

## Cytokine Target Proteins

- Validated by ELISA/SPR/BLI
- Covering ILs, Growth Factors, TNFs, CSFs, and IFNs

Learn  
More



### The Journal of Immunology

RESEARCH ARTICLE | JULY 01 2012

## Pulmonary CCL18 Recruits Human Regulatory T Cells **FREE**

Cécile Chenivresse; ... et. al

*J Immunol* (2012) 189 (1): 128–137.

<https://doi.org/10.4049/jimmunol.1003616>

#### Related Content

Involvement of CCL18 in Allergic Asthma

*J Immunol* (May,2006)

Unique Regulation of CCL18 Production by Maturing Dendritic Cells

*J Immunol* (April,2003)

CCL18 does not require CCR6 for functional activity in mice (44.3)

*J Immunol* (May,2012)

# Pulmonary CCL18 Recruits Human Regulatory T Cells

Cécile Chenivresse,<sup>\*,†,‡,§,¶,1</sup> Ying Chang,<sup>\*,†,‡,§,1</sup> Imane Azzaoui,<sup>\*,†,‡,§</sup> Saliha Ait Yahia,<sup>\*,†,‡,§</sup> Olivier Morales,<sup>||</sup> Coline Plé,<sup>\*,†,‡,§</sup> Arnaud Foussat,<sup>#</sup> André-Bernard Tonnel,<sup>\*,†,‡,§,¶</sup> Nadira Delhem,<sup>||</sup> Hans Yssel,<sup>\*\*</sup> Han Vorng,<sup>\*,†,‡,§</sup> Benoit Wallaert,<sup>\*,†,‡,§,¶</sup> and Anne Tscicopoulos<sup>\*,†,‡,§,¶</sup>

CCL18 is both a constitutively expressed and an inducible chemokine, whose role in the inflammatory reaction is poorly known. The aim of this study was to evaluate whether CCL18 has the capacity to attract human T cells with a regulatory function (regulatory T cells [Treg]). Results from chemotaxis assays performed on different types of Treg showed that CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cells, but neither T regulatory type 1 clones nor Treg differentiated in vitro with anti-CD3/CD46 mAbs, were recruited by CCL18 in a dose-dependent manner. CCL18-recruited memory CD4<sup>+</sup> T cells were enriched in CD25<sup>high</sup>, CD25<sup>+</sup>CD127<sup>low</sup>, latency-associated peptide/TGF-β1, and CCR4-expressing T cells, whereas there was no enrichment in Foxp3<sup>+</sup> cells as compared with controls. Stimulated CCL18-recruited memory T cells produced significantly increased amounts of the regulatory cytokines IL-10 and TGF-β1, as well as IL-4, but not IFN-γ and IL-17. Cell surface CCL18 binding was found predominantly on IL-10<sup>+</sup> (26.3 ± 5.8%) and on a few latency-associated peptide/TGF-β1<sup>+</sup> (18.1 ± 1.9%) and IL-4<sup>+</sup> (14.5 ± 2.9%) memory T cells. In an in vivo model of SCID mice grafted with human skin and reconstituted with autologous PBMCs, the intradermal injection of CCL18 led to the cutaneous recruitment of CD4<sup>+</sup>, CD25<sup>+</sup>, and IL-10<sup>+</sup> cells, but not Foxp3<sup>+</sup> cells. Furthermore, CCL18-recruited memory T cells inhibited the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> effector T cells through an IL-10-dependent mechanism. These data suggest that CCL18 may contribute to maintaining tolerance and/or suppressing deleterious inflammation by attracting memory Tregs into tissues, particularly in the lung, where it is highly and constitutively expressed. *The Journal of Immunology*, 2012, 189: 128–137.

Two main subsets of regulatory T cells (Tregs) have been described, natural and adaptive/induced Tregs, that differ in their development, Ag specificities, and mechanism of action. The phenotypic characteristics of natural Tregs include expression of the transcription factor Foxp3 (1), and constitutive expression of IL-2Rα CD25 (2) and the T cell activation-associated cell surface marker CTLA-4 (3). The suppressive function of natural Tregs reportedly requires cell–cell contact or proximity in vitro, whereas the in vivo function of natural Tregs depends on the secretion of IL-10 and TGF-β (4, 5). Adaptive Tregs include Foxp3<sup>+</sup> cells that develop extrathymically and share most phenotypic and functional features with natural Tregs (6), as well as Foxp3<sup>-</sup> cells that seem to exert their regulatory activity mainly by means of secreted cytokines such as IL-10 or TGF-β, the best

characterized being T regulatory type 1 (Tr1) cells (7). The expression of specific chemokine receptors by Tregs has been linked to the control of their migration into tissues and may reflect functional heterogeneity among Treg subsets that localize to a distinct tissue environment (8). Some chemokine receptors have been reported to be preferentially expressed on freshly isolated resting human Tregs, such as CCR4 and CCR8 (9–12); however, the homing properties of human Tregs are still poorly understood.

The chemokine CCL18 displays characteristic features of inflammatory chemokines. Its production is induced by infectious agents (13) and allergens (14), and is furthermore regulated by inflammatory cytokines, that is, upregulated in vitro by IL-4 and IL-13 and downregulated by IFN-γ (15). Moreover, CCL18 attracts Th2 cells and basophils, both involved in the allergic immune response (14, 15). In vivo, CCL18 has been shown to be overexpressed in numerous inflammatory disorders of skin, lung, and joints (16). Hitherto, it has been difficult to assess CCL18 functionality, as the CCL18 receptor(s) has not yet been identified, and there is no murine ortholog of this chemokine (17). However, since its discovery, the idea has emerged that CCL18 might be implicated in homeostasis and immune tolerance. Indeed, CCL18 has been reported to be constitutively produced by APCs in vitro, in particular by monocytes/macrophages (18), as well as by lung macrophages and lymph node dendritic cells in vivo (19). Furthermore, its expression by APCs is upregulated by the suppressive cytokine IL-10 (15). In addition, CCL18 attracts both naive CD4<sup>+</sup> T lymphocytes (20) and immature monocyte-derived dendritic cells (21), which may lead to the development of tolerogenic immune responses. Moreover, CCL18 has been recently shown to convert effector memory T cells into Tregs (22), as well as induce tolerogenic dendritic cells (23). Because the main function of chemokines consists of activating cell migration, we hypothesized that CCL18 may induce migration of Tregs. We show in this article that CCL18 is able to attract a particular subset of CD25<sup>+</sup>

