

Involvement of CC chemokines in $\gamma\delta$ T lymphocyte trafficking during allergic inflammation: the role of CCL2/CCR2 pathway

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Abstract

In the present study, we show that the intra-thoracic injection of ovalbumin (OVA, 12.5 μ g per cavity) into C57BL/10 mice induced a significant increase in $\gamma\delta$ T lymphocyte numbers in the pleural cavity, blood and thoracic lymph node of challenged mice. Such increase was significant within 12 h, peaked within 48 h and returned to basal counts within 120 h. Levels of CC chemokine ligand (CCL)-2/monocyte chemoattractant protein-1, CCL5/regulated upon activation, normal T cell expressed and secreted, CCL3/macrophage inflammatory protein-1 α and CCL25/thymus-expressed chemokine were above control values in pleural washes recovered 24 h after OVA challenge (OPW) and were likely produced by pleural macrophages and mesothelial cells. Antigenic challenge also induced an up-regulation in CC chemokine receptor (CCR)-2, CCR5 and CCR9 on $\gamma\delta$ T cells from pleural cavities, blood and lymph nodes, suggesting that cells found in mice pleural cavity migrate from secondary lymphoid organs into the inflammatory site via blood stream. The *in vitro* neutralization of CCL2 (but not of CCL3, CCL5 or CCL25) abrogated OPW-induced $\gamma\delta$ T lymphocyte transmigration. Confirming such results, the *in vivo* administration of α -CCL2 mAb inhibited $\gamma\delta$ T lymphocyte accumulation in the pleural cavity of challenged mice, whereas the blockade of CCL3, CCL5 or CCL25 showed no effect on $\gamma\delta$ T cell mobilization. In addition, OVA challenge failed to induce $\gamma\delta$ T lymphocyte accumulation in the pleural cavity of C57BL/6 CCR2 knockout mice, which also showed decreased numbers of these cells in blood and lymph nodes when compared with wild-type mice. Overall, such results demonstrate that CCR2/CCL2 pathway is crucial for $\gamma\delta$ T lymphocyte mobilization during the allergic response.

Introduction

$\gamma\delta$ T cells are unique cells of the immune system, believed to constitute a link between innate and acquired systems. They comprise a minor subset of T lymphocytes and are preferentially localized in non-lymphoid tissues, such as epithelial and mucosal tissues. Such tissue colonization is one of the characteristics that renders resident $\gamma\delta$ T cells crucial roles in first line of defense against infections, tissue damage or stress, providing a rapid and tissue-specific immune response, without the need of antigen presentation or clonal expansion (1). The diversity of TCRs composed by γ and δ chains is usually limited; however, they can exclusively recognize a wide range of pathogenic antigens, such as bacterial phosphoantigens, alkylamines, phosphorylated isoprenoid precursors, lipid molecules and LPS (2). Compelling

data demonstrate the involvement of $\gamma\delta$ T cells in response to infectious diseases; however, its role in non-infectious diseases is also relevant.

Specific subsets of $\gamma\delta$ T cells are shown to be distributed along the airway tissue and play an important role in normal airway function maintenance (3, 4). In addition, $\gamma\delta$ T cells have been shown to accumulate in the airways during allergic inflammation; however, data concerning their contribution to the allergic response is still poor and contradictory, attributing either pro-inflammatory or regulatory roles for these cells. A few reports show that, in animal models and in allergic subjects, $\gamma\delta$ T cells contribute for eosinophil infiltration, as well as for IgE synthesis via the regulation of IL-5 and IL-4 production, as shown by means of the use of knockout (KO)

mice and antibodies treatment (5–8). On the other hand, conflicting results have demonstrated an important anti-inflammatory effect of these cells, through the negative regulation of airway hyperresponsiveness, eosinophil influx and IgE production (4, 9, 10). Subsequent clarifying studies reported by Hahn and coworkers (11, 12) have demonstrated that such distinct effector and regulatory functions might rely on specific subsets of $\gamma\delta$ T cells, on tissue distribution and on stage of the immune response.

The migration of $\gamma\delta$ T lymphocytes into tissues is straightly controlled by chemoattractant factors, such as chemokines. Therefore, in parallel with the pattern of chemokine production, the expression of chemokine receptors is determinant for lymphocyte trafficking during physiological and inflammatory conditions. Several members of the CC and CXC chemokine families and their counterpart receptors have been shown to account for $\gamma\delta$ T lymphocyte recruitment in different responses induced by pathogenic antigens. Following activation, stimuli such as isopentenyl pyrophosphate and *Mycobacterium tuberculosis* are capable to induce the up- or down-regulation of chemokine receptors for CC and CXC ligands on $\gamma\delta$ T cells (13, 14). It has been shown by *in vitro* assays that $\gamma\delta$ T cells migrate towards chemokines, such as CC chemokine ligand (CCL)-5/regulated upon activation, normal T cell expressed and secreted (RANTES), CCL3/macrophage inflammatory protein (MIP)-1 α , CCL4/MIP-1 β and CCL2/monocyte chemoattractant protein (MCP)-1 (15, 16). However, there is only a small amount of *in vivo* evidence concerning the participation of inflammatory CC chemokines in $\gamma\delta$ T lymphocyte migration (17, 18). Moreover, all these data are restricted to pathogenic inflammatory reactions, and the role of CC chemokines in allergic inflammation remains to be investigated. In the present study, we describe the involvement of CC chemokines and their respective receptors on the coordination of $\gamma\delta$ T cell migration during the allergic response, by means of chemokine neutralization and the use of KO mice.

Methods

Animals

C57BL/10 and C57BL/6 mice (18–20 g) were provided by Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil). C57BL/6-CC chemokine receptor (CCR)-2 KO mice were generated by W. A. Kuziel (19; Protein Design Laboratories, Fremont, CA, USA). KO mice were obtained as breeding pairs, with all subsequent breeding and husbandry of animals taken under specific pathogen-free conditions at Fundação Oswaldo Cruz (Fiocruz, Brazil). Mice were caged with free access to food and fresh water in a room at temperature ranging from 22 to 24°C and in a 12-h light/dark cycle at Farmanguinhos experimental animal facility, until used. All experimental procedures were performed according to The Committee on Ethical Use of Laboratory Animals of Fundação Oswaldo Cruz.

Antibodies and reagents

Ovalbumin (OVA), PBS, RPMI 1640, saponin, Tween 20, sodium citrate, *o*-phenylenediamine dihydrochloride, EDTA,

BSA, phosphate citrate buffer, RBC lysing buffer, ionomycin, phorbol myristate acetate (PMA), collagenase, gentamicin, monensin and goat anti-mouse IgG were purchased from Sigma–Aldrich (St Louis, MO, USA). Medium 199 and fetal bovine serum were obtained from Hy-Clone (Logan, UT, USA). Endothelium growth factor was purchased from Invitrogen (Carlsbad, CA, USA). Aluminum hydroxide was purchased from EMS Sigma Pharma (São Paulo, Brazil). Purified and biotinylated anti-murine CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES and CCL25/thymus-expressed chemokine mAbs, and their respective recombinants, were all obtained from R&D Systems (Minneapolis, MN, USA). FITC-conjugated hamster IgG anti-murine CD3 and PE-conjugated hamster IgG anti-murine $\gamma\delta$ TCR mAbs, goat polyclonal IgG anti-murine CCR2, CCR5 and CCR9 bovine anti-goat biotinylated IgG were all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Murine IL-4, streptavidin-conjugated Cy-chrome and rat FITC-conjugated mAb anti-CD11b/MAC-1 were purchased from PharMingen (San Diego, CA, USA).

