

Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity

Iris Bellinghausen, PhD,^a Sebastian Reuter, PhD,^b Helen Martin, PhD,^b Joachim Maxeiner,^{b,c} Uli Luxemburger,^{b,d} Özlem Türeci, MD,^{b,d} Stephan Grabbe, MD,^a Christian Taube, MD,^{b,e} and Joachim Saloga, MD^a Mainz, Germany, and Leiden, The Netherlands

Background: IL-10-treated dendritic cells (DCs) have been shown to inhibit T-cell responses through induction of anergy and regulatory T cells in various model systems, including allergic inflammation, but the factors being involved in this inhibition are still unclear.

Objective: This study set out to analyze such factors produced or induced by IL-10-treated DCs by using gene expression profiling and to explore their function.

Methods: CD4⁺ T cells from allergic donors were stimulated with autologous monocyte-derived allergen-pulsed mature DCs or IL-10-treated DCs. After 24 hours, the transcriptional profile was analyzed by using Affymetrix technology. Results were validated by using quantitative real-time PCR, protein expression, and functional *in vitro* and *in vivo* studies.

Results: In CD4⁺ T-cell/IL-10-treated DC cocultures the expression of several known genes, such as *IL13*, *IL5* and *OX40*, was suppressed. Interestingly, there was only one factor that was strongly upregulated: the DC-derived chemokine *CCL18*.

In vitro addition of CCL18 to cocultures of CD4⁺ T cells and allergen-pulsed DCs resulted in a similar inhibition of T_H2 cytokine production as induced by allergen-pulsed IL-10-treated DCs without exogenous CCL18, whereas T_H1 cytokine production, IL-10 production, and proliferation were not affected. Furthermore, in a humanized mouse model of allergy using PBMC-engrafted NOD-*scid*- γ c^{-/-} mice, CCL18, but not another T_H2-associated chemokine, CCL17, inhibited airway reactivity and lung inflammation. Chemotaxis assays revealed that CCL18 preferentially attracted regulatory T cells and, less efficiently, T_H2 cells.

Conclusion: These data demonstrate that CCL18 might represent a molecule of significant importance in immunoregulation and might be a therapeutic target in patients with allergic airway diseases. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Chemokines, dendritic cells, T_H1/T_H2 cells, allergy, regulation, humanized mice

The balance between allergen-specific T_R1 cells and T_H2 cells plays a key role in the development of allergy: although IL-10- and TGF- β -producing T_R1 cells are more frequent in healthy donors, IL-4-producing T_H2 cells are dominant in allergic patients.^{1,2} T_R1 cells are induced during allergen-specific immunotherapy *in vivo* and by immature or tolerogenic dendritic cells (DCs), such as IL-10-treated DCs, *in vitro*.^{3,4} In contrast to naturally occurring CD4⁺CD25⁺ forkhead box protein 3 (FoxP3)-positive regulatory T (Treg) cells, FoxP3 is not critical for the suppressive function of T_R1 cells, which rather seems to be dependent on the production of TGF- β and IL-27 and on contact-dependent signals, such as programmed death 1, glucocorticoid-induced TNF receptor, and cytotoxic T lymphocyte-associated antigen 4.^{5,6} Recruitment of Treg cells to the inflamed tissue through the chemokine receptors CCR4, CCR5, CCR7, and CCR8 might play an important role as well.⁷ However, the precise mechanisms of suppression still remain elusive. To identify further suppression factors produced or induced by IL-10-treated DCs, we performed a genome-wide analysis of gene expression in cocultures of CD4⁺ T cells from patients with grass pollen allergy and either tolerogenic IL-10-treated DCs or regular mature allergen-pulsed DCs. We showed that the only gene being upregulated in IL-10-treated DC/T-cell cocultures was *CCL18*.

CCL18, also known as DC-derived CC chemokine 1 or pulmonary and activation-regulated chemokine, is a 7.8-kDa chemokine that was generated by fusion of 2 macrophage inflammatory protein 1 α (CCL3)-like genes, which occurred after the diversification of rodents, so that no murine homologue exists. The CCL18 receptor is also unknown thus far.⁸ CCL18 attracts naive CD4⁺ and CD8⁺ T cells, B cells, and immature DCs and is mainly expressed by monocyte-derived DCs, alveolar macrophages, monocytes, and eosinophils.⁹ CCL18 is constitutively expressed in human serum and synovial fluid and upregulated in patients with several diseases,¹⁰ such as rheumatoid arthritis,¹¹ cutaneous contact hypersensitivity,¹² pulmonary fibrosis,¹³ chronic rhinosinusitis,¹⁴ and allergic asthma.¹⁵ The T_H2-associated cytokines, such as IL-4, IL-13, and IL-10, increase the production of CCL18 in alveolar macrophages, whereas IFN- γ inhibits its production.¹⁶ CCL18 is also expressed in the skin of patients with

From ^athe Department of Dermatology, ^bIII Medical Clinic, ^cthe Asthma Core Facility, and ^dthe High Density Microarray Core Facility of the Research Center Immunology, University Medical Center, Johannes-Gutenberg-Universität Mainz, and ^ethe Department of Pulmonology, Leiden University Medical Center.

Supported by Deutsche Forschungsgemeinschaft (DFG) SFB 548 TP A4 and BE 4504/2-1 grant.

Disclosure of potential conflict of interest: I. Bellinghausen has received one or more grants from and has received support for travel from the DFG (grant BE 4504/2-1). J. Saloga has received one or more grants from the German Research Foundation, has received one or more grants from or has one or more grants pending with the pharmaceutical industry, and has received one or more payments for lecturing from or is on the speakers' bureau for the pharmaceutical industry. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 9, 2012; revised August 23, 2012; accepted for publication August 27, 2012.

Corresponding author: Iris Bellinghausen, PhD, Department of Dermatology, University Medical Center, Johannes-Gutenberg-Universität Mainz, Langenbeckstr. 1, 55131 Mainz, Germany. E-mail: iris.bellinghausen@unimedizin-mainz.de.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2012.08.039>

Abbreviations used

AHR: Airway hyperresponsiveness
 APC: Allophycocyanin
 BAL: Bronchoalveolar lavage
 DC: Dendritic cell
 FITC: Fluorescein isothiocyanate
 FoxP3: Forkhead box protein 3
 IMDM: Iscove modified Dulbecco medium
 iTreg: Induced regulatory T
 PE: Phycoerythrin
 Treg: Regulatory T

atopic dermatitis, where it binds to CD3⁺CLA⁺CCR4⁺ T cells and mediates skin homing.¹⁷ In this study we show that CCL18 leads to the inhibition of allergic T_H2 immune responses *in vitro* and *in vivo* using a recently developed humanized mouse model of allergy in immunodeficient NOD-*scid*- γ c^{-/-} mice engrafted with PBMCs from highly sensitized allergic donors.^{18,19}

METHODS**Blood samples**

Heparinized blood was obtained from atopic donors with allergic rhinoconjunctivitis and/or asthma to grass or birch pollen or house dust mite. Specific sensitization was documented by positive skin prick test responses and detection of allergen-specific IgE in sera (CAP class ≥ 2 and CAP class ≥ 5 for studies in humanized mice, as measured with the ImmunoCAP specific IgE blood test; Phadia AB, Uppsala, Sweden). This study was approved by the local ethics committee. Informed consent was obtained from all subjects before the study.

Mice

NOD.CB17-Prkdcscid/J γ c^{-/-} mice 4 to 8 weeks of age were obtained from the Central Animal Facility of the Johannes-Gutenberg-Universität, Mainz, Germany. Experiments were performed in accordance with current federal, state, and institutional guidelines (authorization 23 177-07/G 08-1-009).

