Functional Diversity of T-Cell Subpopulations in Subacute and Chronic Hypersensitivity Pneumonitis

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***Rationale*: Hypersensitivity pneumonitis (HP) exhibits a diverse outcome. Patients with acute/subacute HP usually improve, whereas patients with chronic disease often progress to fibrosis. However, the mechanisms underlying this difference are unknown.**

***Objectives*: To examine the T-cell profile from patients with subacute HP and chronic HP.**

***Methods*: T cells were obtained by bronchoalveolar lavage from 25 patients with subacute HP, 30 patients with chronic HP, and 8 control subjects. T-cell phenotype and functional profile were evaluated by flow cytometry, cytometric bead array, and immunohistochemistry.**

***Measurements and Main Results*: Patients with chronic HP showed higher $CD4^+$:$CD8^+$ ratio (median, 3.05; range, 0.3–15; subacute HP: median, 1.3; range, 0.1–10; control: median, 1.3; range, 0.7–2.0; $P < 0.01$), and a decrease of γδT cells (median, 2.0; range, 0.5–3.4; subacute HP: median, 10; range, 4.8–17; control: median, 15; range, 5–19; $P < 0.01$). Patients with chronic HP exhibited an increase in the terminally differentiated memory $CD4^+$ and $CD8^+$ T-cell subsets compared with patients with subacute HP ($P < 0.05$). However, memory cells from chronic HP showed lower IFN-γ production and decreased cytotoxic activity by $CD8^+$ T lymphocytes. Chronic HP displayed a Th2-like phenotype with increased CXCR4 expression (median, 6%; range, 1.7–36, vs. control subjects: median, 0.7%; range, 0.2–1.4; and subacute HP: median, 2.2%; range, 0.1–5.3; $P < 0.01$), and decreased CXCR3 expression (median, 4.3%; range, 1.4–25%, vs. subacute HP: median, 37%; range, 4.9–78%; $P < 0.01$). Likewise, supernatants from antigen-specific–stimulated cells from chronic HP produced higher levels of IL-4 (80 ± 63 pg/ml vs. 25 ± 7 pg/ml; $P < 0.01$), and lower levels of IFN-γ (3,818 ± 1671 pg/ml vs. 100 ± 61 pg/ml; $P < 0.01$) compared with subacute HP.**

***Conclusions:* Our findings indicate that patients with chronic HP lose effector T-cell function and exhibit skewing toward Th2 activity, which may be implicated in the fibrotic response that characterizes this clinical form.**

Keywords: allergic alveolitis; cytotoxic; hypersensitivity pneumonitis; T cells; Th1/Th2 cells

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanisms by which some patients with hypersensitivity pneumonitis progress to fibrosis are yet unclear.

What This Study Adds to the Field

This study demonstrates that patients with chronic hypersensitivity have different phenotypic/functional lung T-cell subsets compared with patients with subacute disease, likely associated with the fibrotic response.

Hypersensitivity pneumonitis (HP) is a complex syndrome of varying intensity and clinical presentation that results from an immunologically induced lung inflammation in response to a wide variety of inhaled antigens (1). The clinical presentation is heterogeneous and, thus, HP may present as an acute, subacute, or chronic form, depending on, among several factors, the intensity of inhaled antigen and repetitiveness of exposure (1, 2). Importantly, patients with acute or subacute HP usually respond to treatment, whereas patients with chronic disease often progress to an irreversible destruction of the lung, either by fibrosis or emphysematous changes (1, 3, 4).

HP is characterized by a remarkable T-cell alveolitis. However, characterization of lymphocyte phenotypes, primarily $CD4^+$/$CD8^+$ T-cell subsets, has given inconsistent results. Some authors have reported an increase in $CD8^+$ T cells with a decrease in the $CD4^+$:$CD8^+$ ratio, whereas others have shown that both subpopulations accumulate without changes in that ratio, and still others have reported a clear predominance of $CD4^+$ T cells (5–9). Several reasons may account for this variability, including the type of inhaled antigen and the clinical presentation (1, 9).

Studies of other T-cell phenotypes in human HP (i.e., Th1 vs. Th2), and of memory/effector T cells, are scanty, whereas other putative regulatory or effector cells, like natural killer (NK) T (NKT) cells, among others, have not been studied (10). Likewise, immunological memory in the context of recent descriptions based on different homing and effector capabilities of T-cell subpopulations (11–14) has not been examined in this disease.

Importantly, possible differences between subacute and chronic disease have not been studied, and we hypothesized that the exhaustion/skewing of T cells in chronic HP is the principal reason for the inability of the host to eliminate the persisting antigen. Supporting this notion, a specific mechanism of T-cell exhaustion in chronic lymphocyte choriomeningitis virus (LCMV), which ends in a limited proliferative potential and inability to produce cytokines, was recently described (15).

It is well known that naive T helper cells can differentiate to at least two different memory cells during the immune response: Th1 cells, which secrete IFN-γ and tumor necrosis factor-α; and Th2 cells, which secrete IL-4 and IL-13 (16). Interestingly, a growing body of evidence suggests that polarized T cells (i.e., Th1

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or Th2) may regulate the fibrotic response, which may account for patients with chronic HP progressing to fibrosis (17).

In order to better characterize the T-cell subpopulations present in subacute and chronic HP lungs, we analyzed the variety and functional activity of distinct T-lymphocyte subpopulations from cells obtained by bronchoalveolar lavage (BAL). Our results show that patients with chronic disease have a higher $CD4^+$:$CD8^+$ ratio with a Th2-like response, decreased numbers of γδ T cells, and a functional impairment of the local $CD8^+$ memory T-cell subset.

METHODS

Additional details are provided in online supplement. A total of 55 patients with HP were included in this study. Patients were arbitrarily classified as having subacute HP (25 patients; 42.5 ± 11.4 yr [mean ± SD]) and chronic HP (30 patients; 50.3 ± 8.1 yr). None of the patients had been treated with corticosteroids or immunosuppressive drug at the time of the study. Eight healthy subjects (37.6 ± 6.5 yr) were studied as control subjects. The protocol was approved by the ethics committee of the Instituto Nacional de Enfermedades Respiratorias. Diagnosis of HP was obtained as previously described (18, 19). Subacute HP was defined as: (*1*) less than 6 months of symptoms before diagnosis; (*2*) high-resolution computed tomography showing poorly defined nodules and ground glass attenuation; (*3*) biopsy (15 patients) showing an inflammatory infiltrate without fibrosis. Chronic HP was defined as: (*1*) more than 24 months of symptoms before diagnosis; (*2*) high-resolution computed tomography displaying in addition to nodules and ground glass, irregular linear opacities, lobar volume loss, and occasionally cystic lesions; (*3*) biopsy (22 patients) showing more than 20% extent of fibrotic infiltrate. A pathologist and a radiologist, blinded to the clinical data, scored the lesions.

BAL

BAL was performed as previously described (18). Cell aliquots were resuspended in 10% dimethyl sulfoxide and 90% fetal bovine serum and kept in liquid nitrogen until use. Cells were thawed as previously described (20), and only cryopreserved samples showing 95% or greater viability were used.

Determination of T-Lymphocyte Surface Phenotypes

BAL cells were defrosted, washed, resuspended in 50 μl of staining buffer, and incubated with monoclonal antibodies to determine $CD4^+$, $CD8^+$, memory T cells, γδ T cells, NKT, T helper, and T cytotoxic subpopulations (BD Pharmingen, San Diego, CA). *i*NKT were examined with anti-Va24-JaQ TCR chain (BD Pharmingen). Cells were fixed in 1% paraformaldehyde for cytometry analysis (21).

Intracellular Cytokine Staining

BAL cells (2×10^5/ml) were stimulated for 6 hours with either: (*1*) phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (1 μM) to evaluate surface CD107a/b and intracellular perforin; or (*2*) pigeon serum (100 μg/ml) to determine IFN-γ, IL-4, and IL-10.

Determination of Cytokines/Chemokines

IL-2, IL-10, IL-4, and IFN-γ were measured by cytometric bead array (22). IFN-γ–inducible protein 10 (IP-10/CXCL10), monokine induced by IFN-γ (MIG/CXCL9), thymus- and activation-regulated chemokine (TARC/CCL17), regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5, and transforming growth factor-β were measured by ELISA using commercial kits (Immunoassay Quantikine; R&D Systems, Minneapolis, MN).