\*Immunité Pulmonaire, Institut de la Santé et de la Recherche Médicale Unité 1019, F-59019 Lille, France; <sup>†</sup>Institut Pasteur de Lille, Centre d'Infection et d'Immunité de Lille, F-59019 Lille, France; <sup>‡</sup>Centre National de la Recherche Scientifique Unité Mixte de Recherche 8204, F-59000 Lille, France; <sup>§</sup>Université Lille Nord de France, F-59000 Lille, France; <sup>¶</sup>Clinique des Maladies Respiratoires et Centre Hospitalier Régional et Universitaire de Lille, 59037 Lille, France; <sup>||</sup>Unité Mixte de Recherche 8161, Centre National de la Recherche Scientifique, Team Immunorégulation des Cancers Viroinduits, Institut de Biologie, 59019 Lille, France; <sup>#</sup>Txcell, 06560 Valbonne, France; and <sup>\*\*</sup>Institut de la Santé et de la Recherche Médicale Unité 844, 34000 Montpellier, France

<sup>1</sup>C.C. and Y.C. contributed equally to this work.

Received for publication November 2, 2010. Accepted for publication April 22, 2012.

This work was supported by the Fondation pour la Recherche Médicale, Santelyst, and the Agence Nationale de la Recherche Physiopathologie des Maladies Humaines.

Address correspondence and reprint requests to Dr. Anne Tscicopoulos, Pulmonary Immunity, CIIL, Institut National de la Santé et de la Recherche Médicale, Unité 1019, Institut Pasteur, BP245, 59019 Lille Cedex, France. E-mail address: anne.tscicopoulos@pasteur-lille.fr

Abbreviations used in this article: LAP, latency-associated peptide; MFI, mean fluorescence intensity; Tr1, T regulatory type 1; Treg, regulatory T cell.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/\$16.00

CD127<sup>low</sup> and Foxp3<sup>-</sup> Tregs that exerts its regulatory function through the production of IL-10.

## Materials and Methods

### Isolation of memory and naive CD4<sup>+</sup> T cells and culture

Venous blood provided by the Etablissement Français du Sang was obtained from 36 healthy subjects. PBMCs were isolated from venous blood by density centrifugation over Ficoll/Hypaque (Pharmacia, Uppsala, Sweden). Human CD4<sup>+</sup> and CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were isolated from PBMCs by negative selection over a MACS column using CD4<sup>+</sup> and memory CD4<sup>+</sup> T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) with a purity of 96–98%. In some control experiments, naive CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were purified by negative selection using naive CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotec) with a purity of >95%.

After purification, memory or naive CD4<sup>+</sup> T cells (10<sup>6</sup> cells/ml) were kept overnight in RPMI 1640 medium, supplemented with 2 mmol/L glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% FCS.

### Flow cytometry sorting of T cell subsets

CD4<sup>+</sup>CD45RO<sup>+</sup> or CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were purified from PBMCs by magnetic separation (Miltenyi Biotec). Cells were stained with anti-CD4-FITC (BD Pharmingen) and anti-CD25-PE (BD Pharmingen) or anti-CCR4-PE (BD Pharmingen) and with CD127-allophycocyanin (BD Pharmingen) for 30 min on ice, washed in PBS/2% BSA, filtered, and then sorted by flow cytometry (FACSARIA; BD Biosciences, San Jose, CA). CD4<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>high</sup>, CD4<sup>+</sup>CCR4<sup>+</sup>, CD4<sup>+</sup>CCR4<sup>-</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cell purity was >96%.

### Generation of anti-CD3/CD46 Tregs and culture of Tr1 clones

Purified CD4<sup>+</sup> T cells were stimulated (2 × 10<sup>5</sup> cells/well) with 10 μg/ml coated anti-CD3 mAb (OKT3) and 5 μg/ml anti-CD46 mAb (a kind gift of Dr. C. Kemper, London, U.K.) in 96-well, flat-bottom microculture plates (BD, Franklin Lakes, NJ), and were cultured in RPMI 1640 medium supplemented with 2 mmol/L glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FCS, and 10 ng/ml recombinant human IL-2 (Tebu, Le Perray en Yvelines, France) for 3 d and assessed in chemotaxis assays. Two sources of human Tr1 clones were used. The first ones were generated from skin delayed-type hypersensitivity reactions, induced in healthy human volunteers after an initial sensitization and a subsequent challenge with 2,4-dinitrochlorobenzene, as previously described (24). The second ones were OVA-specific clones generated from the PBMCs of patients with Crohn disease, which were cultured in the presence of native OVA in X-Vivo15 and cytokine-enriched *Drosophila* feeder cell supernatant as described previously (25).