Cell-culture reagents

Iscove modified Dulbecco medium (IMDM) containing L-glutamine and 25 mmol/L HEPES supplemented with an antibiotic-antimycotic solution (PAA Laboratories GmbH, Cölbe, Germany) and 1% or 5% heat-inactivated autologous plasma was used for DC culture or DC/T-cell cocultures. Recombinant human IL-4, IL-1 β , and TNF- α were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); IL-10 was purchased from DNAX (Palo Alto, Calif); IL-12 was purchased from Hoffmann-La Roche (Nutley, NJ); GM-CSF (Leukine) was purchased from Immune (Seattle, Wash); TGF- β was purchased from R&D Systems (Wiesbaden, Germany); and prostaglandin E₂ was purchased from Cayman Chemical (Ann Arbor, Mich). Endotoxin-free (<1 ng/mL) grass pollen, birch pollen, and house dust mite allergen extracts were provided by ALK-Abelló (Hamburg, Germany). Recombinant human CCL18 was purchased from R&D Systems, and CCL5 and CCL17 were purchased from Tebu-bio GmbH (Offenbach, Germany).

Antibodies

The following anti-human mAbs were used: fluorescein isothiocyanate (FITC)-conjugated HLA-DR (L243), phycoerythrin (PE)-conjugated CD80 (L307.4), CD83 (HB15e), CD86 (2331[FUN-1]), allophycocyanin (APC)-conjugated CD209 (DCN46), and mouse IgG isotype controls (all from BD Biosciences, San Jose, Calif) for staining of DCs; PE-conjugated CD25 (4 E3, Miltenyi Biotec), FITC-conjugated CD4 (RPA-T4) and IFN- γ (4S.B3),

PE-conjugated IL-4 (MP4-25D2), IL-10 (JES3-19F1), CD294 (BM16), and mouse or rat IgG isotype controls (all from BD Biosciences), FITC- or APC-conjugated CCL18 (64507, R&D Systems), AlexaFluor 647-conjugated CD4 (MT310; Santa Cruz Biotechnology, Santa Cruz, Calif), FITC-conjugated FoxP3 (PCH101; eBioscience, San Diego, Calif), PE-conjugated GARP (G14D9, eBioscience), and fluorescein (DTAF)-conjugated goat anti-rat IgG (Dianova, Hamburg, Germany) for staining of T cells; CCL18 (AF394, R&D Systems), IL-10 (JES3-19F1, BD Biosciences), and goat or rat IgG isotype control for blocking experiments; CD3e (UCHT1, R&D Systems) and CD28 (CD28.2, BD Biosciences) for polyclonal T-cell stimulation; and IL-4 (AB-204-NA) and IL-12 (AB-219-NA, R&D Systems) for generation of T_H1- or T_H2-priming conditions.

PBMC preparation and generation of monocyte-derived human DCs

PBMCs were isolated from heparinized blood by using Ficoll-Paque (1.077 g/mL; PAA, Pasching, Austria) density centrifugation. PBMCs (1×10^7 per well) were incubated for 45 minutes in a 6-well plate (Greiner, Frickenhausen, Germany) in IMDM (3 mL per well) at 37°C to enrich CD14⁺ monocytes. After washing the nonadherent cells, the remaining monocytes (purity >90%) were incubated in IMDM supplemented with IL-4 (1000 U/mL) and GM-CSF (200 U/mL). On day 6, the resulting immature DCs were pulsed with the respective allergen extract (10 μ g/mL) and matured with TNF- α (1000 U/mL), IL-1 β (2000 U/mL), and prostaglandin E₂ (1 μ g/mL). At the same time point, IL-10 (20 ng/mL) was also added to the culture. DCs were harvested 48 hours later, washed twice, and used for T-cell stimulation assays. Mature DCs expressed high levels (>90%) of CD80, CD83, CD86, and MHC class II molecules, whereas the percentage of CD80 and CD83 and the mean fluorescence intensity of CD80, CD83, and CD86 expression was strongly inhibited by the addition of IL-10 during maturation, as shown previously.²⁰

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMCs by using antibody-coated paramagnetic MultiSort MicroBeads (MACS, Miltenyi Biotec), according to the manufacturer's protocols. For isolation of CD4⁺CD25⁺ Treg cells, CD4⁺ T cells were stained with CD25 MicroBeads (Miltenyi Biotec) after detaching; CD4⁺CD25⁺ T cells were positively selected, and CD4⁺CD25⁻ T cells were negatively selected. Separation was controlled by using flow cytometry (purity: >98% CD4⁺ T cells, >98% CD4⁺CD25⁻ T cells, and >95% CD4⁺CD25⁺ Treg cells).

Reconstitution of mice with PBMCs and assessment of airway reactivity by using invasive body plethysmography

Mice were injected intraperitoneally with 2×10^7 PBMCs together with 20 μ g of allergen with or without 0.5 μ g of CCL18 or CCL17 in 500 μ L of 0.9% NaCl (n = 3–4 per group). Eight days later, the mice received an additional intraperitoneal allergen boost of the same concentration in 200 μ L of 0.9% NaCl. Control groups received allergen or NaCl alone. On days 20 to 22, mice were challenged intranasally with 20 μ g of allergen with or without 0.5 μ g of CCL18 or CCL17 on the first day of challenge in 20 μ L of 0.9% NaCl. Forty-eight hours after the last challenge, airway hyperresponsiveness (AHR) was measured invasively by administering increasing doses (3.125–50 mg/mL) of methacholine with a body plethysmograph (Buxco Electronics, Wilmington, NC). Data are reported as airway resistance of greater than the baseline value taken before receiving methacholine.

Sera collection, bronchoalveolar lavage, cell preparation, and histology

After measurement of airway reactivity, sera were collected and stored at -20°C until determination of allergen-specific and total IgE levels by using the ImmunoCAP blood test (Phadia AB). Then lungs underwent lavage through the tracheal tube with 1 mL of PBS. Numbers of bronchoalveolar

lavage (BAL) cells were counted by using trypan blue exclusion. Differential cell counts were made from cytocentrifuged preparations stained with DiffQuick (Dade Behring AG, Düringen, Switzerland).

Spleens and lungs were removed, fixed in formalin or digested with 1 mg/mL collagenase IA (Sigma-Aldrich, Steinheim, Germany) for 45 minutes (only lungs), and dispersed through a 70- μ m nylon cell strainer (BD Biosciences). Erythrocytes were lysed by incubating the cells for 1 minute in lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 10 mmol/L EDTA-sodium, pH 7.4). Then cells were washed twice with PBS and used for flow cytometric staining.

Paraffin-embedded sections were stained with hematoxylin and eosin or anti-human CD45 (2B11 and PD7/26; DakoCytomation, Glostrup, Denmark) or anti-human FoxP3 (236A/E7; Abcam, Cambridge, Mass) and visualized with the Dako REAL EnVision Detection System, Peroxidase/DAB+, Mouse/Rat (DakoCytomation), according to the instructions of the manufacturer, to assess lung inflammation or migration of human cells in lung tissue. Slides were examined with a microscope (BX40; Olympus, Hamburg, Germany) by using the color view soft imaging system camera and software.

Coculture of T cells and autologous allergen-pulsed DCs

For proliferation or cytokine production assays, 1×10^5 or 5×10^5 CD4⁺ T cells were cocultured in 96-well or 48-well plates (Greiner) in triplicate with 1×10^4 or 5×10^4 autologous allergen-pulsed DCs or IL-10-treated DCs in 200 μ L or 1 mL of IMDM. After 5 days, the cells were pulsed with 37 kBq per well of tritiated thymidine (ICN, Irvine, Calif) for 6 hours, and tritiated thymidine incorporation was evaluated in a beta counter (1205 Betaplate; LKB Wallac, Turku, Finland). For detection of cytokines, T cells were restimulated with 5×10^4 newly generated autologous allergen-pulsed DCs or IL-10-treated DCs on day 7, and supernatants were collected 24 hours later.

