

Enhanced ovalbumin-induced airway inflammation in CD26^{-/-} miceShuling Yan^{1,3}, Reinhard Geßner², Corinna Dietel², Ulrike Schmiedek² and Hua Fan^{1#}¹Institut für Molekularbiologie und Biochemie, CBF, Charité-Universitätsmedizin Berlin
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1 List of abbreviations:**2** BAL: Bronchoalveolar lavage**3** DPPIV: Dipeptidyl peptidase IV**4** OVA: Ovalbumin

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Summary

In this study, we investigated the potential role of CD26 in ovalbumin (OVA)-induced airway inflammation using CD26 gene knockout mice. Compared with wild type counterparts, CD26^{-/-} mice showed an obviously enhanced tissue response and denser pulmonary infiltrates containing eosinophils around vessels and in the parenchyma after OVA sensitisation and challenge. Serum IgG, including subclasses IgG1 and IgG2a, was greatly reduced in CD26^{-/-} mice, but serum IgE remained unchanged. CD26^{-/-} mice had increased mRNA expression of the Th2 cytokines IL-4, IL-5 and IL-13 in the lungs compared with wild type mice, while levels of the pro-Th1 cytokine IL-12p40 was similar in both strains. Consequently, enhanced protein secretion of IL-4, IL-5 and IL-13 was detected in bronchoalveolar lavage (BAL) fluid from CD26^{-/-} mice. In agreement with over-expressed Th2 cytokines, both mRNA transcript and protein levels of chemokines eotaxin and RANTES, as well as their receptors CCR3 and CCR5, were elevated in CD26^{-/-} mice. These results suggest a protective role for CD26 in restricting OVA-induced airway inflammation.

1 Introduction

2 CD26 is a multifunctional type II transmembrane glycoprotein with dipeptidyl peptidase IV
3 (DPPIV, EC 3.4.14.5) activity, widely distributed in mammalian tissues, mainly on the
4 surface of epithelial and endothelial cells as well as on fibroblasts and lymphocytes [1, 2].
5 As a costimulatory molecule, CD26 is involved in T-cell activation via its ability to interact
6 with adenosine deaminase (ADA) and CD45 [3-5]. CD26 also mediates cell adhesion by its
7 binding to the extracellular matrix proteins collagen and fibronectin [6, 7]. A well-
8 documented property of CD26 is its protease activity, which is characterised by the specific
9 cleavage of dipeptides from the N-terminus of bioactive peptides carrying proline or alanine
10 at the second position. CD26 is, on the other hand, a rather indiscriminate enzyme as a diverse
11 range of bioactive peptides, such as peptide hormones, growth factors, chemokines and
12 neuropeptides has been determined to be substrates of CD26 [8]. The CD26-mediated
13 truncation leads to inactivation, activation or modification of the specificities of these peptides,
14 and hence, influences different biological processes. Clinical observations have linked CD26
15 to type II diabetes, HIV infection, malignant transformation, transplantation rejection and
16 autoimmune diseases [9-15].

17 Asthma is a chronic inflammatory pulmonary disorder characterized by airway
18 hyperresponsiveness and reversible airway obstruction. The early-phase is associated with IgE
19 production and mast cell degranulation [16]. The critical feature in the late-phase is the
20 accumulation of eosinophilic cells and their subsequent activation in bronchial tissues, which
21 cause severe damage to the bronchiolar epithelium. The infiltration of eosinophils is governed
22 by a network of cytokines and chemokines produced by mast cells, epithelial and endothelial
23 cells, and Th2 cells, or even by eosinophils themselves [17]. Specifically, the interplay
24 between the Th2 cytokines IL-4, IL-5, IL-13, and the chemokine eotaxin is responsible for the
25 selective regulation of the expansion of eosinophil pools in the bone marrow and blood
26 compartments, and of migration of these leukocytes into the allergic lung [18].

Several observations suggest a potential role of CD26 in the pathogenesis of asthma. On the one hand, CD26 serves as a useful marker of Th1 cells [19]. It is able to regulate cytokine secretion and immunoglobulin synthesis as demonstrated by the application of CD26 inhibitors, antibodies and CD26 gene knockout mice [20-24]. On the other hand, CD26 is involved in the processing and degrading of key chemokines in asthma, particularly eotaxin and RANTES (Regulated upon Activation, Normally T-cell Expressed and Secreted). CD26-truncated eotaxin (3-74 aa) and RANTES (3-68 aa) show reduced chemotactic activity for eosinophils in vitro and impaired binding and signalling via the CC chemokine receptor 3 (CCR3), which is mainly expressed in eosinophils, basophils and Th2 lymphocytes [25, 26].

In this study, we compared ovalbumin-induced airway inflammation, analyzed cytokine and chemokine levels in CD26^{+/+} and CD26^{-/-} mice, and demonstrate that CD26 has a protective function in allergic immune response.

