

Original article

Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation

Background: As Th2 type lymphocytes orchestrate the cardinal features of allergic asthma, inhibiting their recruitment to the lungs could be of therapeutic benefit. Although human Th2 cells express the CCR4 chemokine receptor and increased production of CCR4 ligands has been found in asthmatic airways, studies in animals have reached contradictory conclusions on whether blocking this pathway would be beneficial.

Objective: As a lack of efficacy might be due to differences between mouse and man, we readdressed this question using a humanized severe combined immunodeficiency model of asthma.

Methods: Mice received peripheral blood mononuclear cells from house dust mite (HDM) allergic asthmatic patients and then underwent bronchial challenge with HDM.

Results: This resulted in marked allergic inflammation and bronchial hyper-reactivity. Administration of CCR4 blocking antibody abolished the airway eosinophilia, goblet cell hyperplasia, IgE synthesis and bronchial hyperreactivity. In this chimeric system, human CD11c⁺ dendritic cells (DCs) were the predominant source of CCR4 ligands, suggesting that DC-derived chemokines attract Th2 cells. In separate experiments using human DCs, *in vitro* exposure to HDM of DCs from HDM allergic patients but not healthy controls caused CCL17 and CCL22 release that resulted in chemoattraction of polarized human Th2 cells in a CCR4-dependent way.

Conclusions: Taken together, our data provide proof of concept that CCR4 blockade inhibits the salient features of asthma and justify further clinical development of CCR4 antagonists for this disease.

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Key words: animal model; asthma; chemokines; dendritic cells; eosinophils.

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Accepted for publication 26 March 2009

In allergic asthma, the presence of high levels of allergen-specific IgE is a reflection of an aberrant Th2 cell immune response to commonly inhaled environmental allergens, such as house dust mite (HDM), cockroach or pollen (1). Allergens are presented to T cells by dendritic cells (DCs) that continuously sample incoming foreign antigens (2). Upon proper activation by DCs, allergen-specific lymphocytes that are present in diseased airways produce Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 that furthermore control leucocyte extravasation, goblet cell hyperplasia and bronchial hyper-reactivity (3). As lymphocytes are so essential for disease pathogenesis, it is crucial to understand how these cells are attracted to inflamed airways. Certain chemokine receptors are differentially expressed on the surface of effector and central memory cells, Th1 cells expressing CXCR3 and Th2 cells expressing CCR4 and CCR8 (4, 5). Furthermore, thymus and activation-regulated chemokine (TARC)/CCL17 and monocyte-derived

chemokine (MDC)/CCL22 induce the selective migration of Th2 cells but not Th1 cells through triggering of CCR4 (5). The same mechanism might explain why Th2 cells are attracted to asthmatic airways as T cells in bronchial mucosal biopsies or obtained by bronchoalveolar lavage (BAL) of patients with allergic asthma express CCR4 (some co-expressing CCR8) (6–8), and the BAL fluid levels of TARC/CCL17 and MDC/CCL22 but not Th1-selective chemokines are increased upon allergen challenge to the lung (7–10).

Materials and methods

Reagents

Anti-human CCR4 (clone 1D1) was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). Dermatophagoides pteronyssinus (Dpt) extracts were obtained from Stallergenes (Almere, the Netherlands). Purified Dermatophagoides pteronyssinus

allergen 1 (Derp1) was provided by G. A. Stewart (Perth, Australia). Fluorescein isothiocyanate (FITC)-labeled anti-human CD45, phycoerythrin (PE)-labeled anti-human CD3, allophycocyanin (APC)-labeled anti-human CD4, anti-human CD8 and anti-human CD11c were obtained from BD (Breda, the Netherlands). Phycoerythrin-labeled anti-human CRTH2 antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Biotinylated anti-human TARC/CCL17, PE-labeled anti-mouse CCR3, FITC-labeled anti-human CXCR3 and PE-labeled anti-human CCR4 antibodies, recombinant mouse and human TARC/CCL17 and MDC/CCL22, recombinant human IL-4 were obtained from R&D Systems (Abingdon, UK). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) was obtained from Leukomax (Brussels, Belgium).

Mice

Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (5–6 week old) were bred in the animal facility of the Erasmus medical center, and experiments were approved by the ethical board of Erasmus (Rotterdam, the Netherlands).