In some experiments recombinant human CCL18 (200 ng/mL), anti-human CCL18, anti-human IL-10, or goat or rat IgG isotype controls (2 μ g/mL) were added to the cocultures from the beginning, and FCS was used instead of autologous plasma because human plasma already contains high amounts of CCL18, whereas FCS does not, allowing the analysis of exogenous effects of CCL18. For polyclonal T-cell activation, CD4⁺ T cells were stimulated with anti-human CD3 (0.5 μ g/mL) and anti-human CD28 (1 μ g/mL) for 72 hours. For intracellular staining of cytokines and CCL18, cocultures were further stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μ g/mL ionomycin (Sigma-Aldrich, Taufkirchen, Germany), and 0.7 μ L of GolgiStop (BD Biosciences) for 5 hours.

Flow cytometric analysis and fluorescence microscopy

Surface phenotyping was performed by staining 5×10^5 T cells or 5×10^4 DCs with specific mAbs for 20 minutes at 4°C, after which they were washed and analyzed in a FACSCalibur equipped with CellQuest Software (BD Biosciences). For determination of intracellular cytokine, FoxP3, and CCL18 levels, cells were fixed with Fix/Perm Buffer (eBioscience) for 30 minutes at 4°C. Then cells were permeabilized with Permeabilization Buffer (eBioscience) for 5 minutes, and staining was performed for 30 minutes at 4°C.

For fluorescence microscopy, cells were spun on slides by using a cytospin (Tharmac, Waldsolms, Germany) at 500 rpm for 5 minutes before fixation and permeabilization. After staining, cells were covered with fluorescent mounting medium (Dako, Carpinteria, Calif) and analyzed with a fluorescence microscope (Zeiss, Göttingen, Germany). For image acquisition and processing, Cell-P software was used.

Quantification of cytokine production by means of ELISA

Levels of human IL-4, IL-5, IL-13, IL-10, and IFN- γ (BD Biosciences) and human CCL18 (R&D Systems) were measured by using ELISA, according to the instructions of the distributors of the used pairs of antibodies.

RNA isolation and microarray analysis

CD4⁺ T cells (5×10^6) were cocultured in 6-well plates with 5×10^5 allergen-pulsed DCs or IL-10-treated DCs in 3 mL of IMDM. After 24 hours, total RNA was extracted by using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and concentrated to 1 μ g/ μ L by using the RNeasy MinElute Cleanup Kit (Qiagen). A total of 5 μ g of RNA was used for synthesis of biotinylated cRNA and hybridization on the human genome GeneChip U133A (Affymetrix, Santa Clara, Calif) containing 22,283 full-length genes. Microarray analysis was performed according to the manufacturer's instructions, and statistical significance was evaluated by using the software Array Assist (Stratagene, La Jolla, Calif).

Quantitative real-time PCR

Quantitative real-time PCR analysis was performed with QuantiTect Primer Assays (Qiagen) for β -actin (Hs_ACTB_1_SG [QT00095431]) and CCL18 (Hs_CCL18_1_SG [QT00024066]). One hundred nanograms of RNA was reverse transcribed in a 20- μ L reaction containing Oligo-dT primers and Omniscript RT (Qiagen). Then the cDNA was diluted 1:100, and 2 μ L of diluted cDNA was amplified in duplicate by using 45 cycles in a LightCycler apparatus (Roche Diagnostics, Mannheim, Germany) with the SYBR Green PCR Mastermix Kit (Qiagen). Data were collected with LightCycler Software 3.5.3 (Roche Diagnostics), and the relative gene expression of CCL18 was examined by using the $\Delta\Delta$ cycle threshold ($\Delta\Delta$ CT) method.²¹

Chemotaxis assay

CD4⁺CD25⁻ T cells (3×10^6) were cocultured with 3×10^5 allergen-pulsed DCs in 12-well plates in the presence of T_H1 or T_H2 priming conditions (1 μ g/mL anti-IL-4 and 2 ng/mL IL-12 to induce T_H1 cells or 10 ng/mL IL-4 and 1 μ g/mL anti-IL-12 to induce T_H2 cells) or with allergen-pulsed IL-10-treated DCs and 1 ng/mL TGF- β to induce induced regulatory T (iTreg) cells in 1 mL of IMDM. Furthermore, 3×10^6 CD4⁺CD25⁺ Treg cells were cocultured with 3×10^5 allergen-pulsed DCs. After 7 days, all cell populations were harvested, and 1×10^6 T cells were seeded in triplicate into the upper filter plate of a 96-well MultiScreen-MIC Plate (Millipore, Billerica, Mass) containing a 3- μ m-pore polycarbonate filter and selected wells of the receiver plate to obtain 100% migration value. CCL18 or CCL5 (10–100 ng/mL) was added to the receiver plate in 150 μ L of IMDM without plasma or FCS. The plate was incubated for 90 minutes at 37°C. Then the filter plate was removed, and the migrated cells were harvested and counted in a FACSCalibur for 60 seconds. Results were calculated as the percentage of the 100% migration value.

Statistics

ANOVA or the Student *t* test was used to test the statistical significance of the results by using GraphPad Prism version 5.04 software (GraphPad Software, La Jolla, Calif). A *P* value of .05 or less was considered significant.

RESULTS

Transcriptional profile revealed that CCL18 is upregulated in cocultures of CD4⁺ T cells with allergen-pulsed, tolerogenic, IL-10-treated DCs compared with regular allergen-pulsed DCs

CD4⁺ T cells from donors with grass pollen allergy were cocultured with allergen-pulsed DCs or IL-10-treated DCs. After 24 hours, the RNA was extracted for microarray analysis by using Affymetrix technology. Table I shows all the genes transcribed at an enhanced or reduced rate after evaluation of the results from 3 different donors by using Array Assist software (*P* < .05). Although 20 of 21 genes were upregulated in cocultures of CD4⁺ T cells and allergen-pulsed DCs compared with IL-10-treated DCs with a log₂ ratio of greater than 1.5, there was only 1 gene

TABLE I. Differential gene expression in cocultures of CD4⁺ T cells and allergen-pulsed DCs compared with CD4⁺ T cells stimulated with allergen-pulsed IL-10–treated DCs after evaluation of 3 different experiments with 3 different donors

No.	Log ₂ ratio > 1.5	Title	Gene symbol	Location
1	3,029	Signal transducer and activator of transcription 1.91 kDa	<i>STAT1</i>	chr2q32.2
2	2,869	Carcinoembryonic antigen–related cell adhesion molecule 1 (biliary glycoprotein)	<i>CEACAM1</i>	chr19q13.2
3	2,800	Cytokine-inducible SH2-containing protein	<i>CISH</i>	chr3p21.3
4	2,667	Suppressor of cytokine signaling 1	<i>SOCS1</i>	chr16p13.13
5	2,560	Interferon-induced protein 35	<i>IFI35</i>	chr17q21
6	2,542	TNF receptor superfamily, member 4	<i>TNFRSF4</i>	chr1p36
7	2,369	Guanylate binding protein 1, interferon-inducible, 67 kDa	<i>GBP1</i>	chr1p22.2
8	2,256	Chemokine (C-C motif) receptor 2	<i>CCR2</i>	chr3p21
9	2,224	Lymphotoxin α (TNF superfamily, member 1)	<i>LTA</i>	chr6p21.3
10	2,168	Chemokine (C-C motif) ligand 20	<i>CCL20</i>	chr2q33-q37
11	1,948	Interferon-induced protein 44	<i>IFI44</i>	chr1p31.1
12	1,878	Suppressor of cytokine signaling 2	<i>SOCS2</i>	chr12q
13	1,734	IL-2 receptor, α	<i>IL2RA</i>	chr10p15-p14
14	1,706	Tryptophanyl-tRNA synthetase	<i>WARS</i>	chr14q32.31
15	1,645	Radical S-adenosyl methionine domain containing 2	<i>RSAD2</i>	chr2p25.2
16	1,626	2'-5'-Oligoadenylate synthetase 3, 100 kDa	<i>OAS3</i>	chr12q24.2
17	1,604	HemK methyltransferase family member 1	<i>HEMK1</i>	chr3p21.3
18	1,543	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	<i>TAP1</i>	chr6p21.3
19	1,522	Endothelial cell growth factor 1 (platelet-derived)	<i>ECGF1</i>	chr22q13 22q13.33
20	1,511	Mesenchyme homeo box 1	<i>MEOX1</i>	chr17q21
21	–5,486	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	<i>CCL18</i>	chr17q11.2

that was more than 32-fold downregulated, the DC-derived chemokine *CCL18*.