Results

Enhanced OVA-mediated eosinophilic airway inflammation in CD26^{-/-} mice

OVA sensitisation and challenge (Fig. 1) provoked a peribronchiolar and perivascular inflammatory response in both CD26^{+/+} and CD26^{-/-} mice (Fig. 2C and D), which was not observed in animals sham-sensitised with adjuvant alone and challenged with PBS (Fig. 2A and B). In contrast to CD26^{+/+} mice, however, CD26^{-/-} mice showed an obviously enhanced tissue response, denser pulmonary infiltrates containing eosinophils around vessels and in the parenchyma (Fig. 2D and E), a more prominent increase in the number of total inflammatory cells and eosinophils in BAL fluid (Fig. 2F). 36 h after OVA challenge, the numbers of total leukocytes and eosinophils in the BAL fluid from CD26^{-/-} mice were 5- ($4.52 \pm 1.20 \times 10^6$ vs. $0.96 \pm 0.82 \times 10^6$ cells per lung, $p < 0.001$) and 8-fold ($3.21 \pm 0.87 \times 10^6$ vs. $0.41 \pm 0.36 \times 10^6$ cells per lung, $p < 0.001$) higher than that from their wild type counterparts respectively.

Greatly reduced serum IgG in OVA-immunized CD26^{-/-} mice

Both CD26^{+/+} and CD26^{-/-} mice showed increased concentrations of serum IgM and IgG after OVA immunization (Fig. 3). Compared with CD26^{+/+} mice, CD26^{-/-} mice exhibited a lower level of serum IgM, but the difference was not statistically significant (Fig. 3A). In contrast, differences of the IgG concentrations in these two strains were much more pronounced. On day 5, there was still nearly no increase of IgG in both strains. Following the second sensitisation, incremental changes of serum IgG concentrations were detectable. On days 10 and 13, CD26^{+/+} mice yielded 1.5-fold more IgG than CD26^{-/-} mice (3042 vs. 2097 µg/ml, $p < 0.0001$ and 3888 vs. 2532 µg/ml, $p < 0.001$, respectively) (Fig.3B).

Decreased production of immunoglobulin isotypes IgG1, IgG2a, but not IgE in OVA-immunized CD26^{-/-} mice

Immunoglobulin isotypes were determined for OVA immunization on the basis of the serum production of IgG1, IgG2a and IgE. As shown in Fig. 3C and D, the concentrations of serum IgG1 and IgG2a were significantly lower in CD26^{-/-} mice. On days 10 and 13 after primary immunization, the concentrations of serum IgG1 were 1.7- and 2.3-fold higher in CD26^{+/+} mice than in CD26^{-/-} mice (1436 vs. 849 µg/ml, $p < 0.01$ and 2164 vs. 922 µg/ml, $p < 0.0001$) (Fig. 3C). The decrease of IgG2a production in CD26^{-/-} mice was observed as early as day 5 after primary immunization. On days 5, 10 and 13, serum IgG2a concentrations were 2.2-, 2.7- and 2.0-times higher in CD26^{+/+} mice than in CD26^{-/-} mice (650 vs. 290 µg/ml, $p < 0.05$, 1126 vs. 411 µg/ml, $p < 0.0001$ and 1337 vs. 652 µg/ml, $p < 0.001$) respectively (Fig. 3D). However, serum IgE production was no significant difference between both kinds of mice after OVA immunization, although the maximum IgE levels were reached on day 10 (Fig. 3E).

1 **Overexpressed Th2 cytokines in the lungs of OVA-sensitised and -challenged CD26^{-/-}** 2 **mice**

