



## Requirement of L-selectin for $\gamma\delta$ T lymphocyte activation and migration during allergic pleurisy: Co-relation with eosinophil accumulation

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### ABSTRACT

Intra-thoracic antigenic challenge (ovalbumin, 12.5  $\mu$ g/cavity) led to increased numbers of  $\gamma\delta$  T lymphocytes in pleural cavities, blood and thoracic lymph nodes in sensitized mice within 48 h. Part of these cells expressed CD62L, which increased on  $\gamma\delta$  T cell surfaces obtained from lymph nodes after ovalbumin (OVA) challenge. Selectin blockade by fucoidan pre-treatment (10 mg/kg, i.v.) impaired *in vivo* increase in CD25<sup>+</sup> and *c-fos*<sup>+</sup>  $\gamma\delta$  T cell numbers in lymph nodes, indicating a role for selectins on  $\gamma\delta$  T lymphocyte activation and proliferation. *In vivo* selectin blockade by fucoidan or  $\alpha$ -CD62L mAb (200  $\mu$ g/mice, i.p.) also inhibited OVA-induced  $\gamma\delta$  T cell accumulation in pleural cavities. Confirming the direct effect of CD62L on  $\gamma\delta$  T cell transmigration, the migration of i.v. adoptively-transferred CFSE-labeled  $\gamma\delta$  T lymphocytes into pleural cavities of challenged recipient mice was impaired by fucoidan *ex vivo* treatment. It is noteworthy that eosinophil influx was also impaired in those mice, indicating that reduced eosinophil migration by CD62L *in vivo* blockade depended on  $\gamma\delta$  T cell migration via CD62L molecules. Accordingly, pleural  $\gamma\delta$  T lymphocytes from fucoidan-treated mice presented reduced OVA-induced IL-5 and CCL11 production. Supporting these data, the depletion of V $\gamma$ 4 T lymphocytes, which are pulmonary  $\gamma\delta$  T cells, decreased OVA-induced eosinophil influx into allergic site. Such results demonstrate that CD62L is crucial for the activation of  $\gamma\delta$  T cells in lymph nodes, for their migration into inflamed tissue and for the modulation of eosinophil influx during allergic response.

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### 1. Introduction

Lymphocytes expressing  $\gamma\delta$  T cell receptors (TCR) comprise less than 10% of T cell population in lymphoid organs and peripheral blood in human subjects [1]. In contrast with  $\alpha\beta$  T cells,  $\gamma\delta$  T lymphocytes are preferentially localized in epithelial and mucosal tissues and are able to recognize bacterial antigens with no need of processing, which suggests a particular role of these cells in the first line of defense against pathogens [2,3]. These cells are able to produce a multiplicity of cytokines/chemokines and are involved in several infectious diseases, autoimmune disorders and cancer [4].  $\gamma\delta$  T lymphocytes have also been shown to regulate allergic airway inflammation. During allergic reactions, the number of these cells increases in mice bronchoalveolar lavage (BAL) and lung tissue, displaying either effector functions or negative regulatory functions [5].

A few reports show that, in animal models and in allergic subjects,  $\gamma\delta$  T cells that accumulate in the inflammatory site contribute to eosinophil infiltration [6–9]. These cells undertake pro-inflammatory functions and induce immunoglobulin E synthesis via the regulation of IL-5 and IL-4 production, as demonstrated by means of the use of knockout mice or  $\alpha$ - $\gamma\delta$  antibody depletion [6–9]. In these models of lung allergic inflammation, whether  $\gamma\delta$  T cells display pro- or anti-inflammatory profiles seems to be determined by the  $\gamma\delta$  T cell subset involved in inflammatory response as well as by its tissue distribution [10,11]. Specific subsets of  $\gamma\delta$  T cells are shown to be distributed along the airway tissue. Among these subsets, V $\gamma$ 4 T lymphocytes are described as the major pulmonary resident subset in mice [5]. V $\gamma$ 4 T cells are also found in peripheral blood and lymphoid organs, being capable to migrate into inflamed airways and to regulate local immune response [3,10]. The depletion of V $\gamma$ 4 T cells has been shown to suppress lung inflammation induced by infectious agents or allergens [12–14].

$\gamma\delta$  T cells express CD62L, which is a member of the selectin family, and support the rolling and migration on these cells [15–17].  $\gamma\delta$  T lymphocytes exhibit high degree of *in vitro* migration through endothelial cells [18] and are able to bind CD62E and CD62P substrates under physiological flow conditions, a phenomenon mainly mediated by sialyl Lewis<sup>x</sup> (sLe<sup>x</sup>) carbohydrate antigens, cutaneous lymphocyte-

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associated antigen (CLA) and CD62P glycoprotein ligand (PSGL-1) [16,17,19,20]. In addition,  $\gamma\delta$  T cells that express low levels of CD62L are shown to inefficiently accumulate in the tissue under normal conditions or during inflammation, in contrast with CD62L<sup>+</sup> high  $\gamma\delta$  T cells [16], which suggests that CD62L expression patterns can determine the migration of these cells into the tissue.

During the allergic process, blockade or genetic deficiency of CD62L or CD62P has been shown to reduce the number of T lymphocytes in ovalbumin (OVA)-challenged mice BAL and lung tissue [21–23]. Furthermore, CD62L also functions as a co-stimulatory signal in T lymphocytes and is involved in the proliferation of these cells [24]. Moreover, CD62L has also been shown to associate with TCR/CD3 complex, supporting the role of CD62L in T lymphocyte co-stimulatory signaling [25,26].

Nevertheless, the role of selectins in activation and migration of  $\gamma\delta$  T cells during allergic reaction has not been previously shown. In the present study, we investigate the role of members of selectin family in mediating  $\gamma\delta$  T cell activation in lymph nodes and its migration into the inflamed tissue during allergic response, as well as its correlation with eosinophil accumulation in a murine model of allergic pleurisy.

## 2. Materials and methods

### 2.1. Materials

Ovalbumin (OVA), phosphate buffered saline (PBS), RPMI 1640, ethylenediaminetetraacetic sodium salt (EDTA), bovine serum albumin (BSA), sodium azide, gentamicin, monensin, saponin, red blood cell lysing buffer, Histopaque 1077, trypsin/EDTA solution, FITC-conjugated mouse IgG anti-rabbit IgG and *Fucus vesiculosus* fucoidan were purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hy-Clone (Logan, Utah). Aluminum hydroxide was purchased from EMS Sigma Pharma (São Paulo, Brazil). Purified and biotinylated anti-mouse CCL11, IL-4 and IL-5 monoclonal antibodies (mAbs) and their respective recombinants were all obtained from R&D Systems (Minneapolis, MN). Cy-Chrome-conjugated streptavidin, PerCP/PE/FITC-conjugated hamster IgG1 anti-mouse CD3 (145-2C11), PE/FITC-conjugated hamster IgG2 anti-mouse  $\delta$  TCR (GL3), FITC-conjugated hamster IgG1 anti-mouse V $\gamma$ 4 TCR (UC3.10A6), FITC-conjugated hamster IgG2 anti-mouse V $\delta$ 4 TCR (GL2), PE-conjugated hamster IgG2 anti-mouse V $\delta$ 6.3 TCR (8F4H7B7), FITC-conjugated rat IgM anti-mouse CD25 (7D4) mAbs, FITC-conjugated or purified rat IgG2a anti-mouse CD62L (MEL-14), rat IgG1 anti-mouse CD62P (RB40.34) and rat IgG2a anti-mouse CD62E (10E9.6), PerCP/PE/FITC-conjugated hamster IgG1 and IgG2, rat IgM, IgG 1, IgG2a isotype controls were all purchased from BD Pharmingen (San Diego, CA). Carboxyfluorescein diacetate-succinimidyl ester (CFSE) was obtained from Molecular Probes (Carlsbad, CA). Purified rabbit IgG anti-mouse *c-fos* was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

### 2.2. Animals

C57BL/6 or BALB/c mice (18 to 20 g) provided by Oswaldo Cruz Foundation breeding unit (Fiocruz, Rio de Janeiro, Brazil) were used. IL-5 transgenic BALB/c mice were kindly provided by P. F. Weller (Harvard Medical School, Boston, MA) through S. A. C. Perez (Fiocruz, Rio de Janeiro, Brazil). Until used, mice were caged with free access to food and fresh water in a room with temperature ranging from 22 to 24 °C and a 12 h light/dark cycle at Farmanguinhos experimental animal facility. All experimental procedures were performed according to Oswaldo Cruz Foundation's Committee on Ethical Use of Laboratory Animals.

