

CCL25 induces $\alpha_4\beta_7$ integrin-dependent migration of IL-17⁺ $\gamma\delta$ T lymphocytes during an allergic reaction

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Herein, we provide evidence that during allergic inflammation, CCL25 induces the selective migration of IL-17⁺ $\gamma\delta$ T cells mediated by $\alpha_4\beta_7$ integrin. Intrapleural injection of CCL25 into ovalbumin (OVA)-immunized C57BL/6 mice triggered the accumulation of $\gamma\delta$ T lymphocytes expressing CCR9 (CCL25 receptor) and $\alpha_4\beta_7$ integrin in the pleura, but failed to attract $\alpha\beta$ T lymphocytes. CCL25 attracted CCR6⁺ $\gamma\delta$ T cells producing IL-17 (but not IFN- γ or IL-4). OVA challenge triggered increased production of CCL25 followed by the accumulation of CCR9⁺, $\alpha_4\beta_7$ ⁺, and CCR6⁺/IL-17⁺ $\gamma\delta$ T cells into the pleural cavities of OVA-immunized mice, which was inhibited by the in vivo neutralization of CCL25. The in vivo blockade of $\alpha_4\beta_7$ integrin also inhibited the migration of IL-17⁺ $\gamma\delta$ T lymphocytes (but not of $\alpha\beta$ T lymphocytes) into mouse pleura after OVA challenge, suggesting that the CCL25/ $\alpha_4\beta_7$ integrin pathway is selective for $\gamma\delta$ T cells. In addition, $\alpha_4\beta_7$ integrin blockade impaired the in vitro transmigration of $\gamma\delta$ T cells across endothelium (which expresses $\alpha_4\beta_7$ ligands VCAM-1 and MadCAM-1), which was induced by CCL25 and by cell-free pleural washes recovered from OVA-challenged mice. Our results reveal that during an allergic reaction, CCL25 drives IL-17⁺ $\gamma\delta$ T-cell mobilization to inflamed tissue via $\alpha_4\beta_7$ integrin and modulates IL-17 levels.

Keywords: Adhesion molecules • Allergy • CCL25 • Chemokines • T cells



See accompanying Commentary by Silva Santos



Supporting Information available online

Introduction

Lymphocytes bearing the $\gamma\delta$ T-cell receptor (TCR) comprise a minor T-lymphocyte subset in blood and secondary lymphoid tissues and are preferentially localized in epithelial and mucosal tissues [1,2]. This unique subset of lymphocytes can provide rapid tissue-specific immune responses, without the requirement of anti-

gen presentation or clonal expansion, and is able to produce a large repertory of Th1-, Th2-, and Th17-associated cytokines [2–6]. These characteristics make $\gamma\delta$ T cells a crucial first line of defense during infection, tissue damage, or stress.

$\gamma\delta$ T cells have been shown to migrate into the airways during allergic inflammation highly controlled by a chemotactic gradient of chemokines produced in tissue [5,7–11]. We have previously demonstrated that allergen-induced $\gamma\delta$ T-cell accumulation is

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paralleled with a marked production of chemokines in the tissue, including CCL25/TECK [11]. CCL25 is mostly described as a homeostatic chemokine that plays a major role in T-cell development in the thymus and in intraepithelial lymphocytes (IELs) homing into small intestine mucosa [12]. Recent data have provided evidence that CCL25 production increases during inflammatory processes that take place at nonmucosal tissues, such as autoimmune arthritis, atherosclerosis, and allergy [11, 13, 14]. Moreover, we and others have shown that $\gamma\delta$ T lymphocytes migrate in vitro toward CCL25, via its counterpart receptor CCR9 [11, 15]; however, the role of the CCL25/CCR9 pathway in $\gamma\delta$ T-cell migration has not been described.

Chemokine-mediated T-lymphocyte migration into the tissue is a multistep process that requires the action of adhesion molecules. This large group of cell-surface proteins is responsible for cell rolling, firm adhesion, and transendothelial migration. Firm adhesion is achieved by the interaction of leukocyte integrins with their endothelial counter ligands. CCL25 has been shown to induce lymphocyte adhesion to VCAM-1 and MadCAM-1, mediated by $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins [16, 17]. The coexpression of CCR9 and $\alpha_4\beta_7$ integrin has been described on gut-associated T lymphocytes and has been shown to dictate T-cell adhesion and migration to inflamed intestinal mucosa [18–21]. $\gamma\delta$ T lymphocytes also express both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins that mediate the in vitro adhesion to cytokine-activated endothelial cells [22–24].

In the present study, we demonstrate that CCL25/CCR9 is involved in the migration of $\gamma\delta$ T cells via the $\alpha_4\beta_7$ integrin to inflamed tissue during an allergic reaction. In addition, we show that CCL25 modulates IL-17 levels by inducing the specific migration of CCR6⁺/IL-17⁺ $\gamma\delta$ T lymphocytes.

Results

CCL25 triggers selective accumulation of $\gamma\delta$ T lymphocytes

The intrapleural (i.pl.) injection of recombinant mouse CCL25 (rmCCL25) into C57BL/6 mice induced the pleural accumulation of $\gamma\delta$ T lymphocytes (SAL 4.3 versus CCL25 7.0% in CD3⁺ T lymphocytes), with no observation of changes in the numbers of $\alpha\beta$ T lymphocytes (Fig. 1A and B) or other leukocyte populations (not shown) 24 h after stimulation. $\gamma\delta$ T cells recovered from CCL25-stimulated pleura expressed CCR9 and $\alpha_4\beta_7$ integrin, both phenotype markers of gut mucosal lymphocytes (Fig. 1C). It is noteworthy that, even though only approximately 40% of pleural $\gamma\delta$ T cells from i.pl. CCL25-stimulated mice were CCR9⁺ (Fig. 1C), this amount corresponds to the larger portion of newly arrived $\gamma\delta$ T cells (Fig. 1A).

The analysis of activation marker expression revealed that i.pl. rmCCL25 stimulation triggered the accumulation of CD2^{hi} and CD25⁺ $\gamma\delta$ T lymphocytes (Fig. 1C). However, no significant differences were observed in the migration of CD45RB⁺, CD69⁺, and CD122⁺ $\gamma\delta$ T cells recovered from CCL25-stimulated and from nonstimulated mice.

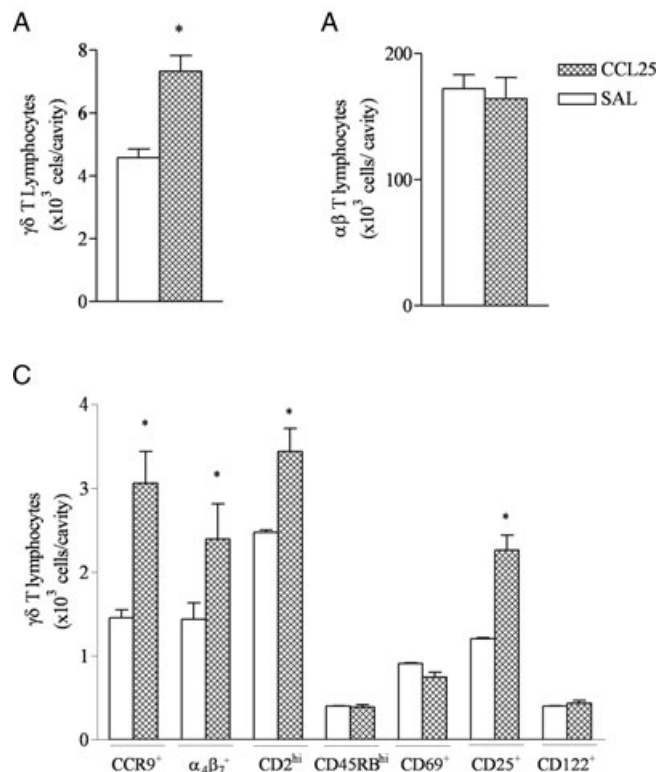


Figure 1. CCL25 triggers the migration of $\gamma\delta$ T lymphocytes expressing CCR9⁺ $\alpha_4\beta_7$ ⁺ into mouse pleura. Accumulation of (A) $\gamma\delta$ T lymphocytes and (B) $\alpha\beta$ T lymphocytes in mouse pleural cavities was assessed 24 h after CCL25 stimulation (200 ng/cavity, i.pl.). (C) Expression of surface molecules on $\gamma\delta$ T lymphocytes 24 h after CCL25 i.pl. stimulation was determined by flow cytometry. Data show mean + SEM of $n \geq 6$ mice/group and are representative of $n \geq 3$ experiments. * $p \leq 0.05$ between nonstimulated and stimulated groups, Student's t-test.