Allergic pleurisy

Active sensitization was achieved by a subcutaneous (s.c.) injection of 200 μ l of a mixture of OVA (50 μ g) and aluminum hydroxide (5 mg). Fourteen days later, pleurisy was induced in mice as described by Henriques *et al.* (20). An adapted needle (13 $\times$ 0.45 mm) was inserted carefully through 1 mm of the right side of the thoracic cavity of mice to enable the intra-thoracic (i.t.) injection of OVA (12.5 μ g per cavity) diluted in sterile PBS to a final volume of 100 μ l. Sensitized mice injected with saline vehicle alone or unsensitized mice injected with OVA (12.5 μ g per cavity) were used as negative control groups. At specific time points after stimulus, mice were killed in a carbon dioxide chamber. Pleural cells were recovered from thoracic cavities after washing with 1 ml of saline containing EDTA (10 mM), pH 7.4. Blood was obtained from mice abdominal aorta and submitted to a Histopaque-1077 gradient (400 $\times$ g for 30 min) for PBMCs separation. Thoracic lymph nodes were harvested and mechanically disrupted into single-cell suspensions after centrifugation (400 $\times$ g for 10 min). For transmigration assays, pleural washes recovered from mice injected with saline (SPW, n = 20) or OVA (OPW, n = 30) were pooled after washing thoracic cavities with 500 μ l of saline/EDTA (10 mM). SPW and OPW were centrifuged (420 $\times$ g for 10 min at 20°C), and cell-free supernatant was recovered and kept at –20°C until used.

Pleural mesothelial cell culture

Mesothelial cells were recovered from C57BL/10 mice pleura 14 days after s.c. sensitization. Animals received an i.t. injection of collagenase (200 IU ml^{–1}, 200 μ l), and 30 min later, their thoracic cavities were washed (sterile medium 199 supplemented with 10% fetal bovine serum, 40 ng ml^{–1} hydrocortisone, 25 μ g ml^{–1} gentamicin and 20 ng ml^{–1} epidermal growth factor). Pleural washes were centrifuged (1100 $\times$ g, 10 min), and the cells were suspended in medium 199 and cultured in Petri dishes for 10 min in 5% CO₂

at 37°C in order to eliminate adherent fibroblasts and macrophages. The enriched mesothelial cell suspension was cultured in 75-cm³ bottles for at least 2 weeks until confluence, when these cells were trypsinized and washed for counting. At least 90% of cells were positive for cytokeratin 7 (data not shown), a cell-surface marker that characterizes mesothelial cells (21). Cells (4×10^5 per well) were stimulated in a 24-well cell culture plate with IL-4 (40 ng ml⁻¹), a representative T_H2 cytokine, shown to favor chemokine production (22). Supernatants were recovered after 12 h for CCL25 evaluation by ELISA.

ELISA

Levels of CCL2, CCL5, CCL3 and CCL25 in the cell-free pleural fluid or supernatant were evaluated by sandwich ELISA by using matched antibody pairs from R&D Systems according to the manufacturer's instructions. Results are expressed as picograms per cavity.

Leukocyte counts

Total leukocyte counts were made in Neubauer chamber, under an optical microscope, after dilution in Türk fluid (2% acetic acid). Counts are reported as numbers of cells per cavity for pleural lymphocytes and as cells per microliter (mm³) for blood lymphocytes. Lymph nodes lymphocyte counts are expressed as cells per milligram of lymphoid tissue.

Flow cytometric analysis of phenotype and chemokine receptor expression

Cells recovered from pleural cavities, thoracic lymph nodes and peripheral blood (10^6 cells per 100 μ l) were incubated with the appropriate concentration of purified goat polyclonal antibodies (pAbs) anti-murine CCR2, CCR5, CCR9 or IgG isotype control for 30 min at 4°C, after incubation with rat serum to block non-specific binding. For CCRs staining, bovine anti-goat biotinylated IgG followed by streptavidin–Cy-chrome (1:250) were added to all wells, including the ones containing IgG used as irrelevant primary antibody. Cells were then incubated with FITC- or PE-conjugated mAbs to CD3, $\gamma\delta$ TCR for 30 min and washed with PBS/0.1% azide. Surface-marker analysis was performed using the Cell Quest program in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). At least 10^4 lymphocytes were acquired per sample. All data were collected, displayed on a log scale of increasing fluorescence intensity and presented as histograms. To determine the percentages of $\gamma\delta$ lymphocyte sub-population, CD3⁺ T lymphocytes were specifically gated. Lymphocyte counts are reported as numbers of cells per cavity after multiplying the percentage of $\gamma\delta$ T lymphocytes by the total number of pleural leukocytes.

Intracellular chemokine immunostaining

Cells recovered from mice pleural cavities were re-suspended in RPMI 1640 supplemented with 10% fetal bovine serum (2.5×10^6 ml⁻¹) and incubated with or without ionomycin (500 ng ml⁻¹), PMA (20 ng ml⁻¹) and monensin (2 μ M) for 4 h at 37°C at 5% CO₂. Then, after incubation with

rat serum to block non-specific binding, cells were incubated with the appropriate concentration of FITC-conjugated mAb anti-CD11b/MAC-1 for 30 min at 4°C. Subsequently, after PBS/0.1% azide washing, cells were fixed with 4% PFA for 20 min at 4°C and incubated with 0.1% saponin in PBS/0.1% azide/10% fetal bovine serum. Goat biotinylated anti-murine CCL2, CCL5, CCL3 and CCL25 antibodies were added (1:100) to the cell suspension and incubated for 30 min at 4°C, followed by streptavidin–Cy-chrome (1:250). Analysis was performed using the Cell Quest program in a FACSCalibur flow cytometer (Becton Dickinson). Macrophages were defined as CD11b/MAC-1⁺ cells in an forward scatter/side scatter gate. At least 10^4 cells were acquired per sample. Chemokine-positive CD11b/MAC-1⁺ macrophages were calculated after multiplying the percentage of FL1/FL3 dot plots by the total number of pleural leukocytes.

In vivo neutralization of chemokines

Animals received an i.p. injection of anti-murine CCL2, CCL3, CCL5 or CCL25 mAbs at 10 μ g per cavity in a final volume of 100 μ l, 45 min before OVA stimulation as previously described (17). Control mice received 10 μ g of rat IgG antibody (i.t.) per cavity.