### 2.3. Treatments

Animals received intravenous (i.v.) injection of fucoidan (10 mg/kg) or intraperitoneal (i.p.) injection of mAb anti-CD62L (200  $\mu$ g/mice). Both treatments were diluted in sterile PBS solution in a final volume of 100  $\mu$ l, 1 h before antigenic challenge, as previously described [27,28].

### 2.4. V $\gamma$ 4 depletion

The anti-V $\gamma$ 4 antibody-secreting hybridoma (UC3) was kindly provided by J. A. Bluestone (University of California, San Francisco, CA) through R. L. O'Brien (National Jewish Medical and Research Center, Denver, CO). Hamster anti-V $\gamma$ 4 monoclonal antibody (mAb) was obtained from ascitic fluid generated in BALB/c nu/nu mice. mAb was purified by chromatography on protein G column (Pharmacia, Piscataway, NJ). Mice were depleted of V $\gamma$ 4<sup>+</sup> cells by i.p. injections of mAb (400  $\mu$ g/mouse 1 day before sensitization and 400  $\mu$ g/mouse per day, every 2 days, until euthanasia). Control mice were similarly sham-treated with 400  $\mu$ g of protein G-purified normal hamster serum IgG.

### 2.5. Allergic pleurisy

Active sensitization was achieved by subcutaneous (s.c.) injection of 200  $\mu$ l of a mixture of endotoxin-free OVA (50  $\mu$ g) and aluminum hydroxide (5 mg). Fourteen days later, mice were challenged by an intrathoracic (i.t.) injection of OVA (12.5  $\mu$ g/cavity) diluted in sterile saline solution to a final volume of 100  $\mu$ l. Sensitized mice challenged with saline vehicle alone were used as a negative control group. At specific time points after stimulus, mice were euthanized in carbon dioxide chamber. Blood samples were immediately withdrawn from mice abdominal aorta and submitted to Histopaque-1077 gradient (400  $\times$ g for 30 min) for peripheral blood mononuclear cell separation. Thoracic lymph nodes were harvested and mechanically disrupted into single-cell suspensions after centrifugation (400  $\times$ g for 10 min). Pleural leukocytes were recovered from thoracic cavities after washing with 0.5 ml of saline solution containing EDTA 10 mM (pH 7.4). For transmigration assays, pleural washes recovered from previously sensitized mice injected with saline (SPW) or OVA (OPW) were used. SPW and OPW were obtained by washing thoracic cavities ( $n=20$  per group) with 0.5 ml of sterile saline solution, pooled and centrifuged (420  $\times$ g for 10 min). Cell-free supernatants were recovered and kept at  $-20$  °C until used.

### 2.6. Leukocyte counts

Total leukocyte counts were made in Neubauer chamber, under an optical microscope, after dilution in Türk fluid (2% acetic acid). Differential counts of mononuclear cells and eosinophils were made by using stained cytopins (Cytospin 3, Shandon Inc., Pittsburgh, PA) by May–Grünwald–Giemsa method. CFSE<sup>+</sup> eosinophils were analyzed by flow cytometry and gated according to FL-1 intensity and physical characteristics, as described [29]. Counts are reported as numbers of cells per cavity for pleural eosinophils and lymphocytes, as well as cells per microliter ( $\text{mm}^3$ ) for blood lymphocytes. Lymph nodes lymphocyte counts are expressed as cells per milligram of lymphoid tissue.

### 2.7. Enzyme-linked immunosorbent assay

Levels of CCL11, IL-4 and IL-5 were evaluated in cell-free pleural washes, recovered 6 h after challenge, by sandwich enzyme-linked immunosorbent assay (ELISA). Matched antibody pairs from R&D (Minneapolis, MN) were used according to manufacturer's instructions. Results are expressed as pg per cavity.

## 2.8. Surface marker immunostaining

Cells recovered from pleural cavities, thoracic lymph nodes and peripheral blood ( $10^6/100\ \mu\text{l}$ ) were incubated with appropriate concentrations of FITC-, PE- or PerCP-conjugated mAbs at  $4\ ^\circ\text{C}$  for 30 min, after incubation with rat serum to block non-specific binding sites. IgG isotypes were used as irrelevant antibodies. Surface marker analysis was performed by using Cell Quest program in FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). 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 and dot plots. Percentages of  $\gamma\delta$  lymphocyte subpopulation were determined in a specific  $\text{CD}3^+$  T lymphocytes gate. Counts are reported as numbers of cells after multiplying the percentage of  $\gamma\delta$  T lymphocytes by the total number of leukocytes.

## 2.9. Intracellular immunostaining

After surface marker staining, cells were fixed with 2% paraformaldehyde at  $4\ ^\circ\text{C}$  for 20 min and incubated with 0.1% saponin in PBS/0.1% azide/10% FBS. Anti-murine c-fos, CCL11, IL-4 or IL-5 antibodies were added to cell suspension and incubated at  $4\ ^\circ\text{C}$  for 30 min, followed by streptavidin-Cy-Chrome or anti-rabbit IgG conjugated with FITC. IgG isotypes were used as irrelevant antibodies. For intracellular cytokine staining, cells recovered from mice pleural cavities were pre-incubated overnight with RPMI 1640 supplemented with 10% FBS containing monensin ( $2\ \mu\text{M}$ ) at  $37\ ^\circ\text{C}$  at 5%  $\text{CO}_2$ . Analysis was performed by using Cell Quest program in FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).  $\text{CD}3^+$  lymphocytes were specifically gated, and at least  $10^4$  cells were acquired per sample. Absolute numbers of cytokine-expressing lymphocytes were calculated by multiplying the percentage of positive cells determined in dot plots by the total number of leukocytes.

## 2.10. Endothelial cell culture

Murine thymic endothelioma cell line (tEnd.1) was grown in RPMI 1640 supplemented with 10% FBS containing  $25\ \mu\text{g/ml}$  of gentamicin. Adherent cells were detached by 5-min incubation in  $4\ ^\circ\text{C}$  trypsin/EDTA solution.

## 2.11. Recovery of spleen cells

Murine spleen cells were recovered from naïve C57BL/6 and IL-5 transgenic BALB/c mice. Briefly, spleens were dissected and macerated in RPMI 1640. For *in vitro* and *in vivo* assays with mononuclear cells, Histopaque 1077 density gradient centrifugation ( $400\ \times\text{g}$ ,  $20\ ^\circ\text{C}$ , 30 min) was performed. For eosinophil adoptive transfer assay, spleen cells from IL-5 transgenic mice were recovered and washed in RPMI 1640 ( $405\ \times\text{g}$ ,  $4\ ^\circ\text{C}$ , 10 min). Leukocyte pellet containing approximately 30% of eosinophils was resuspended and labeled with CFSE, as described below, in Adoptive transfer assay.

## 2.12. Recovery of peripheral lymph node cells

Peripheral lymph nodes were collected from C57BL/6 mice and macerated in PBS/EDTA (10 mM). Cells were centrifuged ( $405\ \times\text{g}$ ,  $4\ ^\circ\text{C}$ , 10 min), and leukocyte pellet was resuspended and stained for  $\text{V}\gamma 4^+$  cells.