3 Regular RT-PCR was performed to analyse the expression profiles of different cytokine genes
 4 in mouse lungs. In both CD26^{+/+} and CD26^{-/-} mice, before exposure to aerosol OVA, only
 5 mRNA of IL-12p40 was detectable among analysed cytokines; after challenge, mRNA
 6 transcripts of IL-2, IL-10 and IFN- γ still remained undetectable, whereas those of IL-4, IL-5,
 7 and IL-13 increased (Fig. 4A). Compared with CD26^{+/+} mice, the mRNA expression of Th2
 8 cytokines, IL-4, IL-5 and IL-13, in the lungs of CD26^{-/-} mice was significantly higher; while
 9 that of IL-12p40 was similar in both strains (Fig. 4A). These results were verified by
 10 quantitative real-time PCR (qRT-PCR) in an independent experiment (Fig. 4B). Whereas IL-
 11 10, IL-12p40 and IFN γ levels were the same for OVA-exposed CD26^{+/+} and CD26^{-/-} mice, IL-
 12 4 mRNA was about 6-fold ($\Delta\Delta\text{Ct} = -2.8$, $p < 0.01$), IL-5 mRNA 5-fold ($\Delta\Delta\text{Ct} = -1.7$, $p <$
 13 0.05) and IL-13 mRNA 1.5-fold higher ($\Delta\Delta\text{Ct} = -0.6$, $p = 0.3$) in OVA-exposed CD26^{-/-} mice
 14 as compared with CD26^{+/+} mice (Fig. 4B). In agreement, enhanced protein secretion of IL-4,
 15 IL-5 and IL-13 was measured in BAL fluid of CD26^{-/-} mice. Notably, the amount of IL-4, IL-
 16 5 and IL-13 in BAL fluid of CD26^{-/-} mice was 2.1-, 2.5- and 2.8-fold higher than in CD26^{+/+}
 17 mice 24 h after aerosol exposure respectively (37.9 ± 10.0 vs. 17.6 ± 10.2 pg/ml, $p < 0.01$,
 18 127.5 ± 51 vs. 51.8 ± 31.5 pg/ml, $p < 0.01$, and 87.3 ± 18.7 vs. 30.8 ± 13.5 pg/ml, $p < 0.01$
 19 respectively) (Fig. 4C). These results indicate that increased secretion of Th2 cytokines in the
 20 lungs contributes to OVA-induced airway eosinophilia and severe inflammation of CD26^{-/-}
 21 mice.

22 **Overexpressed chemokines Eotaxin and RANTES, and their receptors CCR3 and CCR5,** 23 **in the lungs of OVA-sensitised and -challenged CD26^{-/-} mice**

24 The protein levels of the key chemokines eotaxin and RANTES, as well as of their receptors
 25 CCR3 and CCR5 in the lungs were determined by immunohistochemical analysis. In both

CD26^{+/+} and CD26^{-/-} mice these chemokines and receptors were expressed constitutively and increased over 24 h following the exposure to OVA aerosol. However, the difference between these two strains was distinguishable after the challenge (Fig. 5A). In CD26^{-/-} mice, the number of cells that consistently expressed the chemokines or their receptor proteins was obviously higher and the expression levels of those proteins were significantly up-regulated, in comparison to that in CD26^{+/+} mice (Fig. 5A). The gene expression of eotaxin, RANTES, CCR3 and CCR5 in the lungs was measured by quantitative real-time PCR. Peak levels at 6 h after OVA challenge differed significantly in CD26^{-/-} mice from those in CD26^{+/+} mice (Fig. 5B): RANTES mRNA levels were 2-fold higher ($\Delta\Delta Ct = -1.0$, $p < 0.01$), and eotaxin mRNA 1.6-fold higher ($\Delta\Delta Ct = -0.8$, $p \leq 0.05$) in CD26^{-/-} mice. CCR3 mRNA was even expressed 6-fold higher in CD26^{-/-} mice ($\Delta\Delta Ct = -2.8$, $p < 0.05$), whereas CCR5 mRNA increased only 1.5-fold ($\Delta\Delta Ct = -0.7$, $p = 0.14$). These differences diminished after 24 h ($|\Delta\Delta Ct| < 0.3$, data not shown). This finding shows that increased secretion of eotaxin and RANTES as well as enhanced expression of CCR3 and CCR5 receptors correlates to the OVA-induced severe eosinophilia and inflammation in the lungs of CD26^{-/-} mice.

Discussion

Using a murine model, we demonstrated that the lungs of CD26^{-/-} mice display an obviously enhanced infiltration of leukocytes, especially eosinophils, in the pathogenesis of allergic asthma, suggesting that CD26 is required for the control of eosinophilic airway inflammation mediated by the inhaled allergen. It is remarkable that the serum IgG was greatly reduced, while the expression levels of Th2 cytokines, chemokines eotaxin and RANTES, as well as their receptors CCR3 and CCR5 in the lung of CD26^{-/-} mice were increased significantly.

Allergic airway symptoms are initiated by IgE mediated Mast cell activation. Mast cell-derived cytokines and chemokines, including IL-1, IL-3, IL-4, IL-5, IL-13,

1 granulocyte/macrophage colony stimulating factors (GM-CSF), eotaxin, RANTES and
2 monocyte chemotactic protein (MCP), have an immediate impact on the environment of the
3 airway and surrounding lung tissue, and on the recruitment and/or activation of mononuclear
4 cells and eosinophils observed in these disorders [27-29]. These activated and recruited
5 eosinophils and Th2 cells, in turn, amplify the allergic immune response through an autocrine
6 or paracrine feedback loop.

7 CD26 may exert its role in different phases of the allergic immune response. As a
8 costimulatory molecule described by many studies, it is involved in T cell activation, cytokine
9 secretion and B cell antibody production [20-24]. In the present study, loss of CD26 leads to
10 enhanced Th2 cytokine secretion in the lung, which affects both acute phase and cascade
11 reaction in late phase. Reduced serum IgG may counteract IgE induced pulmonary
12 inflammation as suggested by recent studies on blocking IgG antibody [30-33].