CCL25 production increases during allergic response and induces $\alpha_4\beta_7$ ⁺ $\gamma\delta$ T-lymphocyte migration

The i.pl. antigenic challenge with ovalbumin (OVA) into immunized mice induced increased levels of CCL25 in pleural washes recovered within 24 h (Fig. 2A). CCL25 has been shown to be mainly produced by epithelial cells [25]. Considering that the mesothelium is an epithelial-like cell lining that covers the pleural surface, which actively synthesizes inflammatory mediators, we investigated the production of CCL25 by mesothelial cells. Mesothelial cells recovered from previously sensitized mice were stimulated with IL-4, a Th2 cytokine previously shown to favor chemokine production by these cells [26]. As shown in Fig. 2B, CCL25 levels were increased in supernatants of IL-4-stimulated mesothelial cells within 12 h, suggesting that those cells may be an important source of CCL25 during allergic pleurisy. IL-4 stimulation did not induce CCL25 production by mesothelial cells recovered from unsensitized mice. OVA challenge also induced the accumulation of $\gamma\delta$ T cells expressing CCR9 and $\alpha_4\beta_7$ integrin in previously sensitized mice within 48 h (Fig. 2C). Interestingly, the majority (65%) of CCR9⁺ $\gamma\delta$ T cells recovered from OVA-challenged mice coexpressed $\alpha_4\beta_7$ integrin. The representative histograms

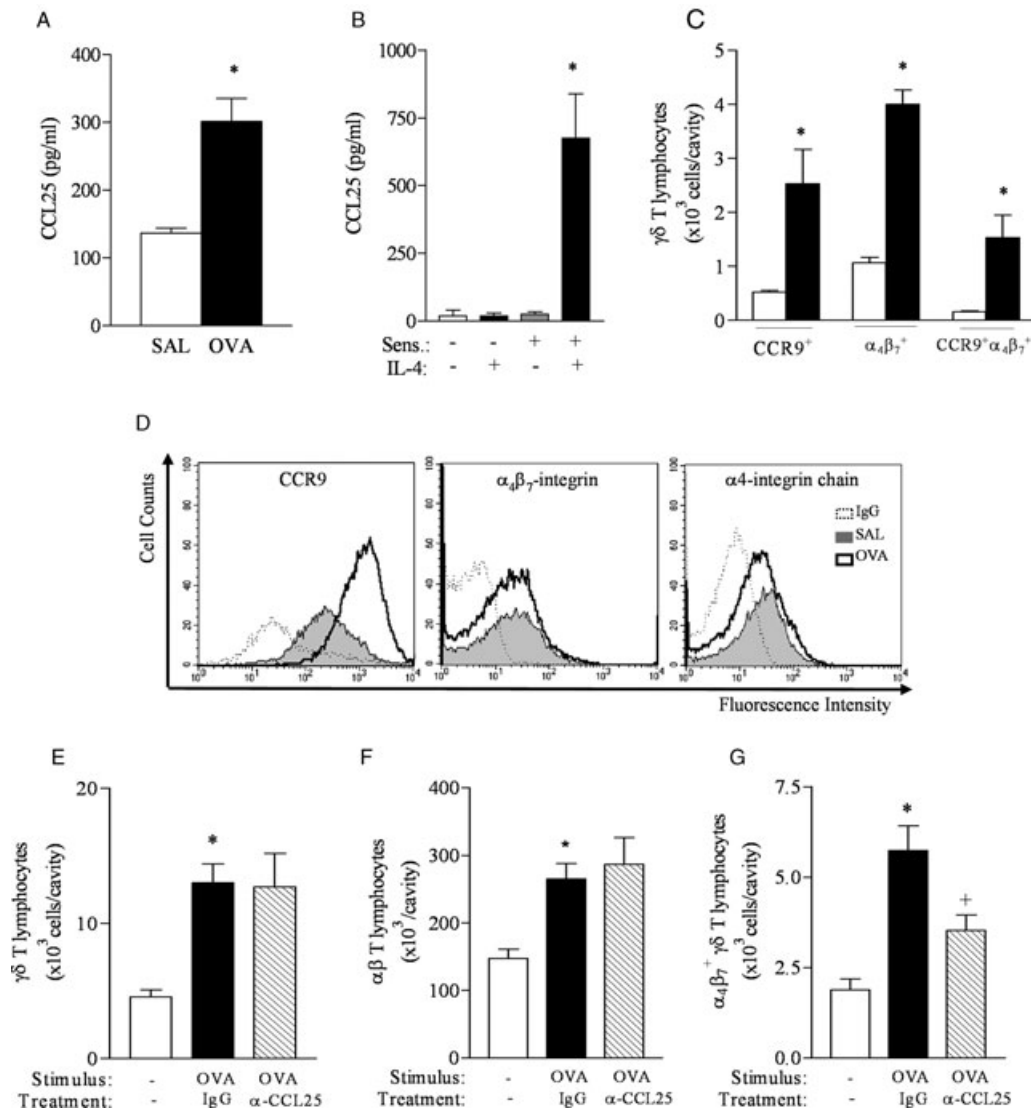


Figure 2. Antigenic challenge triggers CCL25 production and the migration of CCR9⁺ and α₄β₇⁺ γδ T lymphocytes. (A) Mice were injected with saline or OVA and 24 h following stimulation, the CCL25 protein levels in pleural fluid were determined. (B) CCL25 protein levels in the supernatant of pleural mesothelial cells, recovered from sensitized mice, stimulated in vitro with IL-4 (40 ng/mL) for 12 h, are shown. (C) The accumulation of γδ T lymphocytes expressing CCR9 and α₄β₇ integrin in mouse pleura 48 h after OVA challenge (12.5 μg/cavity) is shown. (D) Histograms of CCR9, α₄ integrin chain, and α₄β₇ integrin expression on γδ T cells recovered from mouse pleura 48 h after challenge. (E–G) Effect of in vivo CCL25 neutralization (10 μg/cavity) on the pleural accumulation of (E) γδ T lymphocytes, (F) αβ T lymphocytes, and (G) α₄β₇ integrin⁺ γδ T lymphocytes induced by OVA within 48 h. Data show mean + SEM of *n* ≥ 3 wells/group (A, B) or *n* ≥ 6 mice/group (C, E–G), and are representative of *n* ≥ 2 experiments. **p* ≤ 0.05 between nonstimulated and stimulated groups; ⁺*p* ≤ 0.05 between stimulated and treated groups, Student's *t*-test (A, C) or Newman–Keuls–Student test (E, F, G).

(Fig. 2D) show the increased expression of CCR9 on pleural γδ T cells recovered from OVA-stimulated mice as compared with those from nonstimulated mice (SAL 223.9 ± 36.5 versus OVA 336.1 ± 41.9 mean fluorescence intensity (MFI)). No increase in the expression of α₄ integrin chain (SAL 42.6 ± 1.3 versus OVA 37.5 ± 0.7 MFI) and α₄β₇ integrin (SAL 168.8 ± 6.9 versus OVA 105.5 ± 8.3 MFI) by γδ T cells were observed between groups (Fig. 2D).

The involvement of CCL25 in γδ T-cell migration during an allergic response was assessed. The anti-CCL25 monoclonal antibody (mAb) treatment failed to inhibit γδ or αβ T-cell migration to pleura 48 h after OVA challenge (Fig. 2E and F). However, the

in vivo neutralization of CCL25 specifically impaired the accumulation of γδ T cells expressing α₄β₇ integrin (Fig. 2G).

α₄β₇ integrin is required for γδ T-cell migration induced by CCL25 and during an allergic reaction

Since CCL25 induced the migration of α₄β₇⁺ γδ T lymphocytes, we further evaluated the role of α₄ integrins on γδ T-lymphocyte migration induced by this chemokine. The in vitro blockade of α₄ integrin chain and α₄β₇ integrin by mAbs inhibited γδ T-cell transmigration across endothelial monolayers prestimulated with the Th2 cytokine IL-4 toward CCL25 and cell-free pleural