Patients

The study was approved by the Ethics Committee of the Erasmus Medical Center (Rotterdam, the Netherlands). Each patient provided written informed consent. Fifty ml of blood were obtained from 10 allergic asthmatic patients with a clear history of relevant symptoms and bronchial hyper-reactivity to methacholine (MCH). None of the patients had a history of other respiratory disease. All subjects were clinically free from respiratory infection and glucocorticoid therapy for at least 2 weeks before blood drawing. All subjects had HDM-specific IgE.

Cell preparation

Blood was diluted in RPMI 1640 (Sigma-Aldrich, Zwijndrecht, the Netherlands) and layered over a Ficoll gradient (Pharmacia, Uppsala, Sweden). After centrifugation (1200 g, 20 min), peripheral blood mononuclear cells (PBMCs) were harvested and washed. Cells were resuspended in phosphate-buffered saline (PBS) containing five index of reactivity of Dpt extracts before transfer into SCID mice.

To prepare MDCs, CD14⁺ monocytes were isolated from human PBMCs by using CD14 microbeads and a magnetic MACS separator (Miltenyi). After elution, CD14⁺ cells were resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics (gentamycin) before plating (2×10^6 cells/2 ml per well) into 6-well flat-bottomed culture plates. CD14⁺ cells were differentiated into MDCs for 6 days in medium supplemented with GM-CSF (1000 IU/ml) and IL-4 (200 IU/ml). On day 6, DCs were pulsed or not with 1 µg/ml purified Derp 1 overnight.

To generate Th2 cells, naïve T cells were isolated from PBMCs of healthy donors using CD4 T cell isolation kit and CD45RA⁺ microbeads (Miltenyi). T-cell lines were generated by stimulating the CD4⁺/CD45RA⁺ cells with 5 µg/ml phytohemagglutinin in complete RPMI complemented with 30 U/ml IL-2, 10% FCS in the presence of irradiated 2×10^5 PBMCs plus 250 U/ml rhu IL-4 and 10 mg/ml anti-IL-12 mAb (for type 2 cell polarization). These media were refreshed weekly. After 5–6 weeks of culture, their phenotype was assessed by flow cytometry. T cells were stained with FITC-labeled anti-human CXCR3 and PE-labeled anti-human

CCR4. A large proportion of the cells expressed CCR4 whereas very few cells expressed CXCR3, indicating that the cells generated had a phenotype of Th2 cells.

Reconstitution of SCID mice

On day 0, SCID mice were reconstituted by i.p. injection of 10×10^6 PBMCs. On days 1, 3 and 7, SCID mice were injected i.p. with 100 µg of anti-human CCR4 or isotype control antibodies followed, 30 min later, by an intratracheal (i.t.) administration of Dpt extract. On day 21, SCID mice were killed. After anaesthesia with 2.5% avertin, mice were bled and BAL was performed using 3×1 ml warm PBS containing 0.1 mM EDTA through a canula placed in the trachea. To obtain single lung cell suspensions, lungs were perfused with 20 ml PBS through the right ventricle, minced using iridectomy scissors and homogenized.

Bronchial hyper-responsiveness (BHR) was measured by exposing awake mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized MCH using ultrasonic nebulizers 24 h after the last HDM challenge. PenH (enhanced pause) values were measured for 3 min after each MCH aerosol using a whole body plethysmograph (Buxco, Electronics, Troy, NY, USA). The average PenH values were expressed for each MCH concentration as the percentage increase over baseline PenH values.

Flow cytometry

To detect human T cells, cells were incubated with FITC-labeled anti-human CD45, PE-labeled anti-human CD3 or CRTH2 and APC-labeled anti-human CD4 or CD8. The detection of murine eosinophils was performed by incubating cells with PE-labeled anti-mouse CCR3 antibodies. Cells were then analyzed by flow cytometry on a FACSCalibur (BD).

Immunostaining

Six µm thick paraffin or frozen tissue sections of the lungs were performed. After permeabilization in PBS/Triton 0.3%, the slides were saturated with PBS/20% normal human serum. Sections were incubated with anti-human CD45 (DAKO, Heverlee, Belgium) or an isotype-matched mouse IgG (DAKO) as a negative control. Stainings were revealed using an alkaline phosphatase-anti-alkaline phosphatase technique (DAKO). Slides were counterstained with haematoxylin (Sigma-Aldrich). To detect human TARC/CCL17, frozen sections were incubated with APC-labeled anti-human CD11c antibodies and biotinylated anti-human TARC/CCL17 followed by streptavidin Qdot 525. Slides were analyzed on an LSM 510 confocal microscope (Zeiss, Slidrecht, the Netherlands) equipped with 488 and 633 nm lasers. Images were analyzed using IMARIS software (version 5.0; Bitplane AG, Zurich, Switzerland). The Abs used in this study did not cross-react with murine tissues.