13 Moreover, in human lung, CD26 is expressed strongly in submucosal glands and in the blood
14 vessels of the bronchus [34], suggesting that it is involved in the processing of intravascular
15 and intraluminal bioactive peptides. Among chemokines involved in asthma, CD26/DPP-IV
16 removes the terminal Xaa-Pro dipeptides from full length RANTES, eotaxin, and
17 macrophage-derived chemokine (MDC) [35], which target eosinophils and activate Th2 cells
18 through interaction with CCR3, CCR5 or CCR4 expressed on the surface of these cells. NH₂-
19 terminal truncation greatly affects the biological activities of such chemokines. After CD26-
20 mediated processing, both eotaxin and RANTES exhibit a remarkable reduction in
21 chemotactic potency for eosinophils due to impaired signalling through CCR3 [25, 26]. It was
22 shown recently that CD26/DPP-IV indeed impairs the ability of parenchyma cells (including
23 endothelial and epithelial cells) to establish the cell-cell interactions necessary to sustain
24 leukocyte migration in vitro and tissue infiltration in vivo by metabolizing chemoattractants
25 bound to the endothelial/epithelial cell surface [36]. Coincidentally the highest specific DPP-IV

activity of CD26 was found in the lung among various organs in wild type mice (data not shown). Thus, the aggravated inflammation in the lungs of CD26 deficient mice may result from the inability to degrade the key chemokines involved in the recruitment of eosinophils and activated Th2 cells. Supporting our results, CD26/DPPIV activity has been demonstrated to correlate inversely with the degree of human allergic inflammation [37]. Inhibitor of CD26 enhances eotaxin-mediated recruitment of eosinophils in rats [38], and application of soluble CD26 significantly reduces allergic airway inflammation in rabbit [34].

Taken together, our data suggest that CD26 is required for the control of eosinophilic airway inflammation mediated by the inhaled allergen. Its expression in wild type mice has a protective role in allergic eosinophilic inflammation via its ability as a signal molecule and an enzyme.

Given that CD26 is an abundant and multifunctional protein, it is hard to distinguish CD26 function as an enzyme or as a signal molecule based on static knockout model. Induced and targeted knock out models, allowing temporal and spatial, cell-type or tissue-specific knockout of target gene, will certainly be helpful to characterise further the role of CD26 in asthma. Targeting to T cells or lung-specific epithelial/endothelial cells, including submucosal glands, should be considered, which would give more insights into the underling mechanisms.

These results indicate an important role of CD26 in controlling the allergic immune response and raise the safety question for clinical application of CD26 inhibitors. It is reported that a subset of clinically defined allergic rhinitis subjects exhibited worsening of their symptoms when taking sitagliptin, one of the approved inhibitors of DPPIV. This and other DPPIV inhibitors have been reported for causing “upper respiratory infections” in about 5% of type II diabetics [39]. These reports, together with our finding, suggest that the application of DPPIV inhibitors to patients, especially to those with an immune disorder such as asthma, rhinosinusitis or other eosinophilic diseases, should be carefully controlled.

Materials and Methods

Mice

Homozygous CD26^{-/-} mice on C56BL/6 genetic background [9] and wild type C57BL/6 mice were kept under specific pathogen-free conditions. Male mice, 6 - 8 weeks of age, were used in all experiments. C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were treated according to the German Law on the Protection of Animals, and permission (G 140/01, TVV 11/11) was obtained from the state animal welfare committees.

Sensitisation and airway challenge with ovalbumin

On days 0 and 5, mice were sensitised by intraperitoneal (i.p.) injection of 20 µg of ovalbumin (Sigma) dissolved in 0.3 ml endotoxin-free PBS mixed with 0.2 ml aluminum hydroxide gel (Alu-Gel-S Suspension, Serva electrophoresis, Heidelberg, Germany). On day 12, mice were challenged by exposure to an aerosol of 1% OVA in PBS for 40 min (Fig. 1). Inhalation was carried out in a plastic chamber (27 × 20 × 10 cm). The aerosol was generated via an ultrasonic nebulizer (1-5 µm particles according to the manufacturer's specifications, Model no. SW-966V 230V, Shining World Health Care Co, Taipei).

Bronchoalveolar lavage (BAL)

BAL was performed at indicated time points after aerosol challenge. Mice were killed by cervical dislocation. A median sternotomy was performed. The trachea was dissected free and cannulated. The lungs were lavaged twice with 1 ml-aliquots of PBS. The samples were then pooled and centrifuged. The cell-free BAL fluid was stored at -20°C until analysis. The cell pellet was re-suspended in PBS. Cell numbers were counted in a hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). Smears of BAL fluid cells were stained with

1 Giemsa & May-Grünwald solution (Sigma) and the cell differential was enumerated on the
2 basis of morphology and staining profile.