Validation of enhanced *CCL18* transcription by using quantitative real-time PCR and protein expression

Validation of enhanced *CCL18* transcription by using quantitative real-time PCR revealed that allergen-pulsed IL-10–treated DCs expressed about 10-fold more of the *CCL18* gene than regular mature DCs. *CCL18* mRNA expression was enhanced to the same extent in 24-hour cocultures of CD4⁺ T cells and allergen-pulsed IL-10–treated DCs compared with that seen in regular mature DCs (Fig 1, A). However, expression levels of *CCL18* by DC/IL-10–treated DC cultures were about 60-fold stronger than observed for T-cell–DC/IL-10–treated DC cocultures at a 10:1 ratio (data not shown). CD4⁺ T cells cultured alone showed no signal for *CCL18*, even after stimulation with PHA, anti-CD3/anti-CD28, IL-10, or supernatant from DC cultures (data not shown).

Analyzing *CCL18* protein expression, we could show that production of *CCL18* was increased in allergen-pulsed IL-10–treated DCs on day 8 of DC culture (containing 1.5×10^5 DCs/mL) compared with that seen in regular mature DCs and in 10:1 cocultures of CD4⁺ T cells and allergen-pulsed IL-10–treated DCs (containing 0.5×10^5 DCs/mL) compared with that seen in cocultures of CD4⁺ T cells and regular mature DCs after 24 hours (Fig 1, B). However, neutralization of IL-10 by anti-human IL-10 mAb did not change *CCL18* levels in T-cell/DC cocultures (Fig 1, B). Intracellular *CCL18* expression was measured by using flow cytometry to verify the source of *CCL18*. Again, *CCL18* was significantly increased in cocultures of CD4⁺ T cells and IL-10–treated DCs. However, costaining for CD4 and CD209 showed that *CCL18*⁺ cells expressed both markers because of DC/T-cell clustering (data not shown). Therefore intracellular *CCL18* expression was measured by using fluorescence microscopy, which clearly revealed that *CCL18* was

only produced by DCs (lavender dye) and not by CD4⁺ T cells (green dye; Fig 1, C).

The enhanced *CCL18* gene and protein expression by tolerogenic IL-10–treated DCs was not a specific feature of donors with grass pollen allergy because it was also observed in cocultures of CD4⁺ T cells and tetanus toxoid– or grass pollen–pulsed IL-10–treated DCs derived from healthy donors (data not shown).

Addition of exogenous *CCL18* to cocultures of CD4⁺ T cells with allergen-pulsed DCs strongly inhibited T_H2 but not T_H1 cytokine production, whereas neutralization of *CCL18* had no effect on cytokine production

To analyze the function of *CCL18*, we added exogenous *CCL18* from the beginning to cocultures of CD4⁺ T cells and allergen-pulsed DCs and compared the T-cell proliferation and cytokine production with that induced by allergen-pulsed IL-10–treated DCs. As expected from our previous observations,²⁰ IL-10–treated DC–stimulated CD4⁺ T cells were anergic and showed lower proliferation rates, produced significantly lower T_H1 and T_H2 cytokine levels, and produced higher IL-10 levels than CD4⁺ T cells stimulated with regular DCs (Fig 2, A and B). The addition of exogenous *CCL18* to DC–stimulated CD4⁺ T cells reduced the production of the T_H2 cytokines IL-4 and IL-5 to the same extent as observed for IL-10–treated DCs, whereas proliferation and IL-10 and IFN- γ production were not affected (Fig 2, A and B). Next we addressed the question of whether neutralization of the *CCL18* pathway would restore the proliferation and cytokine production in IL-10–treated DC–stimulated anergic CD4⁺ T cells. However, neither anti-human *CCL18* nor isotype control antibody had a significant influence on proliferative responses or cytokine production (Fig 2, A and B).

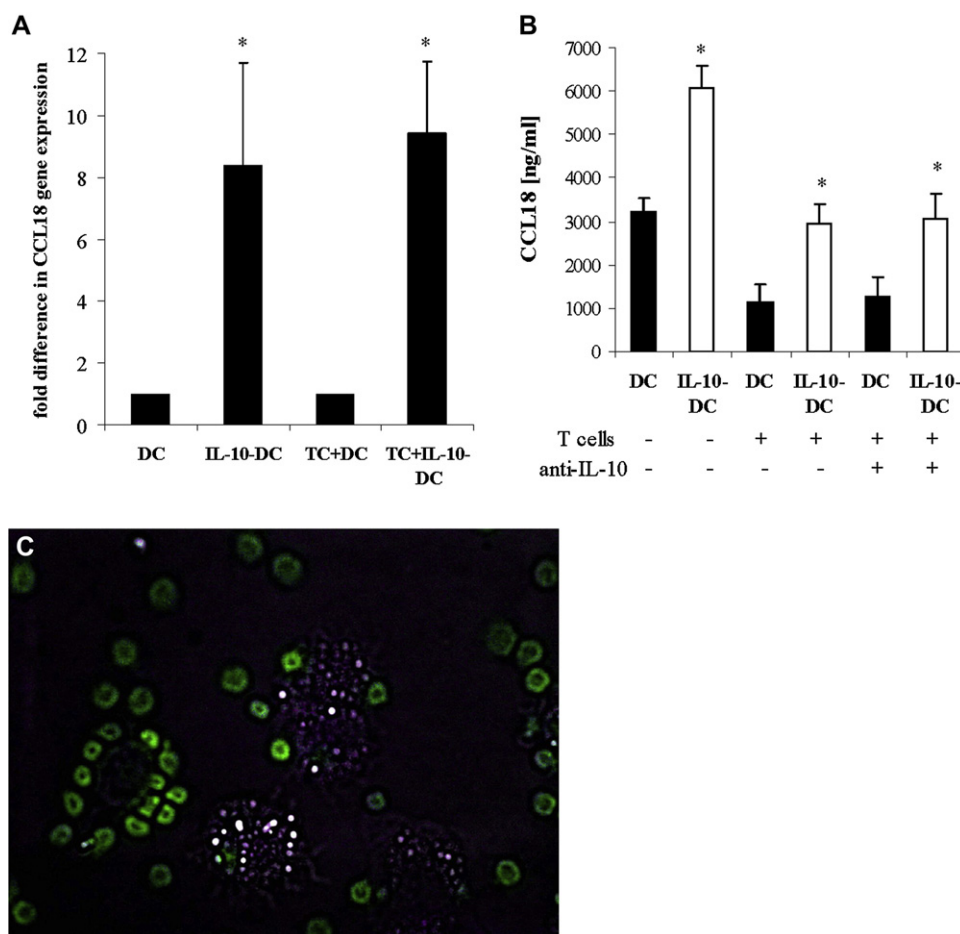


FIG 1. Validation of enhanced transcription of *CCL18* by IL-10-treated DCs. **A**, *CCL18* mRNA expression on day 8 of DC culture. **B**, *CCL18* protein expression of DC cultures on day 8 and of T-cell/DC cocultures after 24 hours (means $\pm$ SEMs, $n = 10$ [$n = 5$ for Fig 1, B]). * $P \leq .05$. **C**, Fluorescence microscopy of a single representative of 6 different T-cell/DC cocultures. Cells were stained with anti-human CD4 FITC and anti-human CCL18 APC.