Transwell migration assay

Transwell migration assay was performed with C57BL/10 mice spleen cells. Spleen was dissected, macerated in washing buffer (HBSS without Ca²⁺/Mg²⁺ containing 30 mM HEPES, 0.25% BSA, pH 7.4) and centrifuged ($420 \times g$ for 10 min at 20°C). The cell pellet was re-suspended in 5 ml of RBC lysing buffer (Sigma–Aldrich) for 2 min. After centrifugation ($200 \times g$ for 10 min at 20°C), the leukocyte pellet was re-suspended in assay buffer (HBSS with Ca²⁺/Mg²⁺ containing 30 mM HEPES and 0.25% BSA, pH 7.4). Spleen leukocytes (3×10^6 cells in 200 μ l of assay buffer) were added to the upper chamber of 3.0- μ m pore diameter transwell tissue culture inserts (Falcon, CA, USA) that were placed in individual wells of a 24-well cell culture plate containing 300 μ l of assay buffer, stimulus (CCL2, 2.5 ng per well; CCL3, 4 ng per well; CCL5, 4 ng per well; CCL25, 40 ng per well, OPW or SPW) or stimulus plus anti-CCL2 mAb (2.5 ng per well), anti-CCL3 mAb (200 ng per well), anti-CCL5 mAb (20 ng per well) or anti-CCL25 mAb (400 ng per well). Plates were incubated for 60 min at 37°C and 5% CO₂. Cells that migrated into the bottom chamber were recovered and labeled as CD3 and $\gamma\delta$ TCR, as described above, and analysed using a flow cytometer (FACSCalibur flow cytometer, Becton Dickinson) by acquiring events at a constant flow for a set time of 180 s. Results are expressed as chemotactic index, generated by using the number of cells that migrated towards buffer as comparison.

Statistical analysis

Data were reported as the mean $\pm$ SEM and were analysed statistically by means of analysis of variance followed by Student–Newman–Keuls test or Student's *t*-test with the level of significance set at $P \leq 0.05$.

Results

Kinetics of OVA-induced CD3⁺ and $\gamma\delta$ T lymphocyte accumulation in mice pleural cavity

The antigenic challenge induced by the i.t. injection of OVA (12.5 μ g per cavity) into previously sensitized mice induced a significant increase in CD3 T lymphocyte numbers 12 h after stimulation (saline: 21.0 ± 3.0 versus OVA: 43.2 ± 9.03 CD3 T lymphocytes $\times 10^3$ per cavity, $P = 0.04$), which was maximal within 48 h and remained significantly above control levels until 6 days (Fig. 1A). Among CD3⁺ T lymphocytes, OVA challenge also triggered a significant accumulation of $\gamma\delta$ T lymphocytes within 12 h that peaked within 48 h, and returned to basal levels 5 days after antigenic challenge (Fig. 1B). Of note, OVA (12.5 μ g per cavity) i.t. administration into non-sensitized mice failed to induce accumulation of total leukocytes (saline: 3.8 ± 0.6 versus OVA: $3.8 \pm 0.0 \times 10^6$ cells per cavity, $P = 1.0$), CD3⁺ T cells (saline: 79.4 ± 9.3 versus OVA: $98.9 \pm 31.5 \times 10^3$ cells per cavity, $P = 0.62$) or $\gamma\delta$ T lymphocytes (saline: 1.2 ± 0.1 versus OVA: $1.3 \pm 0.4 \times 10^3$ cells per cavity, $P = 0.8$), as observed within 24 h.

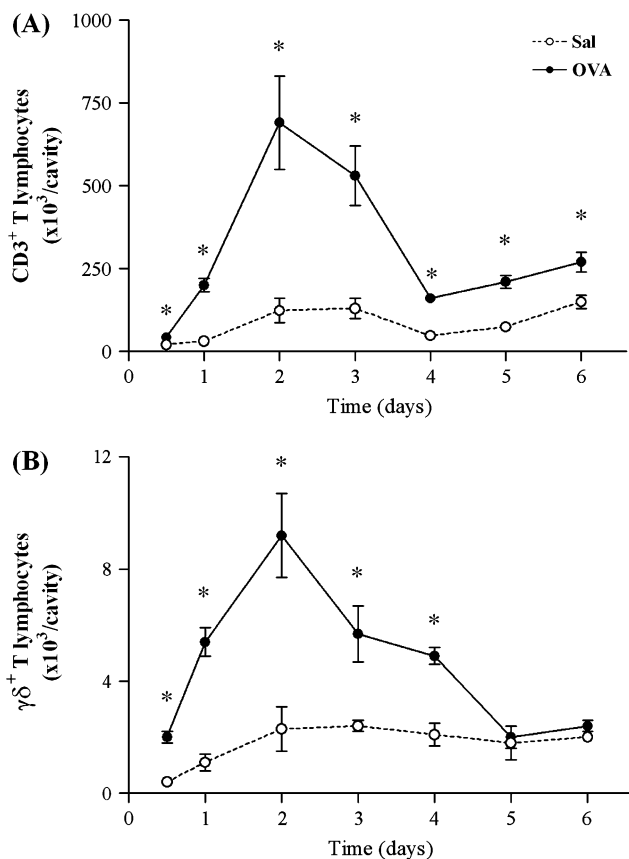


Fig. 1. Time course of CD3 T lymphocyte (A) and $\gamma\delta$ T lymphocyte (B) accumulation after the i.t. injection of OVA (12.5 μ g per cavity) in the pleural cavity of previously sensitized mice. Results are expressed as the mean $\pm$ SEM from at least eight animals per group of two different experiments. Statistically significant differences between saline- and OVA-injected groups ($P \leq 0.05$) are indicated by an asterisk.

Analysis of CC chemokine production in mice pleural cavity after OVA stimulation

Based on the knowledge that chemokines play a crucial role in providing direction for T cell trafficking during the allergic response, we evaluated chemokine levels in 24 h cell-free pleural washes recovered from mice, a time point in which an important increase in $\gamma\delta$ T lymphocyte numbers was observed. As shown in Fig. 2, OVA induced a significant increase in CCL2 (A), CCL5 (B), CCL3 (C) and CCL25 (D) protein levels detected in the 24-h OPW, as determined by ELISA. We further investigated if macrophages produced these chemokines, since they represent an important source of chemoattractant factors during the allergic response. Intracellular immunostaining of CD11b/MAC-1⁺ macrophages revealed an important increase in the numbers of CCL2-, CCL5- and CCL3-positive cells in the pleural cavities of OVA-stimulated mice (Fig. 3A, B and C, respectively). However, macrophages are unlikely the major producer cells of CCL25, since we observed a great variability among challenge animals that led to a slight but not significant increase in such chemokine levels (Fig. 3D). Chemokine intracellular staining in macrophages without PMA/ionomycin treatment showed very weak staining, but still revealed higher values in OVA group than in saline, demonstrating that re-stimulation procedure is effective to amplify the signal. Therefore, we performed an *in vitro* culture of pleural mesothelial cells recovered from previously sensitized mice, and measured CCL25 content in the monolayer supernatant after IL-4 stimulation, a T_H2 cytokine, shown to favor chemokine production by mesothelial cells (22). We detected a significant increase in CCL25 levels within 12 h (from 20 ± 10 to 530 ± 50 pg ml⁻¹ in the supernatant of medium- or IL-4-stimulated mesothelial cells, respectively, $P = 0.006$, $n = 3$ in one out of two experiments), suggesting that mesothelial cells may be an important source of CCL25 during allergic pleurisy. Of note, no differences in CCL25 content in the supernatant were observed between unstimulated (0.02 ± 0.01 pg ml⁻¹) and IL-4-stimulated mesothelial cells from unsensitized mice (0.02 ± 0.01 pg ml⁻¹).