### Chemotaxis and transwell migration

The different T cell populations were harvested and suspended at a concentration of 10<sup>6</sup> cells/ml in RPMI 1640. The chemotaxis protocol was performed as previously described (26) using a 48-well microchemotaxis Boyden chamber (NeuroProbe, Cabin John, MD) with 5-μm pore polycarbonate filters (Nucleopore, Pleasanton, CA). T cells were incubated for 2 h 30 min at 37°C in 5% CO<sub>2</sub> with CCL18 (R&D Systems, Minneapolis, MN) at concentrations ranging from 10<sup>-7</sup> to 10<sup>-11</sup> M. RPMI 1640 and CXCL12 (PeproTech, Rocky Hill, CT) at 10<sup>-7</sup> M were used as negative and positive controls, respectively. Each condition was performed in triplicate. Cells having migrated through the filter were counted in the inferior well, and results were expressed as index of chemotaxis compared with medium. In some experiments, a neutralizing Ab against CCL18 was used to assess the specificity of the recruitment. CCL18 was incubated for 30 min with the neutralizing Ab (R&D Systems), and the mixture was used as chemoattractant in the chemotaxis assay. To recover memory T cells responding to CCL18, we used transwells with 5-μm pore polycarbonate filters (CoStar, Cambridge, MA). T cells were suspended in RPMI 1640 at a concentration of 2 × 10<sup>6</sup> cells/ml and incubated 2 h 30 min at 37°C in 5% CO<sub>2</sub>. RPMI 1640 and CXCL12 were used as controls. Transmigrated memory T cells were recovered in the lower compartment, and stimulated or not (10<sup>5</sup> cells/well) with 10 μg/ml coated anti-CD3 mAb (OKT3) and 2 μg/ml soluble anti-CD28 mAb (BD Biosciences) in 96-well, flat-bottom microculture plates (BD, Franklin Lakes, NJ). As baseline cells, purified memory T cells, not subjected to migration, were also stimulated under the same conditions. After 3 d of stimulation, the culture supernatants and cells were harvested for further experiments.

### Quantification of the cytokine profile

Concentrations of IL-4, IL-10, IFN-γ (Diaclone Research, Besançon, France), IL-17, and TGF-β1 (R&D Systems) in cell culture supernatants were measured by ELISA, according to the manufacturer's recommendations. The level of sensitivity was 1.1 pg/ml for IL-4, 12.5 pg/ml for IL-10 and IFN-γ, and 15.6 pg/ml for IL-17 and TGF-β1. Results were expressed as percentage increase of nonmigrated memory T cells.

### Flow cytometry phenotype analysis

The phenotypes related to Treg were evaluated by flow cytometry using a standardized protocol as previously described (27). For the extracellular markers, cells were stained with anti-CD25-PE, anti-CD127-allophycocyanin (both from BD Pharmingen), anti-CD103-FITC (Beckman Coulter, Fullerton, CA), anti-CTLA-4-FITC, anti-CCR4-PE (both from R&D Systems), and biotinylated anti-latency-associated peptide (anti-LAP) of TGF-β1 mAbs (R&D Systems) followed by streptavidin-PE, or LAP/TGF-β1-PE mAb for double staining (R&D Systems). For intracellular staining, cells were labeled with an anti-human Foxp3-FITC staining set (eBioscience, San Diego, CA), according to the manufacturer's recommendations. For CCL18 receptor expression, cells were labeled with biotin-conjugated CCL18 (Sigma, Steinheim, Germany) followed by streptavidin-allophycocyanin (R&D Systems). The specificity of the biotinylated CCL18 was demonstrated by the inhibition of its binding on naive T cells with nonlabeled CCL18 (data not shown). As a control, we used a small m.w. protein derived from *Plasmodium falciparum* (a kind gift of Dr. S. Tomavo, Lille, France). The complete open-reading frame of a gene encoding the *P. falciparum* TAF10 homolog (28) was cloned in-frame into the pGEX-6P-3 (Amersham, Uppsala, Sweden). The GST was removed from the recombinant protein using preScission protease and glutathione-Sepharose 4B column according to manufacturer's instructions (Amersham). The purified *P. falciparum* TAF10 was subjected to SDS-PAGE followed by Coomassie blue staining and then labeled using a biotinylation kit (Sigma). For concomitant extracellular CCL18R and intracellular cytokine expression, cells were stimulated for 24 h with PMA at 50 ng/ml and ionomycin at 1 μg/ml. Brefeldin was added the last 4 h of the culture. Cells were stained for CCL18R as described earlier, permeabilized using the Cytofix/Cytoperm BD Pharmingen kit according to the manufacturer's recommendations, and subsequently labeled using anti-IL-4-PE and anti-IL-10-PE mAbs (BD Biosciences).

### Real-time quantitative RT-PCR

After removal of the supernatants, the transmigrated memory CD4<sup>+</sup> T cells were suspended in TRI Reagent (Ambion, Austin, TX), and total RNA was extracted according to the manufacturer's procedure. Reverse transcription was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in triplicate by real-time PCR using the SYBR Green PCR kit (Applied Biosystems). The amount of Foxp3 mRNA expression was normalized to β-actin and calculated according to the comparative ΔΔCt method. Primer pairs were as follows: Foxp3, 5'-GAAACAGCACATTCAGAGTTC-3' and 5'-ATGGCCAGCGGATGAG-3'; β-actin, 5'-TCCTCACCTGAA-GTACCCCA-3' and 5'-AGCCACACGCAGCTCATT GT-3'.

### Proliferation suppression assays

FACS-sorted CD4<sup>+</sup>CD25<sup>-</sup> T cells (5 × 10<sup>4</sup>/well) were stimulated in triplicate wells with soluble anti-CD3 mAb (10 ng/ml) and anti-CD28 mAb (10 ng/ml; BD Biosciences) in 96-well round-bottom plates (Corning Costar, Corning, NY), in the absence or presence of CCL18-recruited CD4<sup>+</sup>CD45RO<sup>+</sup> T cells at suppressor/responder ratios of 1:4, 1:2, and 1:1. Irradiated (50 Gy) autologous PBMCs were used as APCs (5 × 10<sup>4</sup>/well). Purified sorted CD4<sup>+</sup>CD25<sup>high</sup> T cells were used as positive control of T cell suppression, and medium- and CXCL12-recruited CD4<sup>+</sup>CD45RO<sup>+</sup> T cells as negative controls of T cell suppression. Proliferation was measured in triplicate after methyl-[<sup>3</sup>H]thymidine (1 μCi/well; GE Healthcare, Bucks, U.K.) incorporation for the last 18 h. Cultured cells were harvested on glass fiber filter (Printed Filtermat A; Wallac, Turku, Finland) using the harvester (Tomtec; Wallac) and sealed in a sample bag after drying and the addition of scintillation liquid (Beckman Coulter, Fullerton, CA). Incorporated radioactive thymidine was detected by scintillation counting using a 1450 Trilux β-counter (Wallac, Turku, Finland), and estimated in cpm. Results were expressed as percentage of proliferation ± SEM of CD4<sup>+</sup>CD25<sup>-</sup> effector T cells alone. In some experiments, 1 μg/ml neutralizing anti-IL-10 (R&D Systems), 1 μg/ml neutralizing anti-TGF-β (R&D Systems), or 1 μg/ml isotype mAb was added to the cultures. To investigate a contact-dependent mechanism, we separated CD4<sup>+</sup>CD25<sup>-</sup> T cells from CD4<sup>+</sup>CD45RO<sup>+</sup> T cells recruited by CCL18 using a 96-well transwell plate with a 0.4-μm polycarbonate membrane (Corning).