Chemotaxis assay

Chemotaxis of Th2 cells was evaluated by measuring their migration through 5-µm pore polycarbonate filters in 48-well transwell chambers (Nuclepore, Tübingen, Germany). Buffer or stimuli were added into the lower compartment wells. Thereafter a 10 µm polycarbonate membrane with a pore size of 5 µm was placed over the wells. Th2 cells (10^5 cells/well) were added to the upper compartment and incubated at 37°C for 60 min. After

removing the cells from the upper side of the membrane, migrated Th2 cells on the lower side of the membrane were fixed in methanol and stained with haematoxylin. Experiments were performed in triplicate. Migrated Th2 cells were counted in five randomly chosen high-power (400) fields and a mean value for each sample was calculated. The chemotactic index was calculated as the ratio between Th2 cells migrated in the presence and in the absence of stimuli.

ELISA

Lung cells were resuspended at a concentration of 100 000 human cells/ml in RPMI supplemented with 10% heat-inactivated FCS and antibiotics. Two hundred μ l of the cell suspension was added to 96-well flat-bottomed plates coated with 1 μ g/ml anti-human CD3 and 2 μ g/ml anti-human CD28. Twenty-four hours later, supernatants were collected and IL-5, IL-13 and interferon (IFN)- γ were measured by enzyme-linked immunosorbent assay (ELISA) (Diaclone, Stamford, CT, USA). The supernatants of DCs pulsed or not *in vitro* with Derp 1 overnight were collected and assayed for the presence of human TARC/CCL17, MDC/CCL22 and interferon-inducible protein 10 (IP-10)/CXCL10 by ELISA (R&D systems).

Statistical analysis

For all experiments, the difference between groups was calculated using the Mann–Whitney *U*-test for unpaired data. Differences were considered significant when $P < 0.05$.

Results

Human CCR4 blockade *in vivo* reduces airway inflammation in a Hu-SCID model

As expected in mice injected (i.t.) with PBMCs from HDM-allergic patients exposed to HDM, there was an accumulation of CD45+ human leucocytes in the lungs starting at day 7 and peaking at day 14 after the last aerosol exposure (Fig. 1). No human leucocytes could be detected in the lungs of mice injected with PBMCs from healthy donors (Fig. 1). For this reason, the rest of the experiments was performed using SCID mice injected with cells from allergic patients. We next tested the effect of blocking anti-CCR4 antibodies on the recruitment of human leucocytes to the lungs of SCID mice. In mice injected with the isotype control antibody, human CD45+ cells could be detected in the lung (Fig. 2A and C) of SCID mice. In mice treated with blocking anti-CCR4 antibodies, there was a substantial reduction in the number of CD45+CD3+ cells detected in the lungs (Fig. 2A). Both CD4+ and CD8+ T-cell numbers were decreased in mice that received the anti-CCR4 antibodies. Interestingly, the injection of CCR4 blocking antibodies also significantly reduced the number of eosinophils in the BAL fluid of the mice compared with animals injected with the control antibodies (Fig. 2B). Bronchial hyper-responsiveness to nonspecific stimuli like MCH is one of the defining symptoms of allergic asthma. Mice injected with PBMCs from healthy donors did not develop BHR