## 2.13. Transendothelial migration assay

A total of  $2 \times 10^5$  tEnd.1 cells were cultured in transwell polycarbonate culture inserts placed in 24-well culture plates ( $8.0\ \mu\text{m}$

pore diameter, BD Falcon, San Diego, CA) and allowed to grow to confluence ( $37\ ^\circ\text{C}$  and 5%  $\text{CO}_2$ ). Cultured tEnd.1 cell monolayers were washed in  $37\ ^\circ\text{C}$  PBS once, then, added to 24-well culture plates containing PBS, SPW or OPW ( $500\ \mu\text{l}$ ). Immediately after, tEnd.1 cells were incubated with fucoidan, with blocking anti-CD62P or with blocking anti-CD62E mAbs, at saturating concentration ( $25\ \mu\text{g/ml}$ ) [30], throughout the duration of transmigration assay. Splenocytes were pre-treated with blocking anti-CD62L mAb ( $25\ \mu\text{g/ml}$ ) or fucoidan ( $25\ \mu\text{g/ml}$ ) for 30 min, and then added ( $10^6$  cells/well in  $200\ \mu\text{l}$  of RPMI 1640) to the tEnd.1 monolayers at  $37\ ^\circ\text{C}$ , 5%  $\text{CO}_2$ , for 3 h. IgG isotypes were used as irrelevant antibodies. Transmigrated cells were collected from lower chambers, stained with antibodies against CD3 and  $\gamma\delta$  TCR, as described above, and analyzed by flow cytometry.

## 2.14. Adoptive transfer assay

Adoptive transfer assay was adapted from previous report [21]. Donor and recipient C57BL/6 mice were previously sensitized and i.t. injected with OVA or saline solution 14 days post sensitization. Splenocytes were recovered from donor mice 24 h after i.t. challenge, as described above. Recovered splenocytes were incubated with CFSE ( $1\ \mu\text{M}$  in RPMI/ $4 \times 10^7$  cells) for 15 min [ $20\ ^\circ\text{C}$ ] under gentle agitation and washed 3 times in RPMI containing 10% FBS ( $405\ \times\text{g}$ , 10 min). Cells were then treated or not with fucoidan ( $25\ \mu\text{g/ml}$ ) in sterile flow chamber [28]. IgG isotype was used as irrelevant antibody. CFSE-labeled splenocytes ( $4 \times 10^7$  cells/ $0.2\ \text{ml}$ ,  $\geq 90\%$  viability) were i.v. injected into recipient mice 24 h after i.t. injection of OVA or saline solution. Recipient mice were euthanized 24 h after adoptive transfer, and their thoracic cavities were rinsed for further analysis.

To assess eosinophil migration, CFSE-labeled spleen eosinophils ( $6 \times 10^6$  cells/ $0.2\ \text{ml}$ , approximately 30% of total splenocytes) recovered from IL-5 transgenic BALB/c mice treated or not with anti-CD62L ( $25\ \mu\text{g/ml}$ ) were adoptively transferred into recipient mice 8 h after i.t. challenge. Recipient mice were euthanized 16 h after adoptive transfer, and their thoracic cavities were rinsed. Pleural leucocytes were counted and stained with antibodies against CD3 and  $\gamma\delta$  TCR. At least  $4 \times 10^5$  events were acquired by FACS. Total cell numbers corresponding to each cell type were calculated based on percentages determined by flow cytometry.

## 2.15. In vitro proliferation assay

Splenocytes from C57BL/6 sensitized mice were pre-treated or not with fucoidan or anti-CD62L ( $25\ \mu\text{g/ml}$ ) 30 min before addition to culture plates. Spleen cells ( $1 \times 10^6$  per well) were stimulated with OVA ( $100\ \mu\text{g/ml}$ ) in RPMI 1640 medium supplemented with 10% FBS for 72 h. Cells were labeled with  $0.5\ \mu\text{M}$  CFSE immediately prior to treatment. After incubation, cells were stained for surface markers (CD3 and  $\gamma\delta$  TCR). Cell division was assessed by the reduction of CFSE mean of fluorescence intensity (MFI) by flow cytometry. It is noteworthy that cells incubated with fucoidan or anti-CD62L ( $25\ \mu\text{g/ml}$ ) and cultured in RPMI medium presented no differences from cells cultured in RPMI without fucoidan. Cell viability was evaluated by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method 24 h after treatment (RPMI: 100%; Fucoidan: 100%;  $\alpha$ -CD62L: 80%).

## 2.16. Statistical analysis

Data were reported as the mean  $\pm$  SEM and were statistically analyzed by means of 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.

### 3. Results

#### 3.1. Effect of selectin blockade on OVA-induced $\gamma\delta$ T lymphocyte accumulation

Intra-thoracic injection of OVA (12.5  $\mu\text{g}/\text{cavity}$ ) into previously sensitized mice increased the number of  $\gamma\delta$  T lymphocytes in thoracic lymph nodes (Fig. 1A), in peripheral blood (Fig. 1B) and in pleural cavities (Fig. 1C), 48 h after antigenic challenge. This increase was inhibited by the *in vivo* blockade of selectins by fucoidan. Accordingly, OVA challenge also triggered a significant increase in the number of  $\gamma\delta$  T cells expressing CD62L in thoracic lymph nodes (~45% of total  $\gamma\delta$  T lymphocytes) within 48 h (Fig. 1D), as it also induced the increase in CD62L expression (Fig. 1G). A slight increase of CD62L<sup>+</sup>  $\gamma\delta$  T lymphocyte numbers was observed in blood (Fig. 1E), which was significant in pleural cavities of challenged mice (Fig. 1F). OVA challenge did not induce significant alterations in CD62L expression on  $\gamma\delta$  T cell surface in peripheral blood (Fig. 1H) or pleural cavities (Fig. 1I). It is noteworthy that OVA (12.5  $\mu\text{g}/\text{cavity}$ ) i.t. administration into non-sensitized mice failed to induce accumulation of total leukocytes (saline:  $3.8 \pm 0.6$  vs OVA:  $3.8 \pm 0.0 \times 10^6$  cells/cavity,  $p=1.0$ ), CD3<sup>+</sup> T cells (saline:  $79.4 \pm 9.3$  vs OVA:  $98.9 \pm 31.5 \times 10^3$  cells/cavity,  $p=0.62$ ) or  $\gamma\delta$  T lymphocytes (saline:  $1.2 \pm 0.1$  vs OVA:  $1.3 \pm 0.4 \times 10^3$  cells/cavity,  $p=0.8$ ) within 24 h.

#### 3.2. Selectins are required for OVA-induced $\gamma\delta$ T cell activation and proliferation in thoracic lymph nodes

Since fucoidan pre-treatment inhibited OVA-induced accumulation of  $\gamma\delta$  T cells in lymph nodes, the effect of selectins in  $\gamma\delta$  T cell activation in mice lymph nodes was investigated. OVA stimulation induced the increase in the number of CD25<sup>+</sup>  $\gamma\delta$  T cells in thoracic lymph nodes within 48 h, which was inhibited by *in vivo* fucoidan pre-treatment (Fig. 2, A and B). OVA injection also induced the increase of c-fos<sup>+</sup>  $\gamma\delta$  T lymphocytes in thoracic lymph nodes 6 h after challenge, which was also inhibited by selectin blockade (Fig. 2, C and D). It is important to emphasize that no changes were observed in total numbers of  $\gamma\delta$  T lymphocytes in lymph nodes 6 h after stimulation (data not shown). Confirming the role of selectins on  $\gamma\delta$  T cell proliferation, spleen  $\gamma\delta$  T lymphocytes recovered from sensitized mice and incubated with fucoidan or with anti-CD62L mAb (which specifically blocks L-selectin) presented impaired proliferation induced by OVA after 72 h (Fig. 2E). Moreover, immobilized anti-CD3 antibody also induced  $\gamma\delta$  T lymphocyte proliferation, which was also inhibited by fucoidan pre-treatment (RPMI  $2.4 \pm 0.5$  vs anti-CD3  $23.9 \pm 6.4$ ,  $p=0.006$ ; anti-CD3 vs anti-CD3+fucoidan  $10.3 \pm 1.5\%$  of proliferating cells,  $p=0.08$ ,  $n=10$ ). Fucoidan or anti-CD62L mAb incubation of non-stimulated cells did not induce significant changes in CFSE fluoresce when compared to control group (data not shown).