### *In vivo model of SCID mouse grafted with human skin*

Skin from human donors was obtained from thorax surgery in which skin was discarded. Skin was kept in sterile normal saline with added penicillin and streptomycin, and transplanted onto SCID mice within 2 h after harvesting. Blood from the same donors was collected on heparin 6 wk after surgery. The protocol was approved by the Centre Hospitalier Régional et Universitaire ethical committee (no. 96102). All donors signed an informed consent form according to the declaration of Helsinki protocols.

Inbred mice with SCID (CB-17 SCID mice) were obtained from breeding pairs originally provided by M. Lieberman (Stanford University, Stanford, CA) maintained at the Institute Pasteur of Lille in sterilized isolators. Leaky mice (displaying spontaneous IgG production after 6 wk of age) were discarded. Mice were housed under pathogen-free conditions. Animals were handled according to the ethical principles of animal experimentation established by the European Center of Tufts University.

Skin grafting was performed as described previously (29, 30). In brief, after anesthesia, 6- to 8-wk-old mice were prepared for grafting by shaving the hair from a 5-cm<sup>2</sup> area on each side of the lateral abdominal region. Two circular graft beds of ~1.5 cm in diameter were prepared by removing shaved mouse skin. Full-thickness human skin grafts of the same size were placed onto wound beds. The use of two skin grafts per mouse allowed each mouse to be its own control. The transplants were held in place using 6–0 silk suture material and covered with an adhesive wound dressing and then with a standard bandage. Dressing material and sutures were removed 10 d after transplantation.

Six weeks after human skin transplantation, antiasialo GM1 (1/20 dilution; Wako, Osaka, Japan) was injected i.p. to neutralize murine NK activity. Twenty-four hours later, SCID mice were reconstituted i.p. with  $20 \times 10^6$  autologous PBMCs purified from the donor's blood and resuspended in PBS. The use of mononuclear cells and skin specimens obtained from the same human donor for each group of mice was of importance in this SCID mouse model because it avoids allogenic reactions during the reconstitution phase. Recombinant human CCL18 was then immediately injected in one of the two skin grafts at a dose of 2.5  $\mu$ g with 5% Evans blue dye (Sigma) to mark the site of injection. On the opposite site, skin graft was injected with a diluent containing an equivalent amount of BSA and 5% Evans blue dye to serve as control. Twenty-four hours later, human skin biopsies were taken at the site of injection marked by the Evans blue dye using a cylindrical sterile punch and were cut into two halves. One half was immediately embedded in OCT compound (Labonord, Villeneuve d'Ascq, France), snap-frozen in isopentane precooled in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . The other half was fixed in 4% paraformaldehyde, washed in 15% PBS/sucrose before OCT embedding, freezing, and storage at  $-80^\circ\text{C}$ .

### *Immunohistochemistry*

Cryostat sections (6  $\mu$ m) of PFA-fixed tissues were cut, air-dried, wrapped in aluminum foil, and stored at  $-20^\circ\text{C}$  for immunohistochemistry. Anti-human CD45, CD25, and CD4 mAbs were purchased from BD Biosciences. Anti-human CD45RO mAb was from Dako (Glostrup, Denmark), anti-human Foxp3 mAb was from Abcam (Cambridge, U.K.), anti-human IL-4, IL-5, and IL-10 mAbs were from Pharmingen (San Diego, CA), and anti-human IFN- $\gamma$  mAb was from Ibt (Hycult Biotechnology, Uden, Netherlands). Fast Red (Fast Red/naphthol ASMX tablets) was from Sigma (St. Quentin Fallavier, France). For all Abs except anti-human IL-4, IL-5, and IL-10 mAbs, immunohistochemistry was performed using a modified alkaline phosphatase anti-alkaline phosphatase method, as previously described (26). In brief, PFA-fixed cryostat sections were incubated in 0.3% Triton X-100 for 20 min, washed in TBS and incubated with the primary Ab overnight at  $4^\circ\text{C}$ , and successively incubated 30 min with rabbit anti-mouse Ig and then monoclonal alkaline phosphatase anti-alkaline phosphatase Abs (both from Dako), diluted in 20% normal human AB<sup>+</sup> serum. The coloration was developed using Fast Red, and sections were counterstained using hematoxylin. Irrelevant primary Ab of the same species was used as negative control. For IL-4, IL-5, and IL-10, immunohistochemistry was performed by using a modified avidin complex method, as previously described (14). In brief, PFA-fixed sections were incubated in 0.3% Triton X-100 for 20 min and with PBS containing 1% hydrogen peroxide. Endogenous biotin was quenched by using a Vector Laboratories kit (Peterborough, U.K.). Sections were preincubated with rabbit serum and incubated overnight with the anti-cytokine mAbs in PBS containing 0.1% saponin (Sigma). Sections were treated with the ABC Vectastain Elite kit (Vector Laboratories) and the color developed by using diaminobenzidine tetrahydrochloride-nickel (Vector Laboratories). Substitution of the primary Ab with an irrelevant Ab of the same species was used as a negative control. Anti-human Abs displayed no cross-reactivity with murine structures, as verified by immunohistochemistry on cryostat sec-

tions from biopsies performed at the border between human and murine tissue. Slides were encoded and counted in a blinded fashion at  $\times 250$  magnification using an eyepiece graticule. For skin sections, the upper edge of the grid was placed at the epidermal junction. For each specimen, at least three sections were evaluated, from which three to six fields were counted for immunohistochemistry. Absolute numbers of positive cells were counted per square millimeter.