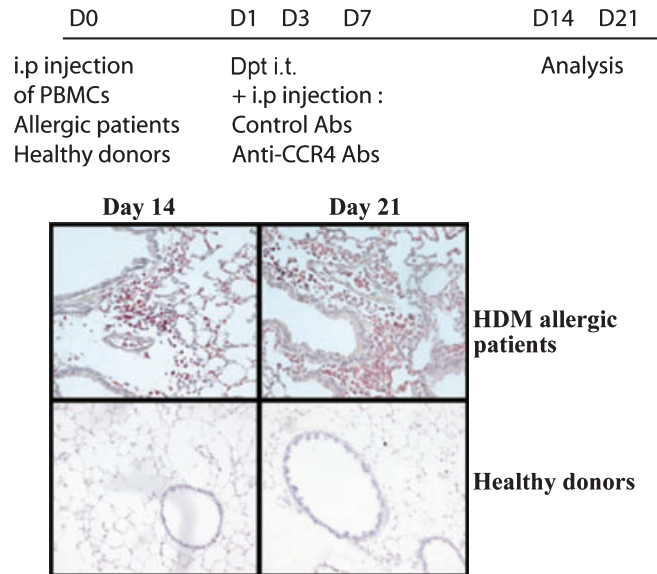


Figure 1. Severe combined immunodeficiency (SCID) mice injected with peripheral blood mononuclear cells (PBMCs) from house dust mite (HDM)-allergic patients develop airway inflammation. Severe combined immunodeficiency mice were injected i.p. with PBMCs from HDM-allergic patients or healthy donors on day 0. On days 1, 3 and 7, SCID mice received an intratracheal injection of HDM extracts. At days 14 and 21, lungs were stained for the presence of human CD45+ cells (in red) and counterstained with H&E.

in response to MCH inhalation. However, mice injected with PBMCs from allergic patients and treated with isotype control antibodies showed significant changes in responsiveness to inhaled MCH compared with mice injected with cells from healthy donors. BHR was strongly attenuated in mice reconstituted with cells from HDM allergic patients and injected with blocking CCR4 antibodies (Fig. 2D). The Th2 marker CRTH2 is a very reliable marker for human Th2 cells *in vivo* (11). Therefore, we also enumerated the number of human CD4+CRTH2+ cells in the lung of mice. This number was also decreased in mice injected with anti-CCR4 antibodies (data not shown). We finally determined the effect of CCR4 blockade on cytokine production by lung T cells in SCID mice injected or not with blocking anti-CCR4 antibodies. T cells obtained from the lungs of mice injected with the control antibodies produced IL-5, IL-13 and IFN- γ (Fig. 3). In mice injected with anti-CCR4 antibodies, the production of all cytokines tested was decreased.

Human CD11c+ DCs are the cellular source of CCR4 ligand in the lung of SCID mice

We next investigated the cellular source of CCR4 ligands. In this chimeric system, both murine epithelial cells and murine and human DCs could produce TARC/CCL17 and MDC/CCL22. We first checked whether murine