Surface expression of chemokine receptors on $\gamma\delta$ T lymphocytes recovered from mice pleural cavity

In order to investigate the direct role of the chemokines detected in pleural washes, we sought to determine the expression of chemokine receptors by $\gamma\delta$ T lymphocytes by two-color flow cytometry. OVA challenge induced a significant increase in the number of $\gamma\delta$ T lymphocyte expressing CCR2, CCR5 and CCR9 in the mice pleural cavity within 12 h that was maximal within 24 h, and returned to basal values 120 h after the stimulus (data not shown). Representative histograms shown in Fig. 4 demonstrate an increase in fluorescence intensity of the three CC receptors on $\gamma\delta$ T lymphocytes 12 and 48 h after OVA challenge when compared with cells recovered from non-stimulated mice and a decrease to normal levels within 144 h.

$\gamma\delta$ T lymphocyte analysis in peripheral blood

$\gamma\delta$ T lymphocytes accomplish a minority of 1–5% of circulating lymphocytes in mice. To establish if peripheral blood

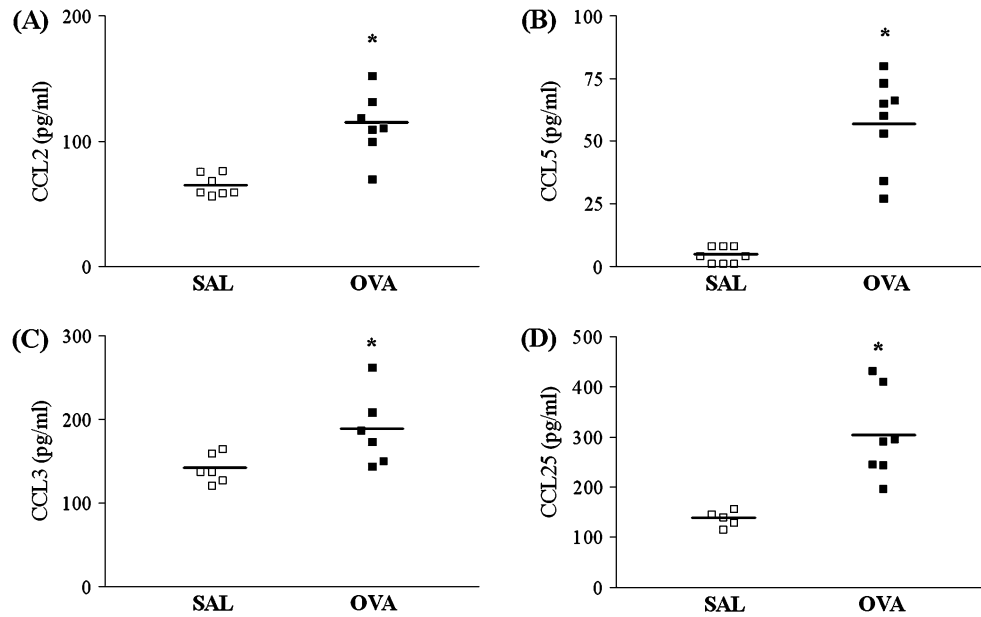


Fig. 2. CCL2 (A), CCL5 (B), CCL3 (C) and CCL25 (D) protein levels in pleural fluid obtained from saline- or OVA-injected mice (12.5 μ g per cavity, i.t.), determined by ELISA in pleural washes recovered at 24 h following stimulation. Results are expressed as the mean $\pm$ SEM from at least six animals per group of three different experiments. Statistically significant differences ($P \leq 0.05$) are indicated by an asterisk.

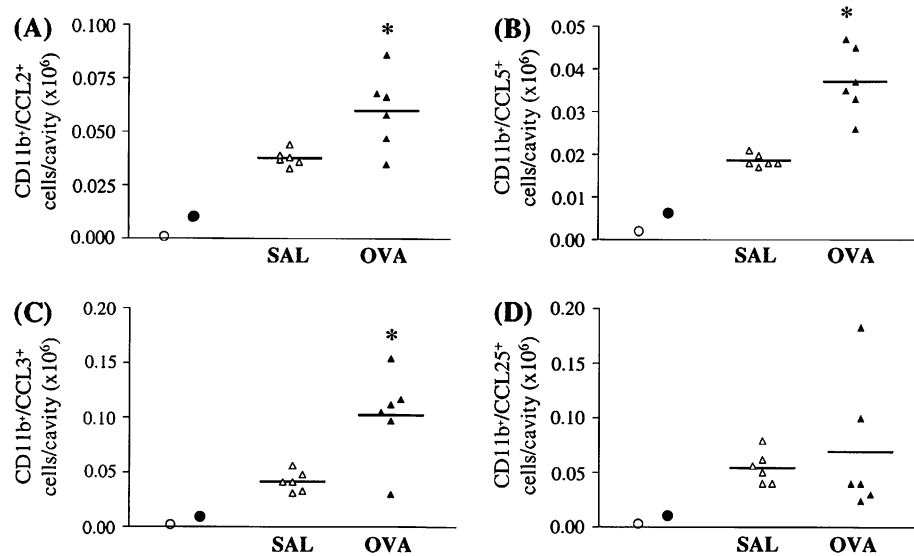


Fig. 3. Flow cytometry analysis of intracellular CCL2 (A), CCL5 (B), CCL3 (C) and CCL25 (D) in CD11b/MAC-1⁺ macrophages recovered from mice pleural cavities 6 h after saline or OVA (12.5 μ g per cavity) i.t. injection. Cells were incubated with the appropriate concentration of FITC-conjugated anti-CD11b/MAC-1 mAb after stimulation with ionomycin/PMA/monensin and fixed with 4% PFA before chemokine staining with biotinylated anti-CCL2, CCL5, CCL3 and CCL25 mAbs, followed by streptavidin-Cy-chrome. Mean values of intracellular chemokine staining of unstimulated pleural macrophages are represented as open (from saline-injected mice) and closed (from OVA-injected mice) circles. Results are expressed as the mean $\pm$ SEM from at least six animals. Statistically significant differences ($P \leq 0.05$) are indicated by an asterisk.

was likely to be a source of $\gamma\delta$ T lymphocytes found in mice pleural cavity after antigenic challenge, we performed an immunostaining of PBMC, recovered 12, 48 and 144 h after the i.t. stimulation. The representative histograms of CCR expression on peripheral blood $\gamma\delta$ T lymphocytes shown in Fig. 4 demonstrate that OVA challenge induced a slight in-

crease in mean fluorescence intensity (MFI) of CCR2 (saline: 278.0 ± 28.1 versus OVA: 389.1 ± 52.7 MFI, $P = 0.089$) and CCR5 (saline: 390.9 ± 21.0 versus OVA: 513.3 ± 105.4 MFI, $P = 0.28$) within 12 h and of CCR9 within 48 h (saline: 343.4 ± 32.7 versus OVA: 507.6 ± 83.9 MFI, $P = 0.02$) that returned to control levels within 144 h. In addition, OVA