### *Statistical analysis*

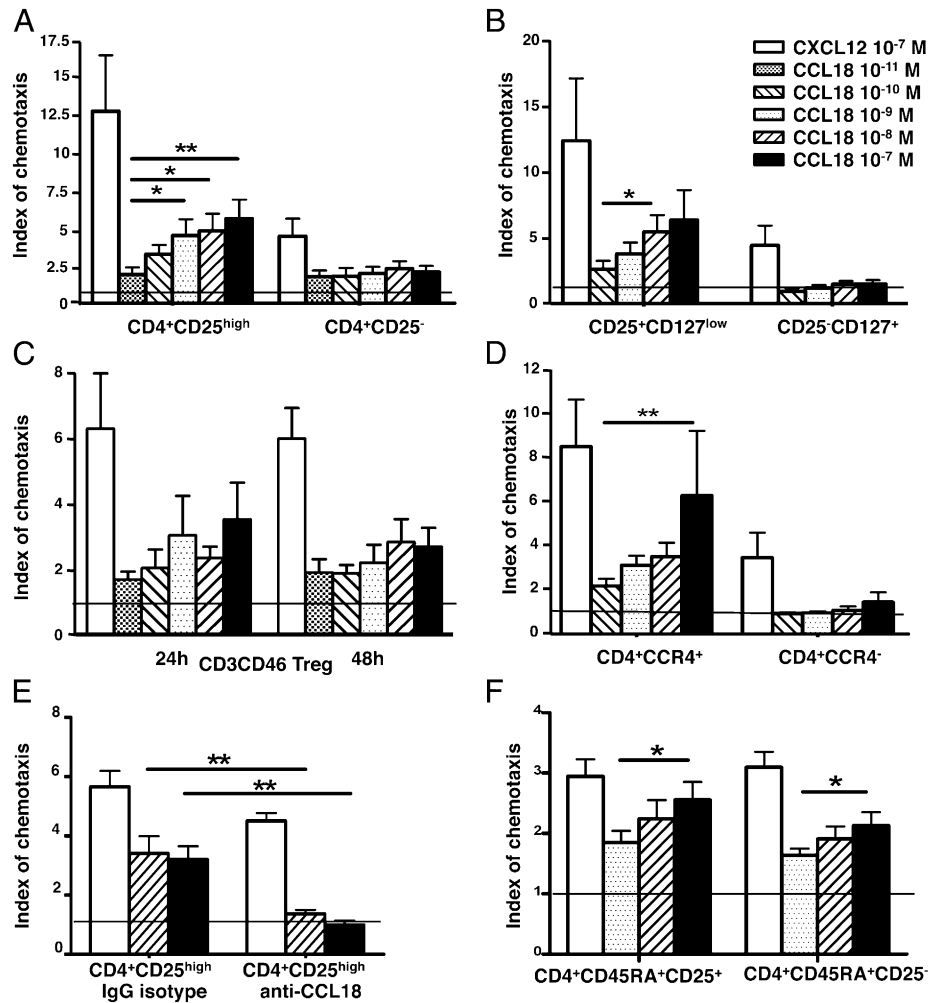
Statistical analysis was first performed within the subgroups by using one-way ANOVA tests and, when significant, followed by post hoc multiple-comparison tests. For normally distributed data, we used Bonferroni's test, and for not normally distributed data, we used Dunnett's test. For the *in vivo* study, differences between diluent and CCL18 injection sites were compared using the Wilcoxon matched pairs test. The *p* values  $<0.05$  were considered as statistically significant. Statistical analysis was performed using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA).

## **Results**

### *CCL18 attracts human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> Tregs, but not Tr1 clones in vitro*

To investigate whether CCL18 is able to attract human Tregs, we performed chemotaxis assays using different types of human Tregs at different concentrations of CCL18 ranging from  $10^{-11}$  to  $10^{-7}$  M. The constitutive expression of CD25 on CD4<sup>+</sup> T cells is a marker of Treg (2), although this receptor is also transiently up-regulated on activated effector T cells. A low expression of IL-7R CD127 has been shown to discriminate between CD4<sup>+</sup>CD25<sup>+</sup> Tregs and activated effector CD4<sup>+</sup>CD25<sup>+</sup> T cells (31). Therefore, highly purified FACS-sorted memory CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> Tregs were assessed, as well as polarized Tr1 type cells, that is, *in vitro* anti-CD3/CD46 polarized IL-10-secreting Tregs (32) and IL-10-producing Tr1 clones. For FACS, purified memory CD45RO<sup>+</sup> T cells were used to discard naive T cells, which are attracted by CCL18 but localize predominantly in lymphoid organs, and because most Tregs are memory cells. For CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cells, we used CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> cells as negative controls, respectively. As shown in Fig. 1A and 1B, sorted CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cells dose-dependently migrated in response to CCL18 with a maximal effect at  $10^{-7}$  and  $10^{-8}$  M, whereas control cells did not. The statistical analysis was performed by comparison with the lowest dose of CCL18 to demonstrate a dose-dependent effect, which is a characteristic feature of chemotaxis as opposed to chemokinesis. In contrast, *in vitro* differentiated Tregs were not attracted by CCL18, either at 24 or 48 h of differentiation (Fig. 1C). To assess the effect of CCL18 on *in vivo* differentiated Tr1 cells, T clones derived from skin and PBMCs were evaluated in chemotaxis assays. Similarly, CCL18 did not attract these cells either in a resting state or after anti-CD3/CD28 stimulation (data not shown). It has been shown that the chemokine receptors CCR4 and CCR8 are expressed on both human Th2 cells and Tregs (9–12). Given the similarity with CCL18 function, the migration of sorted memory CD4<sup>+</sup>CCR4<sup>+</sup> cells in response to CCL18 was assessed. As shown in Fig. 1D, CD4<sup>+</sup>CCR4<sup>+</sup> T cells were dose dependently attracted by CCL18 with a maximal effect at  $10^{-7}$  M. We were unable to evaluate the attraction of CD4<sup>+</sup>CCR8<sup>+</sup> cells by CCL18 because of the lack of specificity of all commercially available anti-CCR8 Abs, which did not allow cell sorting, at least in our hands. To check the specificity of CD4<sup>+</sup>CD25<sup>high</sup> T cell recruitment, we incubated CCL18 with a neutralizing Ab against CCL18 before the chemotaxis assay. As shown in Fig. 1E, a complete inhibition of CD4<sup>+</sup>CD25<sup>high</sup> T cell migration was obtained. Finally, because naive T cells are targets of CCL18 and CD25<sup>+</sup> naive T cells have