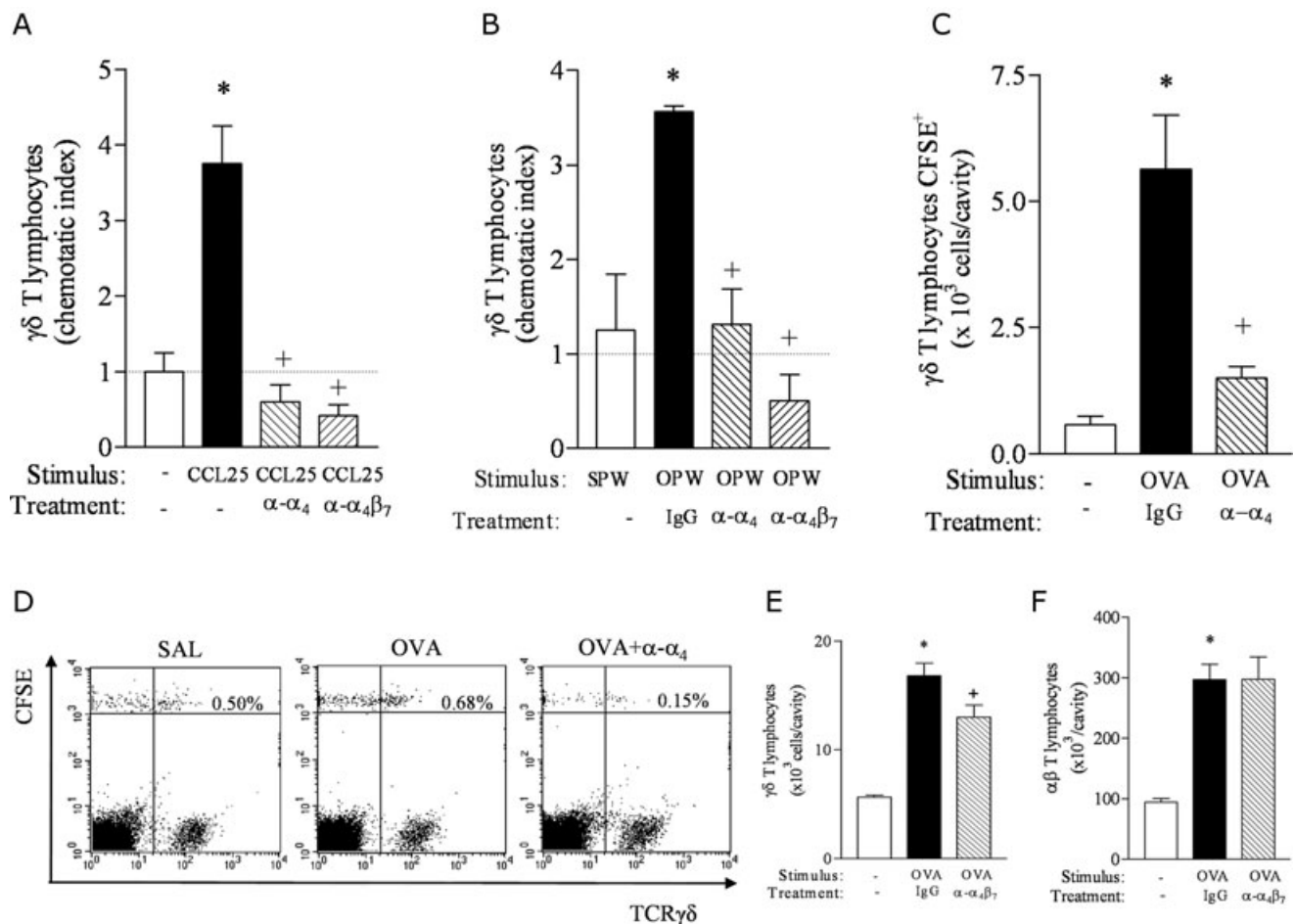


Figure 3. $\alpha_4\beta_7$ integrin is required for $\gamma\delta$ T cell migration induced by CCL25 during an allergic reaction. $\gamma\delta$ T-cell transmigration across endothelial monolayers was induced by (A) CCL25 or (B) OPW and the effect of the in vitro blockade of α_4 integrin chain and $\alpha_4\beta_7$ integrin on migration was determined. Results are expressed as chemotactic index, generated by using the number of cells that migrated toward buffer as comparison. (C) Mice were challenged with OVA and adoptively transferred with CFSE⁺ $\gamma\delta$ T lymphocytes and the effect of ex vivo pretreatment with anti- α_4 integrin mAb (25 μ g/mL) on $\gamma\delta$ T lymphocyte migration into mouse pleural cavities was determined. (D) Representative dot plots of CFSE⁺ $\gamma\delta$ T lymphocytes. Data are representative of $n \geq 2$ experiments. Effect of in vivo $\alpha_4\beta_7$ integrin blockade (100 μ g/mouse) on (E) $\gamma\delta$ T lymphocyte and (F) $\alpha\beta$ T lymphocyte pleural accumulation induced by OVA (12.5 μ g/cavity) within 48 h. Data show mean + SEM of $n \geq 3$ wells/group (A, B) or $n \geq 6$ mice/group (C, E–F), and are representative of $n \geq 2$ experiments. * $p \leq 0.05$ between nonstimulated and stimulated groups, + $p \leq 0.05$ between stimulated and treated groups, Newman–Keuls–Student test.

wash recovered from OVA-challenged mice (OPW; which contains CCL25) (Fig. 3A and B). Cell-free pleural wash recovered from saline-injected mice (SPW) was used as negative control. To confirm that $\alpha_4\beta_7$ integrin mediates $\gamma\delta$ T-cell transmigration from blood into inflamed pleura during allergic response, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes recovered from OVA-challenged mice, ex vivo treated (or not) with anti- α_4 integrin mAb, were adoptively transferred into recipient mice 24 h after OVA i.p. challenge. Adoptively transferred $\gamma\delta$ T cells migrated into challenged mouse pleura in a higher extent than into saline-injected recipient mice, a phenomenon which was reduced by α_4 integrin blockade (Fig. 3C and D). Moreover, the in vivo blockade of $\alpha_4\beta_7$ integrin inhibited the migration of $\gamma\delta$ T lymphocytes into mouse pleural cavities after OVA challenge (Fig. 3E). By contrast, the pretreatment with anti- $\alpha_4\beta_7$ integrin failed to inhibit the migration of $\alpha\beta$ T lymphocyte (Fig. 3F).

Corroborating results demonstrated that tEnd.1 (Murine thymic endothelioma) cells constitutively express VCAM-1 and MadCAM-1 whose expression was increased after IL-4 stimulation, as demonstrated by immunofluorescence staining (Supporting Information Fig. 1).

CCL25 induces the migration of $\gamma\delta$ T lymphocytes expressing CCR6⁺ IL-17⁺ during allergic pleurisy via $\alpha_4\beta_7$

OVA challenge induced the migration of IL-17⁺ $\gamma\delta$ T lymphocytes (Fig. 4A–C). We therefore investigated the role of $\alpha_4\beta_7$ integrin and CCL25 in this phenomenon. Both $\alpha_4\beta_7$ integrin blockade and CCL25 neutralization inhibited the migration of IL-17⁺ $\gamma\delta$ T lymphocytes into mouse pleura during the allergic response (Fig. 4A–D). Likewise, the blockade of CCR9 impaired IL-17⁺ $\gamma\delta$