3 **Lung histology and immunohistochemistry**

4 After BAL, lungs were inflated with 1 ml tissue freezing medium (Leica Instruments,
5 Nossloch, Germany) instilled through a tracheotomy tube. Tissues were frozen immediately in
6 liquid nitrogen and stored at -20°C until sectioning. Sections of $7\text{ }\mu\text{m}$ thickness were mounted
7 on slides and stained with hematoxylin & eosin (Sigma).

8 Immunohistochemistry was performed according to the description of “Cell & Tissue Staining
9 Kit” from R&D System (Abingdon, UK). All procedure was performed at room temperature.
10 Endogenous peroxidase and non-specific Ig-, avidin- or biotin-binding sites were blocked
11 with their respective blocking reagents. Tissue sections were first incubated with primary
12 antibodies for 60 min. After washing with PBS, these sections were further incubated with
13 biotinylated secondary antibody for 60 min. Following washing steps, the sections were
14 incubated with HSS-HRP complex for 30 min and subsequently visualized with DAB
15 chromogen solution for 3-20 min. Finally hematoxylin was applied for counterstain. The
16 antibodies used in this experiment were rat anti-mouse monoclonal antibodies against
17 CCL11/Eotaxin ($25\text{ }\mu\text{g/ml}$), CCL5/RANTES ($25\text{ }\mu\text{g/ml}$), CCR3 ($10\text{ }\mu\text{g/ml}$) and CCR5 (10
18 $\mu\text{g/ml}$) (R&D System, Abingdon, UK). A rat IgG was used as a negative control.

19 **Measurement of immunoglobulin concentration in sera**

20 Blood samples were collected from the mouse tails and clotted at room temperature for 4 h.
21 Sera were transferred into new tubes after centrifugation and were stored at -20°C for further
22 analysis. Serum immunoglobulin concentration was measured by ELISA as described
23 previously [24]. Anti-mouse Ig pAb was used as coating antibody to detect IgM, IgG, IgG1
24 and IgG2a. Anti-mouse IgE mAb was used as coating antibody to detect IgE (PharMingen).

1 Biotin-labelled anti-IgM, anti-IgG (Sigma), anti-IgG1, anti-IgG2a, and anti-IgE mAbs
 2 (PharMingen) were used as detecting antibodies.

3 **Measurement of cytokine and chemokine concentrations**

4 IL-4, IL-5, and IL-13 concentrations in supernatants of BAL were determined by ELISA
 5 (R&D Systems), using the procedure recommended by the manufacturer.

6 **RNA isolation, reverse transcription PCR and quantitative real-time PCR**

7 Total RNA was isolated with trizol (Invitrogen) from lung tissue, checked for integrity by
 8 agarose gel electrophoresis, digested with DNase I (Fermentas), heated to 65 °C to inactivate
 9 DNase I and reverse transcribed into cDNA using SuperScript® Vilo cDNA synthesis kit
 10 (Invitrogen). Real-time PCR was performed in 25 µl 1x PCR buffer containing 1.5 mM
 11 MgCl₂, 100 µM dNTP, 400 nM forward and reverse primer (TIB Molbiol), 100 nM TaqMan
 12 chemistry probe (6FAM/BBQ, TIB MolBiol) and 1.5 U Maxima® Hot Start Taq polymerase
 13 (Fermentas) on a Mx3005P real-time PCR system (Agilent Technologies). Cycle conditions
 14 were 4 min enzyme activation at 95 °C and 50 cycles of 30 s 95 °C, 30 s 60 °C and 20 s 72 °C.
 15 Threshold Ct-values were recorded in independent duplicates or triplicates for each of at least
 16 4 identically treated animals and related to those of GAPDH ($\Delta Ct = Ct_{\text{target}} - Ct_{\text{GAPDH}}$).
 17 Differences between homzygous CD26 knockout and wildtype mice were calculated as $\Delta\Delta Ct$
 18 $= \Delta Ct_{\text{CD26}^{-/-}} - \Delta Ct_{\text{CD26}^{+/+}}$, yielding a negative value for a higher expression. Primer and probe
 19 sequences for IL-2, IL-4, IL-5, IL-10, IL-12p40, IL-13, IFN γ , RANTES, Eotaxin, CCR3,
 20 CCR5 and GAPDH will be provided upon request. Standard PCR was performed accordingly;
 21 the amplification products were separated by 2% agarose gel electrophoresis and visualized
 22 by EtBr staining.