**FIGURE 1.** Chemotactic effect of CCL18 on different CD4<sup>+</sup> Treg subpopulations. **(A)** Flow cytometry sorted memory CD4<sup>+</sup>CD25<sup>high</sup> T cells compared with CD4<sup>+</sup>CD25<sup>-</sup> T cells. **(B)** Flow cytometry sorted memory CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cells compared with CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> cells, **(C)** in vitro differentiated anti-CD3/CD46 Tregs after 24 and 48 h of differentiation, and **(D)** flow cytometry sorted memory CD4<sup>+</sup>CCR4<sup>+</sup> cells compared with CD4<sup>+</sup>CCR4<sup>-</sup> cells. **(E)** Chemotactic effect of CCL18 pretreated with a neutralizing Ab against CCL18 or the isotype control on flow cytometry sorted memory CD4<sup>+</sup>CD25<sup>high</sup> T cells. **(F)** Flow cytometry sorted naive CD4<sup>+</sup>CD25<sup>+</sup> T cells compared with CD4<sup>+</sup>CD25<sup>-</sup> T cells. Chemotaxis assays were performed using a Boyden chamber, with CCL18 at different doses, RPMI 1640 medium as negative control and CXCL12 as positive control. Results are expressed as mean index of chemotaxis related to medium  $\pm$  SEM of  $n = 6-7$  experiments. The medium chemotactic index is shown as the horizontal line at 1 in each graph. \* $p < 0.05$ , \*\* $p < 0.01$ .



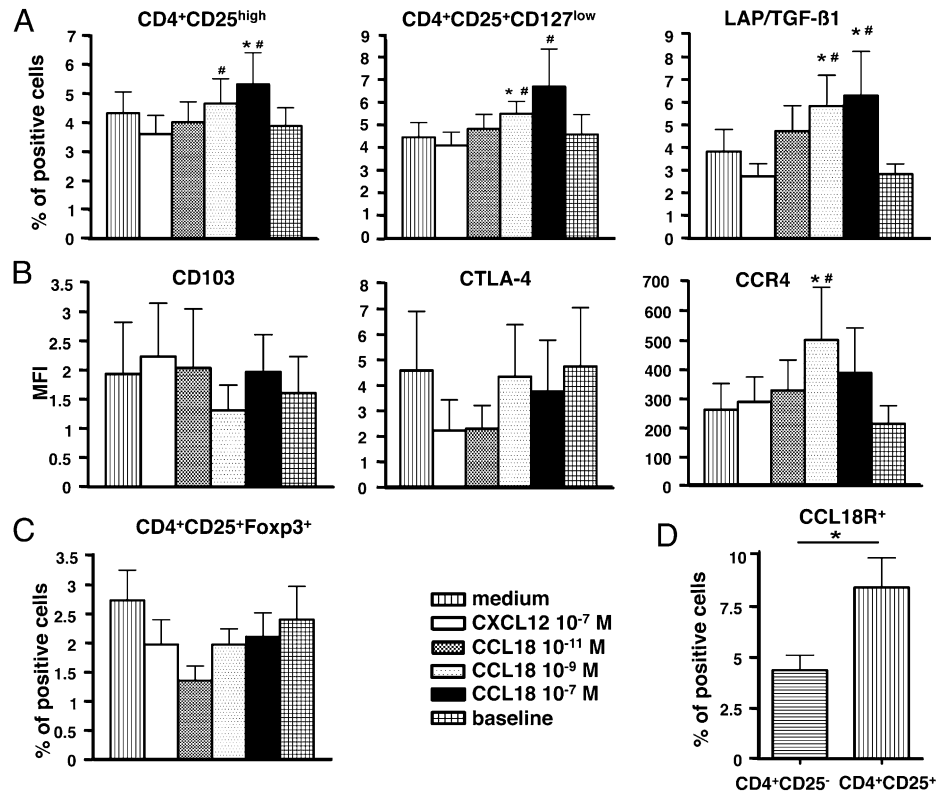
been shown to behave as Tregs (33), we also assessed the recruitment of flow cytometry sorted CD45RA<sup>+</sup>CD25<sup>+</sup> versus CD45RA<sup>+</sup>CD25<sup>-</sup> T cells. As shown in Fig. 1F, these two cell subsets were not differentially recruited by CCL18. Altogether, these findings suggest that CCL18 attracts CCR4-expressing memory T cells and highly purified CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> memory Treg, but that it does not affect Tr1 clones.