To analyze whether CCL18 affects T-cell responses directly, we activated CD4⁺ T cells with anti-CD3/CD28 mAb with or without the addition of CCL18 for 72 hours. Because production of IL-4 and IL-5 was too low or even undetectable in these cultures, we further measured levels of the T_H2 cytokine IL-13. This cytokine was produced at high amounts and its production was significantly inhibited by CCL18, whereas production of IL-10 and IFN- γ was not affected (Fig 2, C).

CCL18 preferentially attracted Treg cells and *in vitro*-polarized T_H2 but not T_H1 cells

To investigate the effect of CCL18 on the migration of T cells, we performed chemotaxis assays with *in vitro*-polarized T_H1 and T_H2 cells, CD4⁺CD25⁺ Treg cells, and IL-10-treated DCs and TGF- β -induced iTreg cells. *In vitro*-polarized T_H1 cells produced more IFN- γ than IL-4 and no IL-10; *in vitro*-polarized T_H2 cells showed a predominant IL-4 production, little IFN- γ , and no IL-10; iTreg cells produced IL-10, IL-4, and IFN- γ ; and CD4⁺CD25⁺ Treg cells secreted almost no cytokines (data not shown). All T-cell populations were dose-dependently attracted by CCL18. Compared with migration to medium alone, T_H2 and iTreg cells showed an enhanced migration toward CCL18,

whereas the highest migration rates were observed for CD4⁺CD25⁺ Treg cells (Fig 3).

Coinjection of CCL18 in PBMC-engrafted NOD-*scid*- γ c^{-/-} mice reduces allergic AHR and lung inflammation

Analysis of the *in vivo* function of CCL18 in common asthma models was not possible because no murine homologue for CCL18 exists. Therefore we used a recently developed humanized mouse model of allergy using PBMC-engrafted NOD-*scid*- γ c^{-/-} mice.^{18,19} In this model a strongly increased airway resistance after intranasal allergen challenge was only observed in mice engrafted with PBMCs from highly sensitized allergic donors in the presence of the respective allergen, whereas engraftment of mice with PBMCs alone was not sufficient to induce AHR compared with control mice receiving no human cells (Fig 4, A). Coinjection of CCL18 together with PBMCs plus allergen at day 1 or during allergen boost at day 9 significantly improved airway resistance. If another T_H2-associated chemokine, CCL17, was co-injected, no significant improvement in airway resistance was observed (Fig 4, B). Intranasal administration of CCL18 at the first day of allergen challenge also led to a significant inhibition

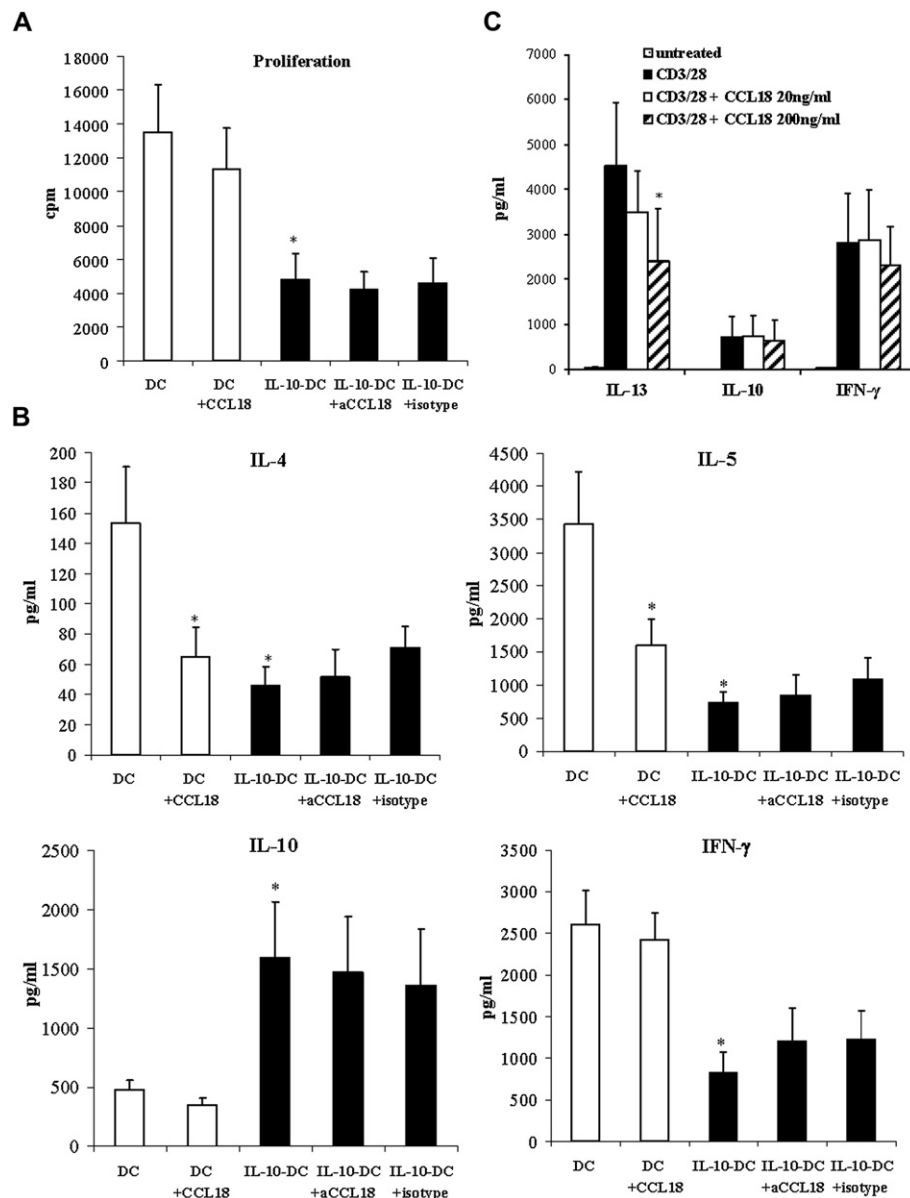


FIG 2. CCL18 inhibits T_H2 cytokine production in T-cell/DC cocultures. **A** and **B**, $CD4^+$ T cells were cocultured with allergen-pulsed DCs with or without CCL18 (open bars) or allergen-pulsed IL-10-treated DCs with or without anti-CCL18 or isotype control antibodies (solid bars) for 5 days (Fig 2, A, proliferation) or for 1 week and restimulated with freshly generated DCs or IL-10-treated DCs for 24 hours (Fig 2, B, cytokine production). **C**, $CD4^+$ T cells were stimulated with anti-CD3/28 mAbs with or without CCL18, and cytokine expression was analyzed after 72 hours. Data represent means $\pm$ SEMs ($n = 10$ [5 for Fig 2, C]). * $P \leq .05$ compared with DCs or CD3/CD28.

of airway reactivity (Fig 4, C). Histologic staining of the lungs revealed enhanced mucus production and goblet cell hyperplasia in PBMC plus allergen-engrafted mice, which was also inhibited by CCL18 but not by CCL17 (Fig 4, D).

Coinjection of CCL18 in PBMC-engrafted NOD-*scid*- $\gamma c^{-/-}$ mice decreases recruitment of neutrophils in the BAL fluid but does not enhance recruitment of T_H2 or activated Treg cells into the lung

Enhanced numbers of murine macrophages and neutrophils were found in the BAL fluid of PBMC plus allergen-treated mice,

which were significantly inhibited by CCL18, whereas CCL17 had no effect (Fig 4, E). In NOD-*scid*- $\gamma c^{-/-}$ mice murine lymphocytes were not present, and only some human lymphocytes were found in PBMC-engrafted mice, without any difference induced by CCL18 or CCL17. Unfortunately, eosinophilia (Fig 4, E) and human T_H2 cytokines (data not shown) were not observed in the BAL fluid of PBMC-engrafted mice.