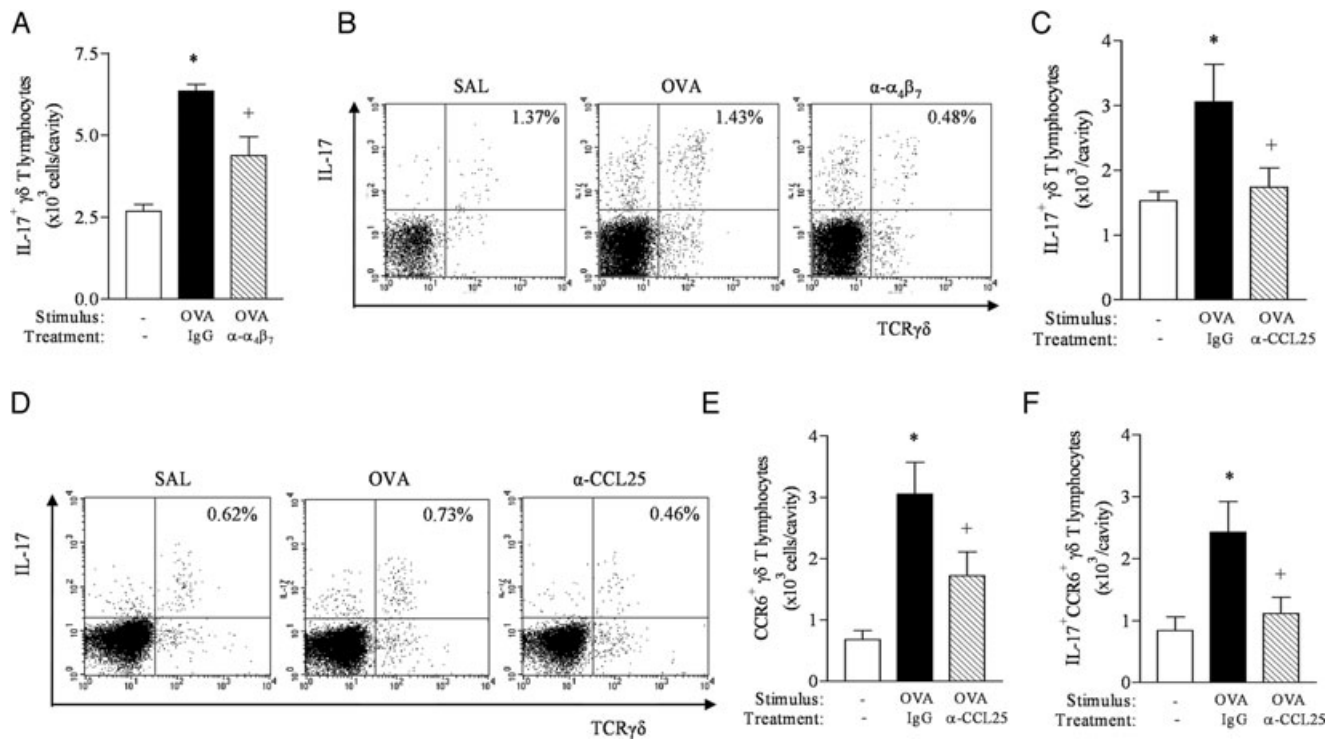


Figure 4. CCL25 induces the migration of CCR6⁺/IL-17⁺ γδ T lymphocytes during allergic pleurisy via α₄β₇ integrin. IL-17⁺ γδ T lymphocyte accumulation was induced in vivo by OVA and the effect of pretreatment with (A, B) anti-α₄β₇ integrin mAb (100 μg/mouse, i.v.) or (C, D) anti-CCL25 mAb (10 μg/cavity i.p.) was determined. (B, D) Representative dot plots of IL-17⁺ γδ T lymphocytes among CD3⁺ T lymphocytes are shown. Effect of in vivo CCL25 neutralization on the pleural accumulation of (E) CCR6⁺ γδ T lymphocytes and on (F) CCR6⁺ IL-17⁺ γδ T lymphocytes induced by OVA within 48 h are shown. Data show mean ± SEM of *n* ≥ 6 mice/group (A, C, E), and are representative of *n* ≥ 2 experiments. **p* ≤ 0.05 between nonstimulated and stimulated groups, ⁺*p* ≤ 0.05 between stimulated and treated groups, Newman-Keuls-Student test.

T lymphocyte in vitro chemotaxis toward OPW (79% of inhibition). Fig. 4B and D show representative dot plots that show that OVA challenge did not increase percentages of IL-17⁺ γδ T lymphocytes (among T lymphocytes), since other T-cell populations also migrate into challenged pleura (data not shown). Of note, OVA challenge also triggered the accumulation of IFN-γ⁺, but not of IL-4⁺, γδ T cells into the pleura of immunized mice. However, anti-CCL25 mAb treatment failed to inhibit IFN-γ⁺ γδ T-cell influx (Supporting Information Fig. 2).

Consistent with the notion that CCR6 is a specific marker of IL-17-producing γδ T cells [6], 80% of IL-17⁺ γδ T cells that migrate into OVA challenge pleura express CCR6. Accordingly, CCL25 neutralization inhibited the migration of CCR6⁺/IL-17⁺ γδ T lymphocytes (Fig. 4E and F). It is important to note that the neutralization of CCR6 ligand, CCL20, slightly inhibited (15%) IL-17⁺ γδ T-lymphocyte chemotaxis toward OPW, suggesting that this chemokine might present additive effects to CCL25 (Supporting Information Fig. 3).

CCL25 modulates IL-17 levels by inducing IL-17⁺ γδ T-lymphocyte migration

In order to evaluate the cytokine profile of CCL25-recruited γδ T cells, we examined the intracellular content of IL-4, IFN-γ, and

IL-17. Figure 5A shows that CCL25 i.p. injection only triggered the in vivo migration of IL-17⁺ γδ T lymphocytes (SAL 74.3 versus CCL25 87.2% in γδ T lymphocytes), but not of IL-4⁺ or IFN-γ⁺ γδ T lymphocytes. Such phenomenon accounted for the increase in IL-17 levels in mouse pleura (Fig. 5B), with no differences observed in the levels of IL-4 (SAL 287.8 ± 53.0 versus CCL25 283.8 ± 73.0 pg/mL) and IFN-γ (SAL 684.5 ± 252.1 versus CCL25 769.9 ± 270.2 pg/mL). In accordance, CCL25 induced the accumulation of CCR6⁺ γδ T lymphocytes (Fig. 5C), which has been correlated to IL-17 production [6]. CCL25 induced IL-17⁺ γδ T lymphocyte in vitro chemotaxis (Fig. 5D); however, it failed to induce IL-17 production by γδ T lymphocytes or to enhance IL-17 production by anti-γδ TCR-stimulated γδ T lymphocytes (Fig. 5E).

Discussion

CCL25 has been acclaimed as a homeostatic chemokine that has also been shown to participate in a few inflammatory processes, mainly in the gut and oral mucosa [25, 27–29]. CCR9⁺ γδ T lymphocytes, which are present in the thymus, peripheral lymph nodes, and spleen, have been shown to be attracted by CCL25 in vitro [6, 11, 15]. Our data demonstrate that CCL25 drives the in vivo migration of CCR9⁺ γδ T lymphocytes during allergic inflammation (or when directly injected), by using an experimental

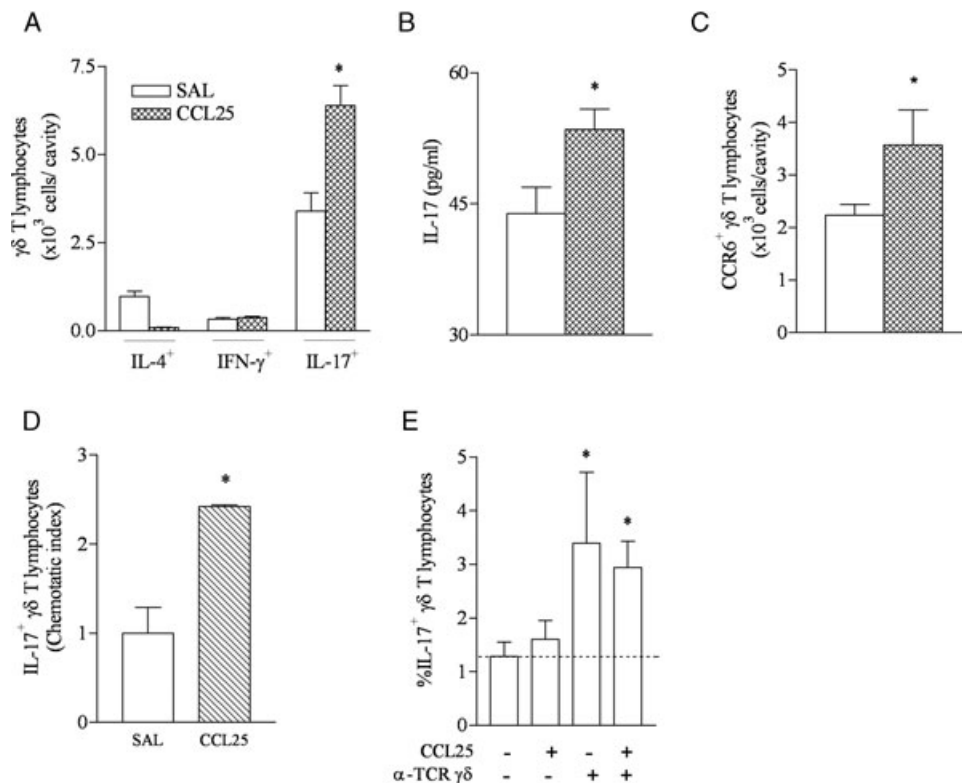


Figure 5. CCL25 modulates IL-17 production by inducing IL-17⁺ $\gamma\delta$ T-lymphocyte migration into mouse pleural cavities. The effect of CCL25 i.pl. stimulation (200 ng/cavity, i.pl.) on (A) IL-4⁺, IFN- γ ⁺, and IL-17⁺ $\gamma\delta$ T lymphocyte migration within 24 h and on (B) IL-17 protein levels in pleural fluid was determined by ELISA. (C) Accumulation of $\gamma\delta$ T lymphocytes expressing CCR6 in mouse pleural cavities 24 h after CCL25 stimulation is shown. (D) In vitro chemotaxis of IL-17⁺ $\gamma\delta$ T lymphocytes induced by CCL25 (100 ng/mL). Results are expressed as chemotactic index, generated by using the number of cells that migrated toward buffer as comparison. (E) IL-17 production by $\gamma\delta$ T lymphocytes after anti-TCR $\gamma\delta$ (10 μ g/mL) and/or CCL25 (100 ng/mL) in vitro stimulation. Data show mean + SEM $n \geq 6$ mice/group (A, C) or $n \geq 3$ wells/group (B, D, E), and are representative of $n \geq 2$ experiments. * $p \leq 0.05$ between nonstimulated and stimulated groups, Student's t-test.

model of murine pleurisy. Interestingly, CCL25 specifically triggered tissue accumulation of a subpopulation of $\gamma\delta$ T lymphocytes that presents Th17 phenotype and expresses CCR9 and $\alpha_4\beta_7$ integrin, which is required for their migration into the tissue.