#### Human memory CD4<sup>+</sup> T cells attracted by CCL18 in vitro display some phenotypic characteristics of Tregs without expression of Foxp3

To assess whether CCL18 was able to preferentially recruit Tregs in a competitive T cell environment, we performed experiments the other way around by evaluating the phenotype of T cells attracted by CCL18 among nonselected memory T cells. For this purpose, CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were isolated by magnetic cell separation and allowed to migrate in a transwell system in response to 10<sup>-11</sup>, 10<sup>-9</sup>, and 10<sup>-7</sup> M CCL18 or to controls (medium and CXCL12 used at 10<sup>-7</sup> M). After 2 h 30 min, cells were harvested from the lower compartment. We obtained a moderate, but significant and dose-dependent, chemotaxis of memory CD4<sup>+</sup> T cells by CCL18 (43.2  $\pm$  5.7% increase at CCL18 10<sup>-7</sup> M compared with medium,  $p < 0.001$ ), which was more important for CXCL12 (226  $\pm$  44% increase; data not shown), confirming previously described results (20). The harvested cells were allowed to recover for 24 h before phenotyping by flow cytometry. As baseline, memory T cells maintained under the same conditions, but not allowed to migrate, were used. Even though there is

no clear specific cell surface marker of Treg, some markers have been shown to be preferentially expressed on human Treg, in particular, CD25<sup>high</sup> (34), CD25<sup>+</sup>CD127<sup>low</sup> (31), LAP/TGF- $\beta$ 1 (35), CD103 ( $\alpha$ E $\beta$ 7 integrin) (36), and CTLA-4 (37). As shown in Fig. 2A, among memory CD4<sup>+</sup> T cells recruited by CCL18 at 10<sup>-7</sup> and 10<sup>-9</sup> M as compared with baseline level before migration, there was a small but significant increased percentage of cells expressing CD25<sup>high</sup> (5.35  $\pm$  1.1 and 4.69  $\pm$  0.86% versus 3.91  $\pm$  0.63%, respectively), CD25<sup>+</sup>CD127<sup>low</sup> (6.9  $\pm$  1.7 and 5.67  $\pm$  0.56% versus 4.73  $\pm$  0.9%) or LAP/TGF- $\beta$ 1 (6.34  $\pm$  1.9 and 5.88  $\pm$  1.37% versus 2.87  $\pm$  0.45%), corresponding to  $\sim$ 30% enrichment for the two first populations and 100% for the latter. Memory T cells recruited either by medium or CXCL12 at 10<sup>-7</sup> M were not enriched in these populations. In contrast, there was no differential expression of the surface markers CD103 and CTLA-4 (Fig. 2B). Although there was no increase in the number of CD4<sup>+</sup>CCR4<sup>+</sup> T cells (data not shown), we observed a strong upregulation of CCR4 mean fluorescence intensity (MFI) in the CCL18-recruited cell population, suggesting a preferential recruitment of T cells strongly expressing CCR4 (504.4  $\pm$  182.3 MFI for CCL18 at 10<sup>-9</sup> M compared with baseline; 214.1  $\pm$  62.4 MFI; Fig. 2B). As another readout of Treg, the T cell transcription factor Foxp3 was assessed by intracellular staining. However, there was no increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells, independently of the chemokine used in the assay (Fig. 2C). To confirm these data, we evaluated mRNA expression for Foxp3 by quantitative RT-PCR. Similar to the results at the protein level, Foxp3 mRNA was not increased in CCL18-recruited cells (data not shown). Although

**FIGURE 2.** Phenotype of memory CD4<sup>+</sup> T cells from healthy subjects recruited by CCL18. Peripheral memory CD4<sup>+</sup> T cells were allowed to migrate in a transwell system against culture medium, CXCL12, or CCL18 for 2 h 30 min. Recovered cells were rested overnight and stained for expression of the surface molecules CD25, CD127, LAP/TGF- $\beta$ 1, CD103, CTLA-4, and CCR4, and for intracellular expression of Foxp3. Results are expressed as (A) mean percentage of positive cells  $\pm$  SEM for  $n = 9$ –11 subjects, (B) MFI  $\pm$  SEM for  $n = 7$ –11 subjects, and (C) mean percentage of positive cells  $\pm$  SEM for  $n = 9$  subjects. (D) Cell surface binding of CCL18 on purified memory CD4<sup>+</sup>CD25<sup>+</sup> cells versus CD4<sup>+</sup>CD25<sup>-</sup> T cells. Results are expressed as mean percentage of positive cells  $\pm$  SEM for  $n = 6$  subjects. \* $p < 0.05$  compared with medium, # $p < 0.05$  compared with baseline nontransmigrated cells.



the nature of the putative CCL18R is as yet unknown, we checked its expression by flow cytometry on memory CD4<sup>+</sup>CD25<sup>+</sup> cells using a biotinylated ligand. Only a few memory CD4<sup>+</sup>CD25<sup>-</sup> T cells bound CCL18 ( $4.28 \pm 0.72\%$ ), whereas the percentage of CCL18-binding cells was doubled in CD4<sup>+</sup>CD25<sup>+</sup> cells ( $8.25 \pm 1.4\%$ ; Fig. 2D). Thus, CCL18-recruited memory CD4<sup>+</sup> T cells display some characteristics of Treg, that is, high expression of CD25, low expression of CD127, some expression of LAP/TGF- $\beta$ 1, but no expression of Foxp3.