Statistical analysis

Data were from three or more separate experiments and were expressed as mean $\pm$ SD. p values were calculated with unpaired, two-tailed Student's test.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. A schematic diagram of immunization procedure. On day 0 and 5, mice were sensitised by intraperitoneal injection ovalbumin and aluminum hydroxide gel as adjuvant. On day 12, mice were challenged by exposure to an aerosol OVA. Blood samples were taken on day 0, 5, 12 and 13. Lung tissue and BALF were obtained 0, 6, 12, 24, 36 h after challenge.

Figure 2. OVA-induced airway inflammation in CD26^{+/+} and CD26^{-/-} mice. Lung tissues

and BAL fluid were obtained 36 h after OVA challenge. Lung sections were stained with hematoxylin and eosin. Smears of BAL cells were stained with Giemsa and May-Grünwald solution. **(A and B)** Lung sections of **(A)** sham-immunized CD26^{+/+} mouse and **(B)** CD26^{-/-} mouse are shown at 100 × magnification. **(C and D)** Lung sections of OVA-sensitised and -challenged **(C)** CD26^{+/+} mouse and **(D)** CD26^{-/-} mouse are shown at 100 × magnification. **(E)** Lung section of OVA-sensitised and -challenged CD26^{-/-} mouse is shown at 1000 × magnification. Arrows indicate eosinophils. Images are representative of photomicrographs performed in triplicate of three experiments. **(F)** Total leukocytes and eosinophils in BAL fluid. Cell differentials were enumerated on the basis of morphology and staining profile. The values represent the mean + SD of eight mice for each group. Statistical significance was determined by unpaired, two-tailed Student's *t*-test. Results shown are representative of three independent experiments.

Figure 3. Serum immunoglobulin concentrations in OVA-immunized mice. Blood was taken at the indicated times after OVA immunization. Serum concentrations of **(A)** IgM, **(B)** IgG, **(C)** IgG1, **(D)** IgG2A and **(E)** IgE were measured by ELISA. The values represent the mean ± SD of fourteen mice at each time point. Statistical significance determined by unpaired, two-tailed Student's *t*-test. The data are representative of three independent experiments.

Figure 4. Cytokine expression profiles in the lungs of OVA-sensitised and -challenged mice. **(A)** Cytokine mRNA expression determined by RT-PCR in the lung tissues 6 and 24 h after challenge. Only mRNA of IL-12p40 was detected in unchallenged samples. After challenge, mRNA of Th2 cytokines IL-4, IL-5 and IL-13 was increased and detected. P: positive control provided in the detection kit; +/+ : CD26^{+/+} mice; -/- : CD26^{-/-} mice. **(B)** The mRNA expression levels of the indicated cytokines were independently quantified by real

time PCR in lung tissues 6 h after challenge. The values represent the mean
 duplicates/triplicates + SEM of 4 to 5 mice in each group. (C) IL-4, IL-5 and IL-13
 concentrations were measured by ELISA in BAL fluid 24 h after challenge. The values
 represent the mean + SD of eight mice for each group. * $p < 0.05$, ** $p < 0.01$ as determined
 by unpaired, two-tailed Student's *t*-test. Results shown are representative of three independent
 experiments.

Figure 5. Expression of eotaxin, RANTES, CCR3 and CCR5 in the lungs of OVA-sensitised and -challenged mice. (A) Immunohistochemical staining of eotaxin, RANTES, CCR3 and CCR5 in the lungs of PBS- or OVA-sensitised and -challenged mice (a, c, e, g, CD26^{+/+} mice and b, d, f, h, CD26^{-/-} mice). Lung tissues were obtained 24 h after challenge and immunohistochemical staining was performed in frozen sections from PBS- (a, b, c and d, n = 5 per group) or OVA- (e, f, g and h, n = 8 per group) treated mice with monoclonal antibodies against mouse eotaxin, RANTES, CCR3 and CCR5, and then counterstained with hematoxylin. The cell nuclei are blue and immunopositive positions are in dark brown as indicated by arrows. As a control, the secondary goat anti-rat mAb was used without primary antibodies but showed no non-specific reactions (data not shown). Images a, b, e and f were taken at original magnification 100× and images c, d, g, h were taken at original magnification 400×. (B) Quantitative real-time PCR analysis of RANTES, eotaxin, CCR3 and CCR5 expression levels in CD26^{+/+} and CD26^{-/-} mice 6 h after OVA airway challenge. The lower ΔC_t values of the CD26^{-/-} mice indicate a higher mRNA expression of the analyzed proteins. Data are shown as mean + SEM of n=4/5 mice. * $p < 0.05$, ** $p < 0.01$ as determined by unpaired two-tailed Student's *t*-test. Results shown are representative of three independent experiments.