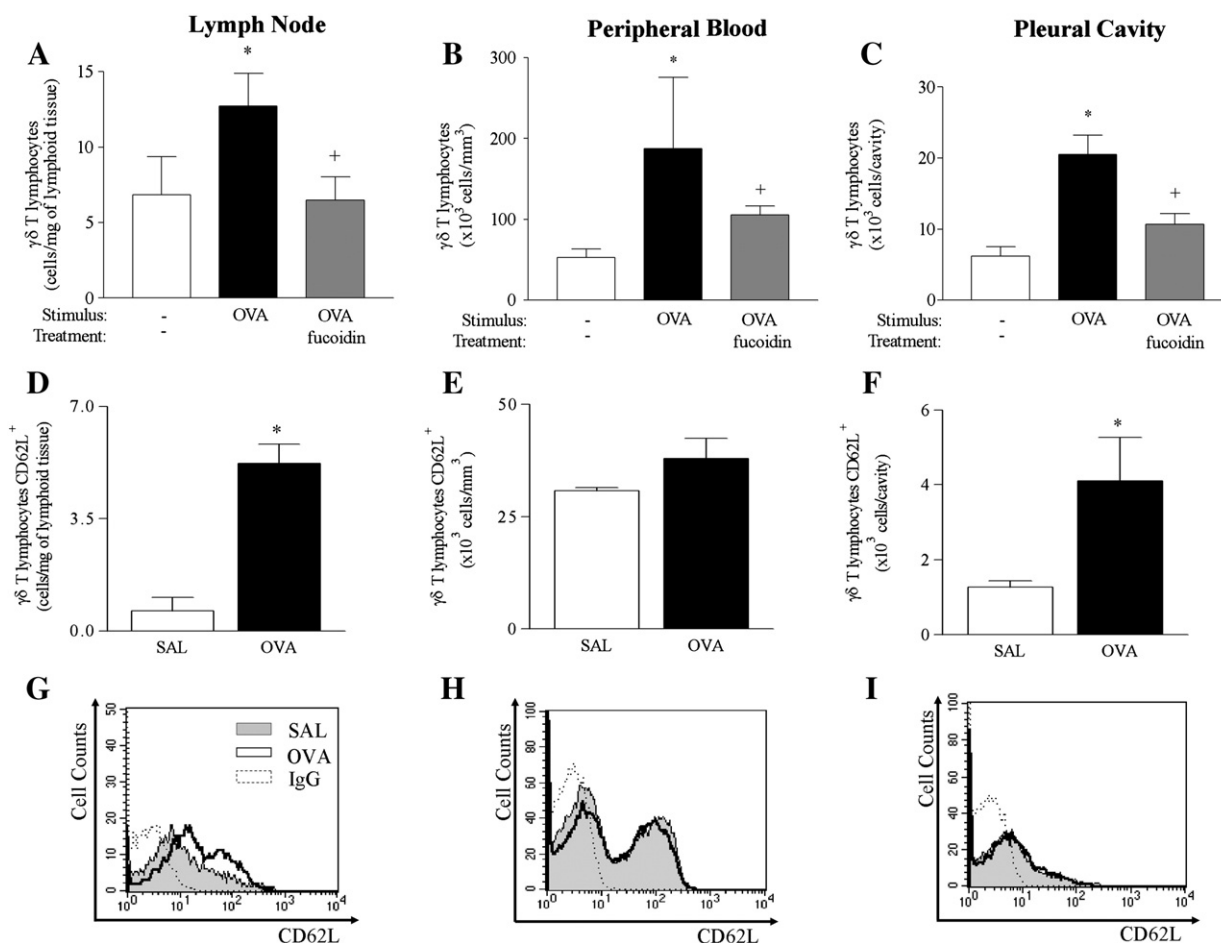


**Fig. 1.** Effect of fucoidan (10 mg/kg, i.v.) pre-treatment on  $\gamma\delta$  T cell accumulation in mice thoracic lymph node (A), peripheral blood (B) and pleural cavity (C) 48 h after OVA-challenge (12.5  $\mu\text{g}/\text{cavity}$ , i.t.). Total  $\gamma\delta/\text{CD62L}^+$  lymphocyte numbers in mice thoracic lymph node (D), peripheral blood (E) and pleural cavity (F) 48 h after i.t. injection of OVA. Representative histograms of CD62L expression on  $\gamma\delta$  T cells recovered from mice thoracic lymph node (G), peripheral blood (H) and pleural cavity (I) after challenge. Results are expressed as the mean  $\pm$  SEM from at least 6 animals per group of two different experiments. \* indicates statistically significant differences ( $p \leq 0.05$ ) between non-stimulated and stimulated animals, whereas + indicates significant differences between stimulated and treated groups.

