are induced that prevent the uncontrolled progression of the disease. Moreover, by comprehensive gene expression profiling of autoreactive CD4⁺ T cells isolated from the inflamed lung, we identified for the first time a variety of genes differentially expressed in airway inflammation. Together, our new *in vivo* model might help to understand the requirements for, and consequences of, chronic T cell-mediated lung injury. Some of the results of these studies have been previously reported in the form of abstracts (11–13).

METHODS

Mice

BALB/c mice were obtained from Harlan (Borchen, Germany). T cell receptor (TCR)-HA transgenic (6.5) mice expressing a major histocompatibility complex class II-restricted TCR $\alpha\beta$ specific for peptide HA110–120 derived from A/PR8/34 influenza HA presented by the MHC class II molecule I-E^d have been described previously (14). SPC-HA transgenic mice were generated in our laboratory using a construct containing the SPC promoter to achieve lung specific expression. Additional detail about mice is provided in an online supplement. Transgene expression in various organs was studied by real-time (RT)-polymerase chain reaction (PCR).

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Quantitative RT-PCR was performed on selected tissues of three SPC-HA transgenic mice in an ABI PRISM cyclor (Applied Biosystems, Foster City, CA) using the SYBR Green PCR kit from Applied Biosystems. If not stated explicitly, 3–4-month-old mice were used for the experiments.

Histology

Lungs were perfused (additional data on the perfusion method are available in the online supplement), fixed with neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry for T and B lymphocytes and HA using paraffin-embedded sections was performed with biotinylated antibodies to CD3 (CD3–12; Serotec Ltd., Düsseldorf, Germany) and CD45R/B220 (RA3–6B2, BD Biosciences, Heidelberg, Germany) as well as fluorescein isothiocyanate (FITC)-conjugated H36–4–52 antibody (purified from hybridoma supernatants) in combination with a secondary biotinylated rabbit anti-FITC antibody and the avidin-biotin-complex method with diaminobenzidine as chromogen. All immunohistochemistry sections were counterstained with hematoxylin. Iron deposits in the tissues were detected using the Turnbull stain with nuclear fast red as counterstain. Histologic examinations were performed on the lungs of 22 SPC-HA/TCR-HA mice of different ages. Three SPC-HA transgenic mice were analyzed with immunohistochemistry for HA expression.

Isolation of Lymphocytes from the Lung

Perfused lungs were excised and finely minced on ice, followed by a 60–90 minute digestion at 37°C with collagenase/dispase (0.2 mg/ml of each) in RPMI medium with 5% fetal calf serum (FCS), in the presence of 25 µg/ml DNase. To improve tissue disintegration, lungs were pipetted every 5 minutes using a Pasteur pipet. Ethylenediaminetetraacetic acid was added to a final concentration of 5 mM, followed by an additional 5 minute incubation at 37°C. Cells were passed through a 70 µm cell strainer, washed, and lung lymphocytes were isolated by density centrifugation (for additional detail on the density centrifugation method see the online supplement).

Antibodies and Flow Cytometry

Monoclonal antibodies 6.5 (anti-TCR-HA) (14) and CD62L (Mel-14) (15) were purified from hybridoma supernatants by protein G affinity chromatography. All other antibodies are from BD Biosciences. Two- and three-color flow cytometry was performed on a FACSCalibur (BD Biosciences). For gene expression profiling, CD4⁺6.5⁺ T cells were sorted with a MoFlow cell sorter (Cytomation, Fort Collins, CO).

Adoptive Transfer

Splenocytes from TCR-HA mice were stimulated *in vitro* with 10 µg/ml HA110–120 peptide. After 5 days of culture, dead cells were removed by density gradient centrifugation and 7×10^6 HA-specific CD4⁺ T cells were injected intraperitoneally into either SPC-HA mice or nontransgenic littermates (n = 6). Alternatively, naive CD4⁺ T cells from the spleen of TCR-HA mice were isolated by negative selection by AutoMACS using the CD4⁺ T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), followed by intravenous injection of 10^6 – 10^7 antigen-specific CD4⁺ T cells into transgenic and control mice (n = 3). Four weeks after transfer, animals were killed; lungs were perfused with phosphate-buffered saline before excision, sectioning, and histologic analysis.

Proliferation Assay and Cytometric Bead Array

Lung lymphocytes (10^5) together with 5×10^5 irradiated BALB/c splenocytes were cultured in 96-well flat-bottom plates in the presence or absence of the HA110–120 peptide in a final volume of 200 µl of Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS. After 48–72 hours, culture supernatants were collected for cytokine measurement, and the remaining cells were pulsed with [³H] thymidine for the final 6–18 hours. Quantification of cytokines in culture supernatants was performed using the CBA kit (Becton Dickinson, Heidelberg, Germany) following the manufacturer's recommendations. Acquired data were analyzed using the Becton Dickinson Cytometric Bead Array software. Cytometric bead array and proliferation experiments were done with pooled cells from two mice. Stimulation was performed in triplicate, and thymidine incorporation and cytokine measurements were done

Figure 1. Targeted expression of hemagglutinin in the lung. (A) Construct used for the generation of surfactant protein C (SPC)–hemagglutinin (HA) transgenic mice. PolyA = polyadenylic acid (B) Real-time (RT)–polymerase chain reaction (PCR) to detect HA mRNA expression in different tissues of SPC-HA transgenic mice (He: heart; Lu: lung; Sp: spleen; Ki: kidney; Th: thymus; Sln: small intestine; Lln: large intestine; St: stomach; Li: liver). RPS9 housekeeping gene specific primer served as internal control. (C) Quantification of HA mRNA expression levels in selected tissues by RT-PCR. (D) Immunohistochemical detection of influenza HA antigen on lung sections taken from SPC-HA transgenic (upper panel) and wild type (lower panel) animals using DAB stain (brown) as chromogen and hematoxylin (blue) as nuclear counterstain. Bars = 30 µm. (E) Adoptive transfer of HA-specific CD4⁺ T cells into wild type (top panel) and SPC-HA transgenic mice (bottom panel). Hematoxylin and eosin (H&E) stain, bar = 100 µm.

in two independent experiments. One representative experiment from these experiments is shown.