Using the experimental model of allergic pleurisy, we have previously demonstrated that CCL25 levels increase during allergic response [11]. Here, we show that mesothelial cells are likely the major source of CCL25 during pleural allergic reaction. Indeed, mesothelial cells are epithelial-like cells that have been shown to play an active role in inflammation via the release of cytokines and chemokines [30]. In accordance, it has been shown that CCL25 is predominantly expressed by epithelial cells from mouse gut and thymus stroma [25]. It is interesting to note that IL-4 induced the CCL25 production by mesothelial cells recovered from immunized mice (but not from naïve mice), suggesting that these cells might be more responsive due to priming during immunization. In fact, the correlation between CCL25/CCR9 axis with Th2 response has been previously exposed in a few reports. IL-4 has been shown to drive increased expression of CCR9 on murine T lymphocytes when cocultured with dendritic cells, which was mediated by dendritic cell-derived retinoic acid [31, 32].

The involvement of CCR9 in allergy has been shown in allergic asthma patients, whose bronchial biopsies present higher numbers of CCR9⁺ natural killer T (NKT) cells than the ones of nonasthmatic subjects. In addition, CCR9⁺ NKT cells recovered from the peripheral blood of these patients migrated in vitro toward CCL25 [33]. Herein, we found that during allergic reaction, CCR9⁺ $\gamma\delta$ T lymphocytes accumulated in mouse pleura, suggesting the involvement of CCR9/CCL25 in $\gamma\delta$ T-cell migration and/or activation during allergy. Interestingly, the in vivo neutralization of CCL25 selectively inhibited the migration of a subpopulation of $\alpha_4\beta_7$ ⁺ $\gamma\delta$ T lymphocytes, but failed to diminish total $\gamma\delta$ or $\alpha\beta$ T-cell counts in the pleura during allergic inflammation. Indeed, in a previous report, we demonstrated that CCR2/CCL2 is mainly required for $\gamma\delta$ T-cell migration during allergy [11]. To address this issue, we analyzed the expression pattern of chemokine receptors by the $\alpha_4\beta_7$ ⁺ $\gamma\delta$ T-cell population from OVA-challenged mouse pleura. We observed that 40% of such population expresses CCR9, whereas only 10% of those cells express CCR2, and 20% expresses CCR6. In accordance, CCL25 i.pl. injection only attracted $\alpha_4\beta_7$ ⁺ $\gamma\delta$ T lymphocytes expressing CCR6 and CCR9, but not CCR2 (Supporting Information Fig 4). CCR9/CCR6 coexpression has been

previously demonstrated [6, 34] and characterized as a phenotype of IL-17 producers in the intestine [35]. However, whether the impairment of CCR6⁺ $\gamma\delta$ T-lymphocyte accumulation by anti-CCL25 mAb during allergic pleurisy occurred via a direct mechanism (due to the coexpression with CCR9) or via an indirect mechanism is yet to be defined. It is noteworthy that the participation of CCL20 in IL-17⁺ $\gamma\delta$ T-cell migration during allergy cannot be ruled out. The fact that CCL20 neutralization slightly diminished IL-17⁺ $\gamma\delta$ T-cell chemotaxis toward OPW suggests that CCL20/CCR6 and CCL25/CCR9 might cooperate for their attraction to the allergic site.

Even though the CCR9/ $\alpha_4\beta_7$ expression determines a phenotype of intestinal mucosa population [18], we detected the presence of CCR9⁺/ $\alpha_4\beta_7$ ⁺ lymphocytes in the pleura during the allergic response. It has been demonstrated that CCL25 induces T-cell adhesion via $\alpha_4\beta_7$ integrin [17] and preferentially induces the migration of $\alpha_4\beta_7$ ⁺ T cells via CCR9 [36]; even though the mechanisms involved in this phenomenon remain to be elucidated. $\gamma\delta$ T cells express several integrins, such as $\alpha_4\beta_1$ and $\alpha_4\beta_7$, which are known to be important for the adhesion to the endothelium and transmigration into inflamed tissue [22, 23, 37–39]. Indeed, selective blocking mAbs against $\alpha_4\beta_1$ integrin inhibited human $\gamma\delta$ T-lymphocyte adhesion to cytokine-activated endothelial cells [24]. Moreover, CCL25 has been shown to induce T lymphocyte adhesion via the interaction of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins to MadCAM-1 and VCAM-1, respectively [16, 17]. These data corroborate our findings that CCL25 induced the transmigration of $\gamma\delta$ T lymphocytes through endothelial cells, via the interaction of $\alpha_4\beta_7$ integrin to MadCAM-1/VCAM-1. During allergy, the expression of VCAM-1 (but not ICAM-1) by mouse endothelium has been shown to be upregulated [40]. In addition, the *in vitro* stimulation of HUVECs by the Th2 cytokine IL-4 also induced the expression of VCAM-1, failing to alter ICAM-1 expression [41], which is in accordance with our observations that IL-4 triggered increased expression of VCAM-1 and MadCAM-1 on mouse endothelial cells, but not of ICAM-1 (not shown). Previous reports have shown the importance of the α_4 integrin chain for the *in vivo* migration of T lymphocytes that are shown to preferentially migrate via α_4 integrin/VCAM-1 pathway rather than via $\alpha_L\beta_2$ or ICAM-1 [40, 42]. However, no data specifically concerned the role of $\alpha_4\beta_7$ integrin on $\gamma\delta$ T-lymphocyte migration during an allergic response. Our results demonstrate the relevance of $\alpha_4\beta_7$ integrin for $\gamma\delta$ T-cell migration during an allergic reaction, which was reinforced by the fact that the *ex vivo* blockade of α_4 chain impaired the migration of adoptively transferred CFSE⁺ $\gamma\delta$ T lymphocytes into the allergic site. Moreover, we observed that $\alpha_L\beta_2$ blockade failed to inhibit $\gamma\delta$ T lymphocyte *in vitro* transmigration toward OPW (not shown). It is also noteworthy that OVA immunization induced a sevenfold increase on the numbers of $\gamma\delta$ T cells expressing $\alpha_4\beta_7$ integrin in the spleen (not shown).

Our data that demonstrate that CCL25 modulates IL-17 production during allergy reveal an interesting role for this chemokine. It is important to call attention to the fact that CCL25 induced the migration of IL-17⁺ cells without affecting IL-17 production *in vitro*.

Therefore, the modulation of pleural IL-17 levels by CCL25 is actually a result of the *in vivo* migration of IL-17⁺ $\gamma\delta$ T lymphocytes. IL-17⁺ $\gamma\delta$ T lymphocytes are committed as IL-17 producers within the fetal thymus, via ROR γ t transcriptional factor- and Notch-Hes1-dependent mechanisms, independently of TCR signaling [43–46] and CD27 costimulation [47]. In the immune site, their activation is determined by multiple cytokines, among which a chief role is attributed to IL-1 β and IL-23 in mice and humans [48–50]. It has been shown that TCR $\gamma\delta$ ⁺/CCR6⁺ cells that produce IL-17 coexpress CCR9 (which was not observed for $\gamma\delta$ ⁺/NK1.1⁺ cells, which produce IFN- γ) [6, 34]. Accordingly, CCL25 induces the specific chemotaxis of Th17 CD4⁺ T cells polarized by retinoic acid (which is an important regulatory signal in the intestine), but not of Th0 lymphocytes [51]. These T cells were shown to express CCR9/ $\alpha_4\beta_7$ integrin and preferentially migrate to the intestine and regulate inflammation. Moreover, it has been demonstrated that, throughout Th17 differentiation, CD4⁺ T lymphocytes are shown to increase the expression of chemokine receptors, including CCR9 [52]. Interestingly, IL-17-producing $\gamma\delta$ T cells have been shown to be CD25⁺ and CD122[−] [43, 44], a phenotype observed by us on $\gamma\delta$ T cells recovered from CCL25-stimulated mouse pleura. It is noteworthy that, since CCL25 *i.p.l.* injection failed to trigger CD122⁺ T-cell migration, the percentage of CD122⁺ $\gamma\delta$ T lymphocyte population in the pleura of CCL25-stimulated mice slightly decreased (SAL 11.8% versus CCL25 5.0% among $\gamma\delta$ T lymphocytes). Similar to our data, it has been demonstrated that IL-17-producing $\gamma\delta$ T cells did not produce IFN- γ or IL-4 and specifically expressed CD25 but not CD122 (whereas CD122⁺ $\gamma\delta$ T lymphocytes produced IFN- γ). Moreover, IL-17-producing $\gamma\delta$ T lymphocyte maintenance was shown to depend on CD25 and IL-2 [44]. It has been also shown that CD122^{lo} $\gamma\delta$ T cells recovered from mouse spleen, lymph nodes, and thymus produced high levels of IL-17 but small amounts or no IFN- γ upon TCR *in vitro* stimulation [43]. An inverse correlation between CD122 and CCR9 has also been demonstrated on $\gamma\delta$ lymphocytes from mouse thymus [53]. This work demonstrates that $\gamma\delta$ thymocytes that express high levels of CCR9 are CD122^{lo}, whereas CCR9^{lo} express high levels of CD122. It is important to note that the CCL25 neutralization and $\alpha_4\beta_7$ integrin blockade during allergic pleurisy did not inhibit $\alpha\beta$ T lymphocyte recruitment, whereas the *i.p.l.* stimulus with CCL25 selectively triggered $\gamma\delta$ T-cell migration. These data corroborate and reinforce the hypothesis that CCL25 is important for the migration of a specific $\gamma\delta$ T-cell subset that produces IL-17 during an allergic reaction, via $\alpha_4\beta_7$ integrin. Therefore, it is possible that the relevance of CCL25 in the context of allergic responses is correlated to IL-17 production in the allergic site. Whether this phenomenon contributes to the enhancement or regulation of allergy is still unclear, since contrasting roles for IL-17 have been described [54–57].