Functional Cytotoxic Assay for CD107a/b Expression

Peripheral blood mononuclear cells (PBMCs) and BAL cells from five patients with subacute and five patients with chronic HP patients were stimulated with PMA and ionomycin. Conjugated antibody to CD107a/b was added to the culture before stimulation. The cultures were incubated for 5 hours in the presence of monesin and brefeldin-A (BD Biosciences) (23).

Flow Cytometry

Samples from all assays were analyzed in a FACS Aria flow cytometer (Becton Dickinson, San Jose, CA) by using FACS Diva software. Typically, 10,000 events from double-positive $CD3^+CD4^+$ or $CD3^+CD8^+$ gates for surface staining cells were acquired. For single-cell cytokine production, 40,000 events were acquired from $CD3^+CD4^+$ and $CD3^+CD8^+$ gates.

Immunohistochemistry

Tissue sections from patients with idiopathic pulmonary fibrosis and control subjects were treated as described previously (18, 19). Human anti–IP-10 and MIG polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were applied and incubated at 4°C overnight. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase–conjugated streptavidin (BioGenex, San Ramon, CA) was used according to manufacturer's instructions. 3-Amino-9-ethyl-carbazole (BioGenex) in acetate buffer containing 0.05% H_2O_2 was used as substrate. The sections were counterstained with hematoxylin. The primary antibody was replaced by non–immune serum for negative control slides.

Statistical Analysis

Results were expressed as mean (±SD), and differences among groups were analyzed through analysis of variance followed by Tukey's test. Data lacking normal distribution were reported as median and range, and differences were evaluated through the nonparametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. Statistical significance was set at P less than 0.05 bimarginally.

RESULTS

Baseline Characteristics of Both HP Subgroups

Demographic data, pulmonary function tests, BAL differential cell counts, and outcome are summarized in Table 1. All patients exhibited clinical, radiological, and functional evidence of interstitial lung disease, with variable degrees of dyspnea, decreased lung capacities, and hypoxemia at rest that worsened with exercise. Patients with chronic HP were older, presented more severe hypoxemia and more frequently with digital club-

TABLE 1. DEMOGRAPHIC, CLINICAL, PHYSIOLOGIC, AND BRONCHOALVEOLAR LAVAGE CHARACTERISTICS OF SUBACUTE AND CHRONIC HYPERSENSITIVITY PNEUMONITIS

Characteristic	Subacute HP (*n* = *25*)	Chronic HP (*n* = *30*)	*P* Value
Age, yr	42.1 ± 10.9	50.3 ± 8.1	<0.01
Gender, female/male	22/3	28/2	
Time of symptoms before diagnosis, mo	3.8 ± 1.6	46.4 ± 16.6	<0.01
Smoking	3/25	5/30	
Clubbing	3/25	14/30	<0.01
FVC % predicted	61.9 ± 18.7	59.6 ± 17.9	
FEV_1 % predicted	64.5 ± 17.4	61.8 ± 16.0	
FEV_1/FVC %	92.2 ± 6.9	86.6 ± 15.6	
Pa_{O_2}	53.2 ± 8.2	48.8 ± 8.9	< 0.05
BAL macrophages, %	26.9 ± 12.9	41.5 ± 17.2	< 0.01
BAL lymphocytes, %	71.2 ± 13.1	56.7 ± 17.3	< 0.01
BAL neutrophils, %	0.9 ± 1.2	1.0 ± 1.8	
BAL eosinophils, %	0.9 ± 2.0	0.6 ± 1.1	
Follow-up at 2 yr			
Healed/improved	16	5	
Stable	6	13	
Worsened	3	8	
Died	0	4	

Definition of abbreviations: BAL = bronchoalveolar lavage; HP = hypersensitivity pneumonitis.

Data expressed as mean ± SD.

bing than the patients with subacute HP. Both subgroups were characterized by marked BAL lymphocytosis, but significantly higher levels were observed in the subacute group (71.2 ± 13.1 vs. 56.7 ± 17.3; $P < 0.01$).

Follow-up

All patients received three boluses of methylprednisolone (1 g/d) and then were treated with (*1*) prednisone alone (0.5 mg/kg/d), reducing to a maintenance average daily dose of 10 mg, or (*2*) inhaled corticosteroids (beclomethasone, 1,000 μg twice a day). There were no differences between the types of initial treatment among both groups (data not shown). Patients were followed for 2 years and outcome was classified as resolved (disappearance of symptoms and normalization of the respiratory function tests), improved (>10% increase in FVC plus >4 mm Hg increase in Pa_{O_2}), stable, worse (>10% decrease in FVC plus >4 mm Hg decrease in Pa_{O_2}), or dead of disease. After 2 years of follow-up, most patients with subacute HP had improved or were stable, whereas 40% of the patients with chronic HP worsened or died from the disease (Table 1).

$CD4^+$:$CD8^+$ Ratio

$CD4^+$:$CD8^+$ ratio was analyzed in 25 patients with subacute HP, 30 patients with chronic HP, and 8 healthy control subjects. This ratio was widely variable, ranging from 0.1 to 10.0 in subacute cases (median, 1.3), from 0.3 to 15.0 in chronic cases (median, 3.05), and from 0.7 to 2.0 in control subjects (median, 1.3) (Figure 1). However, a significant increase in BAL $CD4^+$:$CD8^+$ ratio was observed in patients with chronic HP compared with patients with subacute HP and control subjects ($P < 0.01$). Interestingly, the three patients with subacute HP who worsened showed higher $CD4^+$:$CD8^+$ ratio (4.1, 6.0, and 10, respectively). The change in $CD4^+$:$CD8^+$ ratio in patients with chronic HP was due to the increase in absolute number of $CD4^+$ T cell per milliliter. No differences were found in $CD4^+$:$CD8^+$ ratio among biopsied and nonbiopsied patients (subacute: biopsied [n = 15; 1.7 ± 1.6]; nonbiopsied [n = 10; 2.3 ± 2.8; $P = 0.5$]; chronic: biopsied [n = 22; 4.3 ± 4.2] nonbiopsied: [n = 8; 4.6 ± 3.8; $P = 0.8$]).

Lung-specific Memory CD4 and CD8 T Cells

To characterize memory cells, the surface markers, CD45RA, CD62L, CD4, CD8, and CD3, were studied in T lymphocytes from BAL and PBMCs from 8 control subjects, 21 patients with subacute HP, and 20 with chronic HP. Based on their phenotype, three distinct populations of $CD4^+$ T cells and $CD8^+$ T cells were detected: a central memory population ($CD45RA^-$ $CD62L^{high}$) and two effector memory populations; namely, the $CD45RA^-$ $CD62L^{low}$ and the terminally differentiated effector memory T cells (TEMRA) $CD45RA^+$ $CD62L^{low}$. As illustrated in Figure 2, patients with chronic HP showed a significant