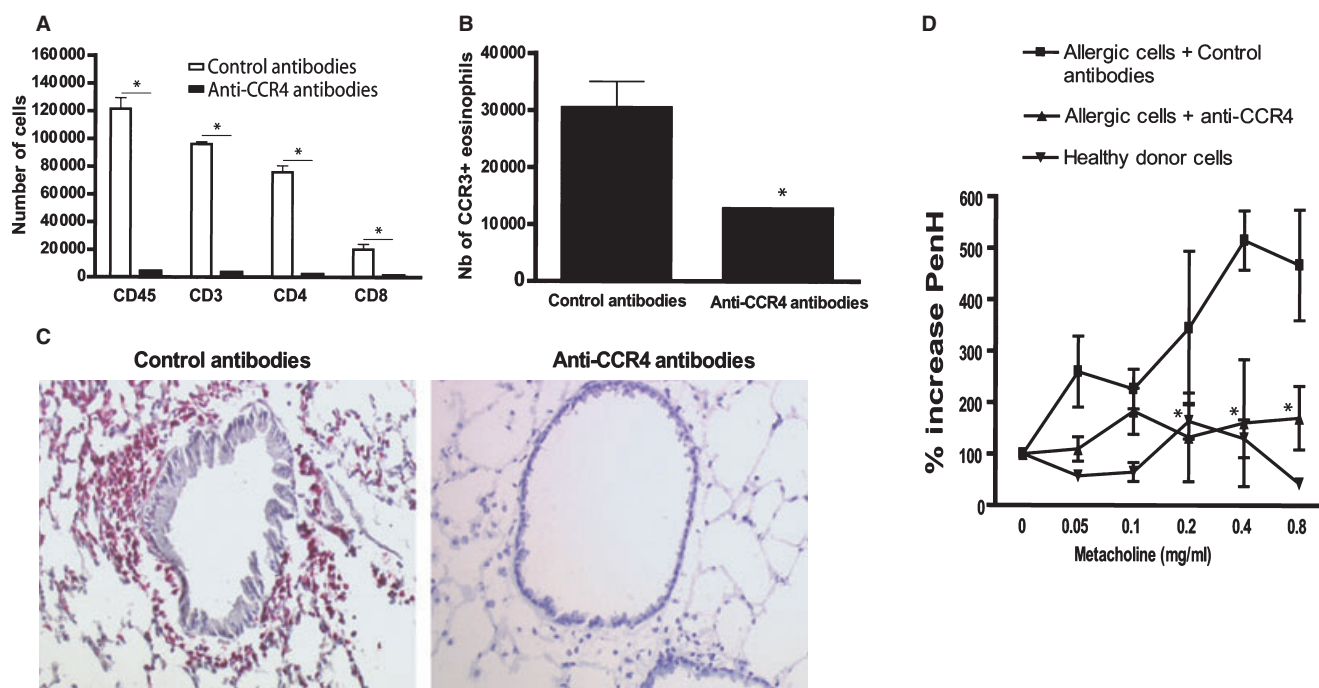


Figure 2. CCR4 suppresses the cardinal features of asthma in humanized severe combined immunodeficiency (SCID) mice. Severe combined immunodeficiency mice were injected i.p. with peripheral blood mononuclear cells (PBMCs) from house dust mite (HDM)-allergic patients on day 0. On days 1, 3 and 7, SCID mice were injected i.p. with 100 µg of anti-human CCR4 or isotype control antibodies followed, 30 min later, by an intratracheal administration of dermatophagoides pteronyssinus. At day 21, mice were killed. (A) Number of human leucocytes in the lung. (B) Number of murine CCR3+ eosinophils in the bronchoalveolar lavage. (C) Immunostaining for human CD45+ leucocytes in the lung with control (right panel) or with anti-CCR4 (left panel) antibodies on lung sections. Also note the thickness of the airway epithelium in the isotype control group, signifying goblet cell hyperplasia (D). Airway obstruction to increasing doses of inhaled methacholine was measured as increase in PenH values (% increase from saline) 24 h after the last Ag exposure. * $P < 0.05$ compared with control antibodies.

CCR4 ligands could recruit human Th2 cells *in vitro*. Mouse MDC/CCL22 poorly recruited human Th2 cells even at the highest dose of chemokine tested compared with human MDC/CCL22 (Fig. 4A). We next addressed whether human infiltrated cells could produce CCR4 ligand *in vivo*. As shown in Fig. 4B, in SCID mice treated with isotype control Abs, human TARC/CCL17 colocalized with human CD11c.

DCs from HDM-allergic patients increase the production of TARC/CCL17 and MDC/CCL22 in response to Derp 1 and recruit Th2 cells in a CCR4-dependent way

Because human CCR4 ligands were found in human DCs in the lung of mice exposed to HDM aerosols and treated with isotype control Abs, we addressed whether CCR4 ligand production by human DCs was regulated by the allergen. We therefore exposed MDCs from allergic patients or healthy donors to the HDM allergen Derp 1. Unpulsed DCs from both healthy donors and allergic patients produced TARC/CCL17 and MDC/CCL22 (Fig. 5A). After overnight pulsing with Derp 1, DCs from HDM allergic asthmatic patients strongly increased

their production of both TARC/CCL17 and MDC/CCL22 whereas DCs from healthy donors pulsed with the same allergen failed to do so and instead produced the CXCR3 ligand IP-10/CXCL10. We next investigated whether supernatants of DCs from allergic patients exposed to Derp 1 would preferentially recruit Th2 cells *in vitro*. Th2 cells were generated *in vitro* and as shown in Fig. 5B, a large proportion of the cells expressed CCR4 whereas very few cells expressed CXCR3, indicating that the cells generated had a phenotype of Th2 cells. We next checked whether these generated Th2 cells would migrate in response to culture supernatants of Derp 1-exposed DCs from HDM-allergic patients or from healthy donors. As expected, migration of Th2 cells in response to supernatants of Derp1-pulsed DC from allergic patients was strongly increased compared with supernatants obtained from unpulsed DCs. The pretreatment of Th2 cells with blocking anti-CCR4 antibodies strongly reduced their capacity to migrate in response to supernatants of Derp 1-pulsed DCs from allergic patients (Fig. 5C). Supernatant of DCs from healthy donors pulsed or not with Derp 1 showed the same weak attracting capacity towards Th2 cells.