DNA Microarray Hybridization and Analysis

The entire data set of this microarray experiment is in Minimum Information About a Microarray Experiment (MIAME) format and accessible online at www.gbf.de/array under Download, and then under Bruder *et al.*, 2004.

RESULTS

Lung-specific HA Expression

To characterize the immunologic reactivity to lung-specific antigen expression, we generated transgenic mice expressing A/PR8/34 HA under control of the SPC promoter (Figure 1A). The HA expression pattern of SPC-HA transgenic lines was evaluated by RT-PCR on total RNA isolated from various organs. We found high pulmonary HA mRNA expression, relatively lower expression levels in the thymus, but essentially no expression in other organs (Figures 1B and 1C). Immunohistochemical staining of lung sections of transgenic mice and nontransgenic littermates revealed HA expression in type II alveolar epithelial cells in SPC-HA mice, but not in type I cells or any other cell type (Figure 1D).

To test whether HA expressed by type II alveolar epithelial cells in SPC-HA transgenic mice results in presentation of a class II-restricted epitope to CD4 T cells and lung pathology, we adoptively transferred HA-specific (6.5⁺) CD4⁺ T cells isolated from TCR-HA mice, previously activated *in vitro*, into SPC-HA mice and nontransgenic littermates. Whereas the lungs of control mice showed no abnormalities, lungs of SPC-HA transgenic mice were consolidated and had severe multifocal/coalescing lymphocytic infiltration predominantly in the interstitium and around bronchial walls and the vasculature (Figure 1E). We found that even the administration of as few as 10^6 naive CD4⁺6.5⁺ T cells was sufficient to cause prominent perivascular lymphocytic infiltration in the lungs of SPC-HA mice (data not shown).

Development of Severe Immune-mediated Lung Disease

To analyze the impact of HA expression in the lung on HA-specific T cells, SPC-HA transgenic mice were crossed to TCR-HA mice. Despite the fact that HA is expressed not only in the lung but also to some extent in the thymus of SPC-HA mice, we observed no reduction in the frequency of CD4⁺6.5⁺ T cells in peripheral lymphoid tissue of SPC-HA/TCR-HA double transgenic mice (data not shown), indicating inefficient thymic deletion of potentially autoreactive T cells. In addition, the proliferative capacity of CD4⁺ T cells from double transgenic mice in response to *in vitro* antigenic stimulation was comparable to that of CD4⁺