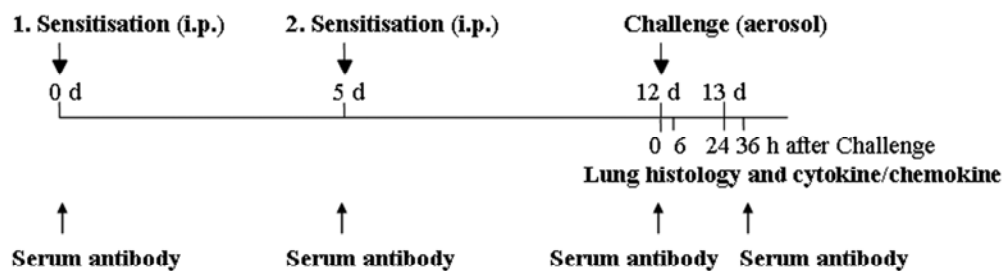


Figure 1

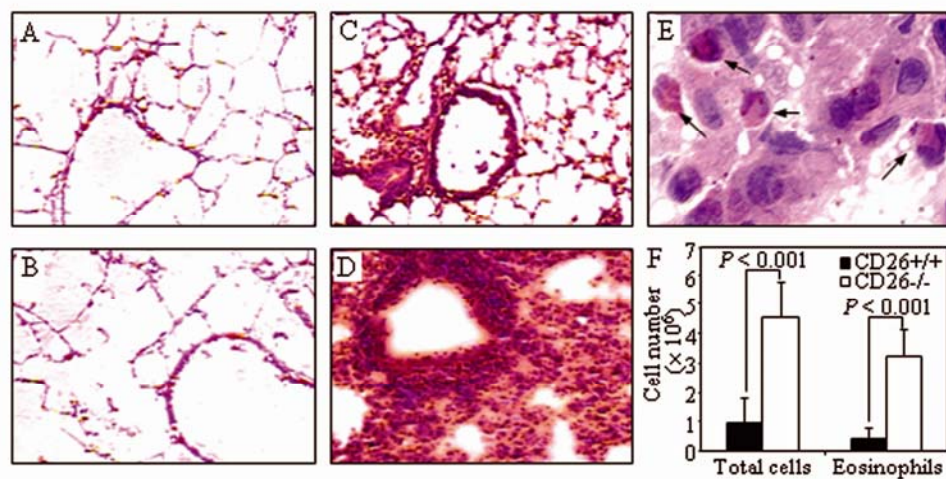


Figure 2

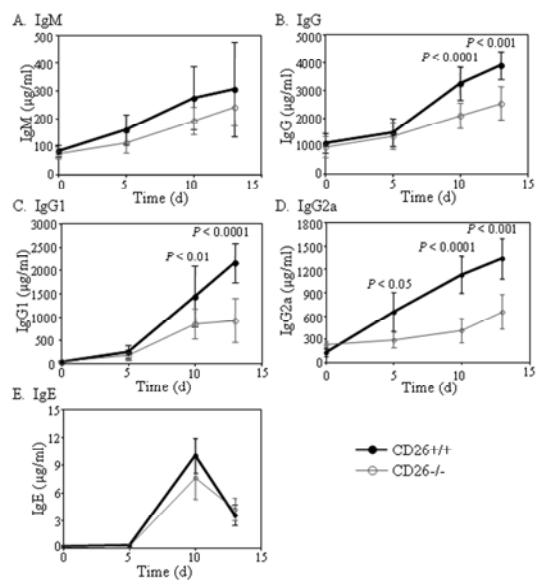
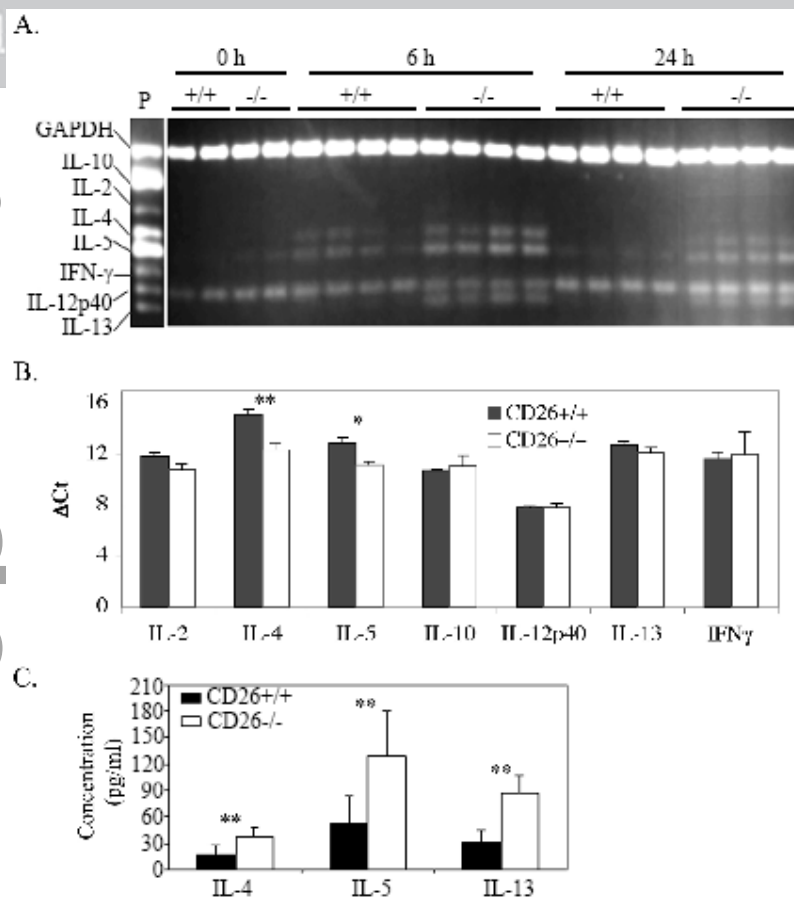