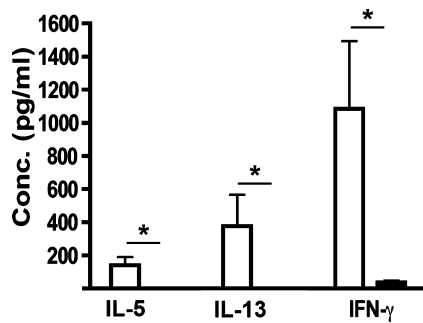


Figure 3. CCR4 treatment suppresses Th2 cytokines in the lungs of humanized model of severe combined immunodeficiency (Hu-SCID) mice. Mice were treated as in Fig. 2. Human T cells obtained from lungs of Hu-SCID mice were stimulated for 24 h with anti-CD3 and anti-CD28 antibodies. Cytokine levels were measured in the supernatants (data are representative of two independent experiments from a total of five different allergic donors). * $P < 0.05$ compared with control antibodies. IL, interleukin; IFN, interferon.

Discussion

Recent studies in animal models of asthma have revealed that T helper 2 lymphocytes interact with antigen presenting DCs in the airways, leading to triggering of effector Th2 cytokine production and *in situ* maturation of DC phenotype (3, 12). Not surprisingly, airway eosinophilia, goblet cell hyperplasia

and BHR does not develop in ovalbumin (OVA)-sensitized mice conditionally depleted of airway DCs at times of OVA allergen challenge (3, 13). The precise reason why DCs are so critical for mounting inflammatory responses in the airways of mice are currently unknown, but could involve the provision of costimulatory molecules to primed T cells or the attraction of effector Th2 cells to exposed sites of allergen challenge, through release of chemotactic factors (2).

Asthma is characterized by infiltration of Th2 lymphocytes and eosinophils and by the production of Th2 cytokines. Th2 cells play an important role in the development of airway inflammation in asthma. Expression of the Th2 cytokines IL-4, IL-5 and IL-13 was increased in the bronchial epithelium and in cells of BAL fluid obtained from patients with allergic asthma (14). Trafficking of activated T cells into inflammatory sites is a complex process directed by several molecules such as adhesion molecules and chemokines (15). The interaction of CCR4 with its ligands TARC/CCL17 and MDC/CCL22 is crucial in controlling the trafficking of Th2 lymphocytes into sites of allergic inflammation. Several papers have described the involvement CCR4 and its ligands in the migration of T cells to the skin in atopic dermatitis models (16–19) and to the airways (9, 20, 21) where the levels of TARC/CCL17 and MDC/CCL22 are increased (10, 22). The exact contribution of CCR4 pathway to lung inflammation has been a matter of debate for a long time and a lot of controversial data have been obtained in mice and in humans. Kawasaki et al.

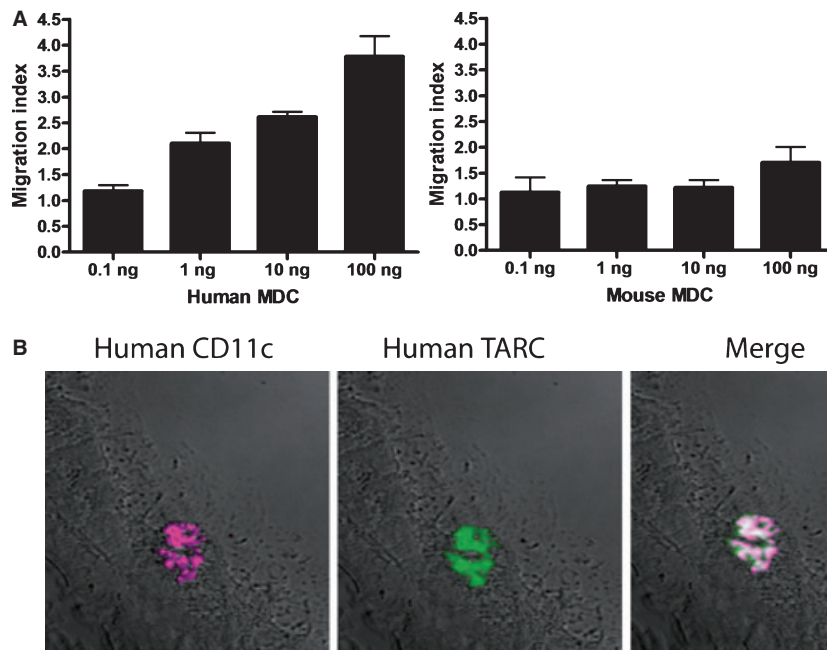


Figure 4. (A) Human and murine thymus and activation-regulated chemokine (TARC)/CCL17 and monocyte-derived chemokine (MDC)/CCL22 were tested for their capacity to recruit human Th2 cells *in vitro*. (B) Dendritic cells are the main source of CCR4 ligands in the lungs of humanized model of severe combined immunodeficiency (Hu-SCID) mice. Lung sections were stained with fluorescein isothiocyanate-anti human CD11c and with biotinylated anti-human TARC/CCL17 followed by streptavidin-Qdot 525.