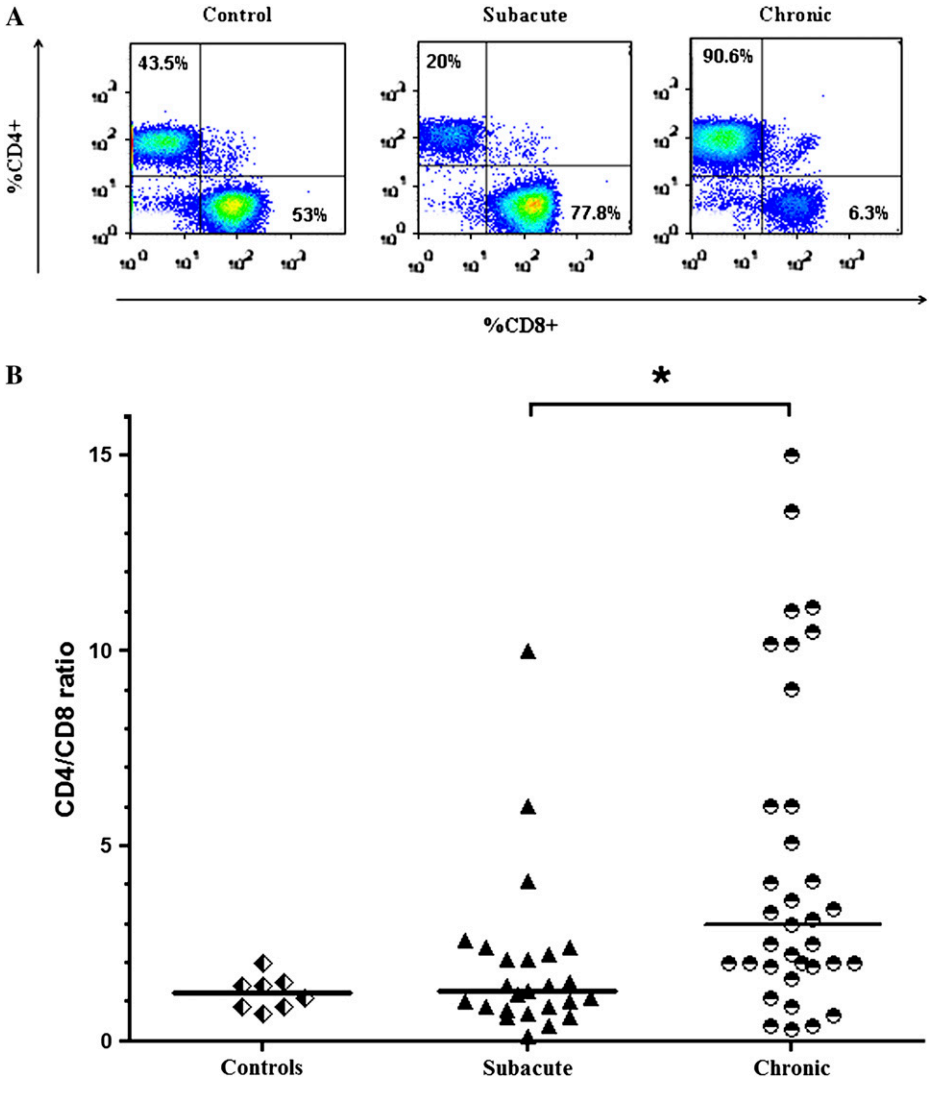

Figure 1. $CD4^+$:$CD8^+$ ratio from healthy control subjects, patients with subacute hypersensitivity pneumonitis (HP), and those with chronic HP. (*A*) Representative flow cytometry analyses of bronchoalveolar lavage T lymphocytes showing the percentage of CD4- and CD8-positive cells. (*B*) $CD4^+$:$CD8^+$ ratio from control subjects and subacute and chronic groups. *Lines* represent median values. *$P < 0.01$.

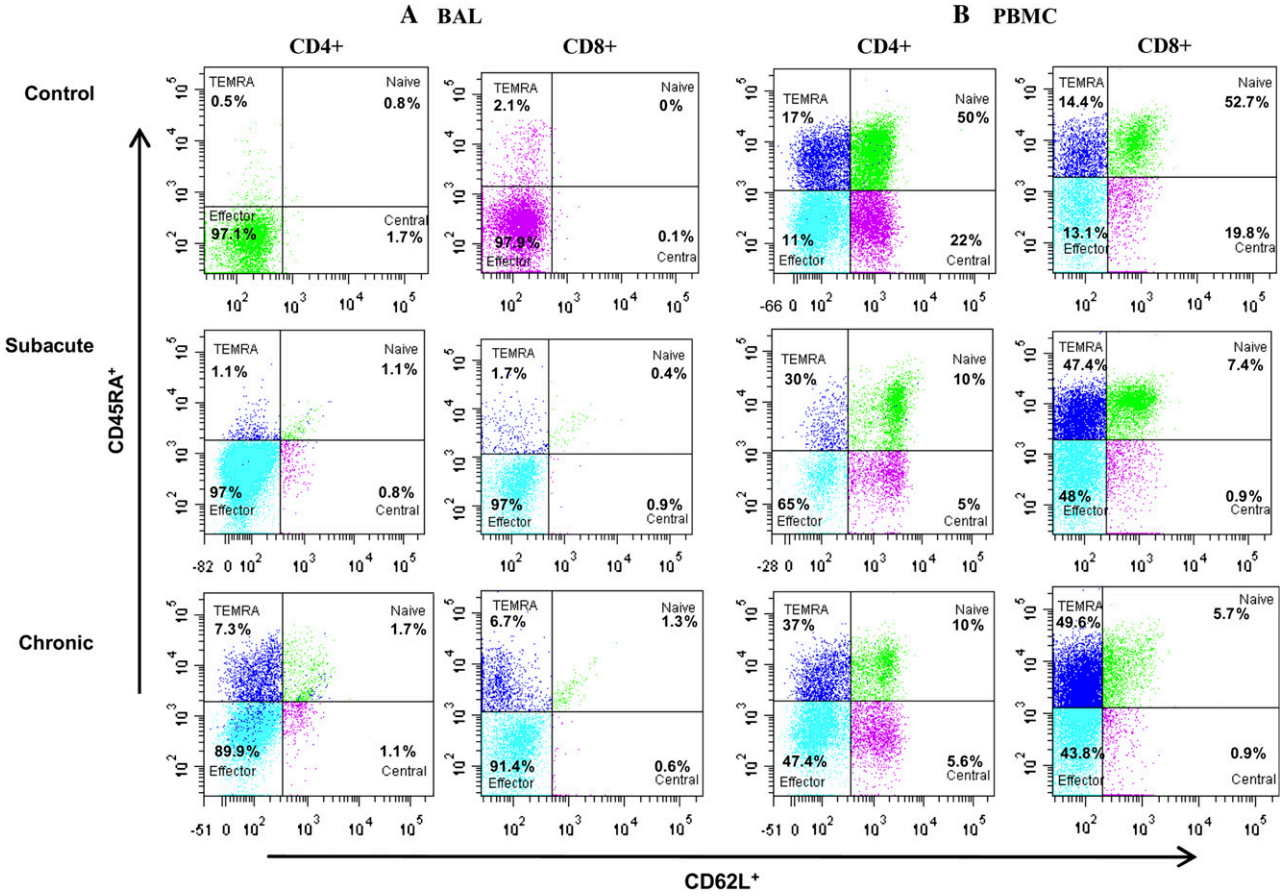

Figure 2. Phenotypic profile of memory CD4$^+$ and CD8$^+$ T cell subsets using multicolor flow cytometry. Bronchoalveolar lavage (BAL) (*A*) and peripheral blood mononuclear cells (PBMCs) (*B*) from 8 control subjects, 21 patients with subacute HP, and 20 with chronic HP were stained with directly conjugated monoclonal antibodies against CD3, CD4, CD8, CD45RA, and CD62L in a five-color experiment. Cells were first identified based on side scatter and CD3 properties, followed by gating on CD4$^+$ or CD8$^+$ cells. Differential gating on CD4$^+$ or CD8$^+$ T cells based on CD62L and CD45RA basal expression revealed four populations: naive T cells (CD45RA$^+$CD62L$^+$), central memory T cells (CD45RA$^-$CD62L$^+$), effector T cells (CD45RA$^-$CD62L$^-$), and terminally differentiated effector memory T cells (TEMRA) (CD45RA$^+$CD62L$^-$). At least 10^5 events were analyzed.

increase in BAL TEMRA CD4$^+$ and CD8$^+$ compared with the subacute HP group (CD4$^+$: 6.7 ± 1.4% vs. 1.8 ± 1%; $P < 0.05$, CD8$^+$: 5 ± 3.3% vs. 2.4 ± 0.8%; $P < 0.05$). Most of the PBMC from both patients with subacute HP and those with chronic HP were effector and TEMRA memory cells, without significant differences between them; however, these last populations were significantly increased when compared with the control cells (Table 2).

Functional Analysis of Memory CD4$^+$ and CD8$^+$ T Cells

For functional analyses, PBMC and BAL cells from five patients with subacute HP and five with chronic HP were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 6 hours. As shown in Figures 3A and 3B, patients with subacute HP had a significantly higher percentage of BAL CD4$^+$ and CD8$^+$ effector and TEMRA memory cells producing IFN-γ after stimulation compared with patients with chronic HP. In contrast, memory cell subpopulations obtained from PBMC expressed low and similar levels IFN-γ in both patients with subacute HP and those with chronic HP (data not shown).

We next evaluated the cytotoxic activity of memory CD8$^+$ T cells from PBMC and BAL from patients with subacute HP and those with chronic HP. For this purpose, we analyzed simultaneously the loss of intracellular perforin and the surface expression of CD107a/b (22). Our results showed that only BAL cells from patients with subacute HP exhibited a correlation between the increased expression of surface CD107a/b and loss of intracellular perforin, indicating functional cytotoxic activity of CD8$^+$ T cells (Figure 4). However, the major difference was related to the ability to express CD107a/b. By contrast, there were not significant differences in the loss of perforin. No differences in PBMC memory CD8$^+$ T cells were detected.