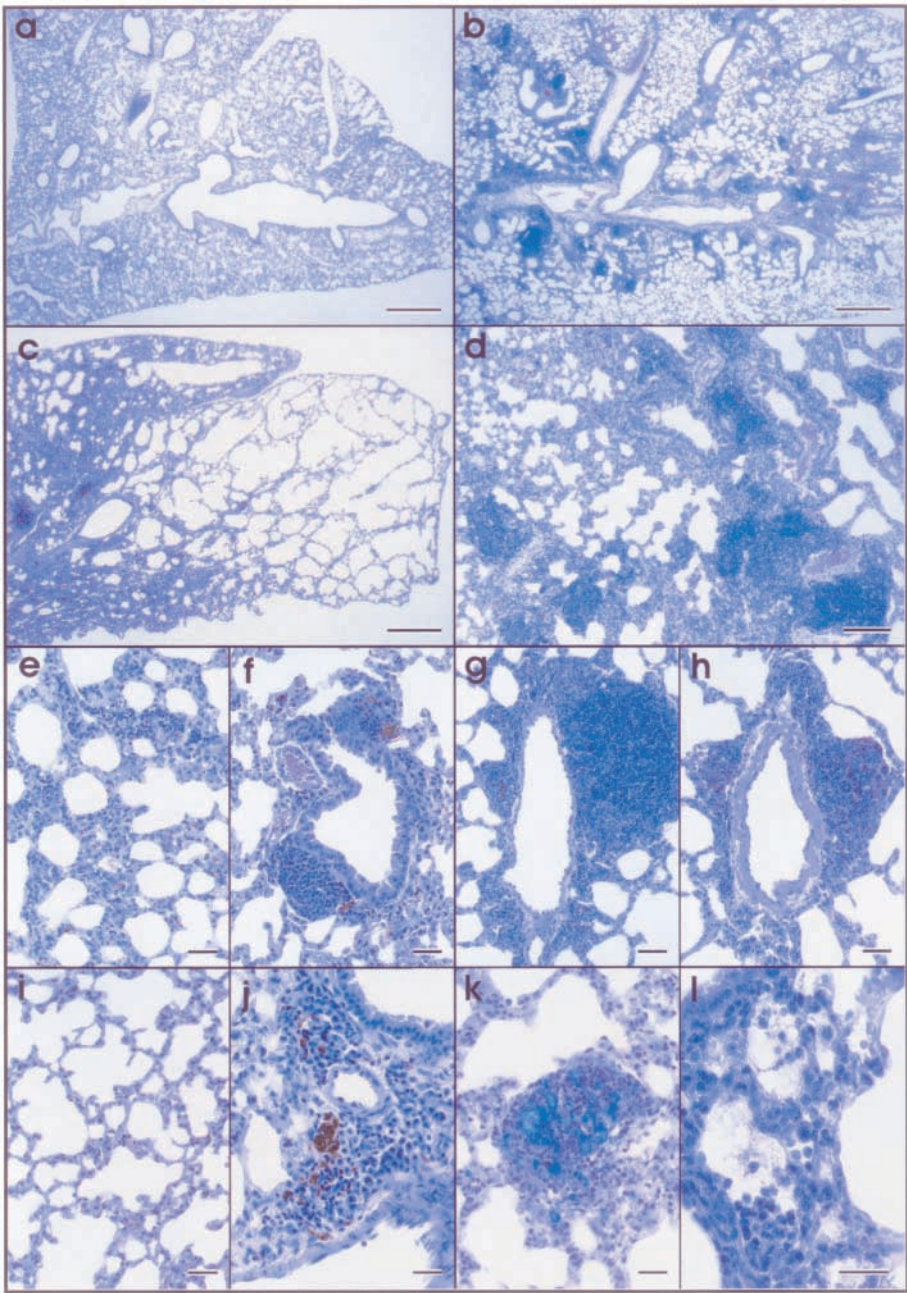
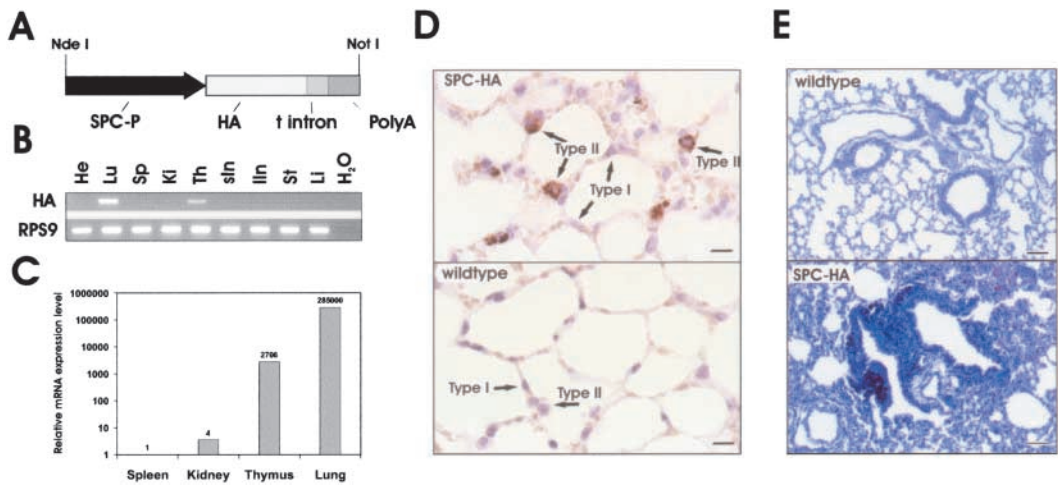


Figure 2. Lung of a healthy SPC-HA mouse (a) compared with a SPC-HA/TCR-HA mouse (b). Alveolar emphysema in an SPC-HA/TCR-HA mouse (c). Lymphocytic infiltration in a double transgenic mouse (d) distinct lymphocytic infiltration patterns in the interalveolar (e), around bronchi (f), veins (g) and arteries (h). Normal interalveolar septa of a SPC-HA control mouse (i; compare with e). Lymphocytic aggregates containing brownish pigment-loaded macrophages (j) suggestive of hemosiderin. Turnbull blue stain for iron revealed significant iron deposition (k; blue color). Lung of a 9-days-old double transgenic mouse (l) with acute tissue damage. H&E stain (a–j, l) and turnbull blue stain for the detection of iron (k). Bars = 500 μ m (a–c), 200 μ m (d) 100 μ m (e–j) and 50 μ m (j–l).