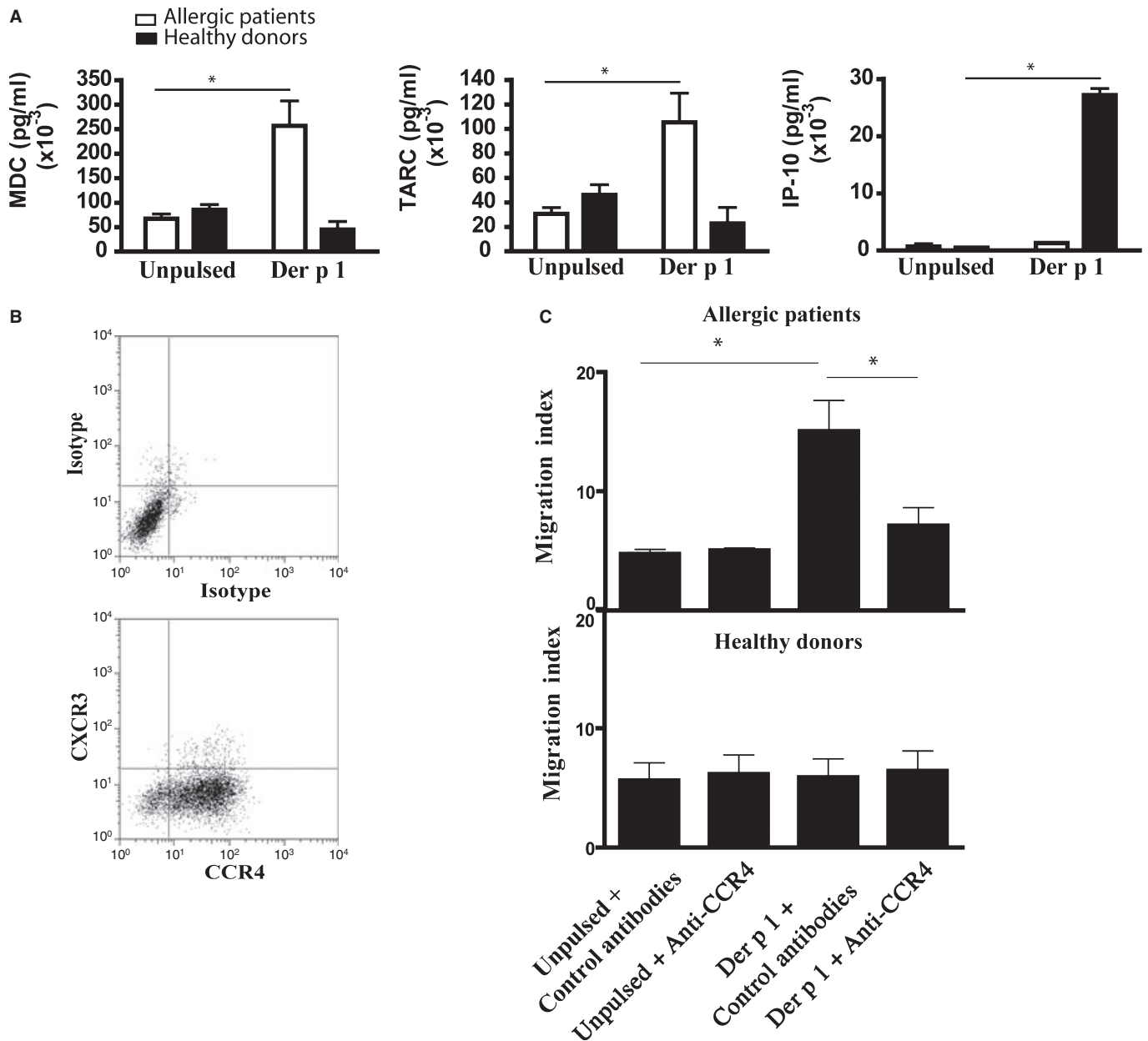


Figure 5. Dendritic cells (DCs) from house dust mite (HDM) allergic patients increase the production of thymus and activation-regulated chemokine (TARC)/CCL17 and monocyte-derived chemokine (MDC)/CCL22 in response to Derp 1 and recruit Th2 cells in a CCR4-dependent way. (A) Monocyte-derived DCs from HDM-allergic patients or from healthy donors were stimulated overnight with 1 μ g/ml Derp 1. The levels of TARC/CCL17, MDC/CCL22 and IP-10/CXCL10 were measured in the supernatants. (B) Chemokine receptor expression on *in vitro*-generated human Th2 cells. (C) Supernatants of Derp 1 pulsed DCs from HDM-allergic patients or from healthy donors were tested for their capacity to recruit human Th2 cells *in vitro*. In this assay, Th2 cells were incubated by blocking anti-CCR4 antibodies or with control antibodies before they were added to the assay. * $P < 0.05$ compared with unpulsed cells and to anti-CCR4 antibodies. IP, interferon-inducible protein; Derp1, Dermatophagoides pteronyssinus allergen 1.

(23) showed in a murine model of asthma that treatment with anti-TARC/CCL17 antibodies reduced the number of CD4⁺ T cells and eosinophils in BAL fluid, the production of Th2 cytokines and airway hyper-responsiveness after allergen challenge. Moreover, corticosteroids used as a standard treatment of asthma attenuated TARC/CCL17 production in a mouse model

of asthma (24). On the contrary, CCR4-deficient mice showed no protection against airway inflammation and BHR (25). A major caveat is that most of the experiments addressing the role of CCR4 in allergic asthma were performed *ex vivo* or in murine models of the disease where the CCR4 receptor had been constitutively knocked out. It was therefore important to address the

role of CCR4 in the human immune system using a relevant natural allergen such as HDM. By using a unique humanized SCID mouse model of allergic airway inflammation, we showed that the blockade of CCR4 prior to allergen challenge reduced allergic airway inflammation as well as the levels of Th2 cytokines in the lungs. Our *in vivo* data therefore provide the proof that CCR4 blockade is a feasible strategy to suppress allergic inflammation. However, CCR4 blockade did not completely block cell recruitment to the airways, suggesting that molecules other than TARC/CCL17 and MDC/CCL22 might be involved. Supporting this idea, CCR8 has been described on the surface of Th2 cells. However, only a small number of CCR4+ Th2 cells recruited to the lungs of asthmatic patients co-expressed CCR8 (7) and CCR8-deficient mice did not show any reduction of pulmonary eosinophilia and Th2 cytokines (26).