γδ T Lymphocytes, NK, and NKT Cells

As shown in Figure 5, patients with chronic HP (n = 20) had a significant decrease in the percentage of BAL γδ$^+$ T cell percentage compared with patients with subacute HP (n = 21) and control subjects (n = 8) (median, 2.0% [range, 0.5–3.4%] vs. subacute: median, 10% [range, 4.8–17%] and control subjects: median, 15% [range, 5–19]; $P < 0.01$]. By contrast, intraepithelial γδ$^+$ T cells (CD103$^+$) were significantly increased in patients with subacute HP ($P < 0.01$) when compared with control subjects and patients with chronic HP.

The proportion of conventional NKT cells (CD16$^+$CD56$^+$) was significantly decreased in patients with subacute HP and those with chronic HP (control [n = 8]: 13.1 ± 3.0%; subacute [n = 21]: 2 ± 2.6%; chronic [n = 20]: 7.3 ± 4.1%; $P < 0.01$ and $P < 0.05$, respectively). Additionally, we determined the subset of NKT cells that expressed the invariant Vα24 TCR chain (Vα*i*NKT cells) in BAL from 3 control subjects, 5 patients with

TABLE 2. NAIVE AND MEMORY T-CELL SUBSETS IN BRONCHOALVEOLAR LAVAGE AND PERIPHERAL BLOOD FROM CONTROL SUBJECTS, PATIENTS WITH SUBACUTE HYPERSENSITIVITY PNEUMONITIS, AND THOSE WITH CHRONIC HYPERSENSITIVITY PNEUMONITIS

	Control ($n = 8$)	Subacute HP ($n = 21$)	Chronic HP ($n = 20$)
Subset CD4$^+$			
BAL			
Naive	1.8 ± 1.1	1.6 ± 1.3	2 ± 1.8
Central	4.1 ± 2.8	1.4 ± 0.9	2.1 ± 1
Effector	93.4 ± 8.9	95 ± 8	90 ± 9.3
TEMRA*	0.65 ± 0.1	1.8 ± 1	6.7 ± 1.4†
PBMC			
Naive*	52.6 ± 3.7	7 ± 3	13 ± 4
Central*	20.8 ± 4	2.3 ± 2.2	3.4 ± 2.6
Effector*	10.3 ± 1.5	56 ± 10.4	50 ± 8.7
TEMRA*	16.1 ± 1.8	35 ± 8	34 ± 13
Subset CD8$^+$			
BAL			
Naive	0	0.4 ± 0.3	1 ± 0.4
Central	0.3 ± 0.1	1.1 ± 0.9	1.8 ± 0.6
Effector	97.8 ± 2.1	96 ± 7	92 ± 3.4
TEMRA	1.8 ± 1.7	2.4 ± 0.8	5 ± 3.3†
PBMC			
Naive*	53 ± 6.1	5 ± 2.4	6.2 ± 3
Central*	21.7 ± 3.8	1 ± 0.9	1.6 ± 1.2
Effector*	10.5 ± 3.1	48 ± 11	45 ± 6
TEMRA*	15.2 ± 1.8	47 ± 11	48 ± 5

Definition of abbreviations: BAL = bronchoalveolar lavage; HP = hypersensitivity pneumonitis; PBMC = peripheral blood mononuclear cell; TEMRA = terminally differentiated effector memory T cell.

Data expressed as mean ± SD.

* $P < 0.01$, control subjects versus patients with subacute HP and those with chronic HP.

† $P < 0.05$, patients with chronic HP compared with patients with subacute HP and control subjects.

subacute HP, and 15 with chronic HP. We found that a small subset of NKT cells expressed this chain, without differences being found between the groups (control: 0.5 ± 0.1%; subacute: 0.5 ± 0.4%; chronic: 0.5 ± 0.8%). Also, no differences were observed in the percentage of NK cells (control: 5.4 ± 1.5%; subacute: 4.0 ± 3.0%; chronic: 4.8 ± 2.7%).

Th1 and Th2 Cell Profile

In this study, Th1- versus Th2-like polarization was evaluated through different phenotypic and functional assays. It is known that some surface receptors are differentially expressed on Th1 and Th2 cells. For instance, CXCR3 and CCR5 are highly expressed on Th1 cells and down-regulated on Th2 cells, whereas CCR4 and CXCR4 are primarily seen on Th2 cells (24, 25). Thus, the frequency of these chemokine receptors was analyzed in BAL CD4$^+$ T cells from five control subjects, 14 patients with subacute HP, and 23 with chronic HP. As illustrated in Figure 6, CD4$^+$ T cells expressing CXCR3 were significantly increased in patients with subacute HP (median, 37% [range, 4.9–78%] vs. control: median, 1.7% [range, 1.4–2.3%] and chronic: median, 4.3% [range, 1.4–25%]; $P < 0.01$), whereas CD4$^+$ T cells expressing CXCR4 were significantly augmented in patients with chronic HP (median, 6% [range, 1.7–36%] vs. control: median, 0.7% [range, 0.2–1.4%] and subacute: median, 2.2% [range, 0.1–5.3%] $P < 0.01$). No differences were found in CD4$^+$ T cells expressing CCR5 and CCR4 between patients with subacute HP and those with chronic HP, although both HP groups exhibited an increased percentage of these T lymphocytes compared with control subjects.

We next evaluated the presence of the CXCR3 ligands IP-10/CXCL10 and MIG/CXCL9, the CCR4 ligand, TARC/CCL17, and the CCR5 ligand, RANTES/CCL5 in BAL fluids in the same patients and control subjects in which the receptors were measured. As illustrated in Figure 7, CXCR3 ligands were significantly increased in patients with subacute HP compared with those with chronic HP and control subjects (IP-10/CXCL10: 1147 ± 837 pg/ml vs. 265 ± 181 pg/ml [chronic] and 27.1 ± 10.2 pg/ml [control]; $P < 0.01$; MIG/CXCL9: 646 ± 389 pg/ml vs. 237 ± 182 pg/ml and 24.7 ± 5.63 pg/ml; $P < 0.01$). By contrast, TARC was marginally but significantly increased in patients with chronic HP compared with the subacute group (control: 25 ± 13.6 pg/ml; subacute: 17.4 ± 7.2 pg/ml; chronic: 49.3 ± 29.1 pg/ml; $P < 0.05$). No differences were found in RANTES/CCL5 levels between patients with subacute HP and those with chronic HP, although patients with subacute HP showed a significant increase compared with control subjects (control: 19.8 ± 9 pg/ml; subacute: 337.8 ± 181.6 pg/ml; chronic: 199.3 ± 161.3 pg/ml). In addition, we also measured total transforming growth factor-β1 (active and latent), a strong profibrotic mediator in the BAL fluid from 5 control subjects, 15 patients with subacute HP, and 26 with chronic HP. No differences between the groups were found (control: 43.4 ± 3.3 pg/ml; subacute: 57.1 ± 15.9 pg/ml; chronic: 67.6 ± 43.8 pg/ml).

To assess which cells express CXCR3 ligands, we performed immunohistochemistry in lung biopsies, and found that MIG/CXCL9 was mainly located in endothelial cells and smooth muscle vascular cells (Figure 8A), whereas IP-10/CXCL10 was observed in alveolar epithelial cells and alveolar macrophages (Figures 8B and 8C). Immunohistochemical staining for the two ligands was negative in normal lungs, as exemplified for IP-10 in Figure 8D.

We also evaluated the Th1/Th2 intracellular cytokine profile in BAL lymphocytes after antigen-specific stimulation in nine patients with subacute HP and nine with chronic HP. BAL from patients with subacute HP stimulated with pigeon serum showed higher percentages of CD4$^+$ lymphocytes expressing IFN-γ (median, 6.0%; range, 4.1–19%) compared with patients with chronic HP (median, 2.2%; range, 0.4–5.2; $P < 0.01$) (Figure 9). By contrast, no differences were detected in the percentage of CD4$^+$ T cells expressing IL-4 (subacute: median, 1.8% [range, 0–2.8%]; chronic: 2.8% [0.4–5.1%]). On the other hand, a significant increase of CD8$^+$ lymphocytes expressing IFN-γ was detected in patients with subacute HP (median, 12% [range, 7.6–22.4%] vs. chronic: median, 7.8% [range 1.7–13.7%]; $P < 0.01$), whereas patients with chronic HP showed a significant increase of CD8$^+$ lymphocytes expressing IL-4 (chronic: median, 6.3% [range, 1.4–9.7%]; subacute: median, 0.1% [range, 0–1.7%]; $P < 0.05$) (Figure 9).