The role of IL-17⁺ $\gamma\delta$ T lymphocytes (and of IL-17) in infection, tumor immunity, and autoimmunity has been reported, and it is still controversial [50, 58–63]. A clear involvement of IL-17⁺ $\gamma\delta$ T lymphocytes in autoimmunity has been evidenced in experimental arthritis and autoimmune encephalomyelitis, in which these cells

have been shown to amplify CD4⁺ Th17 cell responses, to suppress Foxp3⁺ Treg cells, and to contribute to the development of the response [48, 62–64]. In regard to the participation of IL-17⁺ $\gamma\delta$ T lymphocytes in airway inflammation, it has been recently demonstrated that those cells downmodulate central features of an allergic reaction, including Th2 response and lung eosinophilia [65]. Although these regulatory lymphocytes have been shown to express V γ 4 TCR chain, we observed that, in the model of allergic pleural inflammation, V γ 4 T lymphocyte migration was not affected by CCL25 neutralization (not shown). It is noteworthy that, in this experimental model, CCL25 neutralization also failed to alter the accumulation of mononuclear cells, T lymphocytes, and eosinophil in the allergic site, which are major cells that orchestrate the allergic response. Increased levels of CCL25 in synovial fluid from arthritis patients have been reported [13]; however whether CCR9/CCL25 play a role in autoimmune and infectious diseases by mediating IL-17⁺/CCR6⁺ $\gamma\delta$ T lymphocytes is yet to be addressed.

Our results reveal a particular *in vivo* migration pathway for IL-17⁺ $\gamma\delta$ T lymphocytes, which requires CCL25/CCR9 axis and is mediated by $\alpha_4\beta_7$ integrin. Here, we provide evidence that CCL25 plays a pivotal role for IL-17⁺ $\gamma\delta$ T-cell trafficking in allergic response; however, the relevance of this chemokine in Th17-mediated immune responses is yet to be defined.

Material and methods

Animals

C57BL/6 (18–20 g) provided by Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil) were used. All experimental procedures were performed according to The Committee on Ethical Use of Laboratory Animals of Oswaldo Cruz Foundation (Fiocruz, Brazil).

Treatments

Animals received an i.p. injection of mAb anti-CCL25 (89818; 10 μ g/cavity; R&D Systems [Minneapolis, MN, USA]) or an intravenous (i.v.) injection of mAb anti- $\alpha_4\beta_7$ integrin (DATK32; 100 μ g/mouse; BD Pharmingen), 1 h before antigenic challenge.

Pleurisy

Fourteen days after active immunization (50 μ g OVA/5 mg aluminum hydroxide, subcutaneously [s.c.]), mice were challenged by an i.p. injection of OVA (12.5 μ g/cavity; grade V, Sigma-Aldrich) or rmCCL25 (200 ng/cavity; R&D Systems). Sensitized mice challenged with saline vehicle were used as a negative control group. At specific time points after stimulus, pleural leukocytes were recovered and counted. For chemotaxis and transmigration assays, cell-free pleural wash recovered from mice injected with

saline (SPW) or OVA (OPW) 24 h after challenge were pooled ($n = 10$).

Flow cytometry analysis

Leukocytes (10^6) were stained with the appropriate concentration of the following antibodies: CCR6 (29-2L17; Biolegend, Inc.), CCR9 (polyclonal; Santa Cruz), CD3 (145-2C11), TCR δ chain (GL3), PE TCR β chain (H57-597), α_4 integrin chain (R1-2), $\alpha_4\beta_7$ integrin (DATK32), CD25 (7D4), CD2 (RM2-5), CD45RB (16A), CD69 (H1.2F3), CD122 (TM-Beta1) (BD Pharmingen). For intracellular cytokine staining, cells were preincubated for 4 h with PMA (20 ng/mL), ionomycin (500 ng/mL), and brefeldin A (10 μ g/mL) at 37°C at 5% CO₂. After surface marker staining, cells were fixed, permeabilized, and stained with anti-IL-17, anti-IFN- γ , and anti-IL-4 antibodies (BD Pharmingen). IgG isotypes were used as irrelevant antibodies. Analysis was performed by using Cell Quest program in FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Counts are reported as numbers of cells after the multiplication of the percentage of T lymphocyte population by the total number of leukocytes. Gating strategy is shown in Supporting Information Fig. 5.

Enzyme-linked immunosorbent assay

Levels of CCL25, IL-17, IFN- γ , and IL-4 in cell-free pleural washes, recovered 24 h after challenge, were evaluated by sandwich enzyme-linked immunosorbent assay (ELISA) by using matched antibody pairs from R&D, according to manufacturer's instructions.

Pleural mesothelial cell stimulation

Mesothelial cells were recovered from C57BL/6 mice pleura 14 days after s.c. sensitization as previously described [11] and yielded at least 90% of cytokeratin 7⁺ cells (data not shown), a cell surface marker that characterizes mesothelial cells [66]. Cells (4×10^5 /well) were stimulated with rmIL-4 (40 ng/mL; R&D Systems) and supernatants were recovered after 12 h, for CCL25 evaluation.

Transendothelial migration assay

Murine thymic endothelioma cells (tEnd.1) were cultured in transwell polycarbonate culture inserts placed in 24-well culture plates (8.0- μ m pore diameter, BD Falcon) and allowed to grow to confluence. tEnd.1 cell monolayers were prestimulated with rmIL-4 (40 ng/mL; R&D Systems), SPW or OPW for 30 min, washed and added to 24-well culture plates containing PBS, rmCCL25 (100 ng/mL; R&D Systems), SPW, or OPW. Spleen T lymphocytes (85% purity) were pretreated for 30 min with mAb anti- α_4

integrin chain and anti- $\alpha_4\beta_7$ integrin at saturating concentration (25 $\mu\text{g/mL}$; BD Pharmingen) and added (10^6 cells/well) to the tEnd.1 monolayers and allowed to migrate for 3 h (37°C, 5% CO_2). IgG isotypes were used as irrelevant antibodies. Transmigrated cells were counted and stained for flow cytometry, as described above. Results are expressed as transmigration index, generated by using the number of cells that migrated toward buffer as comparison.

Adoptive transfer assay

Donor and recipient C57BL/6 mice were i.p. injected with OVA 14 days post immunization. T lymphocytes were recovered from donor mice 24 h after challenge, labeled with CFSE (1 $\mu\text{M}/4 \times 10^7$ cells), and treated or not with anti- α_4 integrin chain (25 $\mu\text{g/mL}$). IgG isotype was used as irrelevant antibody. CFSE-labeled T lymphocytes (4×10^7 cells $\geq 90\%$ viability) were i.v. injected into recipient mice 24 h after the i.p. injection of OVA or saline solution. Recipient mice were euthanized 24 h after adoptive transfer and their pleural cavities were rinsed.

Chemotaxis assay

Spleen T lymphocytes (3×10^6) were placed in the upper chamber of 3.0 μm pore diameter transwell tissue culture inserts (Falcon). Transwell inserts were placed in the individual wells of a 24-well cell culture plate containing assay buffer or the following stimuli: rmCCL25 (100 ng/mL); rmCCL20, 5 ng/mL (R&D Systems); OPW or OPW plus anti-CCL20 mAb (5 $\mu\text{g/mL}$) and incubated for 2 h (37°C, 5% CO_2). In a set of experiments, T lymphocytes were preincubated with anti-CCR9 blocking Ab (5 $\mu\text{g/well}$; Santa Cruz) for 30 min at 37°C. Migrated cells were labeled as described above, and analyzed by using a flow cytometer (FACScalibur flow cytometer, Becton Dickinson). Results are expressed as chemotactic index, generated by using the number of cells that migrated toward buffer as comparison.