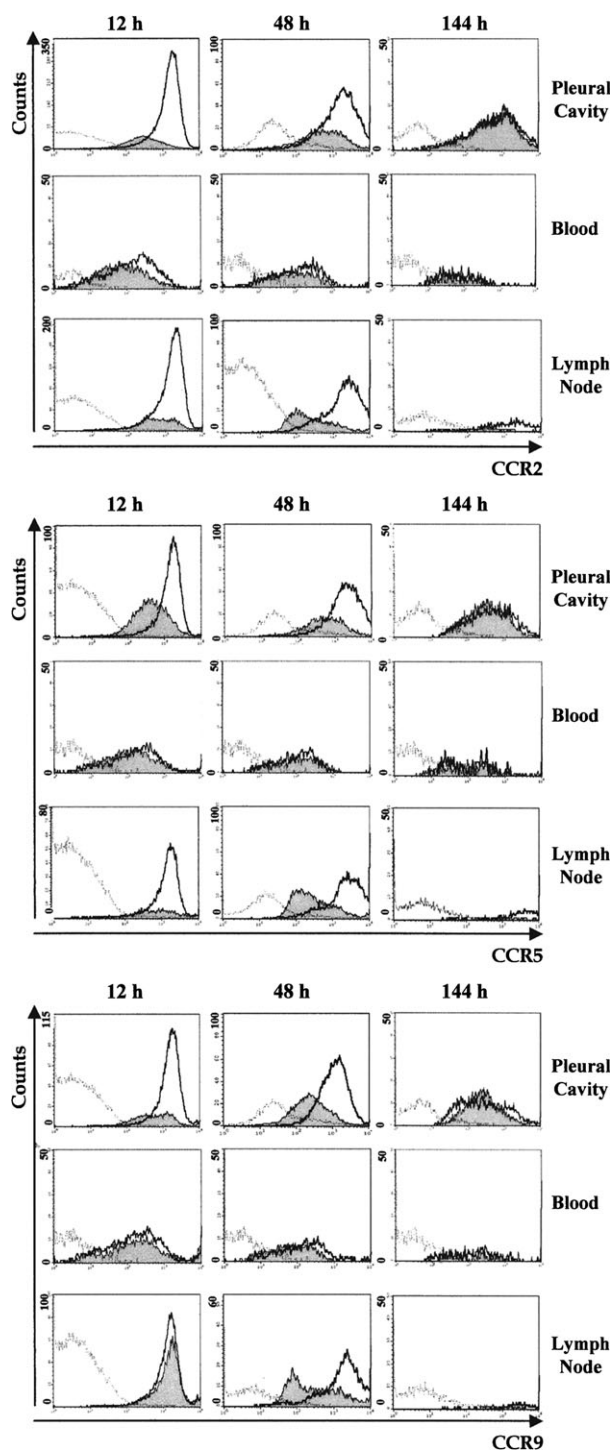


Fig. 4. Representative histograms of the expression of chemokine receptors CCR2 (A), CCR5 (B) and CCR9 (C) by $\gamma\delta$ T lymphocytes recovered from mice pleural cavities, peripheral blood and thoracic lymph nodes 12, 48 and 144 h after saline (shaded histograms) or OVA (12.5 μ g per cavity; bold lines) i.t. injection. Control IgG is represented by dotted lines. Cells were stained with FITC-conjugated anti- $\gamma\delta$ TCR plus purified goat anti-chemokine receptor pAbs anti-murine CCR2, CCR5 or CCR9 (followed by biotinylated IgG, plus streptavidin-Cy-chrome). Chemokine receptor expression was analysed on gated $\gamma\delta$ T lymphocytes. At least 10^4 lymphocytes were acquired per sample. Representative histograms of one animal out of six per group are shown.

challenge induced an increase in the number of $\gamma\delta$ T lymphocyte in the blood within 12 and within 48 h (saline: 7.4 ± 1.8 versus OVA: 18.0 ± 1.0 $\gamma\delta$ T lymphocytes mm^{-3} , $P = 0.002$), which was no longer above normal values within 144 h (data not shown). Of note, we also observed an increase in the number of $\gamma\delta$ T lymphocyte expressing CCR2 (saline: 7.6 ± 2.0 versus OVA: 16.3 ± 3.0 $\gamma\delta$ T lymphocytes mm^{-3} , $P = 0.003$) and CCR9 (saline: 5.1 ± 2.0 versus OVA: 20.9 ± 3.9 $\gamma\delta$ T lymphocytes mm^{-3} , $P = 0.007$). An apparent augmentation in the number of $\gamma\delta$ T lymphocyte expressing CCR5 was observed within 48 h, even though it was not statistically different from the numbers found in non-stimulated mice (saline: 7.2 ± 1.8 versus OVA: 16.0 ± 5.0 $\gamma\delta$ T lymphocytes mm^{-3} , $P = 0.14$).

$\gamma\delta$ T lymphocyte analysis in thoracic lymph nodes

Flow cytometric analyses were performed on $\gamma\delta$ T lymphocytes for surface expression of CCRs in this lymphoid site at different time points of the antigenic response. The representative histograms shown in Fig. 4 demonstrate that OVA challenge induced an increase in MFI of CCR2 and CCR5 within 12 h that was maximal within 48 h (CCR2—saline: 992.9 ± 282.6 versus OVA: 2486.7 ± 188.0 MFI, $P = 0.019$; CCR5—saline: 1185.1 ± 294.6 versus OVA: 2894.5 ± 134.8 MFI, $P = 0.002$). It is interesting to note that even though the expression of CCR9 was similar in saline- and OVA-injected mice 12 h after stimulation, an important increase was observed in stimulated mice 48 h after the challenge (saline: 1466.7 ± 188.5 versus OVA: 2636.3 ± 208.7 MFI, $P = 0.006$). The fluorescence mean of the three CCRs returned to basal levels within 144 h.

Effect of in vivo CC chemokine neutralization on $\gamma\delta$ T lymphocyte accumulation induced by OVA challenge

Since we demonstrated the increased production of CCL2, CCL3, CCL5 and CCL25 during the allergic response by OVA, as well as the expression of the counterpart receptors on $\gamma\delta$ T cells, we further investigated the involvement of such chemokines in the recruitment of $\gamma\delta$ T cells into the mice pleural cavity. Mice were pre-treated with neutralizing anti-murine CCL2, CCL5, CCL3 or CCL25 at 10 μ g per cavity, followed by the i.t. administration of OVA (12.5 μ g per cavity). The neutralization of CCL2 significantly inhibited $\gamma\delta$ T cell accumulation observed 24 h after OVA i.t. injection in mice (Fig. 5A). By contrast, the neutralization of CCL3, CCL5 and CCL25 failed to inhibit OVA-induced $\gamma\delta$ T cell influx (Fig. 5B, C and D, respectively). It is noteworthy that the pre-treatments with anti-CCL5 and anti-CCL3 mAbs were able to impair eosinophil accumulation in the mice pleural cavity of OVA challenge mice within 24 h (saline: 10.0 ± 0.0 ; OVA: 220.0 ± 30.0 ; α CCL5: 60.0 ± 2.0 eosinophils $\times 10^3$ per cavity; $P = 0.002$), as did anti-CCL3 mAb (α CCL3: 10.0 ± 3.0 eosinophils $\times 10^3$ per cavity, $P = 0.04$). Anti-CCL3 pre-treatment also inhibited the increase in CD3 T lymphocyte numbers (saline: 26.9 ± 7.5 ; OVA: 146.3 ± 20.2 ; α CCL3: 76.0 ± 25.4 CD3 T lymphocytes $\times 10^3$ per cavity, $P = 0.02$), ensuring the neutralizing activity of the antibodies.