Finally, we evaluated the cytokine production from culture supernatants of the same BAL cells where the intracellular cytokine profile was performed. Supernatants from patients with chronic HP showed significantly higher levels of IL-4 compared with those obtained from patients with subacute HP (80 ± 63 vs. 25 ± 7 pg/ml; $P < 0.01$). By contrast, IFN-γ production was significantly higher in patients with subacute HP compared with those with chronic HP (3,818 ± 1,671 vs. 100 ± 61 pg/ml; $P < 0.05$).

DISCUSSION

HP is caused by repeated inhalation of many different environmental antigens by susceptible individuals. It can result in acute or subacute clinical forms, or evolve to a chronic and potentially lethal disorder (1–3). However, the phenotypic diversity and functional activity of different T-cell subpopulations

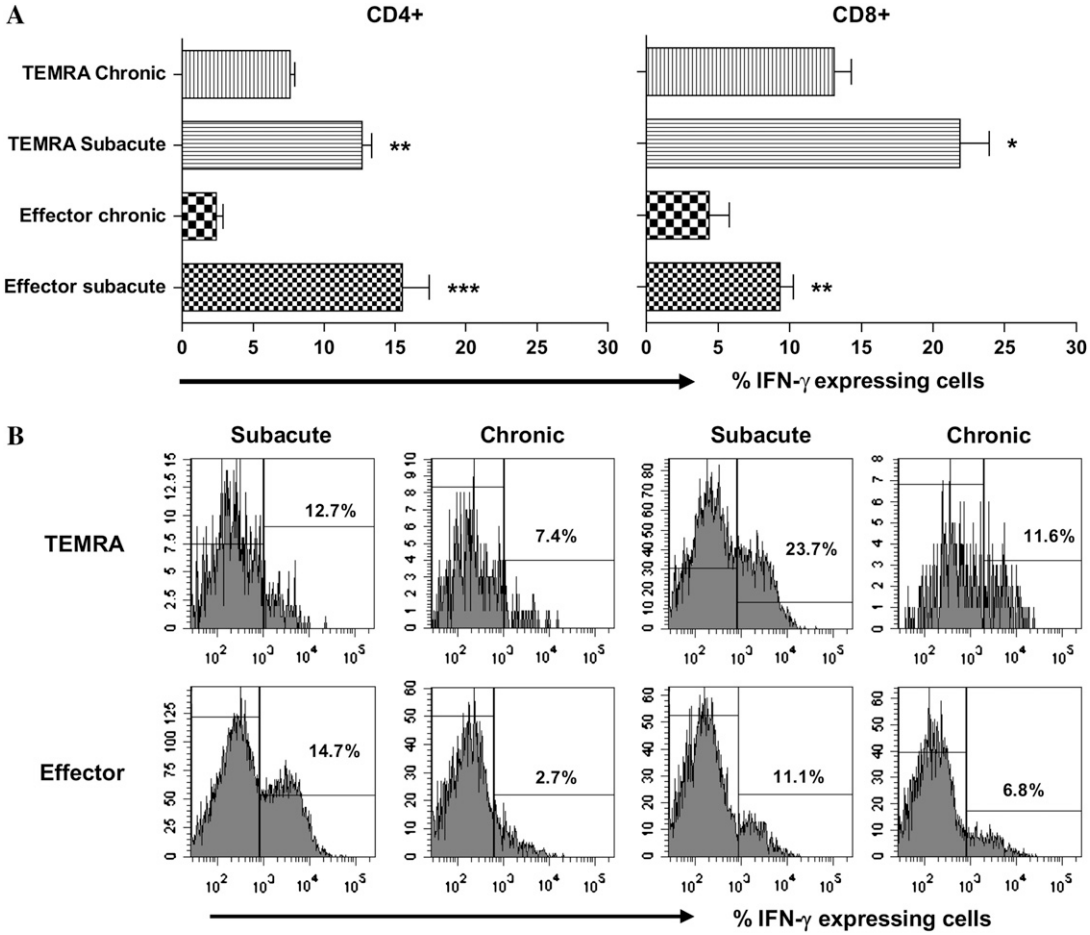

Figure 3. Functional assessment of effector and TEMRA bronchoalveolar lavage (BAL) T cells. (*A*) The effector and TEMRA subsets were gated and analyzed for IFN-γ expression after 6 hours stimulation with phorbol myristate acetate/ionomycin. IFN-γ–positive cells are expressed as a percentage of $CD4^+$ or $CD8^+$ effector and TEMRA BAL T cells. *Error bars* represent 1 SD of five experiments. (*B*) A representative plot illustrating the increase of IFN-γ–positive cells in patients with subacute HP. $*P < 0.001$; $**P < 0.01$; $***P < 0.05$.

in patients with diverse clinical forms of the disease have not been characterized.

The results from this study reveal a number of likely pathogenic-associated differences in the T-cell subsets among patients with subacute and chronic HP. Patients with chronic HP exhibited lower BAL lymphocytosis, with an increase of the $CD4^+$:$CD8^+$ ratio compared with those with subacute disease. Furthermore, patients with subacute HP who worsened dis-

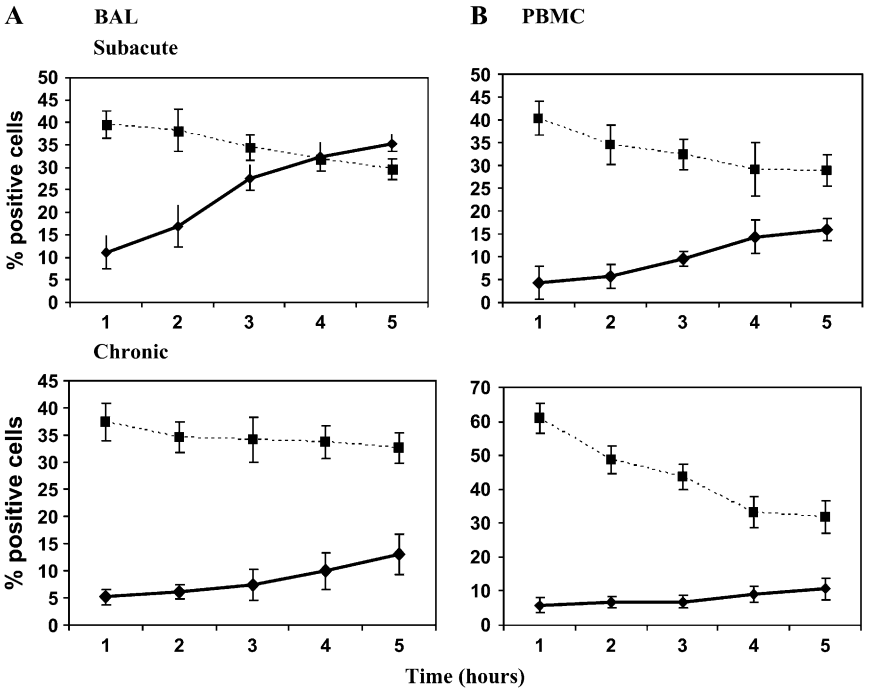

Figure 4. Perforin release and its correlation with surface expression of CD107 from bronchoalveolar lavage (BAL) (*A*) and peripheral blood mononuclear cells (PBMCs) (*B*). T cells from patients with subacute hypersensitivity pneumonitis (HP) and those with chronic HP were stimulated with phorbol myristate acetate/ionomycin *in vitro* for up to 5 hours in the presence of anti-CD107a/b APC and brefeldin/monesin. At time points designated, aliquots were removed, washed, and permeabilized, followed by staining for perforin, CD3, and CD8. Accumulative data of four independent experiments are shown. *Error bars* represent 1 SD. *Solid diamonds, solid line*: CD107a/b+; *solid squares, dotted line*: perforin+.