T cells from TCR-HA single transgenic T cells (data not shown). Therefore, we extensively examined the lungs of double transgenic mice for histologic evidence of inflammation. Fetuses and mice up to 9 months of age were included in this study. In every case, single transgenic littermates served as controls. Although double transgenic mice appeared clinically normal and did not show a diminished life span compared with control mice, immunohistologic analyses of the lungs revealed a severe progressive interstitial pneumonitis characterized by massive lymphocytic and plasmacytic infiltration (Figure 2). Specifically, whereas lungs of SPC-HA transgenic mice (Figure 2A) were normal, lungs of mice were severely consolidated with multifocal interstitial, perivascular, and peribronchial infiltration (Figure 2B). In addition, several of the double transgenic mice demonstrated severe alveolar distension and hyperinflation consistent with alveolar emphysema (Figure 2C) presumably due to interstitial damage, leading to a reduction of elastic fibers, in combination with airway obstruction due to massive peribronchial space-occupying lymphoid follicles. In some cases, more than half of the lung tissue was entirely consolidated due to lymphocytic infiltration (Figure 2D). Four distinct lymphocytic infiltration patterns were observed: in the interalveolar interstitium (Figure 2E), and around bronchi (Figure 2F), veins (Figure 2G) and arteries (Figure 2H). By comparison, the interalveolar septa of SPC-HA mice were normal (Figure 2I; [compare with Figure 2E]). In addition, multiple hemosiderin-loaded macrophages were prominently interspersed among the lymphocytic infiltrates, suggestive of chronic-active capillaritis with hemorrhage (Figures 2J and 2K). Of note, the airways and alveolar spaces were entirely unaffected. In sharp contrast to the lungs of older mice that had primarily diffuse to follicular lymphocytic infiltrations, lungs of 9-day-old SPC-HA/TCR-HA mice had multifocal acute alveolar necrosis with hemorrhage, intraalveolar fibrin deposition, and few macrophages (Figure 2L), consistent with acute tissue damage. Lungs of unborn mice showed no signs of inflammation (data not shown). Inflammation was restricted exclusively to the lung. Importantly, although the early inflammatory lesions observed in the lungs of newborn and young SPC-HA/TCR-HA mice were quite severe, there was no evidence of uncontrolled progression of the inflammation leading to complete tissue destruction or death in elder mice. In particular, no chronic alveolar wall thickening or fibrosis was seen at later stages as a potential sequel to severe acute alveolar wall inflammation.

Reduced Proliferative Capacity and Activated Phenotype of HA-specific CD4⁺ T Cells from the Lung of SPC-HA/TCR-HA Mice

To confirm that the observed morphologic changes are directly linked to infiltration and activation of antigen-specific CD4⁺ T cells, lymphocytes were isolated from inflamed tissue and analyzed for the percentage and activation status of CD4⁺6.5⁺ T cells. The total number of lymphocytes (data not shown) and the proportion of HA-specific CD4⁺ T cells were reproducibly increased in the lungs of double transgenic mice (Figure 3A). When stimulated *in vitro*, the proliferative capacity of lung lymphocytes from SPC-HA/TCR-HA mice was reduced by approximately 50% at low dose of antigen when compared with lung T cells from TCR-HA control mice (Figure 3B). Furthermore, CD4⁺6.5⁺ T cells from inflamed lungs exhibited an activated/memory phenotype, as indicated by upregulation of CD25 and CD69, as well as downregulation of CD45RB and CD62L surface expression (Figure 3B). To further evaluate the responsiveness of lung derived CD4⁺6.5⁺ T cells to antigenic stimulation, culture supernatants from *in vitro*-activated SPC-HA/TCR-HA as well as TCR-HA lymphocytes from the lung were analyzed for cyto-

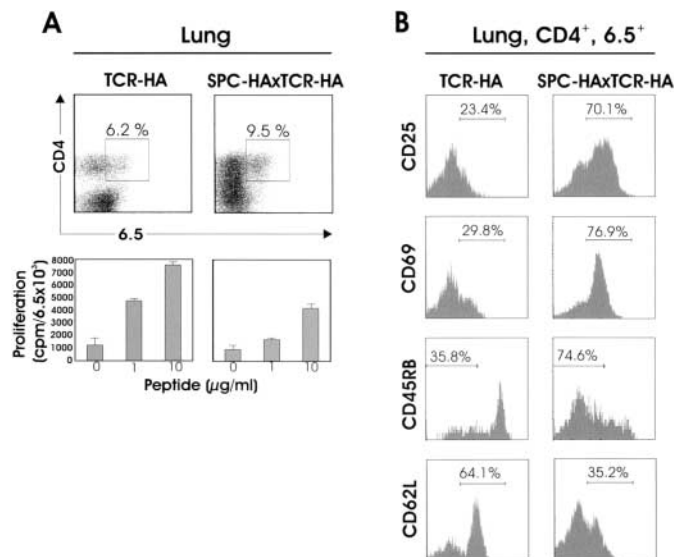


Figure 3. (A) Increased number of HA-specific CD4⁺ T cells in the lung of SPC-HA/TCR-HA mice. Percentages of CD4⁺6.5⁺ T cells isolated from the lung were determined by fluorescence-activated cell sorter (FACS) analysis. Proliferative capacity of lung lymphocytes from single and double transgenic mice was compared in an *in vitro* stimulation assay; cpm = counts/minute. (B) Activated phenotype of CD4⁺6.5⁺ lung lymphocytes from SPC-HA/TCR-HA mice. CD4⁺6.5⁺ T cells isolated from the lung of SPC-HA/TCR-HA and TCR-HA mice were analyzed by FACS for the expression of CD25, CD69, CD45RB, and CD62L.

kine secretion. Autoreactive T cells from the lung of SPC-HA/TCR-HA mice secreted lower amounts of IFN- γ , interleukin (IL)-2, IL-6, and monocyte chemoattractant protein-1, but increased amounts of IL-5 upon *in vitro* stimulation. Tumor necrosis factor- α secretion was not changed (Figure 4A). Because there was evidence for increased expression of IL-10 in lung lymphocytes from diseased mice (*see below*), we tested for IL-10 expression by RT-PCR and intracellular staining. Both measurements clearly showed elevated IL-10 levels in autoreactive CD4⁺ T cells isolated from SPC-HA/TCR-HA mice (Figure 4B and 4C). However, significant IL-4 production was not detectable (data not shown).