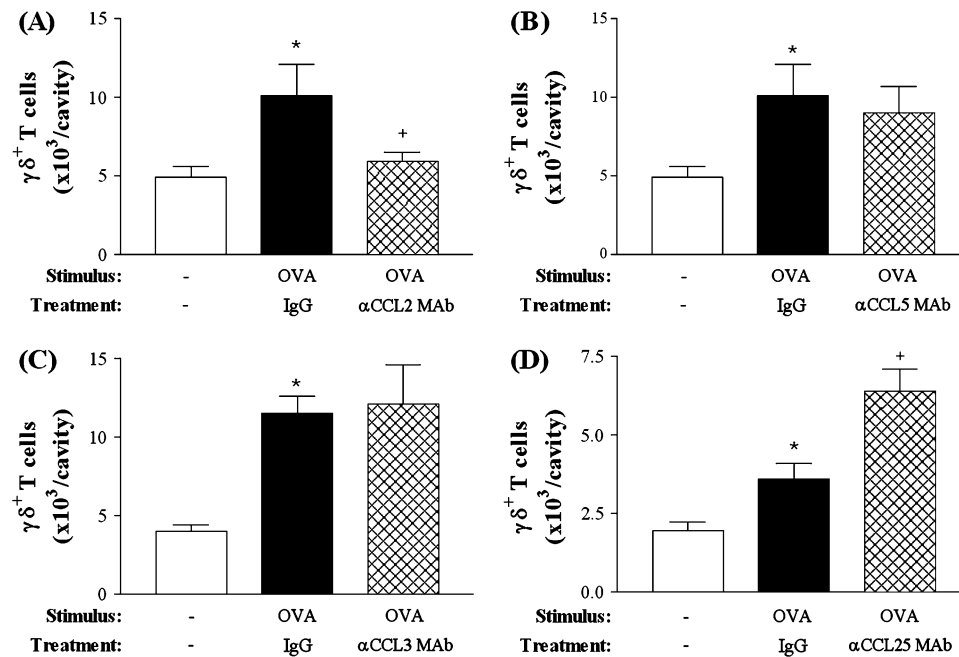


Fig. 5. Effect of *in vivo* chemokine neutralization on $\gamma\delta$ T lymphocyte accumulation induced by OVA (12.5 μ g per cavity, i.t.) within 24 h. Antibodies anti-CCL2 (A), anti-CCL5 (B), anti-CCL3 (C) and anti-CCL25 (D) were administered i.p. at 10 μ g per cavity 45 min before stimulation. Results are expressed as the mean $\pm$ SEM from six animals per group of two different experiments. Statistically significant differences between saline- and OVA-injected groups ($P \leq 0.05$) are indicated by an asterisk, whereas '+' indicates significant differences between stimulated and treated groups.

Effect of CC chemokine neutralization on OPW-induced $\gamma\delta$ T lymphocyte *in vitro* chemotaxis

Since CCL2 *in vivo* neutralization caused a marked decrease in $\gamma\delta$ T lymphocyte numbers in the pleural cavity of challenged mice, we further investigated the effect of *in vitro* neutralization of CCL2 in pleural washes recovered from mice 24 h after antigenic challenge (OPW) on $\gamma\delta$ T lymphocyte *in vitro* chemotaxis. As shown in Fig. 6, $\gamma\delta$ T lymphocytes migrated towards 24-h OPW in a higher extent than towards SPW. As expected, $\gamma\delta$ T lymphocytes failed to migrate towards OPW incubated with neutralizing mAb anti-CCL2 (Fig. 6A), demonstrating that CCL2 present in OPW is crucial for $\gamma\delta$ T lymphocyte chemotaxis. Of note, the *in vitro* neutralization of CCL3, CCL5 and CCL25 failed to inhibit OPW-induced $\gamma\delta$ T cell chemotaxis (Fig. 6B, C and D), confirming the results obtained *in vivo*.

Analysis of $\gamma\delta$ T lymphocyte accumulation in CCR2 KO mice after OVA challenge

In order to confirm the role of CCL2 signaling via CCR2 in $\gamma\delta$ T lymphocyte migration into the allergic site, we induced allergic pleurisy in CCR2 KO mice. In accordance with the results obtained with the neutralization of CCL2, we observed that OVA i.t. injection failed to induce $\gamma\delta$ T lymphocyte accumulation in CCR2 KO mice pleural cavities 24 h after antigenic challenge (Fig. 7A), in contrast with the important increase in $\gamma\delta$ T cell numbers found in the pleural space of wild-type mice. It is noteworthy that $\gamma\delta$ T lymphocyte numbers found in OVA i.t.-injected CCR2 KO mice were

statistically lower than the numbers found in OVA i.t.-injected wild-type mice. Although CCR2 KO mice showed increased numbers of $\gamma\delta$ T lymphocytes in peripheral blood after OVA challenge when compared with non-stimulated CCR2 KO mice, $\gamma\delta$ T cell numbers were significantly lower than those found in challenged wild-type mice (Fig. 7B). OVA challenge induced a significant increase in $\gamma\delta$ T cell numbers in wild-type mice, but failed to induce $\gamma\delta$ T lymphocyte expansion in thoracic lymph nodes of KO mice (Fig. 7C). Interestingly, CCL2 levels in pleural washes of CCR2 KO mice were higher than in wild-type mice 24 h after saline or OVA injection (saline wild type: 1.3 ± 1.3 versus OVA wild type: 24.9 ± 5.1 ; saline KO: 16.2 ± 4.2 versus OVA KO: 148.6 ± 38.3 pg ml $^{-1}$, $n \geq 5$ per group).

Discussion

$\gamma\delta$ T lymphocytes are a minor subset of T cells described to accumulate in inflammatory sites and are capable to display either effector or negative regulatory functions during allergic responses (5, 12, 23). However, the mechanisms by which $\gamma\delta$ T cells accumulate in the allergic site have not been characterized yet. In the present report, we demonstrate an accumulation of $\gamma\delta$ T lymphocytes expressing CCR2, CCR5 and CCR9 in the allergic site, among which CCR2, via CCL2 interaction, plays a crucial role for $\gamma\delta$ T lymphocyte mobilization during allergic response.