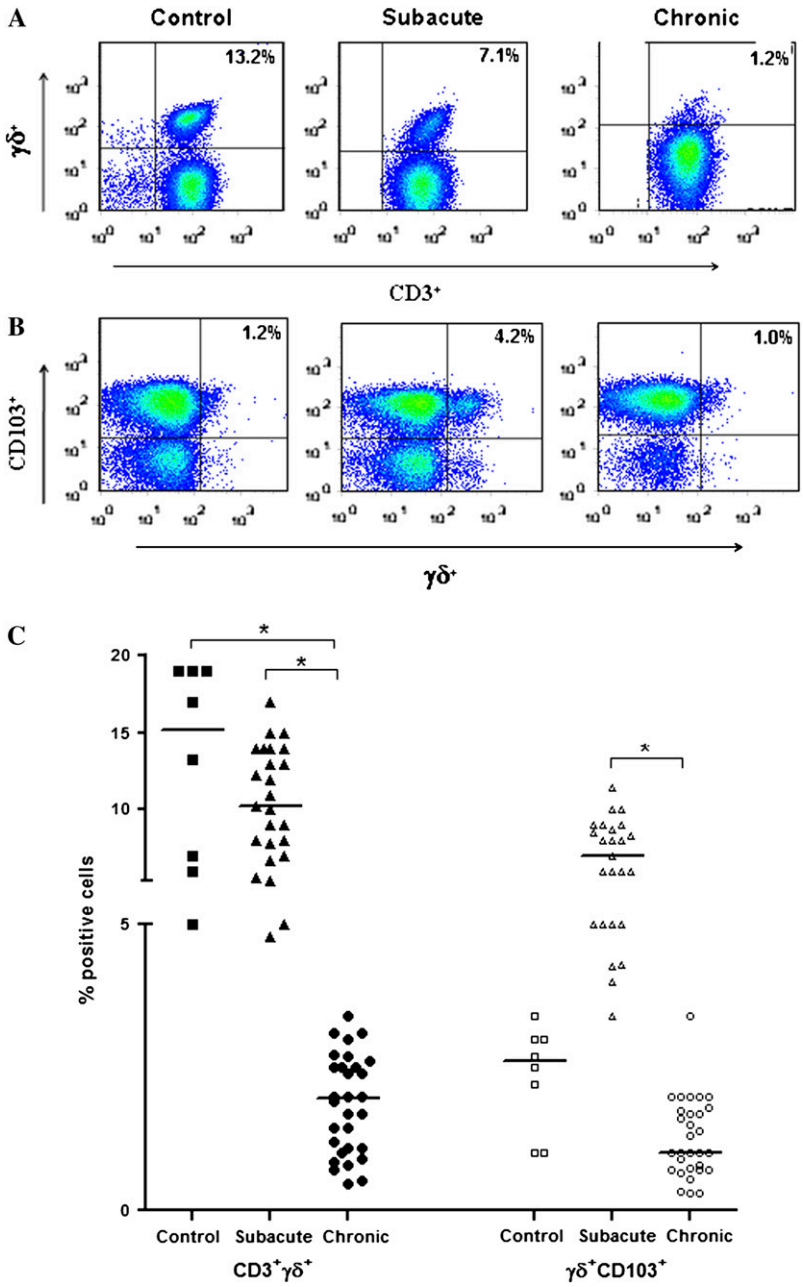

Figure 5. γδT cells and intraepithelial γδT cells in bronchoalveolar lavage. (*A* and *B*) Representative dot plots of γδT cells and CD103$^+$ subsets from 8 healthy control subjects, 25 patients with subacute hypersensitivity pneumonitis (HP), and 30 with chronic HP. (*C*) Vertical scatter plot showing the individual percentages of γδT cells and γδT intraepithelial (CD103$^+$) cells. *Horizontal lines* represents median values; *$P < 0.01$.

played high CD4$^+$:CD8$^+$ ratios. Our findings suggest that the predominance of CD8$^+$ T cells, previously reported in this disease, relates primarily to the more active (predominantly inflammatory and potentially treatable) phase of the disease (6). Supporting this hypothesis, it has been reported that CD8$^+$ T lymphocytes are significantly increased in patients with HP with acute onset, whereas insidious onset was related to lung fibrosis and relatively increased CD4$^+$ T lymphocytes in BAL fluids (26). The reason that an increase of lung CD4$^+$ T cells may be associated with chronic/fibrotic HP is unclear, but it may be related to a concomitant Th2 CD4$^+$ T-cell–dependent mechanism. Actually, a Th2-like response was observed in patients with chronic HP compared with those with subacute HP. The increase of the BAL CD4$^+$:CD8$^+$ ratio and the switch from Th1 to Th2 microenvironment in patients with chronic HP appears to be a pattern that characterizes a late stage of the disease.

In addition, patients with chronic HP also showed a decrease of γδT cells without a change in the percentage of intraepithelial γδT cells, whereas a significant increase of the latter was observed in subacute disease. γδT cells constitute a separate lineage of T lymphocytes, which differ from conventional αβT lymphocytes with regard to T-cell receptor repertoire and tissue localization. The γδT-cell subset accounts for a number of immunoregulatory activities and constitutes an integral part of the epithelial defense mechanisms (27). Therefore, the decrease of γδT-cell subpopulation in chronic disease may have a deleterious effect on lung reepithelialization, as it has been found in idiopathic pulmonary fibrosis (28). Also, a defect in this T-cell subpopulation activity may have an effect on the CD4$^+$ and CD8$^+$ T lymphocyte functions. In this regard, functional assays in normal and γδT-cell–deficient mice have demonstrated that chronic inhalational exposure to bacterial antigens results in the development of peribronchovascular inflammation, with large numbers of CD4$^+$

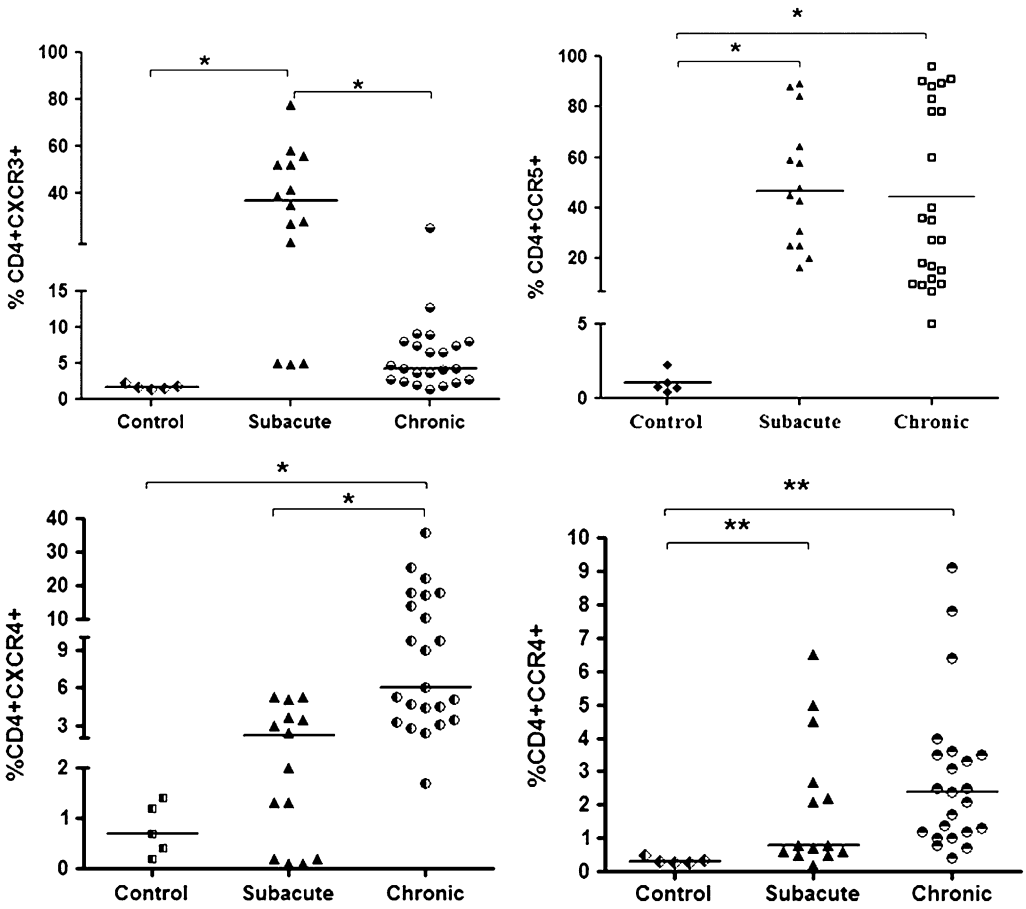

Figure 6. Expression of CXCR3, CCR5, CXCR4, and CCR4 in bronchoalveolar lavage (BAL) T cells from healthy control subjects, patients with subacute hypersensivity pneumonitis (HP), and those with chronic HP. BAL T cells were incubated with the specific monoclonal antibodies and fixed in 1% paraformaldehyde for analysis in six-color flow cytometry. *Horizontal lines* represent median values; $*P < 0.01$; $**P < 0.05$.

and $CD8^+$ T cells (29). Moreover, γδT-cell–deficient mice treated with live *Bacillus subtilis* showed an accelerated collagen deposition, suggesting that these cells play a protective antifibrotic role (29).