Finally, we analyzed whether CCL18 also attracts Treg or T_H2 cells *in vivo*. High numbers of human $CD45^+$ cells were detected in lung tissues of PBMC plus allergen-treated mice, which mainly consist of T cells and some FoxP3 $^+$ and CD294 (CRTH2)-positive cells among them. Addition of CCL18 or CCL17 did not change

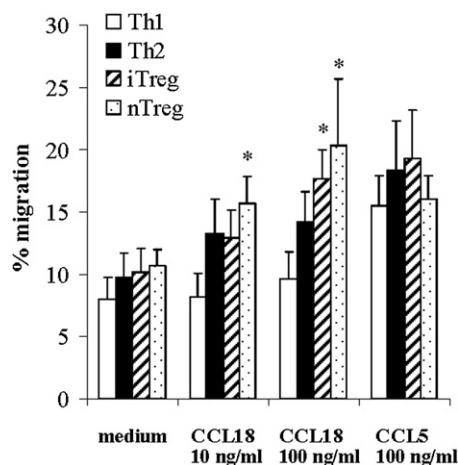


FIG 3. Chemotactic activity of CCL18 on T_H and Treg cells. *In vitro*-primed T_H1 or T_H2 cells, naturally occurring Treg cells, or *in vitro*-induced iTreg cells were analyzed for their migration toward CCL18 and CCL5 in a 96-well MultiScreen-MIC Plate, as described in the methods section. Results were calculated as percentages of the 100% migration value. Data represent means $\pm$ SEMs ($n = 8$). * $P \leq .05$ compared with migration to medium alone.

the number of human cells in the lung (Fig 5, A and B) or spleen (Fig 5, B). Because FoxP3 is not only expressed by Treg cells but also by activated T effector cells,^{22,23} we further used the recently described marker GARP, which is exclusively expressed on Treg cells and not on T effector cells, for staining.²⁴ Fig 5, C, shows that 59% to 77% of the human $CD4^+FoxP3^+$ T cells in the lung coexpressed GARP, whereas only 32% to 57% of GARP⁺ cells were detected among the $CD4^+FoxP3^+$ T cells in the spleen. Additionally, we observed a slight increase in GARP⁺ Treg cell numbers after treatment of mice with CCL18 or CCL17 in both organs independently of the route of application of these chemokines.

DISCUSSION

In the present study we have investigated the gene expression profile of $CD4^+$ T cells from allergic donors cocultured with autologous allergen-pulsed DCs or tolerogenic IL-10-treated DCs. We found that the gene encoding the chemokine *CCL18* was the only gene significantly upregulated in cocultures of $CD4^+$ T cells and IL-10-treated DCs. In functional analysis we have shown that enhanced CCL18 production is associated with recruitment of Treg cells and inhibition of T_H2 responses *in vitro*. Most importantly, we have demonstrated for the first time that CCL18 also prevents allergic airway inflammation *in vivo* by using a recently developed humanized mouse model of asthma.¹⁸

Validation of the microarray results by using quantitative real-time PCR and fluorescence microscopy confirmed that CCL18 was only expressed by DCs, whereas it was not expressed by $CD4^+$ T cells. The finding that IL-10 increases CCL18 production in mature DCs is in line with a study from Vulcano et al²⁵ demonstrating that CCL18 production was upregulated by 2 known inhibitors of DC maturation: IL-10 and vitamin D3. Furthermore, it was shown by Gunther et al¹⁷ that among PBMCs, only DCs and monocytes produced CCL18, whereas T and B cells did not. Neutralization of IL-10 did not inhibit production of CCL18 in DC/T-cell cocultures, suggesting that factors other than IL-10 maintain enhanced CCL18 expression by DCs. Similar results have recently been shown by de Nadai et al,¹⁵ in whose

study stimulation of PBMCs from asthmatic patients with the allergen *Dermatophagoides pteronyssinus* upregulated CCL18 secretion in an IL-4- and IL-13- but not IL-10-dependent manner. In general, CCL18 has been shown to play an important role in allergic diseases because it is preferentially expressed in the lung or nasal mucosa, attracts *in vitro*-polarized T_H2 cells and basophils, and is secreted by PBMCs from asthmatic patients but not from healthy donors after allergen stimulation.^{10,15,26}

More recently, an enhanced production of CCL18 by monocyte-derived DCs from allergic compared with nonallergic subjects was also reported.²⁷ However, in our study monocyte-derived DCs from allergic donors produced similar amounts of CCL18 compared with those from healthy subjects. This discrepancy might be explained by different purification methods for monocytes and by different DC culture conditions. Interestingly, the same group has further demonstrated that CCL18 can generate Treg cells from memory $CD4^+$ T cells of healthy but not allergic donors, as well as tolerogenic DCs.^{27,28} They explained this phenomenon by decreased CCL18 receptor binding on the surface of memory $CD4^+$ T cells from allergic subjects using biotin-conjugated CCL18 for staining, but because the nature of CCL18R is still unknown, the exact mechanisms concerning CCL18 receptor expression cannot be resolved thus far.

In further experiments we demonstrated that addition of exogenous CCL18 led to a similar inhibition of T_H2 cytokine production by allergen-pulsed, DC-stimulated $CD4^+$ T cells as observed with allergen-pulsed, IL-10-treated, DC-stimulated $CD4^+$ T cells, whereas proliferation levels, T_H1 cytokine production, and IL-10 levels were not affected. However, neutralization of CCL18 could not inhibit IL-10-treated DC-induced anergy of $CD4^+$ T cells. The reason for this might be that additional factors could be involved in the induction of anergy. Additionally, CCL18 can directly inhibit T_H2 cytokine production of polyclonally activated T cells, especially IL-13. Chang et al²⁸ described no inhibition of T_H2 cells after anti-CD3/CD28 stimulation, probably because of different cell-culture conditions (CCL18 was washed out after 2.5 hours in their study, whereas it was present the whole time in our study). Furthermore, they did not investigate production of IL-13, the cytokine most strongly affected in our study.

Our finding that CCL18 attracts polarized T_H2 cells better than T_H1 cells is in accordance with a recent report from de Nadai et al,¹⁵ demonstrating that CCL18 among other chemokines preferentially attracted T_H2 cells and basophils but not eosinophils and *in vitro*-polarized T_H1 cells. However, naturally occurring $CD4^+CD25^+$ cells and, to a lesser extent, IL-10-treated DCs plus TGF- β -induced iTreg cells showed the highest migration rates toward CCL18. This recruitment of Treg cells together with the finding that immature rather than mature DCs are attracted by CCL18^{10,25} and that CCL18 even induces Treg cells and tolerogenic DCs, at least in healthy nonallergic donors,^{27,28} suggests that CCL18 is not only a proinflammatory chemokine but also plays an important role in tolerance induction. In this context it was shown by Nibbs et al²⁹ that CCL18 exhibits antagonistic activity for CCR3, a chemotactic receptor for eosinophils and basophils, as well as T_H2 cells, and therefore might preferentially dampen allergic T_H2 responses. Indeed, in our recently developed humanized model of asthma, we demonstrated that systemic or intranasal administration of CCL18 in NOD-*scid*- $\gamma c^{-/-}$ mice that had been engrafted with PBMCs from allergic donors prevented AHR after allergen challenge, whereas another T_H2 -attracting chemokine, CCL17, had no significant effect. However,