It is interesting to note that the increase in $\gamma\delta$ T lymphocyte numbers paralleled with the accumulation pattern observed for CD3 lymphocytes; however, $\gamma\delta$ T cell numbers

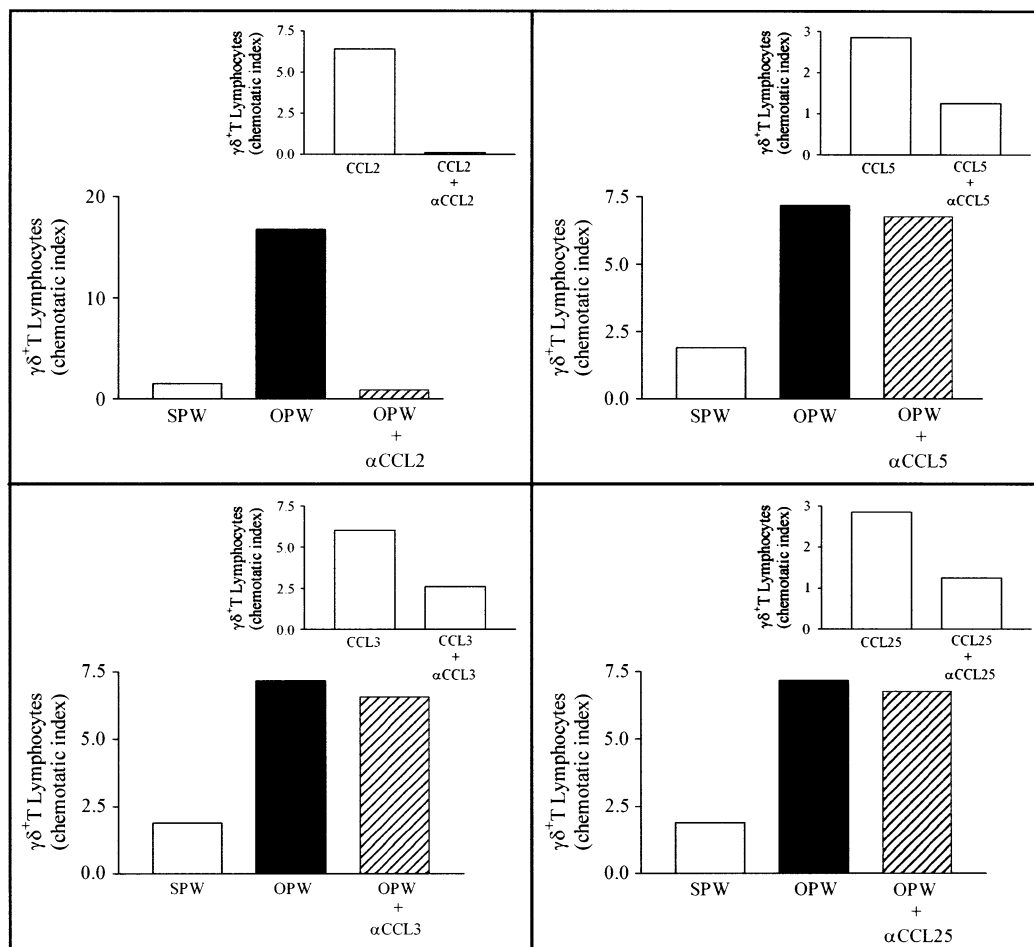


Fig. 6. Effect of chemokine neutralization on $\gamma\delta$ T lymphocyte *in vitro* chemotaxis induced by OPW. OPW recovered from mice 24 h after OVA i.t. injection (12.5 μ g per cavity) was incubated in the presence or absence of mAbs anti-CCL2 (A, 2.5 ng per well), anti-CCL5 (B, 20 ng per well), anti-CCL3 (C, 200 ng per well) or anti-CCL25 (D, 400 ng per well) for 30 min at 37°C, and plated into a 24-well cell culture plate (300 μ l). Spleen leukocytes (3×10^6 per well) were placed in the upper chamber of 3.0- μ m pore diameter transwell inserts and incubated for 60 min at 37°C and 5% CO₂. Cells that migrated into the bottom chamber were recovered and labeled for CD3 and $\gamma\delta$ TCR for FACS analysis. Results are expressed as chemotactic index. Assay buffer and SPW were used as controls. As control, inserts show CCL2- (A), CCL5- (B), CCL3- (C) and CCL25-induced (D) $\gamma\delta$ T lymphocyte transmigration and the effect of their respective neutralization. Data represent one of three different experiments.

returned to basal levels within 5 days after stimulation, while CD3 T lymphocytes were still above control values in the pleural cavities of allergic mice. The increase in $\gamma\delta$ T cell numbers was partially due to an accumulation in V γ 4+ T lymphocytes (Penido, C., manuscript in preparation), a subset described as lung resident $\gamma\delta$ T cells, shown to expand in different mouse models of lung infection (1). Even though resident $\gamma\delta$ T lymphocytes may accomplish their surveillance functions with no need to traffic through secondary lymphoid organs, we observed that $\gamma\delta$ T cells involved in the allergic response triggered by OVA seem to migrate from blood into inflamed tissue. The first compelling evidence was the increased levels of lymphotactic mediators in the pleural washes of challenged mice. Indeed, chemokines and their counterpart receptors are up-regulated during inflammatory conditions and coordinate the recruitment of T lymphocytes (24). Intra-nasal OVA challenge in mice has been shown to induce increased mRNA levels for a variety of CC chemo-

kines (25) and macrophages represent an important source of CC chemokines under different stimulation, including IgE (16, 26). We observed increased levels of CCL2, CCL5 and CCL3 content in OPW that were partially produced by pleural macrophages, according to previous data that describe macrophages as an important source of CC chemokines under different stimulation, including mycobacterial antigens and IgE (24, 25). Although pleural macrophages seem not to be a relevant source of CCL25, *in vitro*-cultured mesothelial cells showed to be able to produce CCL25 after stimulation with IL-4, a T_H2 cytokine involved in allergy. It is known that pleural mesothelial cells are metabolic active cells disposed in a monolayer that lines the thoracic cavity and play a main role in initiating and regulating the innate immune response when in contact with pathogenic organisms (27). During pleural inflammation, mesothelial cells are capable to produce several inflammatory factors, including chemoattractants for monocytes, such as CC chemokines (28, 29).