Global Gene Expression Profiling of Self-reactive CD4⁺ T Cells

To provide new insights into gene expression in CD4⁺ T cell dysregulation underlying mucosal inflammation of the lung, we performed a whole genome transcriptome analysis on HA-specific T cells from healthy and diseased mice. CD4⁺6.5⁺ T cells from the lungs of seven individual mice from both SPC-HA/TCR-HA and TCR-HA lines were isolated by a fluorescence-activated cell sorter. We also included peripheral CD4⁺6.5⁺ T cells that we isolated from the spleens of the same donors. RNA was prepared from the different cell pools for extensive gene expression profiling using Affymetrix MG-U74Av2 oligonucleotide arrays (Affymetrix, High Wycombe, UK). The advantage of this technology is that every gene analyzed is represented by 16 independent probe pairs, which together establish the basis for statistical evaluations of the respective signals. In addition to genes differentially expressed in lung or periphery of double transgenic diseased mice versus single transgenic healthy mice, we also did an alignment of lung- versus spleen-derived CD4⁺6.5⁺ T cells from TCR-HA mice to get an impression of the general expression level of selected genes in these particular organs under normal condi-

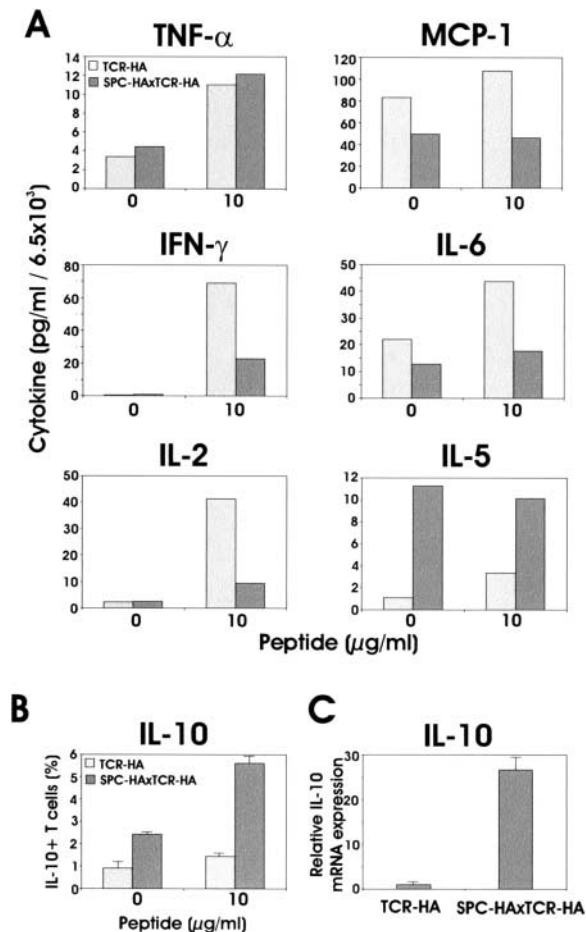


Figure 4. (A) Altered cytokine profile in lung lymphocytes from SPC-HA/TCR-HA mice. Lung lymphocytes from SPC-HA/TCR-HA and TCR-HA mice were stimulated *in vitro* with the HA110–120 peptide. Cytokines in culture supernatants were quantified using cytometric bead array from Becton Dickinson. (B) Intracellular staining for interleukin (IL)-10. Lung lymphocytes from single and double transgenic mice were stimulated *in vitro* with 10 μg/ml HA110–120 peptide, and stained for intracellular IL-10 following the instructions of the BD Cytotfix/Cytoperm protocol. Percent IL-10 positive cells was obtained by gating on CD4⁺ T cells. (C) RT-PCR to quantify IL-10 mRNA expression. RNA was prepared from CD4⁺ T cells isolated from the lungs of SPC-HA/TCR-HA and TCR-HA mice and used as PCR template using IL-10 specific primers. Results were normalized to the housekeeping control RPS9. TNF-α = tumor necrosis factor-α; MCP-1 = monocyte chemoattractant protein-1.

tions. Based on this approach, we identified a wide variety of regulated genes belonging to several functional classes, such as surface receptors, secreted or signaling molecules, transcription and translation factors, as well as genes involved in cell cycle, apoptosis, and survival. Coregulated genes were combined in clusters, as summarized in Figure 5. Four clusters (A–D) were found to be of special interest, combining genes that are either specifically downregulated (A) or upregulated (B–D) in lung CD4⁺ T cells upon airway inflammation. Selected genes are highlighted, and a complete list of genes can be found at www.gbf.de/array under Download, and then under Bruder *et al.*, 2004.

Among the genes most significantly upregulated in association with airway inflammation (up to 26-fold) were genes encoding for integrin α_Eβ₇, secreted phosphoprotein 1 (also termed osteopontin), and for the peptide neurotransmitter, preproenkepha-