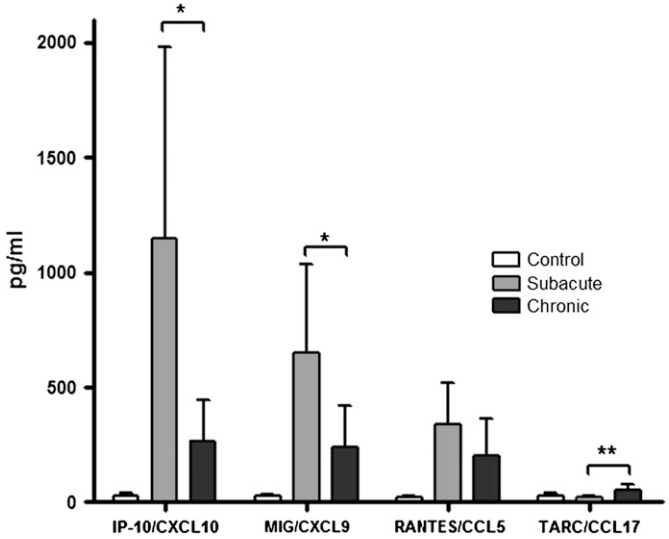

Figure 7. Bronchoalveolar lavage (BAL) concentrations of IFN-γ–inducible protein (IP)-10/CXCL10, monokine induced by IFN-γ (MIG)/CXCL9, RANTES (regulated upon activation, normal T-cell expressed and secreted)/CCL5, and thymus- and activation-regulated chemokine (TARC)/CCL17 in healthy control subjects, patients with subacute hypersensitivity pneumonitis (HP), and those with chronic HP. BAL levels of chemokines were measured by ELISA as described in METHODS. Data represent means and SD; $*P < 0.01$; $**P < 0.05$.

Recent experimental and clinical evidence suggest that a Th1-type cytokine network plays an important role in HP (10, 30, 31). Likewise, it has been suggested that Th1 cell activity may decline while Th2 activity increases (Th1/Th2 switch hypothesis) in HP lungs evolving to fibrosis (1, 32). It is well known that the Th2 cytokines, IL-4 and IL-13, enhance the fibrotic process by induction of fibroblast proliferation and collagen production (33, 34), whereas IFN-γ, a Th1 cytokine, inhibits these processes (17). In this context, it has been recently demonstrated that mice overexpressing GATA binding protein 3 (GATA-3), a transcription factor that encourages Th2 responses (35–37), develop a more severe bleomycin-induced pulmonary fibrosis concomitant with a significant decrease of IFN-γ in the lungs (38). With this hypothesis in mind, we evaluated the predominance of Th1 or Th2 profiles in subacute and chronic HP using several approaches. Our results showed that patients with subacute disease have a Th1-like response, whereas patients with chronic HP express predominantly a Th2-like phenotype. Thus, BAL lymphocytes from patients with subacute HP, stimulated *in vitro* with avian antigens, expressed and released significantly higher levels of Th1 cytokines (IFN-γ) and lower levels of Th2 cytokines (IL-4) compared with those with chronic disease. Likewise, we also found a significant increase of lymphocytes expressing CXCR3 on T cells, as well as the levels of the CXCR3 ligands, IP-10/CXCL10 and MIG/CXCL9, in BAL supernatants of patients with subacute HP. These chemotactic ligands might contribute to the directional migration of activated T cells and accumulation of cytotoxic T lymphocytes in the lung in early stages of the disease. By contrast, T cells expressing CXCR4 (Th2) and TARC were increased in patients with chronic HP. Taken together, these findings raise the possibility that a Th1-to-Th2 switch may play

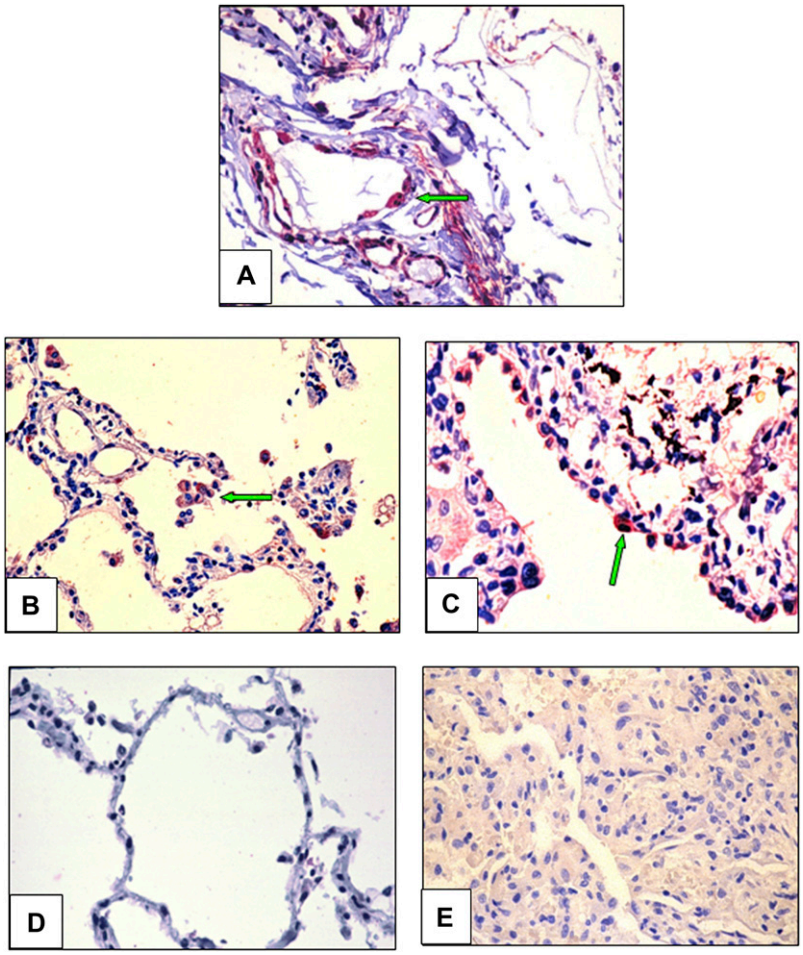

Figure 8. Immunolocalization of IP-10/CXCL10 and MIG/CXCL9 in hypersensitivity pneumonitis (HP) lungs. Immunoreactive CXCL9 and CXCL10 proteins were revealed with 3-amino-9-ethyl-carbazole, and biopsy samples were counterstained with hematoxylin. (*A*) CXCL9 was observed primarily in endothelial cells (*arrow*). (*B*) CXCL10 staining in alveolar macrophages (*arrow*). (*C*) Strong CXCL10 staining in alveolar epithelial cells (*arrow*). (*D*) Normal lung showing no CXCL10 labeling. (*E*) Negative control section from HP lung in which the primary antibody was omitted. Original magnification, ×40.

a role in the fibrotic response that characterizes chronic HP (3, 39).

In the present study, both patients with subacute HP and those with chronic HP exhibited a decrease in the percentage of NKT cells without changes in the subset, *i*NKT (Vα24*i*NKT). Also no changes in the number of BAL NK cells were observed. The physiologic role of NKT cells remains uncertain, but they have been associated with a number of beneficial immune responses, including host protection from a variety of infectious agents, the prevention of autoimmune diseases, and the maintenance of self-tolerance (40). NKT cells may skew the immunity to Th1- or Th2-like reaction, enhancing, in each case, a favorable antiinflammatory response (41, 42). Therefore, the decrease of this T-cell subset may also have an important role in the development of the uncontrolled immune reaction that characterizes HP. Supporting this hypothesis, it has been recently demonstrated that NKT cells attenuate bleomycin-induced pulmonary inflammation and fibrosis, and furthermore, that deficiency of NKT and *i*NKT cells in mice aggravates *Saccharopolyspora rectivirgula*–induced HP (43, 44). However, no differences were found with the subset of Vα24*i*NKT among control subjects and either patients with subacute HP or those with chronic HP. This subpopulation recognizes glycolipids (α-GalCer) presented by the major histocompatibility complex–like molecule CD1d. By contrast, the conventional NKT are major histocompatibility complex class I– or class II–restricted T cells that up-regulate NK-cell marker upon activation (45). NKT cells appear to regulate immunosurveillance and a variety of immunopathological processes, but the mechanisms and the ligands involved remain unknown. Thus, whether the decreased number of NKT cells (without changes in *i*NKT cells) contributes to the development of HP requires further study. Recent findings suggest the existence of different subsets of NKT cells that may have distinct functional capabilities, although these functional differences remain unclear (46). In this context, our findings suggest that decreased numbers of NKT cells in patients with HP may be due to a decreased number of other NKT cell subpopulations (i.e., type II, noninvariant NKT cells).