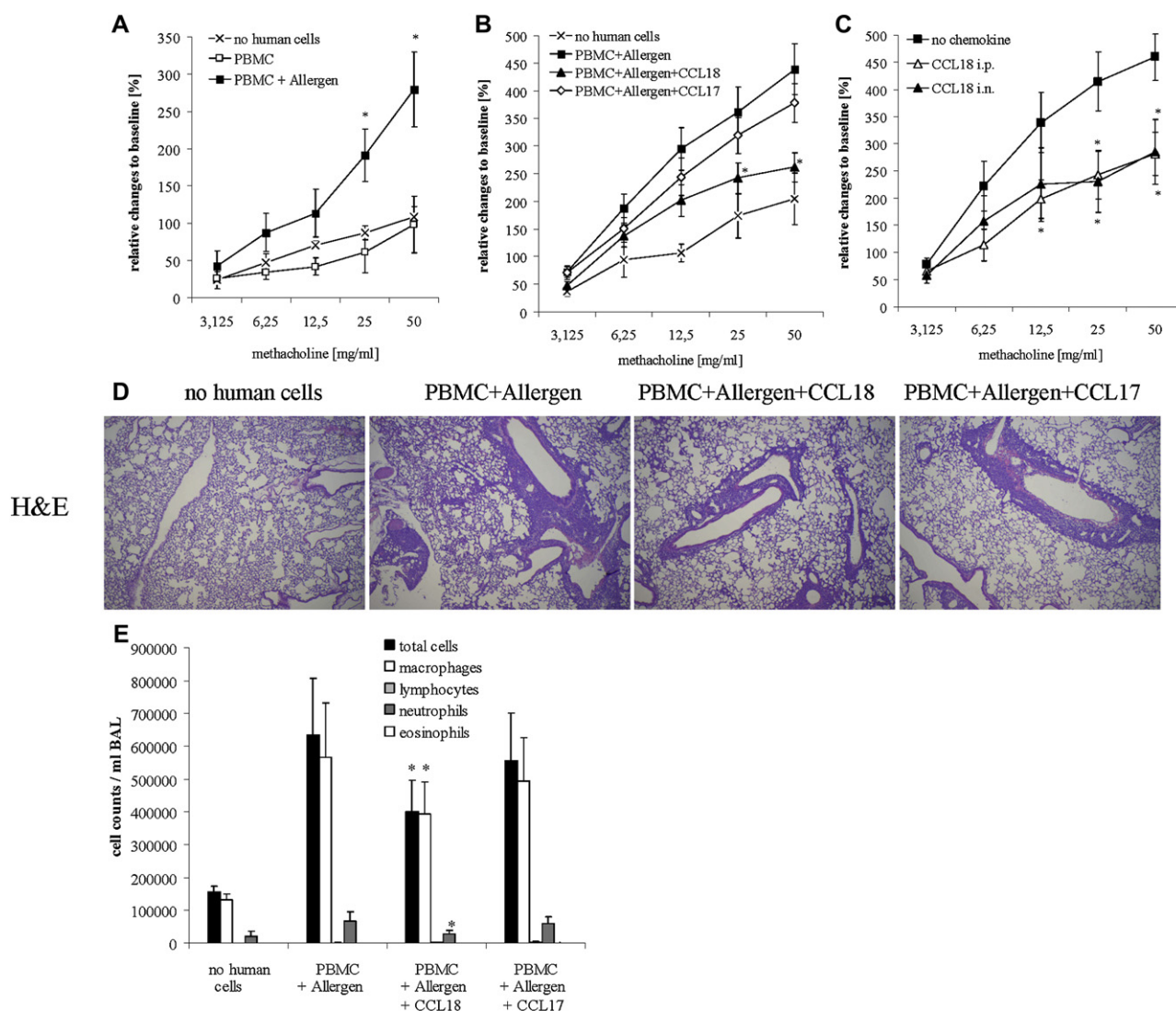


FIG 4. CCL18 inhibits airway reactivity and lung inflammation in a humanized mouse model of asthma. **A-C**, NOD-*scid-γc*^{-/-} mice were engrafted with 2×10^7 human PBMCs from allergic donors injected together with or without the respective allergen. Three weeks later, airway resistance in response to methacholine was measured after intranasal allergen challenge. CCL18 and CCL17 were injected intraperitoneally on day 1 or administered intranasally on day 20. Shown are means $\pm$ SEMs of the relative change to baseline value of 3 (Fig 4, **A**), 9 (Fig 4, **B**), or 6 (Fig 4, **C**) independent experiments with at least 2 mice per group. * $P \leq .05$ compared with mice receiving no human cells (Fig 4, **A**), PBMC plus allergen-treated mice (Fig 4, **B**), or mice receiving no chemokines (Fig 4, **C**). **D**, Hematoxylin and eosin (H&E) staining of the lungs from 1 representative experiment (magnification $\times 40$). **E**, Means $\pm$ SEMs of the numbers of eosinophils, neutrophils, macrophages, and lymphocytes in the BAL fluid ($n = 10$). * $P \leq .05$ compared with PBMC plus allergen-treated mice.

migration of human cells into the lungs or other organs was not affected by CCL18 either intraperitoneally or intranasally, except for a slight increase in activated GARP and FoxP3⁺ Treg cell numbers. Whether this slight increase in activated Treg cell numbers is sufficient for the inhibition of lung inflammation or whether CCL18 might also induce IL-10-producing T_H1 cells, which do not overexpress FoxP3, remains to be investigated. In preliminary experiments restimulation of human CD4⁺ T cells isolated from the lungs of humanized CCL18-treated mice did not lead to an enhanced IL-10 production.

Taken together, the role of CCL18 in patients with allergic diseases needs to be reconsidered. It might also act as a negative regulator to limit allergic immune responses. Although CCL18

does not exist in mice, administration of human CCL18 has recently been shown to attract murine B and T lymphocytes.³⁰ Thus further studies in rodent and humanized mouse models, as shown here, will help to clarify the exact *in vivo* role of this chemokine.

We thank Petra Schuster, Anke Heinz, and Claudia Braun for excellent technical assistance.

Clinical implications: In a humanized mouse model allergic airway inflammation is inhibited by CCL18, which might represent a novel therapeutic approach in the treatment of airway diseases.