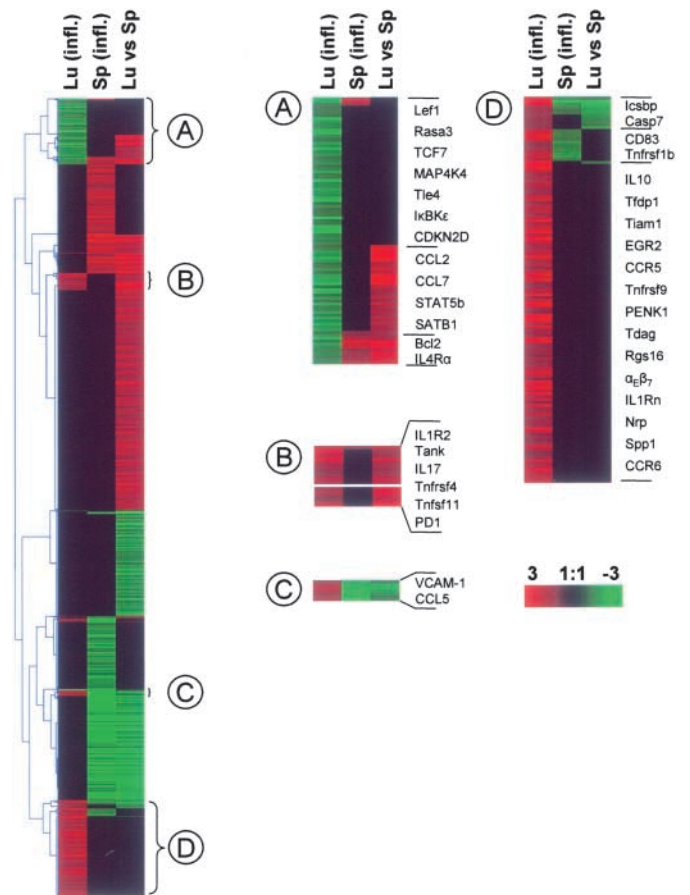


Figure 5. Cluster analysis of genes differentially expressed in CD4⁺6.5⁺ T cells isolated from lungs and spleens of diseased SPC-HA/TCR-HA as well as healthy TCR-HA mice: Red indicates induction of gene expression, green indicates repression (+3: bright red; -3: bright green). Black indicates no changes. Genes in which expression was at least twofold increased or decreased were considered to be regulated. Lu (infl.), lung inflamed, genes differentially expressed in CD4⁺6.5⁺ T cells from the inflamed lung of SPC-HA/TCR-HA mice compared with the lung of healthy TCR-HA donors. Sp (infl.), spleen inflamed, represents genes differentially expressed in CD4⁺6.5⁺ T cells from the spleen of SPC-HA/TCR-HA mice compared with TCR-HA. Lu versus Sp, lung versus spleen, defines basal level expression of genes in the lung. The left lanes represent an overview and the middle and right lanes are an enlarged view outlining clusters of special interest. Cluster A: genes downregulated in the lung of SPC-HA/TCR-HA mice upon airway inflammation, although being, in part, higher expressed in lung than periphery in healthy mice. Cluster B: genes with high basal level expression in the lung of healthy donors, being upregulated during pulmonary disease. Cluster C: genes being expressed at lower levels in the lung than in the spleen under normal conditions, which are upregulated in lung CD4⁺ T cells, but downregulated in the periphery during inflammation. Cluster D: genes that are upregulated in self-reactive CD4⁺ T cells in the inflamed lung, but partially downregulated in the periphery.

lin. Members of the tumor necrosis factor receptor superfamily known to be expressed at the surface of activated T cells, such as Tnfrsf4 (Ox40) and Tnfrsf9, showed increased expression in the inflamed lungs. RNA transcription of the chemokine receptors CCR5 and CCR6, as well as the chemokine ligand for CCR5, CCL5 (RANTES), were upregulated in CD4⁺6.5⁺ lung lymphocytes from double transgenic mice. Other cytokine genes upregulated in autoreactive lung CD4⁺ T cells from diseased SPC-HA/TCR-HA mice

were those encoding IL-10 and IL-17 and the tumor necrosis factor superfamily cytokine Tnfsf11 (Rank-L, TRANCE). Interestingly, the expression of genes involved in the prevention of apoptosis, such as those encoding for B cell leukemia/lymphoma 2 related protein (Bcl2) and the transcription factor, AT-rich sequence binding protein-1 (SATB1), were downregulated in autoreactive CD4⁺ T cells from the inflamed lung tissue. In contrast, proapoptotic genes, such as the T cell death-associated gene Tdag (also termed PHLDA1) and the gene encoding for caspase 7, were upregulated. Other interesting genes found to be differentially expressed upon airway inflammation were those encoding for transcription factors Lef1, Tcf7, and EGR-2, a signaling molecule (Tiam), as well as surface molecules like CTLA-4, PD-1 and Nrpl.

DISCUSSION

We have shown here that due to inefficient thymic deletion, autoaggressive CD4⁺ T cells accumulate in the periphery of SPC-HA/TCR-HA mice, where the recognition of a single epithelial antigen is sufficient to trigger an inflammatory cascade resulting in the manifestation of interstitial pneumonia. Although the severity of inflammation in SPC-HA/TCR-HA mice was quite impressive, autoimmune disease did not progress uncontrolled, but was found to be thoroughly regulated, as demonstrated by reduced proliferative capacity, altered cytokine profile, and broad changes in the gene expression profile of autoreactive CD4⁺ T cells isolated from the inflamed lung. Moreover, despite the very early onset of disease with acute alveolar tissue damage in young mice, the abating progression of airway inflammation in elder mice raises the intriguing possibility that regulatory mechanisms may exist and/or develop under autoimmune conditions to counteract the progression of inflammatory processes in the lung, thus preventing uncontrolled tissue destruction and lethal physiologic derangement.