Memory T-cell differentiation proceeds along distinct pathways after an acute versus chronic viral infection (47). Memory T cells generated after an acute infection are highly functional and constitute an important component of protective immunity. In contrast, chronic infections are often characterized by varying degrees of functional impairment of antigen T-cell responses, and this defect is a principal reason for the inability of the host to eliminate the persisting pathogen. Although functional effector T cells are initially generated during the early stages of infection, they gradually lose function during the course of the chronic infection. This exhaustion of virus-specific T cells was first shown during persistent LCMV infection of mice (48, 49). However, these findings were quickly extended to other model systems, as well as to chronic infections in humans (50–52).

Data reported here show quantitative and qualitative differences in the distribution of these different T-cell subsets between patients with subacute HP and those with chronic HP. Patients with chronic disease displayed an increase in the percentage of the terminally differentiated subset of memory

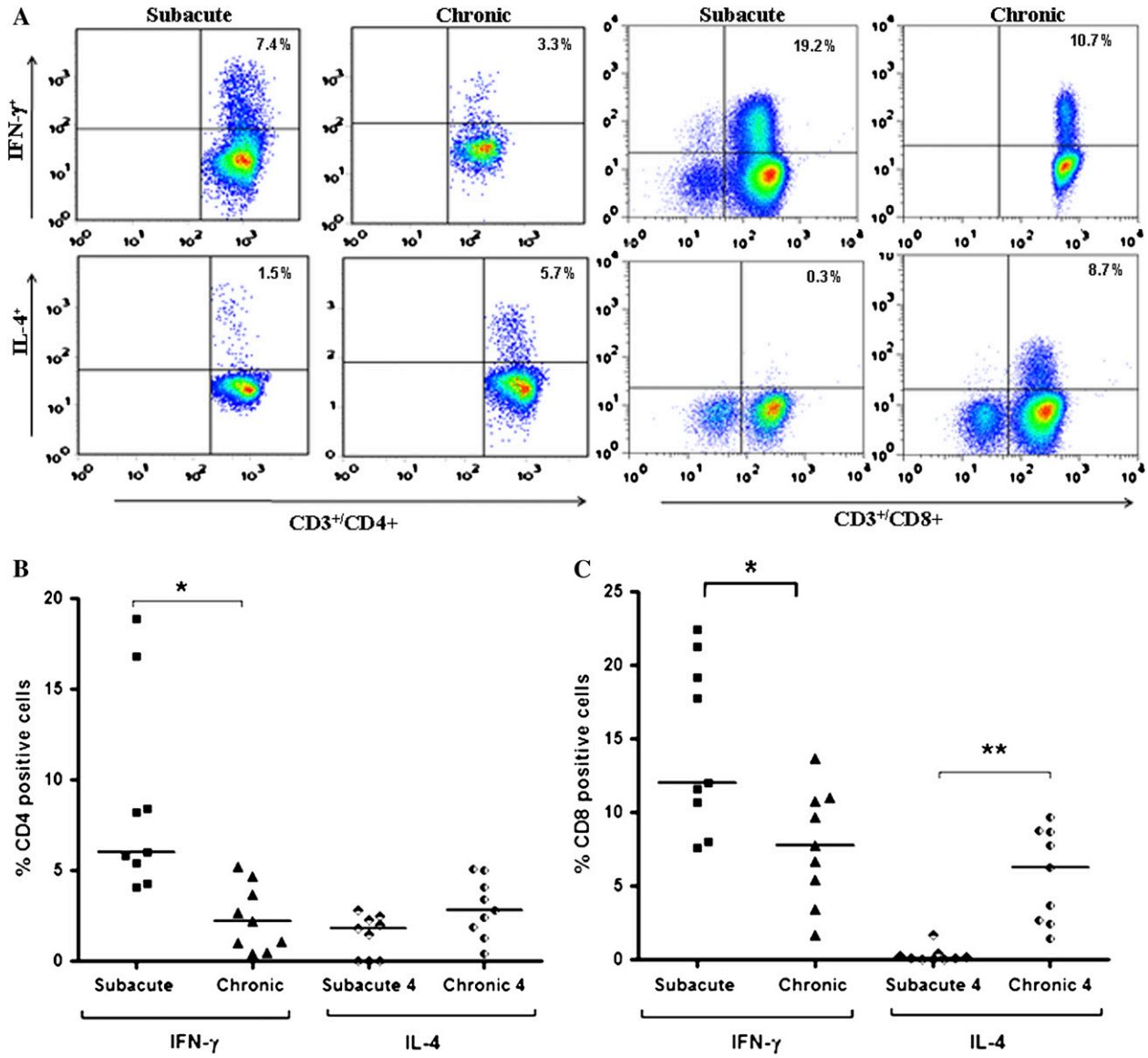

Figure 9. Intracellular cytokine expression in patients with subacute hypersensitivity pneumonitis (HP) and those with chronic HP. (*A*) Representative plots of IFN-γ and IL-4 production within $CD4^+$ and $CD8^+$ T lymphocytes from bronchoalveolar lavage in patients with subacute HP and those with chronic HP. Results are presented as percentage of double-positive cytokine expressing $CD4^+$ T lymphocytes. (*B* and *C*) Percentage of $CD4^+$ and $CD8^+$ T cells producing IFN-γ and IL-4 upon antigen-specific stimulation with pigeon serum after 6-hour incubation. Each *symbol* characterizes one individual patient. *Lines* represent median values; $*P < 0.01$; $**P < 0.05$.

(TEMRA) $CD4^+$ and $CD8^+$ cells compared with patients with subacute disease, but with a decrease of effector and TEMRA $CD4^+$ and $CD8^+$ memory cells expressing intracellular IFN-γ. This finding suggests that, in spite of the increase in number of this memory population, it becomes an exhausted antigen-specific T-cell lineage. Interestingly, increased numbers of TEMRA cells were found in peripheral blood compared with the lung, suggesting systemic immune activation. Similar findings have been recently described in other inflammatory diseases (53).

We also measured the ability of $CD8^+$ T cells to degranulate by monitoring the appearance of lysosomal markers, CD107a/b, on the cell surface after stimulation and the loss of intracellular perforin. This correlation was only observed in patients with subacute HP. Similar behavior of T-cell dysfunction was described in an LCMV mouse model, and was attributed to a selective up-regulation of the receptor, programmed death-1 (17). Likewise, a recent report demonstrated that, in adult T-cell leukemia/lymphoma, $CD4^+$ T cells are the main programmed death-1–expressing cells, and may also be found exhausted as a result of its expression (54).

Our data suggest that patients with chronic HP (exposed patients with > 2 yr of symptoms) may show skewing/exhaustion of $CD8^+$ T cells and $CD4^+$ T cells, which may occur because of the chronicity of the pathological process or because patients continue to be exposed to avian antigens. Unfortunately, despite emphasis to remove the antigen source (typically birds), some patients remain exposed. Also, there are many public spaces in Mexico where birds arrive and stay, and some patients may have additional chronic exposure. Importantly, experimental studies have demonstrated that clonally exhausted virus-specific cytotoxic T lymphocytes are unable to control or eliminate viral infection and lose their cytotoxic and cytokine secretion activity (55). These observations in chronic infections and our findings in antigen-driven lung inflammation may help

us to understand the dynamics between the T-cell cytotoxic populations and chronic antigen exposure in human lung disease.

In summary, the results of this study indicate that patients with chronic HP have different phenotypic and functional BAL T-cell subsets compared with patients with subacute disease. These differences include an increase in $CD4^+$:$CD8^+$ ratio, a decrease of γδT cells, a skewing toward Th2 as opposed to Th1, and exhaustion of effector $CD8^+$ and $CD4^+$ T cells. These findings may provide insight in the development of new therapeutic strategies, such as the inhibition of GATA-3 transcription factor to modify the Th1/Th2 cell balance to enhance a Th1-like lung microenvironment.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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