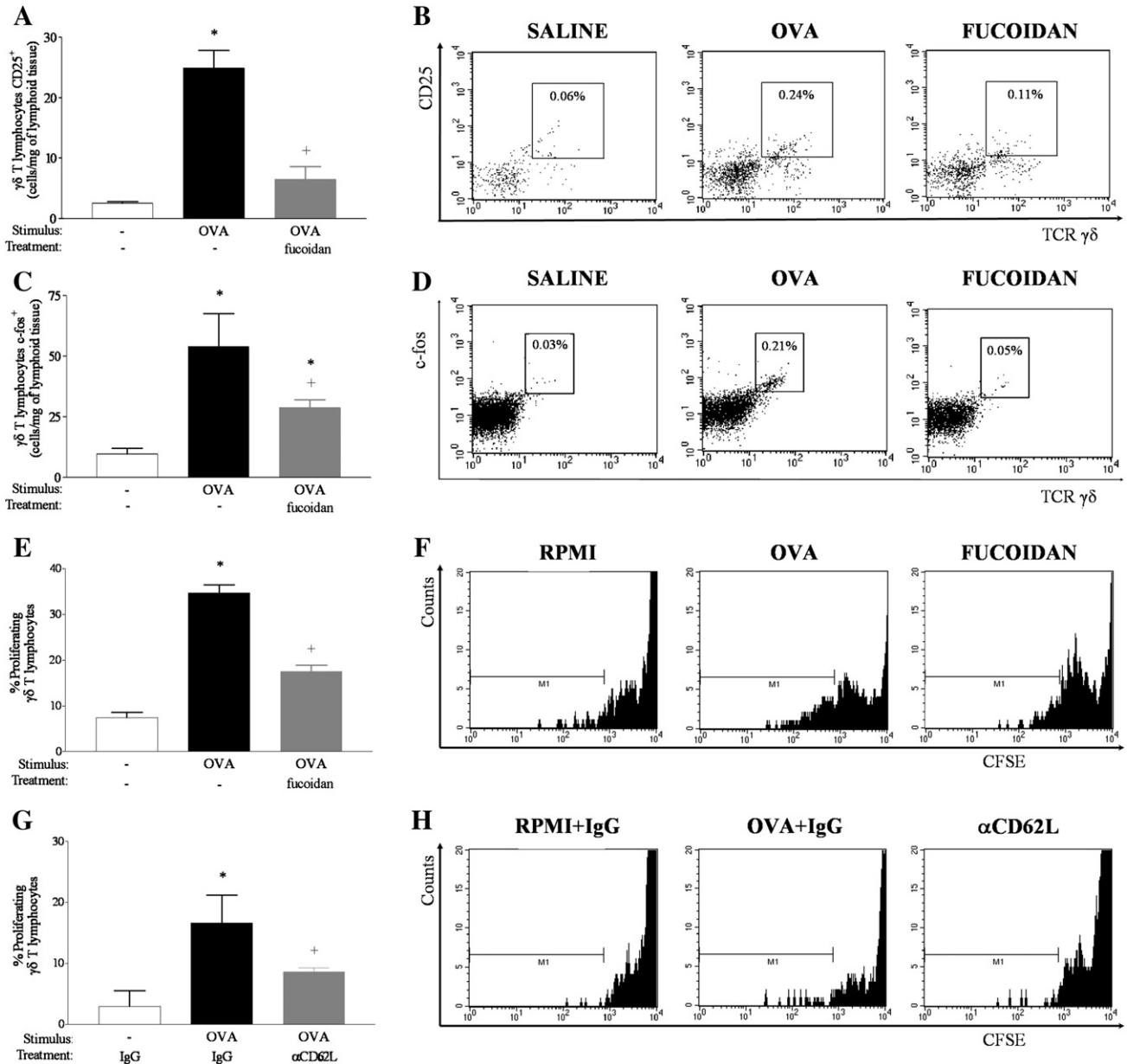


**Fig. 2.** *In vivo* selectin blockade by fucoidan (10 mg/kg, i.v.) impaired  $\gamma\delta$  T cell activation in thoracic lymph nodes after OVA challenge. (A, B) Total  $\gamma\delta$ /CD25<sup>+</sup> T cell numbers in the mice thoracic lymph node 48 h after i.t. injection of OVA (12.5  $\mu$ g/cavity). (C, D)  $\gamma\delta$ /c-fos<sup>+</sup> T cell numbers in the mice thoracic lymph node 6 h after i.t. OVA challenge. Results are expressed as the mean  $\pm$  SEM from at least 6 animals per group out of three different experiments. The values shown in dot plots correspond to percentage of positive cells among CD3<sup>+</sup> T lymphocytes. (E, F) Fucoidan (25  $\mu$ g/ml) or (G, H)  $\alpha$ -CD62L mAb (25  $\mu$ g/ml) incubation of sensitized splenocytes ( $1 \times 10^6$  per well) inhibited proliferation of  $\gamma\delta$  T cells induced by *in vitro* OVA stimulation (100  $\mu$ g/ml), within 72 h. Cell division was assessed by the reduction of CFSE MFI by using a flow cytometer. \* indicates statistically significant differences ( $p \leq 0.05$ ) between non-stimulated and stimulated animals, whereas + indicates significant differences between stimulated and treated groups.

### 3.3. CD62L mediates $\gamma\delta$ T lymphocyte transmigration *in vitro* and *in vivo*

To assess the involvement of the three members of selectins in  $\gamma\delta$  T cell migration, it was performed an *in vitro* transmigration across endothelial monolayers towards cell-free pleural washes recovered from OVA-injected mice (OPW). OPW increased the number of transmigrated  $\gamma\delta$  T cells when compared to SPW (Fig. 3A) or PBS ( $32 \pm 4$   $\gamma\delta$  T lymphocytes/well,  $n=3$ ). The *in vitro* blockade of selectins by fucoidan inhibited  $\gamma\delta$  T cell transmigration towards OPW, as much as the pre-incubation with anti-CD62L mAb; however the pre-incubation of endothelial cells with anti-CD62P and anti-CD62E mAbs failed to inhibit such phenomenon (Fig. 3A). Corroborating results demonstrated that tEnd.1 cells constitutively express L-selectin ligand MadCAM (64.5%

of MadCAM<sup>+</sup> non-stimulated cells [MFI 75.4]; 70% of MadCAM<sup>+</sup> OPW-stimulated cells [MFI 90.8], within 2 h). *In vivo* pre-treatment with fucoidan or with anti-CD62L mAb 1 h before OVA i.t. stimulation inhibited the increase of  $\gamma\delta$  T lymphocytes in mice pleural cavities of challenged mice within 48 h (Fig. 3B). It is noteworthy that *in vivo* blockade of selectins also inhibited eosinophil accumulation in mice pleural cavities after OVA challenge (Fig. 3C). Remarkably, eosinophils did not require CD62L for migration during allergic pleurisy, since *ex vivo* treatment of CFSE-labeled eosinophils with anti-CD62L mAb prior adoptive transfer failed to impair the accumulation of these cells in pleural cavities of OVA challenged recipient mice (Fig. 3D). It is important to emphasize that such *ex vivo* incubation was effective to impair spleen mononuclear cells, demonstrating the effectiveness of the antibody treatment.

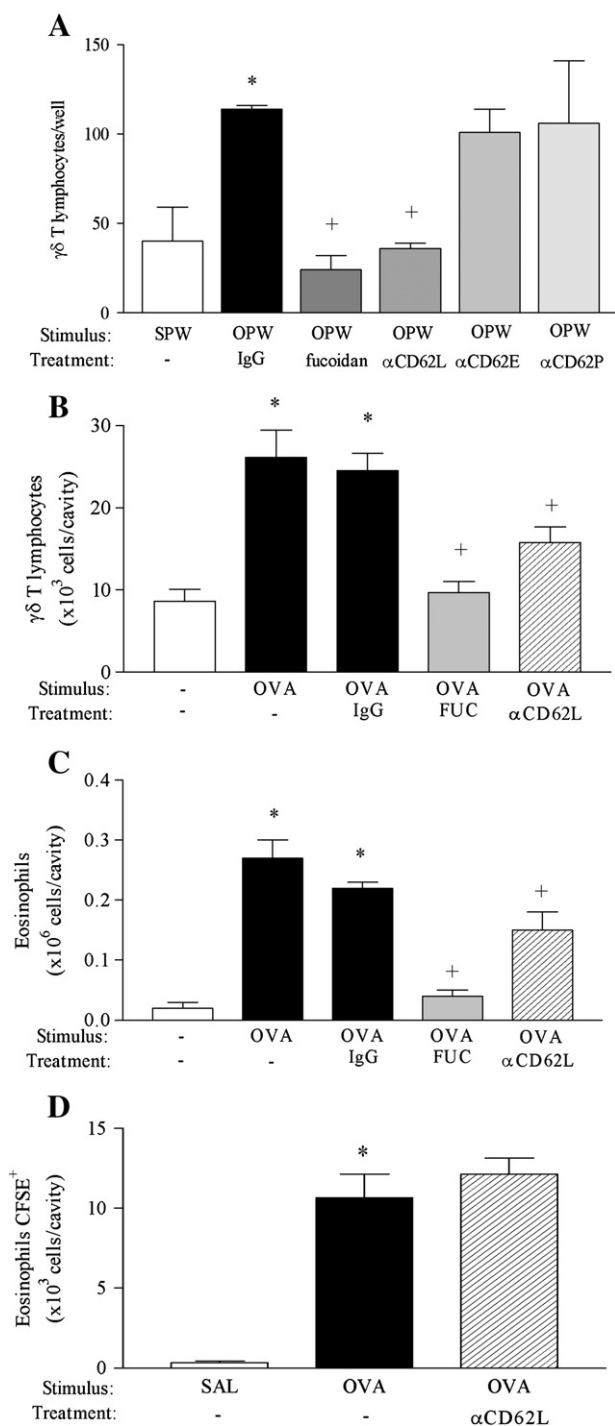


**Fig. 3.** (A) The *in vitro* blockade of CD62L inhibited  $\gamma\delta$  T cell transmigration across endothelial monolayers induced by OPW. tEnd.1 cell monolayers were incubated with fucoidan, anti-CD62P or anti-CD62E mAbs (25  $\mu$ g/ml), whereas splenocytes were treated with fucoidan or anti-CD62L (25  $\mu$ g/ml) for 30 min before the assay. Splenocytes were allowed to transmigrate towards OPW for 3 h, stained for CD3 and  $\gamma\delta$  TCR and analyzed by FACS. Results are expressed as the mean  $\pm$  SEM from triplicate wells of one out of two separate experiments. Effect of selectin blockade by *in vivo* pre-treatment with fucoidan (10 mg/kg, i.v.) or by anti-CD62L mAb (200  $\mu$ g/mice, i.p.) on  $\gamma\delta$  T cell (B) and eosinophil (C) accumulation in C57BL/6 mice pleural cavity 48 h after the i.t. injection of OVA (12.5  $\mu$ g/cavity). (D) Effect of *ex vivo* pre-treatment with anti-CD62L mAb on CFSE-labeled eosinophil migration into recipient mice pleural cavity after i.t. OVA challenge. Eosinophils were recovered from IL-5 transgenic BALB/c mouse spleens, labeled with CFSE-labeled, treated or not with anti-CD62L (25  $\mu$ g/ml) and i.v. injected ( $2 \times 10^7$  cells/0.2 ml) into recipient challenged BALB/c mice. Results are expressed as the mean  $\pm$  S.E.M. from 6 animals per group. \* indicates statistically significant differences ( $p < 0.05$ ) between non-stimulated and stimulated animals, whereas + indicates significant differences between stimulated and treated groups. Data represents one of two different experiments.