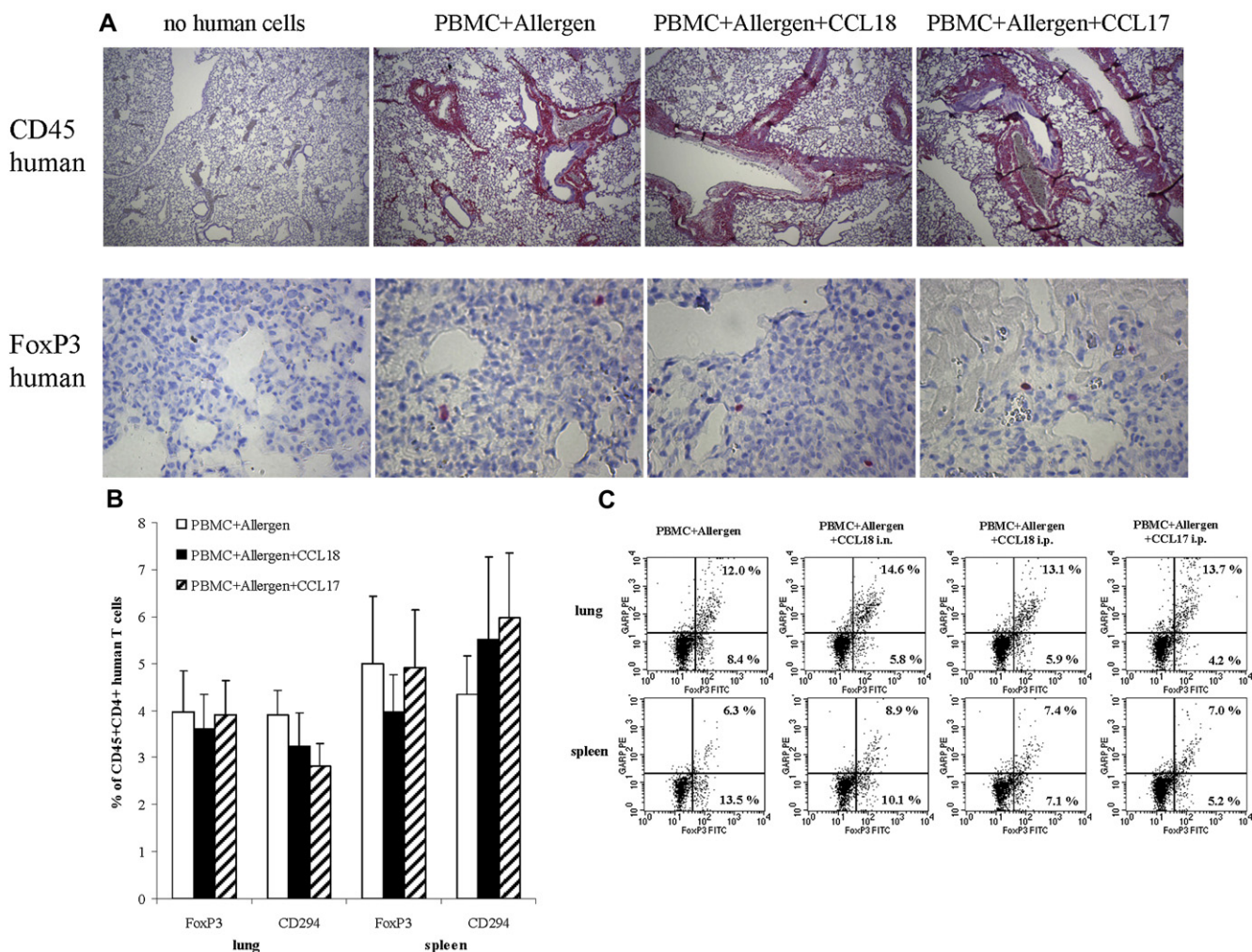


FIG 5. CCL18 does not enhance migration of Treg cells into the lung. NOD-*scid-γc*^{-/-} mice were treated and challenged as described in Fig 4. **A**, Anti-human CD45 (magnification ×40) and FoxP3 staining (magnification ×400) of the lungs from 1 of 9 independent experiments. **B** and **C**, Flow cytometric analysis of anti-human CD45, CD4, CD294, FoxP3, and GARP expression in the lung and spleen. Shown are means ± SEMs from 8 independent experiments (Fig 5, **B**) and 1 of 5 independent stainings of human CD4⁺ T cells in the lung and spleen with anti-human FoxP3 and GARP (Fig 5, **C**).

REFERENCES

- Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.
- Ozdemir C, Akdis M, Akdis CA. T regulatory cells and their counterparts: masters of immune regulation. *Clin Exp Allergy* 2009;39:626-39.
- Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* 2005;105:1162-9.
- Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Inhibition of human allergic T-helper type 2 immune responses by induced regulatory T cells requires the combination of interleukin-10-treated dendritic cells and transforming growth factor-beta for their induction. *Clin Exp Allergy* 2006;36:1546-55.
- Wu K, Bi Y, Sun K, Wang C. IL-10-producing type 1 regulatory T cells and allergy. *Cell Mol Immunol* 2007;4:269-75.
- Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in immune regulation of allergic diseases. *Eur J Immunol* 2010;40:1232-40.
- Terashima A, Watarai H, Inoue S, Sekine E, Nakagawa R, Hase K, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med* 2008;205:2727-33.
- Tasaki Y, Fukuda S, Iio M, Miura R, Imai T, Sugano S, et al. Chemokine PARC gene (SCYA18) generated by fusion of two MIP-1alpha/LD78alpha-like genes. *Genomics* 1999;55:353-7.
- Adema GJ, Hartgers F, Verstraten R, de Vries E, Marland G, Menon S, et al. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 1997;387:713-7.
- Schutyser E, Richmond A, Van Damme J. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J Leukoc Biol* 2005;78:14-26.
- Auer J, Blass M, Schulze-Koops H, Russwurm S, Nagel T, Kalden JR, et al. Expression and regulation of CCL18 in synovial fluid neutrophils of patients with rheumatoid arthritis. *Arthritis Res Ther* 2007;9:R94.
- Goebeler M, Trautmann A, Voss A, Brocker EV, Toksoy A, Gillitzer R. Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. *Am J Pathol* 2001;158:431-40.
- Pochetuen K, Luzina IG, Lockett V, Choi J, Todd NW, Atamas SP. Complex regulation of pulmonary inflammation and fibrosis by CCL18. *Am J Pathol* 2007;171:428-37.
- Peterson S, Poposki JA, Nagarkar DR, Chustz RT, Peters AT, Suh LA, et al. Increased expression of CC chemokine ligand 18 in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2012;129:119-27.
- de Nadai P, Charbonnier AS, Chenivess C, Senechal S, Fournier C, Gilet J, et al. Involvement of CCL18 in allergic asthma. *J Immunol* 2006;176:6286-93.
- Kodjelja V, Muller C, Politz O, Hakij N, Orfanos CE, Goerd S. Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *J Immunol* 1998;160:1411-8.

17. Gunther C, Bello-Fernandez C, Kopp T, Kund J, Carballido-Perrig N, Hinteregger S, et al. CCL18 is expressed in atopic dermatitis and mediates skin homing of human memory T cells. *J Immunol* 2005;174:1723-8.
18. Martin H, Reuter S, Dehzad N, Heinz A, Bellinghausen I, Saloga J, et al. CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. *J Allergy Clin Immunol* 2012;129:521-8.
19. Weigmann B, Schughart N, Wiebe C, Sudowe S, Lehr HA, Jonuleit H, et al. Allergen-induced IgE-dependent gut inflammation in a human PBMC-engrafted murine model of allergy. *J Allergy Clin Immunol* 2012;129:1126-35.
20. Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop J, Saloga J. Inhibition of human allergic T cell responses by interleukin-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* 2001;108:242-9.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-8.
22. Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol* 2006;24:209-26.
23. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev* 2011;241:260-8.
24. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009;106:13439-44.
25. Vulcano M, Struyf S, Scapini P, Cassatella M, Bernasconi S, Bonecchi R, et al. Unique regulation of CCL18 production by maturing dendritic cells. *J Immunol* 2003;170:3843-9.
26. Pardo A, Smith KM, Abrams J, Coffman R, Bustos M, McClanahan TK, et al. CCL18/DC-CK-1/PARC up-regulation in hypersensitivity pneumonitis. *J Leukoc Biol* 2001;70:610-6.
27. Azzaoui I, Yahia SA, Chang Y, Vorng H, Morales O, Fan Y, et al. CCL18 differentiates dendritic cells in tolerogenic cells able to prime regulatory T cells in healthy subjects. *Blood* 2011;118:3549-58.
28. Chang Y, de NP, Azzaoui I, Morales O, Delhem N, Vorng H, et al. The chemokine CCL18 generates adaptive regulatory T cells from memory CD4+ T cells of healthy but not allergic subjects. *FASEB J* 2010;24:5063-72.
29. Nibbs RJ, Salcedo TW, Campbell JD, Yao XT, Li Y, Nardelli B, et al. C-C chemokine receptor 3 antagonism by the beta-chemokine macrophage inflammatory protein 4, a property strongly enhanced by an amino-terminal alanine-methionine swap. *J Immunol* 2000;164:1488-97.
30. Bruna-Romero O, Schmieg J, Del Val M, Buschle M, Tsuji M. The dendritic cell-specific chemokine, dendritic cell-derived CC chemokine 1, enhances protective cell-mediated immunity to murine malaria. *J Immunol* 2003;170:3195-203.