Autoimmune diseases are believed to be under complex genetic regulation, but all require some form of escape from self-tolerance. Despite low level expression of HA in the thymus of all SPC-HA lines tested, central (thymic-dependent) tolerance to the HA transgene is far from complete. In SPC-HA/TCR-HA double transgenic mice, the percentage of mature CD4⁺6.5⁺ T cells in thymus and periphery is similar to that observed in TCR-HA mice (data not shown). This finding was not unexpected, as it has been described previously that expression of the HA-antigen in pancreas (16, 17) and in hematopoietic cells (18) of TCR-HA mice does not lead to complete deletion of 6.5⁺ T cells. A possible explanation for the escape from tolerance might involve coexpression of two different T cell receptors by the same cell. Due to allelic inclusion of TCR α genes, self-reactive T cells may leave the thymus resulting in induction of autoimmunity in the periphery (19). Insufficient thymic deletion of self-reactive CD4⁺ T cells was also observed in mice expressing the A/Japan/57 HA under the lung-specific SPC promoter (20). Here it was speculated that the absence of CD4 T cell tolerance may be due to inefficient processing and presentation of the membrane glycoprotein HA in thymic cells. Contrary to this theory, we found that in TCR-HA mice crossed to another SPC-HA transgenic mouse line, which shows likewise alveolar and thymic HA expression, the number of autoreactive CD4⁺ T cells was 80–90% decreased in the periphery (data not shown), suggesting that mechanisms other than impaired major histocompatibility complex class II antigen processing are involved in insufficient thymic deletion. Interestingly, even those drastically reduced CD4⁺6.5⁺ T cell numbers were sufficient to trigger ILD as severe as that observed in the SPC-HA/TCR-HA line we described here (data not shown). This is in line with the finding

that adoptive transfer of as few as 10⁶ naive HA-specific CD4⁺ T cells leads to the development of interstitial pneumonia in SPC-HA transgenic mice, indicating a highly specific and sensitive CD4⁺ T cell response toward mucosal antigen expression in the lung.

The induction of peripheral tolerance could result from antigen presentation by major histocompatibility complex class II expressing but costimulatory molecule B-7 deficient parenchymal cells. A possible mechanism for the primary stimulation of autoreactive T cells in our model might involve B-7⁺ dendritic cells that take up the epithelial-derived HA and transport it to the local lymph nodes where efficient T cell activation takes place. Recently activated autoreactive T cells may migrate to the lung tissue where they reencounter their ligand on epithelial cells that may not necessarily express the costimulatory molecule B-7. Under these conditions, self-recognition in the periphery might result in a number of potential outcomes, including the induction of T cell unresponsiveness rather than T cell activation (21), or the costimulation-independent activation of terminal effectors in the periphery. Clearly, the potential for either or both exists, and depends to a great extent on the specific activation state of the T cells in question. Our data suggest the induction of regulatory T cells that actively suppress auto-aggressive tissue destruction.

Chronic antigen stimulation *in vitro* has been described previously to result in the induction of T_R1 regulatory T cells that efficiently prevent mucosal pathology in the gut (22). We have shown here that chronic antigen exposure via the lung mucosa obviously also favors the establishment of such a cytokine profile, which is accompanied by drastically reduced secretion of the proinflammatory cytokines IL-2 and IFN- γ , increased levels of IL-10 and IL-5, but no IL-4 production. Moreover, global gene expression profiling of alveolar HA-specific CD4⁺ T cells from SPC-HA/TCR-HA revealed upregulation of many genes known to be induced upon TCR stimulation, or that have been described previously in the context of lung diseases, such as sarcoidosis, chronic obstructive pulmonary disease, or asthma (23–29). Also, a considerable number of genes previously associated with regulatory CD4⁺ T cells were found to be differentially expressed upon airway inflammation. These include those for transcription factors (SATB1, Lef1, Tcf7, EGR-2), surface molecules ($\alpha\beta\gamma$, CTLA-4, PD-1, Nrpl, CCR5), secreted molecules (IL-10, CCL5), signaling molecules (Tiam), and molecules involved in the cell cycle and apoptosis (Bcl2) (30–35). With the intent to get more insight into the complex molecular changes occurring under autoimmune conditions, we also compared the gene expression pattern of lung derived HA-specific CD4⁺ T cells from diseased and healthy mice with anergic CD4⁺ T cells isolated from immunoglobulin kappa (IG)-HA–TCR-HA mice that have been extensively studied at the immunologic and molecular level before (18, 31, 36). This alignment revealed that the majority of genes found to be differentially expressed in autoreactive lung lymphocytes are similarly regulated as those of *in vivo* anergized HA-specific CD4⁺ T cells from IG-HA–TCR-HA double transgenic mice (unpublished observation).