#### 3.4. Selectin *in vivo* blockade reduces the level of eosinophilotactic cytokines

CCL11, IL-5 and IL-4 are important mediators involved in the pathogenesis of allergic reactions, as in eosinophil accumulation in inflamed tissue [31]. Therefore, it was further analyzed if fucoidan impairment of  $\gamma\delta$  T cell activation and migration into the pleural cavity of OVA-challenged mice could alter local production of such mediators. As shown in Fig. 4A–C, levels of CCL11 and IL-5, but not IL-4, were increased in pleural cavities of challenged mice within 6 h as compared to control mice. Fucoidan i.v. pre-treatment inhibited OVA-induced increase of CCL11 and IL-5 levels in pleural washes. Moreover, increased numbers of  $\gamma\delta$  T cells expressing intracellular CCL11 were present in pleural cavities of challenge mice. This increase was also inhibited by selectin blockade (Fig. 4D). Although the increase of  $\gamma\delta$  T cells IL-5<sup>+</sup> (Fig. 4E) and IL-4<sup>+</sup> (Fig. 4F) was not significant in OVA challenged mice, a tendency to increase was observed. Fucoidan treatment slightly decreased such tendency. These results suggest that  $\gamma\delta$  T cells are not the primary source of these mediators in the pleural cavities of challenged mice.

#### 3.5. CD62L-mediated $\gamma\delta$ T cell accumulation in pleural cavities of challenged mice is required for eosinophil influx

To confirm that selectins mediate  $\gamma\delta$  T cell transmigration from blood into inflamed pleural cavities during allergic response, CFSE-labeled splenocytes recovered from challenged mice, *ex vivo* treated or not with fucoidan, were adoptively transferred into recipient mice 24 h after OVA i.t. challenge. Adoptively transferred  $\gamma\delta$  T cells migrated into pleural cavities of challenged mice in higher extent than into saline-injected recipient mice, a phenomenon reduced by fucoidan incubation (Fig. 5, A and B). Recipient mice also presented higher numbers of pleural eosinophils 48 h after challenge, which was reduced in mice injected with fucoidan-treated CFSE-labeled splenocytes (Fig. 5B). These data suggest that eosinophil impairment by selectin blockade relies, at least partially, on  $\gamma\delta$  T cell influx into the inflammatory site.

#### 3.6. $\gamma\delta$ T lymphocyte depletion impairs eosinophil accumulation in pleural cavities of challenged mice

V $\gamma$ 4 T lymphocytes are described as a major  $\gamma\delta$  T cell subset in mice lung [5], being also shown to modulate allergic eosinophil accumulation in the airways [14]. We found that V $\gamma$ 4 T lymphocytes comprised around 40% of total  $\gamma\delta$  T lymphocytes recovered from naïve mice pleural cavities and were also distributed in peripheral lymph nodes (Fig. 6, A and B). OVA i.t. challenge in previously sensitized mice induced significant increase in the number of V $\gamma$ 4 T cells in pleural cavities of non-treated mice within 48 h, but not in pleural cavities of V $\gamma$ 4 T lymphocyte-depleted mice (Fig. 6A). The anti-V $\gamma$ 4 mAb treatment reduced approximately 95% of targeted cells (Fig. 6B), with no changes observed in other T lymphocyte populations (Table 1). As shown in Fig. 6C, OVA challenge failed to induce pleural eosinophil accumulation in V $\gamma$ 4 T lymphocyte-depleted mice, confirming that  $\gamma\delta$  T cells are crucial for eosinophil influx into the allergic site.

## 4. Discussion

We have previously shown that i.t. OVA challenge induces  $\gamma\delta$  T lymphocyte accumulation in pleural cavities of sensitized mice through a mechanism that relies on T cell migration induced by CCR2/CCL2 pathway, which starts at 12 h, peaks at 48 h and returns to basal levels 5 days after stimulation [32]. Indeed,  $\gamma\delta$  T lymphocytes are shown to accumulate in airways of allergic human patients and experimental animals submitted to antigenic challenge [7,8,33–35], as

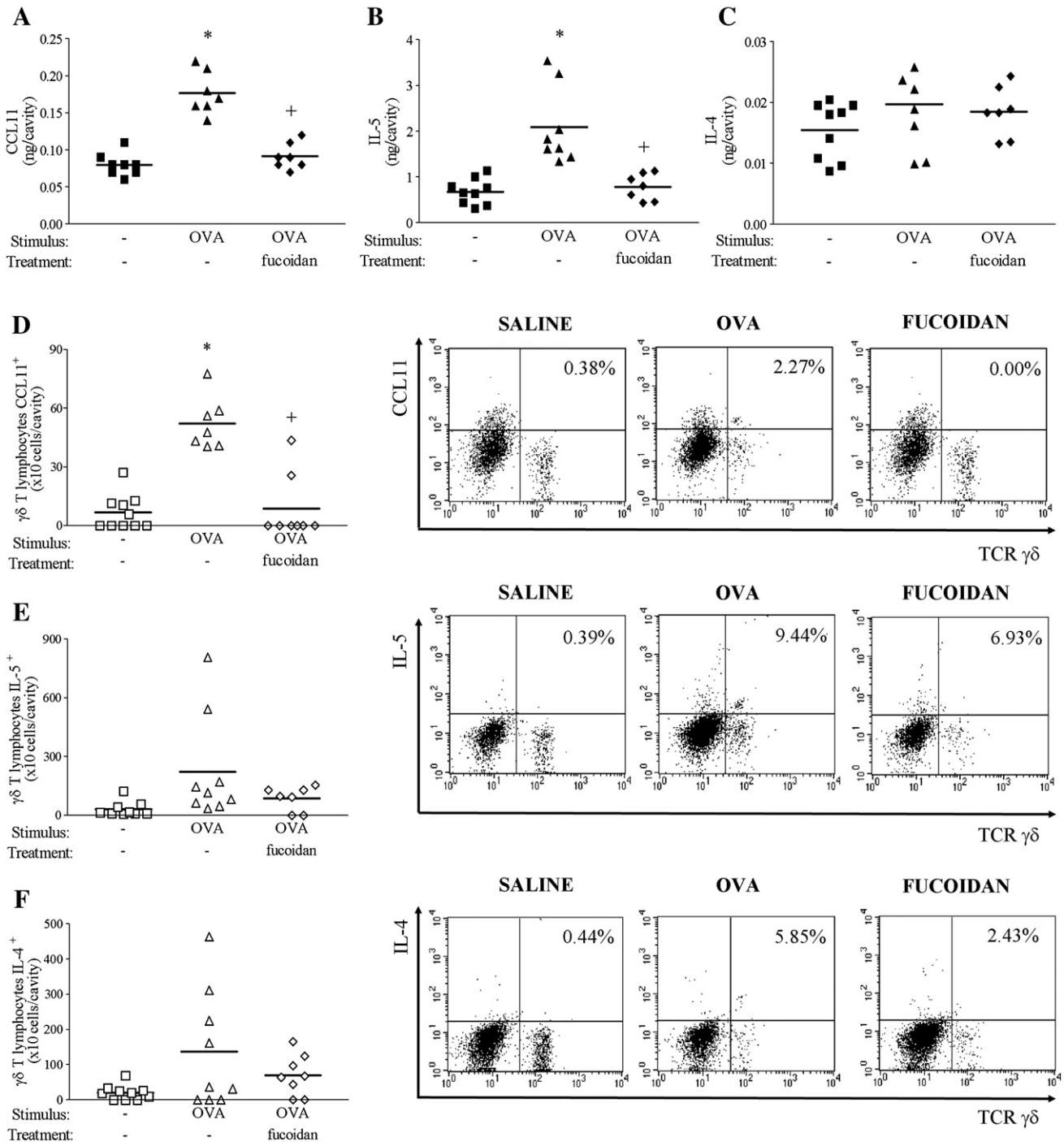


**Fig. 4.** Effect of fucoidan pre-treatment (10 mg/kg, i.v.) on CCL11 (A), IL-5 (B) and IL-4 (C) protein levels in pleural washes recovered at 6 h following OVA stimulation (12.5  $\mu$ g/cavity, i.t.), determined by ELISA. CCL11<sup>+</sup>  $\gamma\delta$  T cell (D), IL-5<sup>+</sup>  $\gamma\delta$  T cell (E), IL-4<sup>+</sup>  $\gamma\delta$  T cell (F) numbers in mice pleural cavities 6 h after i.t. injection of OVA were determined by intracellular cytokine staining, as described in Material and Methods. Results are expressed as the mean  $\pm$  SEM from at least 6 animals per group. \* indicates statistically significant differences ( $p \leq 0.05$ ) between non-stimulated and stimulated animals, whereas + indicates significant differences between stimulated and treated groups. On the right, representative dot plots show percentages of pleural CCL11<sup>+</sup>  $\gamma\delta$ , IL-5<sup>+</sup>  $\gamma\delta$  and IL-4<sup>+</sup>  $\gamma\delta$  T lymphocytes.

well as to modulate several features of allergic responses, including eosinophil influx into inflamed tissue [6–9]. Our results show that i.t. antigenic challenge also increased the number of  $\gamma\delta$  T cells in mice peripheral blood and thoracic lymph nodes, suggesting that the  $\gamma\delta$  T cells that are involved in allergic response triggered by OVA migrate from lymph nodes into inflamed tissue through bloodstream, which is also supported by the pattern of chemokine receptor expression by these cells during allergic pleurisy [32]. Moreover, the increase of  $\gamma\delta$  T cells expressing high levels of CD62L in mice lymph nodes, together

with the low expression of CD62L by  $\gamma\delta$  T cells that migrated into pleural cavities after antigenic challenge, might suggest that such adhesion molecule might have a relevant role in the migration and/or activation of these cells.