In vitro activation assay

T lymphocytes recovered from previously immunized mouse spleens (10^6 per well) were stimulated with rmCCL25 (100 ng/mL) or anti-TCR $\gamma\delta$ mAb (10 $\mu\text{g/mL}$) in RPMI 1640 medium supplemented with 10% FBS for 18 h in the presence of brefeldin A (10 $\mu\text{g/mL}$). After incubation, cells were stained for flow cytometry.

Statistical analysis

Data are reported as the mean $\pm$ SEM and were statistically evaluated by analysis of variance (ANOVA) followed by Newman–

Keuls–Student test or Student's *t*-test. Values of $p \leq 0.05$ were regarded as significant.

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- Abbreviations:** i.pl.: intrapleural · OPW: cell-free pleural wash recovered from OVA-challenged mice · rmCCL25: recombinant mouse CCL25 · SPW: cell-free pleural wash recovered from saline-injected mice · TECK: thymus-expressed chemokine
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Supplementary Materials and Methods

Immunofluorescence staining

tEnd.1 cells (10^5) were cultured in acid-washed coverslips, allowed to grow for 48 h, stimulated with rmIL-4 (40 ng/ml; R&D) for 4 h, and fixed with 4% paraformaldehyde. Cells were then stained with biotin conjugated mAb anti-VCAM-1 (429 [MVCAM.A]) or purified mAb rat IgG anti-MadCAM-1 (MECA-89), followed by FITC-conjugated streptavidin or FITC-conjugated mAb anti-rat IgG (BD Pharmingen). Controls consisted of slides in which the primary antibody was omitted. Cells were examined with a laser-scanning confocal microscope (Fluoview FV300, Olympus, Japan) under oil immersion objective lens (40x). Cells were identified by differential interference contrast microscopy (DIC), and the mean of fluorescence intensity was calculated per cell from at least five high power fields out of three samples per group. Images were obtained and processed using Fluoview 3.3 (Olympus) and Image J (public domain) software programs. Quantitative data are shown as the mean of fluorescence intensity per cell $\pm$ SEM.

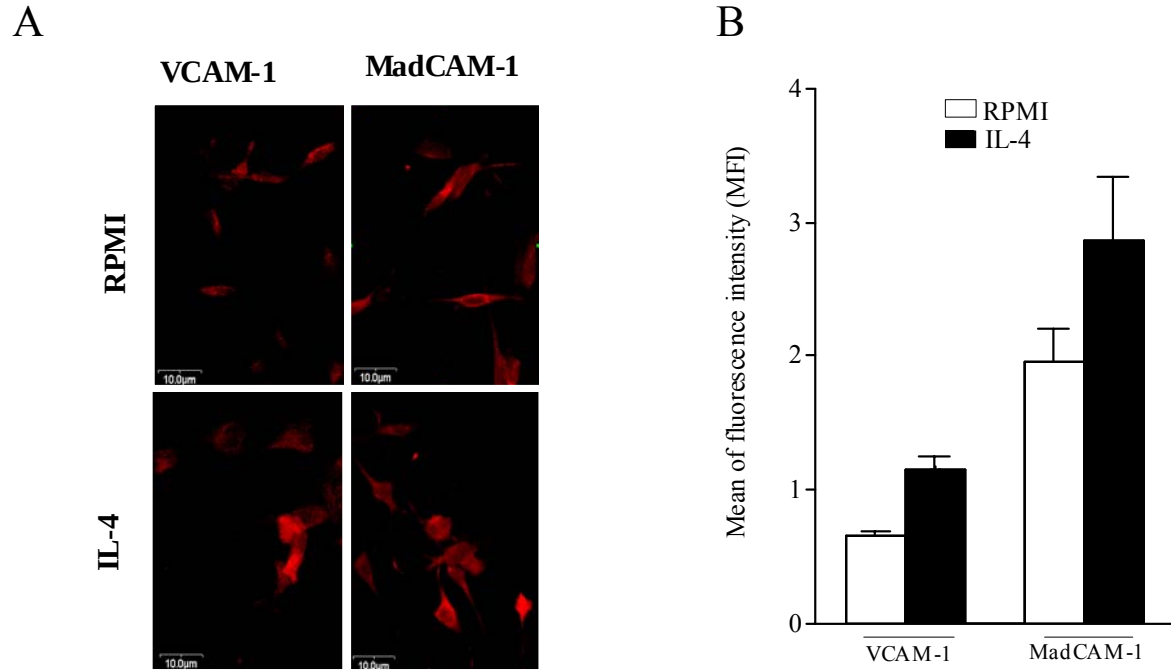
Supplemental Figure 1. CCL25 induces $\gamma\delta$ T cell transmigration mediated by $\alpha_4\beta_7$ -integrin.

Supplemental Figure 2. Effect of *in vivo* pre-treatment with anti-CCL25 mAb on OVA-induced IFN- γ^+ or IL-4 $^+$ $\gamma\delta$ T lymphocyte accumulation.

Supplemental Figure 3. CCL20 neutralization decreases IL-17 $^+$ $\gamma\delta$ T cell chemotaxis towards OPW.

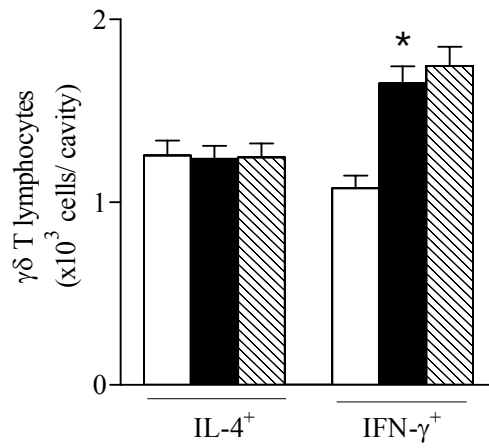
Supplemental Figure 4. Expression of the chemokine receptors CCR2, CCR6 and CCR9 by $\alpha_4\beta_7^+$ $\gamma\delta$ T lymphocytes.

Supplemental Figure 5. Gating strategy used for flow cytometry analysis of $\gamma\delta$ cells expressing CCR6, CCR9 and $\alpha_4\beta_7$ -integrin.

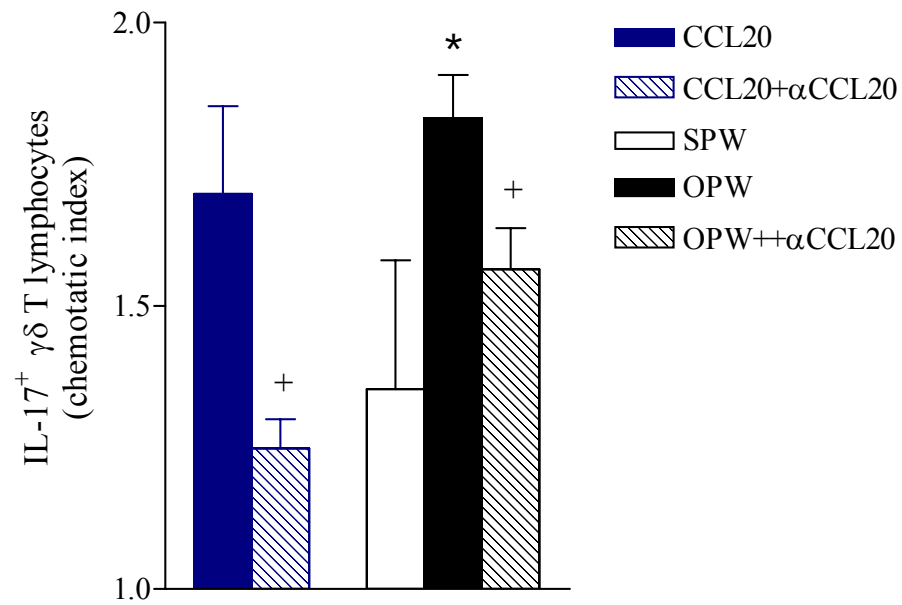


Supplemental Figure 1. CCL25 induces $\gamma\delta$ T cell transmigration mediated by $\alpha_4\beta_7$ -integrin.