Although the relevance for regulatory T cells in controlling development of asthma and allergy is well established (37, 38), the immunologic mechanisms that downmodulate and protect against the development of autoimmune-mediated disorders are poorly characterized. Despite the obvious existence of peripheral tolerance mechanisms preventing uncontrolled disease progression, the severity of airway inflammation, especially in young SPC-HA/TCR-HA mice, was quite impressive. This is in line with results recently published by Huang and colleagues who have shown in an adoptive transfer system that CD4⁺ T cells undergoing peripheral tolerance induction express effector function before

reaching a tolerant state. In a first step, self-antigen encounter leads to transient activation of these cells, accompanied by the secretion of proinflammatory cytokines, such as IL-2 and IFN- γ , and the induction of autoimmune pathology within the organ that expresses the cognate antigen. However, after passing through this early effector phase, tolerance is induced, as demonstrated by the inability to secrete IL-2 and IFN- γ as well as to proliferate upon antigenic stimulation (39, 40). In our double transgenic mouse model, there is a permanent output of relatively high numbers of naive HA-specific T cells and, certainly, also a high proportion of self-reactive CD4⁺ T cells recently activated upon encountering their antigen at the lung mucosa. Therefore, we propose that due to transient activation of self-reactive T cells, we observe pathologic signs of acute inflammation in the lung of young mice before tolerance can be established. As has been shown before *in vitro* and *in vivo*, continuous antigen stimulation results in anergic T cells that fail to proliferate upon antigenic stimulation, secrete high amounts of IL-10 (22, 36), and interfere with the immune response of naive T cells, reflecting the acquisition of immunoregulatory properties (41). This is exactly what we observe in the lung of double transgenic mice, where permanent antigen exposure via the lung mucosa results in drastically decreased IL-2 and IFN- γ production, reduced proliferation, and induction of IL-10 secretion, which in turn may influence neighboring immune responses. IL-10 is a central modulator or effector molecule of tolerance, as it downregulates costimulation by APC (42), DC-driven IFN- γ production by T cells (43), and T cell responses to antigen through the inhibition of IL-2 production and IL-2R α chain expression (44, 45), and is known to play a complex role in autoimmunity (46). Whereas in the early phase of inflammation the impact of recently activated T cells secreting proinflammatory cytokines might prevail, with long standing inflammation T_R1 regulatory T cells and naive T cells might compete for access to antigen presented by APCs (47). As a consequence, broad changes in the cytokine milieu in the lung of SPC-HA/TCR-HA mice might occur, dominated by the pleiotropic immunosuppressor IL-10.

Although there is evidence that IL-10 is not essentially needed for the development of T cell anergy *in vivo* (48), IL-10 has been shown to be essential to induce T_R1 cell differentiation *in vitro* (22). A potential source for IL-10 in our model is pulmonary DC, which has been shown to produce IL-10 under inflammatory conditions. This in turn drives the generation of a population of IL-10-producing regulatory T cells in draining lymph nodes that are capable of suppressing responses to antigenic challenge (49). Further characterization of DC that induces tolerance and T_R cell differentiation *in vivo* revealed that IL-10 is not absolutely required but clearly enhances the differentiation of tolerogenic DCs in the lung (50). In conclusion, we propose that primary changes in the cytokine pattern and functionality of initially activated T cells lead to profound changes in the inflammatory environment in the lungs of diseased mice; supporting the induction of regulatory T cells that on their part control autoimmune-mediated inflammation likely by the release of immunosuppressive IL-10.

Although IL-10 might be an important effector molecule in establishing tolerance in our system, it is unlikely to be the only one. It has to be taken into consideration that many cells of the lung, such as macrophages, eosinophils, dendritic cells, mast cells and granulocytes, interact with lymphocytes and vice versa via a number of cytokines and chemokines (51). Chemokines and their receptors guide T lymphocyte recruitment in lung inflammation, and inflammatory diseases of the lung are characterized by the selective accumulation of inflammatory cells, a process controlled by the expression of distinct cytokines. Upon inflammation, autoreactive CD4⁺ T cells derived from SPC-HA/TCR-HA showed increased expression of IL-10 and IL-5 cytokines, as

well as decreased levels of monocyte chemotactic protein-1, IFN- γ , IL-2, IL-6 and IL-17. Moreover, these T cells showed increased expression of chemokine receptors CCR5 and CCR6, as well as CCL5 (RANTES), the chemokine ligand for CCR5, all of which have been shown to participate in the development and progression of pulmonary inflammation (14–16). It remains to be elucidated whether and to what extent the cytokines and chemokines found to be regulated upon inflammation are mechanistically associated with inflammation and tolerance induction in our system.

To dissect the pathologic and tolerogenic mechanisms underlying pulmonary dysregulation in more detail, we and others are currently trying to identify surface molecules that reliably discriminate regulatory from naive and recently activated T cells. Surface expression of numerous regulatory T cell markers, such as CD25, CTLA-4, $\alpha\text{E}\beta_7$, or PD-1 (30–34), is induced upon T cell activation, thereby making it difficult to determine whether HA-specific CD4⁺ T cells isolated from the site of inflammation are activated or regulatory, i.e., whether they participate in the progression of autoimmune tissue destruction or are involved in the maintenance of immunologic balance. Recently, we described neuropilin-1 as an activation independent marker exclusively expressed on regulatory T cells (35). HA-specific T cells from the lung of diseased -HA/TCR-HA mice show elevated expression of neuropilin-1, further supporting that chronic mucosal antigen exposure leads to the development of regulatory T cells.

In summary, the SPC-HA/TCR-HA mouse combines important features of a variety of lung diseases and will contribute to a better understanding of the requirements for, and consequences of, chronic T cell-mediated lung injury. Information gained in understanding the mechanisms of regulatory cell suppression of chronic inflammation may help to understand why under certain disease conditions there may be a failure to achieve this. The genome-wide transcriptome of self-reactive lung lymphocytes provides a focused starting point for the further elucidation of genetic and mechanistic aspects of autoimmune-mediated lung disease, and offers potentially new molecular markers suitable for the detection and treatment of airway inflammation in human patients.

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