Figure 3

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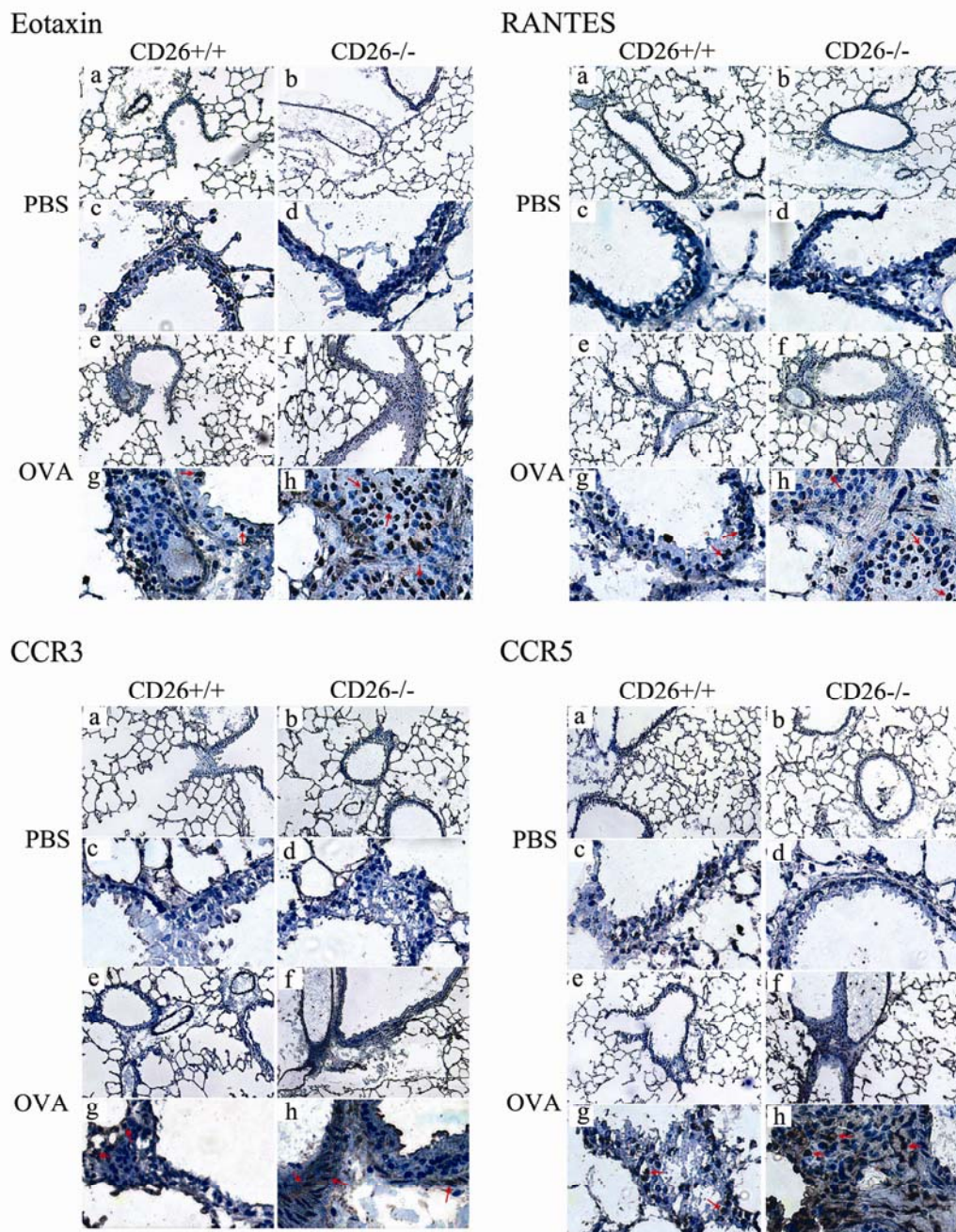


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B.