How human T cells are recruited to the lungs of SCID mice following allergen challenge and orchestrate airway inflammation remain unclear. We therefore investigated the cellular source of CCR4 ligands. In this chimeric system, epithelial cells (murine), DCs (murine and human) and T cells stimulated with IL-4 or with the allergen (human) can produce TARC/CCL17 and MDC/CCL22 (22, 27, 28). Our data clearly show that although CCR4 ligands were expressed by murine epithelium, murine TARC/CCL17 and MDC/CCL22 poorly recruited human cells *in vitro*, making a murine source less likely. However, human TARC/CCL17 colocalized

with CD11c+ DCs in the lung of allergic SCID mice, suggesting that these cells would be the primary source of Th2-attracting chemokines. In support, we showed CCR4 ligand production was regulated by allergen stimulation of DCs. Indeed, MDCs from HDM allergic patients exposed to Derp 1 produced higher amounts of TARC/CCL17 and MDC/CCL22 compared with unstimulated DCs, and their supernatants induced Th2+ cell recruitment in a CCR4-dependent way. Our findings suggest that DCs are important pro-inflammatory cells in asthma that control recruitment of disease-causing Th2 lymphocytes as previously reported in animal models of asthma (3). However, as naive T cells and T cells stimulated with the relevant allergen or with IL-4 can also produce TARC/CCL17 and MDC/CCL22, a feedback loop for T-cell recruitment cannot be excluded (29, 30). Our findings in this unique humanized SCID model of asthma provide definitive proof that CCR4 blockade is a feasible strategy to suppress allergic inflammation. Taken together, our data identify CCR4 blockade as a potent way of inhibiting allergic inflammation in humans.

Funding

Frédéric Perros is supported by a long-term Fellowship grant of the European Respiratory Society. Hamida Hammad and Bart Lambrecht are supported by an Odysseus grant from the Flemish Science Organisation (FWO).

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