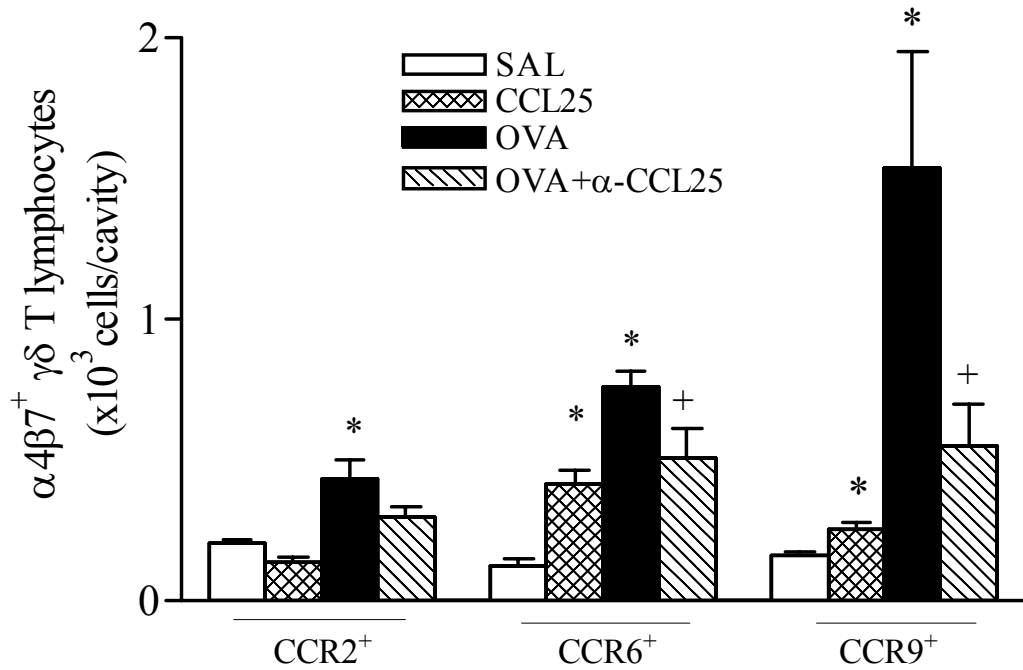
(A) Representative pictures of fluorescent staining of VCAM-1 and MadCAM-1 expression on IL-4 *in vitro* stimulated endothelial cells within 4h (Olympus; 40 x). (B) Quantitative data are shown as the mean of fluorescence intensity per cell $\pm$ SEM (≥ 5 high power fields out of 3 samples/group). * indicates $p \leq 0.05$ between non-stimulated and stimulated groups.



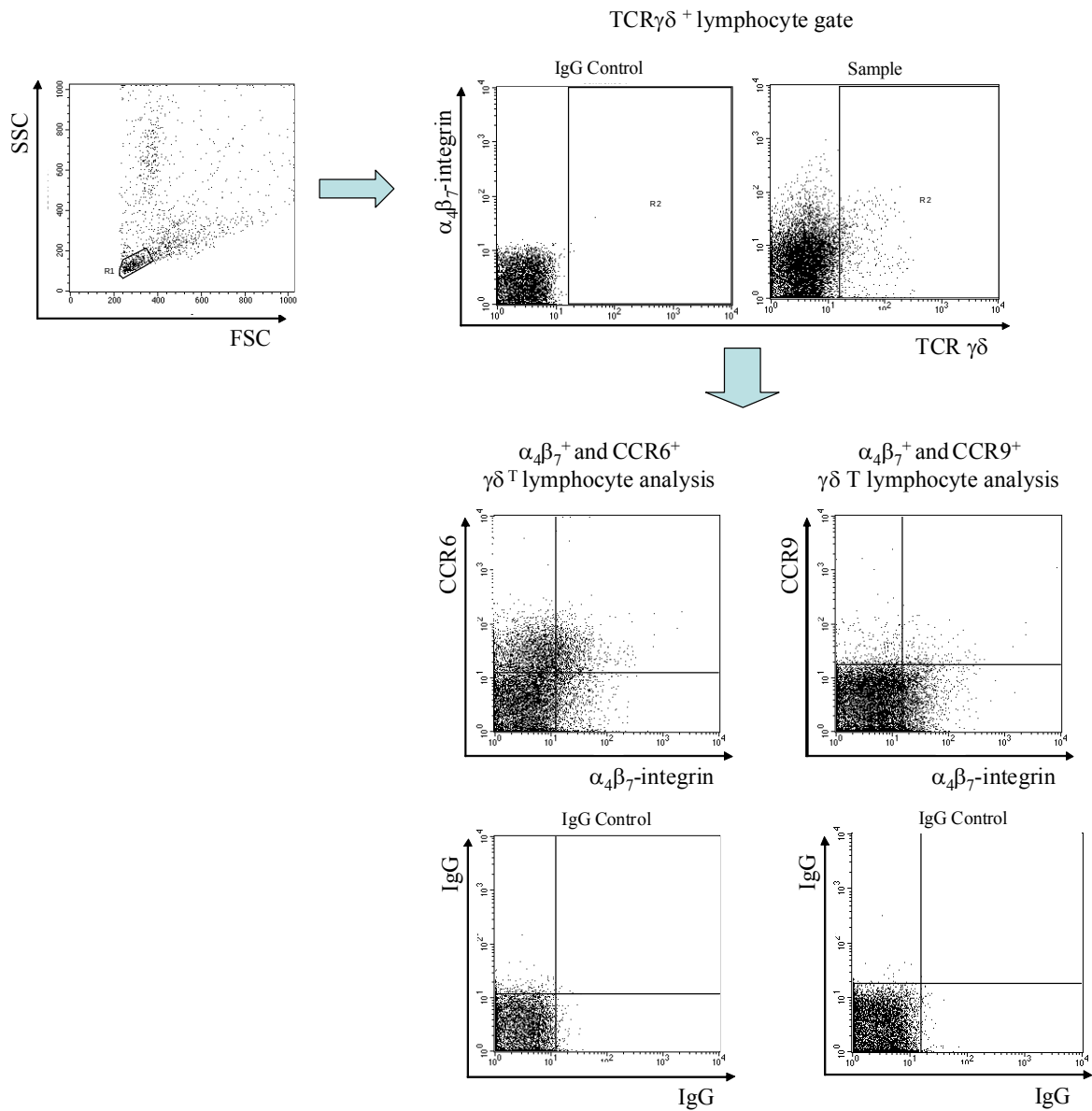
Supplemental Figure 2. Effect of *in vivo* pre-treatment with anti-CCL25 mAb (10 µg/cavity i.pl.) on OVA-induced IL-4⁺ and IFN-γ⁺ γδ T lymphocyte accumulation within 48 h. Data show mean ± SEM (n≥6 mice/group or n≥3 wells/group). * indicates p≤0.05 between non-stimulated and stimulated groups. + indicates p≤0.05 between stimulated and treated groups.



Supplemental figure 3. CCL20 neutralization decreases IL-17⁺ γδ T cell chemotaxis towards OPW. CCL20 (5 ng/ml) or OPW recovered from mice 24 h after OVA i.pl. injection (12.5 μg per cavity) were incubated in the presence of mAb anti-CCL20 (5 μg/ml) for 30 min at 37°C. Cells that migrated into the bottom chamber were recovered and labeled for CD3, γδ TCR and IL-17 for FACS analysis. Results are expressed as chemotactic index, generated by using the number of cells that migrated towards buffer as comparison. Data represent one of two different experiments. * indicates $p \leq 0.05$ between non-stimulated and stimulated groups. + indicates $p \leq 0.05$ between stimulated and treated groups.



Supplemental figure 4. Expression of the chemokine receptors CCR2, CCR6 and CCR9 by $\alpha_4\beta_7^+ \gamma\delta$ T lymphocytes. Pleural lymphocytes were recovered from C57BL/6 mice 24 h after CCL25 (200 ng/cavity, i.pl.) or 48 h after OVA (12.5 μ g/cavity, i.pl.) and stained for $\gamma\delta$ TCR, $\alpha_4\beta_7$, CCR2, CCR6 and CCR9. Mice were pre-treated with anti-CCL25 mAb (10 μ g/cavity, i.pl.) 1 h before OVA challenge. Data show mean $\pm$ SEM ($n \geq 6$ mice/group). * indicates $p \leq 0.05$ between non-stimulated and stimulated groups. + indicates $p \leq 0.05$ between stimulated and treated groups.



Supplemental Figure 5. Gating strategy used for flow cytometry analysis of $\gamma\delta$ cells expressing CCR6, CCR9 and $\alpha_4\beta_7$ -integrin. A lymphocyte gate (R1) was defined based on the cells' Forward Scatter (FSC) and Side Scatter (SSC), further gated on $\text{TCR}\gamma\delta^+$ lymphocytes (R2) and analyzed for CCR9, CCR6 and $\alpha_4\beta_7$ -integrin levels of expression.