Besides mediating the rolling and the adhesion of T lymphocytes to the endothelium, selectins also provide stimulatory signals to activate T lymphocytes, mainly when shed from cell surface [35]. During antigen recognition by T cells, selectins can act as co-stimulatory molecules. This phenomenon is also supported by the fact that such

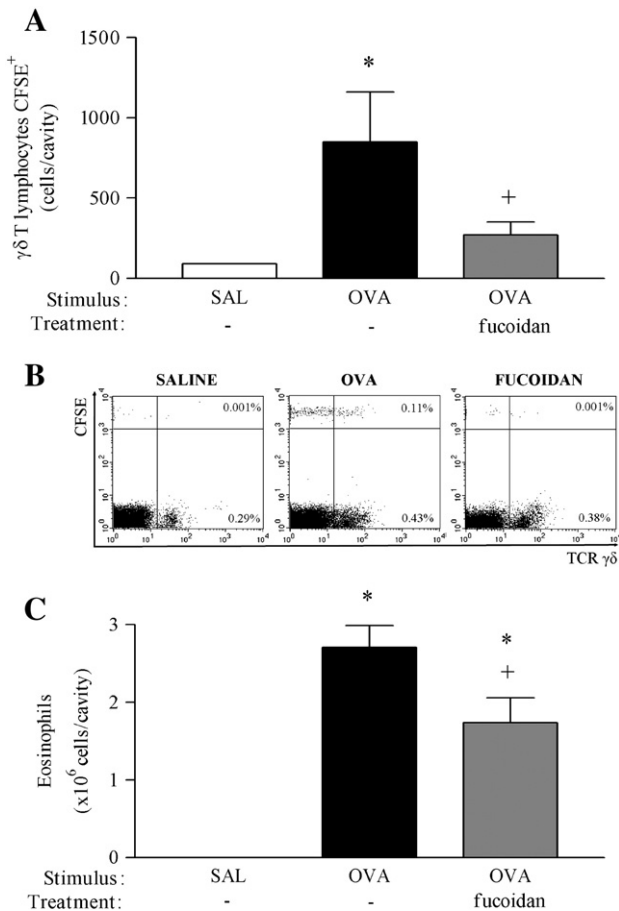


**Fig. 5.** (A) *Ex vivo* pre-treatment of CFSE-labeled spleen mononuclear cells with fucoidan inhibited the migration of adoptively transferred γδ T lymphocytes into C57BL/6 mice pleural cavities after OVA (12.5 μg/cavity) i.t. challenge. (B) Representative dot plots of CFSE<sup>+</sup> γδ T lymphocytes. (C) Eosinophil counts in mice pleural cavities after adoptive transfer of spleen mononuclear cells. CFSE-labeled spleen mononuclear cells were recovered from C57BL/6 donor mice 24 h after OVA i.t. challenge, treated or not with fucoidan (25 μg/ml) and i.v. injected (4 × 10<sup>7</sup> cells/0.2 ml) into recipient mice 24 h after the i.t. injection of OVA or saline.

molecules are capable to associate with TCR/CD3 complex [24–26]. The fact that *in vivo* selectin blockade both impaired OVA-induced increased numbers of γδ T lymphocytes in thoracic lymph nodes and reduced the numbers of γδ T cells expressing the proliferating phenotype IL-2α receptor chain CD25 and *c-fos* transcriptional factor suggests diminished activation and primary proliferating responses [36–38]. Accordingly, low levels of IL-2 and impaired proliferation have already been reported to occur in lymph nodes of CD62L KO mice submitted to delayed-type hypersensitivity [39]. Selectin blockade could affect several phenomena that can indirectly prevent T cell activation *in vivo*, such as i) impairment of migration of antigen presenting cells into lymph nodes [40]; ii) lack of naïve T cell recirculation preventing antigen encounter [41–43]; iii) inability to present the antigen by defective cell–cell interactions after lymph node entry [44,45].

Consequently, we aimed at investigating the direct effect of selectin blockade on T cell activation. Our *in vitro* studies confirmed that CD62L mediates OVA-induced γδ T lymphocyte proliferation, suggesting defective co-stimulatory signaling during antigen presentation. Supporting reports demonstrated that CD62L co-stimulation with specific antibody enhanced α-CD3 mAb-induced proliferation and expression of CD25 on T lymphocytes [24]. In addition, the inhibition of CD62L signaling blocked α-CD3 mAb-induced T cell proliferation, due to impaired activation, as determined by calcium

influx and ZAP-70 phosphorylation [35]. Accordingly, results obtained by the present study showed that fucoidan pretreatment also reduced α-CD3 mAb-induced γδ T lymphocyte proliferation *in vitro*.

Here we show that CD62L is also required for *in vitro* γδ T lymphocyte transmigration through endothelial cell monolayers towards OPW, which was used as chemotactic stimulus due to the presence of CCL2, shown by us to be responsible for OVA-induced γδ T lymphocyte *in vitro* and *in vivo* migration [32]. Even though γδ T cells express CD62E and CD62P ligands that mediate interaction with