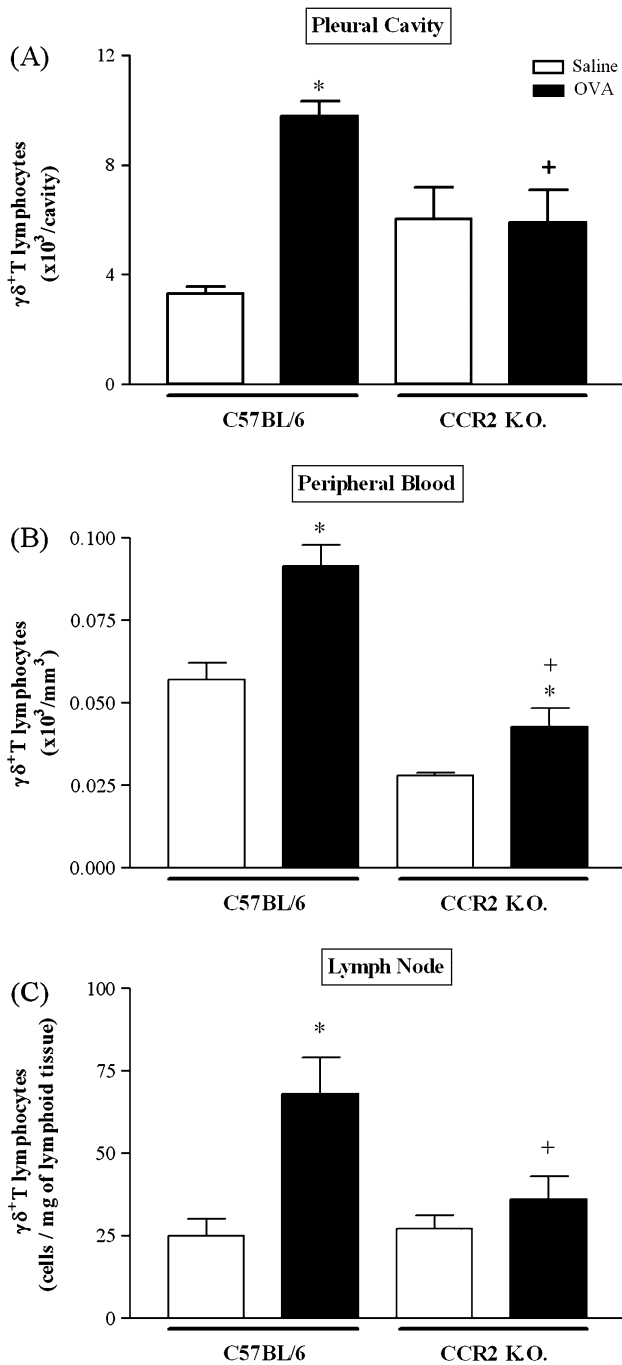


Fig. 7. Effect of OVA (12.5 μ g per cavity) i.t. injection on $\gamma\delta$ T lymphocyte numbers in the pleural cavity (A), blood (B) and thoracic lymph nodes (C) of previously sensitized C57BL/6 or CCR2 KO mice 24 h after stimulation. Results are expressed as the mean $\pm$ SEM from at least five animals. Statistically significant differences between saline- and OVA-injected groups ($P \leq 0.05$) are indicated by an asterisk, whereas '+' indicates significant differences between stimulated wild-type and KO mice. Data represent one of two different experiments.

In addition, these cells express cell-surface molecules, such as intercellular adhesion molecule-1, the expression of which is increased in patients during tuberculous pleural inflammation, facilitating monocyte transmigration (30). Here we show

for the first time that mesothelial cells are an important source of chemokines during allergic reactions; however, further analyses are still needed to better understand this phenomenon.

Transendothelial *in vitro* assays have demonstrated that isolated $\gamma\delta$ T cells transmigrate towards CC chemokines, including CCL2 and CCL5 (15). Indeed, the expression of CCRs by $\gamma\delta$ T cells has been previously demonstrated (13, 14, 17). According to our results, it has been shown that following activation, $\gamma\delta$ T cells up- or down-regulate chemokine receptors (13, 16, 17). The fact that the pattern of expression of such receptors by $\gamma\delta$ T lymphocytes accompanied its kinetic of accumulation in the mice pleural cavity during allergic pleurisy suggested that such chemokine/chemokine receptor binding plays a role in the mobilization and/or in the activation of $\gamma\delta$ T cells during the allergic response. As yet, the expression of CCRs in inflamed tissue, in blood and in lymph nodes characterizes a possible migratory route of $\gamma\delta$ T lymphocytes.

Multiple expression of chemokine receptors indicates that cellular response results from an integrated and complex signal provided by numerous receptors. Data obtained with the *in vivo* neutralization of chemokines showed that $\gamma\delta$ T cell migration during allergic pleurisy is coordinated by CCL2, since its neutralization (but not of CCL3, CCL5 and CCL25) impaired $\gamma\delta$ T cell (among other CD3⁺ T cell population) accumulation in the pleural cavity of challenged mice. The accumulation of T lymphocytes in the inflamed tissue driven by CCL2 has been well demonstrated; however, published data concerning $\gamma\delta$ T cell migration are scarce and contradictory (16, 17). Previous results obtained by us showed that $\gamma\delta$ T cell mobilization into the mice pleural cavity induced by LPS and Bacillus Calmette-Guérin depends on CCL2 (17), in contrast with data obtained by Uezu *et al.* (31), who showed that CCL2 failed to account for *in vivo* $\gamma\delta$ T cell migration into the lungs of *Cryptococcus neoformans*-infected mice. Here we show for the first time that CCL2 plays a key role in $\gamma\delta$ T lymphocyte trafficking during *in vivo* allergic response and also suggest a role for such chemokine on $\gamma\delta$ T cell *in vitro* transmigration towards pleural washes recovered from challenged mice (OPW). The role of CCL2/CCR2 activation in $\gamma\delta$ T cell trafficking during the allergic response was reinforced in CCR2 KO mice i.t. challenged with OVA, in which the lack of CCR2 (but not the suppression of CCL2 production), impaired $\gamma\delta$ T cell influx triggered by allergen. Our results are in accordance with data previously shown (32), which demonstrate that the lack or blockade of CCR2 was associated with high levels of CCL2 in inflamed lungs.

The fact that CCL5, CCL3 and CCL25 neutralization failed to inhibit $\gamma\delta$ T lymphocyte migration, even though $\gamma\delta$ T cells express the counterpart receptors, suggests a putative role in $\gamma\delta$ T lymphocyte activation by such chemokines. Chemokines can also work as co-stimulators during host response promoting T cell activation, notably by proliferation and differentiation (33–38). However, unquestionable, further experiments are required to clarify the involvement of CCR5/CCL3, CCR5/CCL5 and CCR9/CCL25 receptor-ligand systems on $\gamma\delta$ T cell activation. It is very interesting to note that *in vivo* CCL2 neutralization was also able to indirectly inhibit eosinophil accumulation in the mice pleural cavity triggered by OVA injection. It is also noteworthy that OVA i.t. injection

induced eosinophil recruitment into CCR2 KO mice pleural cavity; however, in a lower extent to that observed in wild-type mice (Penido, C., manuscript in preparation). Such results support data published by other authors, who showed that $\gamma\delta$ T cells account for eosinophil accumulation during the allergic process (5, 8, 12).

In conclusion, our data demonstrate that antigenic challenge into mice pleural cavity leads to increased levels of CCL2, CCL3, CCL5 and CCL25 in pleural washes, produced by pleural macrophages and most likely by mesothelial cells. During the allergic response, an up-regulation of CCRs CCR2, CCR5 and CCR9 occurs on $\gamma\delta$ T cells from thoracic lymph node, blood and pleural cavity, suggesting that $\gamma\delta$ T lymphocytes migrate from lymph nodes into blood to achieve the inflamed tissue. Moreover, we provide evidence that CCL2/CCR2 activation is crucial for $\gamma\delta$ T cell trafficking into the inflammatory site during the allergic response.

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Abbreviations

CCL	CC chemokine ligand
CCR	CC chemokine receptor
i.t.	intra-thoracic
KO	knockout
MCP	monocyte chemotactic protein
MFI	mean fluorescence intensity
MIP	macrophage inflammatory protein
OPW	pleural washes recovered after ovalbumin i.t. injection
OVA	ovalbumin
pAbs	polyclonal antibodies
PMA	phorbol myristate acetate
RANTES	regulated upon activation, normal T cell expressed and secreted
s.c.	subcutaneous
SPW	pleural washes recovered after saline i.t. injection

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