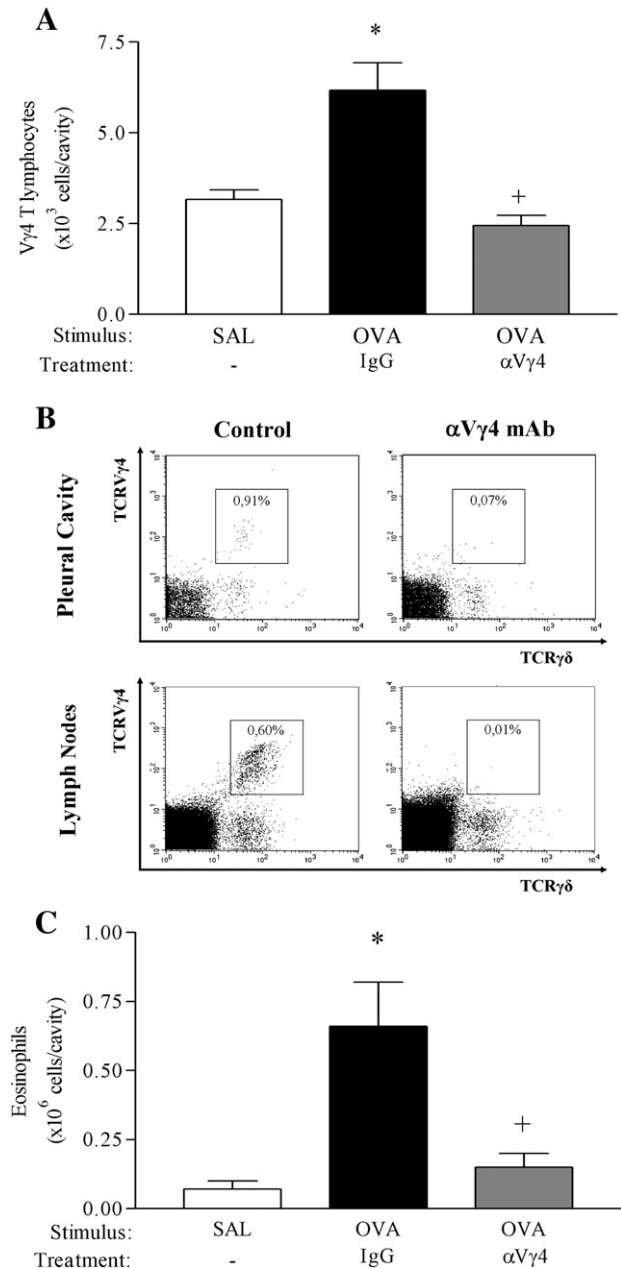


**Fig. 6.** (A) Effect of α-Vγ4 mAb treatment on Vγ4 T lymphocyte numbers in pleural cavities 48 h after OVA i.t. challenge. (B) Representative dot plots of Vγ4<sup>+</sup> lymphocytes from pleural cavity and peripheral lymph nodes from naïve mice treated with α-Vγ4 mAb or vehicle. (C) Depletion of Vγ4 T lymphocytes inhibited eosinophil influx induced by antigenic challenge. Antibody anti-Vγ4 mAb (UC3) was administered i.p. at 400 μg/mouse 1 day before sensitization and 400 μg/mouse per day every 2 days. Results are expressed as the mean ± S.E.M. from 6 animals per group. \* indicates statistically significant differences (*p* ≤ 0.05) between non-stimulated and stimulated animals, whereas + indicates significant differences between stimulated and treated groups. Data represents one of two different experiments.

**Table 1**  
Effect of  $\alpha$ -V $\gamma$ 4 mAb treatment on T lymphocyte populations in C57BL/6 mice

|                                              | Peripheral lymph nodes (%) |                            |
|----------------------------------------------|----------------------------|----------------------------|
|                                              | Naïve                      | $\alpha$ -V $\gamma$ 4 mAb |
| V $\delta$ 6.3 <sup>+</sup> T lymphocytes    | 0.3                        | 0.6                        |
| V $\delta$ 4 <sup>+</sup> T lymphocytes      | 0.6                        | 0.8                        |
| CD4 <sup>+</sup> $\alpha\beta$ T lymphocytes | 57                         | 57                         |
| CD8 <sup>+</sup> $\alpha\beta$ T lymphocytes | 41                         | 37                         |

Values of V $\delta$ 6.3<sup>+</sup> and V $\delta$ 4<sup>+</sup> cells represent percentages among  $\gamma\delta$  T lymphocytes. Values of CD4<sup>+</sup> and CD8<sup>+</sup> cells represent percentages among  $\alpha\beta$  T lymphocytes. Cells were recovered from mice pleural cavities and peripheral lymph nodes from naïve and V $\gamma$ 4-depleted mice.  $\alpha$ -V $\gamma$ 4 mAb treatment is described in [Materials and methods](#).

endothelial cells [16,19,20], our data showed that CD62E and CD62P seem to be of minor importance for  $\gamma\delta$  T lymphocyte transmigration towards OPW *in vitro*. However, a possible role of these molecules in *in vivo* rolling and transmigration of  $\gamma\delta$  T cells into allergic sites cannot be excluded. The requirement of CD62L for *in vivo*  $\gamma\delta$  T lymphocyte accumulation in allergic sites was confirmed by the *i.v.* pre-treatment of challenged mice with anti-CD62L mAbs.

Even though eosinophils did not directly require CD62L to migrate into the allergic site, the fact that *in vivo* blockade of CD62L also diminished OVA-induced eosinophil influx suggests a possible role for  $\gamma\delta$  T lymphocytes on eosinophil accumulation. The reduced production of eosinophilotactic mediators CCL11 and IL-5 in pleural cavities of fucoidan-treated mice reinforced that selectins indirectly imply in allergic pleural eosinophilia. We failed to observe any significant increase in IL-4 in pleural cavities of challenged mice; however, IL-4 involvement in eosinophil accumulation in the airways during allergy [46,47] occurs mainly via the production of CCL11 [48–51] and IL-5 [52].  $\gamma\delta$  T lymphocytes have been shown to actively produce a wide array of cytokines and chemokines and present a unique plasticity to produce both Th2 and Th1 cytokines and chemokines [53]. Our results show a significant increase in the number of  $\gamma\delta$  T lymphocytes expressing intracellular CCL11, but only a slight increase of IL-5 and IL-4. However,  $\gamma\delta$  T lymphocytes may be indirectly modulating the production of IL-5 and IL-4 by other cell populations, such as pleural macrophages,  $\alpha\beta$  T lymphocytes and endothelial cells, in a cross-talk previously demonstrated to occur in LPS-induced pleurisy [54,55] and other reactions [2,14].

An important question to address was whether reduced  $\gamma\delta$  T cell accumulation in OVA-challenged pleural cavities by CD62L blockade was brought about by impaired  $\gamma\delta$  T cell activation in lymph nodes (which would therefore reduce its migration into the allergic site), by the direct effect on cell transmigration, or by both phenomena. To specifically investigate CD62L mediation of  $\gamma\delta$  T cell transmigration, we performed the adoptive transfer assay of splenocytes. The finding that *ex vivo* incubation with fucoidan impaired the migration of adoptively transferred CFSE-labeled  $\gamma\delta$  T lymphocytes into the pleural cavities of challenged recipient mice confirmed that CD62L directly mediates  $\gamma\delta$  T cell trafficking from bloodstream into inflammatory site during allergic pleurisy. It is noteworthy that eosinophil influx induced by OVA challenge was reduced in pleural cavities of recipient mice that received adoptively transferred fucoidan-treated spleen mononuclear cells. These data corroborate the concept that  $\gamma\delta$  T lymphocytes are required for positive regulation of eosinophil influx. Selectins are shown to also mediate eosinophil *in vivo* migration into the inflamed tissue [27,56]; however, during allergic responses, eosinophil migration is described to be independent of CD62L [28,57]. Therefore, although *in vivo* CD62L blockade inhibited OVA-induced eosinophil accumulation in mice pleural cavities, overall data demonstrate that such impairment occurs via an indirect pathway mediated by  $\gamma\delta$  T lymphocytes. However, since total splenocytes, but not purified  $\gamma\delta$  T lymphocytes, were adoptively transferred due to the extremely limited low numbers of  $\gamma\delta$  T cells recovered from donor

mice, the involvement of other T cell populations in this phenomenon can not be excluded.

According to the knowledge that V $\gamma$ 4 T cells comprehend the predominant lung resident  $\gamma\delta$  T cell subset in mice [5], we found V $\gamma$ 4 T lymphocytes in mice pleural cavities, which also increased in numbers after antigenic challenge. Other reports also describe that V $\gamma$ 4 T cells are found in peripheral blood and lymphoid organs, being capable to migrate into inflamed airways [3,10]. Although a well established role has been described for V $\gamma$ 4 T cells in a murine model of airway hyperresponsiveness [10,14], the regulation of eosinophil influx into the airways by these cells has also been demonstrated [14]. This report shows that the depletion of V $\gamma$ 4 by aerosolized mAbs impaired airway eosinophilia in OVA-challenged mice within 48 h. Our data of eosinophil impairment during allergic pleurisy in V $\gamma$ 4 T lymphocyte depleted mice reinforces the notion that this T cell subset plays a crucial role in eosinophil recruitment into inflamed tissue. Pro-inflammatory effects of V $\gamma$ 4 T lymphocytes during virus infections are shown to be immunoregulatory. These cells do not present competent effector functions, as those observed in  $\alpha\beta$  T cells [12]. Therefore, in the murine model of allergic pleurisy, it remains to be elucidated whether V $\gamma$ 4 subset directly regulates allergic eosinophilia or it results from indirect regulation of other cell population, as  $\alpha\beta$  T lymphocytes and macrophages.

In summary, our findings demonstrate that CD62L is crucial for  $\gamma\delta$  T lymphocyte migration into inflamed tissue, as well as for  $\gamma\delta$  T cell activation and proliferation during allergic response. Furthermore, our results demonstrate that eosinophil accumulation in the allergic site relies on CD62L-dependent  $\gamma\delta$  T cell activation and migration.

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