#### Human memory CD4<sup>+</sup> T cells attracted by CCL18 in vitro produce IL-10 and TGF- $\beta$ 1 upon activation

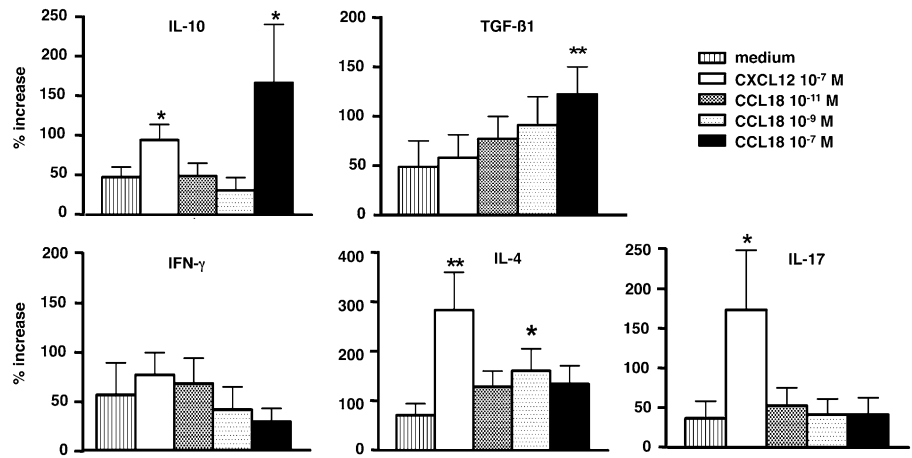
To evaluate the polarization profile of memory T cells recruited by CCL18, we examined their cytokine production. Using the same transwell system described earlier, we stimulated the recovered cells with anti-CD3/CD28 Abs for 3 d, and the supernatants were harvested and analyzed for production of IL-4, IFN- $\gamma$ , IL-10, TGF- $\beta$ 1, and IL-17 by ELISA. As baseline, memory T cells stimulated under the same conditions, but not having migrated, were used. As compared with this baseline, memory T cells recruited in response to culture medium only generally exhibited a 50% increase in their cytokine production, showing that transmigration nonspecifically activated these cells (Fig. 3). Cells recruited by CXCL12 exhibited an increased production of IL-4, IL-10, and IL-17, as compared with medium, which is consistent with the ubiquitous chemoattractant effect of CXCL12 on diverse T cell subpopulations. In contrast, memory T cells recruited in response to CCL18 showed a significant increase in the production of regulatory cytokines, that is, IL-10 and TGF- $\beta$ 1 at the highest concentration of CCL18 ( $169.2 \pm 75.1$  and  $122 \pm 27.4\%$  increase, respectively). IL-4-producing cells were also more attracted by CCL18 at  $10^{-9}$  M concentration ( $161.9 \pm 44.4\%$  increase), although to a lesser extent than for CXCL12 ( $284.9 \pm 76.9\%$  increase), whereas no significant differences were observed for IFN- $\gamma$ - and IL-17-producing cells. To evaluate the proportion of cells expressing

CCL18R among IL-10- and IL-4-producing cells, double immunostaining was performed on baseline memory CD4<sup>+</sup> T cells stimulated with PMA and ionomycin, and evaluated by flow cytometry. Double immunostaining was also performed for cell surface CCL18R and LAP/TGF- $\beta$ 1 on nonstimulated memory T cells. The mean percentage of CCL18R<sup>+</sup> cells was  $14.5 \pm 2.9\%$  in IL-4<sup>+</sup> cells,  $26.3 \pm 5.8\%$  in IL-10<sup>+</sup> cells, and  $18.1 \pm 1.9\%$  in LAP/TGF- $\beta$ 1<sup>+</sup> cells in 5–9 subjects (data not shown). These data show that, in addition to the known chemoattraction of Th2 cells (14), CCL18 also recruits cells able to produce immunosuppressive cytokines.

#### Human CD4<sup>+</sup> T cells attracted by CCL18 in vivo express CD25, but not Foxp3, and produce IL-10

To confirm the phenotype of CCL18-recruited CD4<sup>+</sup> T cells in vivo, we used a humanized model consisting in a SCID mouse model grafted with human skin and reconstituted with autologous mononuclear cells. CCL18, as well as its diluent, were injected into the skin grafts, and biopsies were performed 24 h later and analyzed by immunohistochemistry. First, the cell infiltrate generated by the injection of CCL18 was assessed as compared with the control diluent. T cells recruited by CCL18 included memory (CD45RO<sup>+</sup>,  $p < 0.01$ ; Fig. 4A) and CD4<sup>+</sup> T cells, whereas CD8<sup>+</sup> T cells were not found (data not shown). Consistent with the in vitro findings, the CD25 marker was up-regulated in CCL18-injected graft ( $p < 0.02$ ), whereas no Foxp3<sup>+</sup> cells were evidenced (Fig. 4A). Next, we examined the cytokine profile expression of CCL18-injected grafts and found an elevated percentage of IL-10<sup>+</sup> cells in comparison with the diluent injection site ( $p < 0.03$ ; Fig. 4A). In contrast, no immunostaining was observed for IFN- $\gamma$ , and a small, nonstatistically significant increase was observed for IL-4 and IL-5. Examples of immunostaining are shown in Fig. 4B. These results confirm the ability of CCL18 to recruit in vivo CD4<sup>+</sup> T cells having a phenotype compatible with a regulatory function.

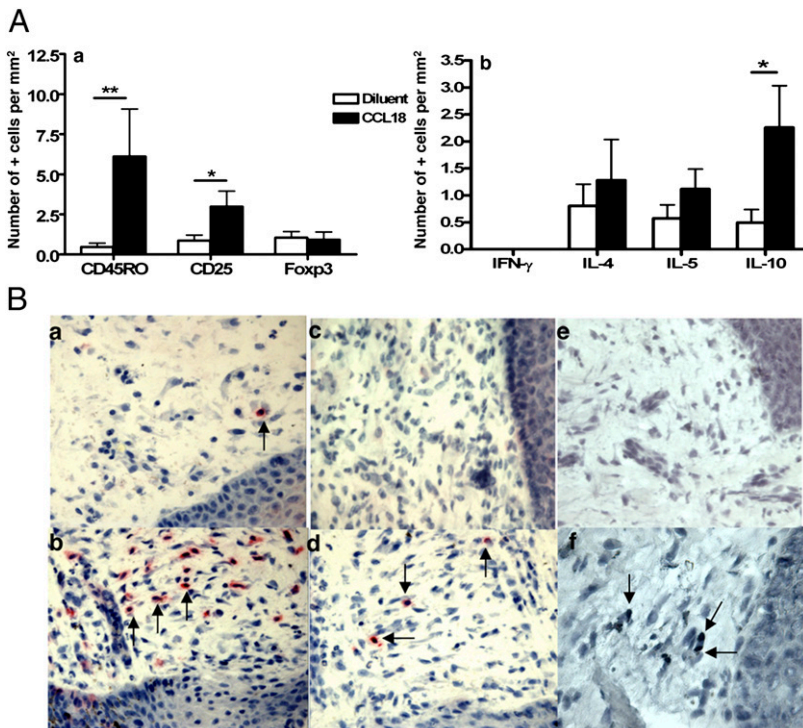
**FIGURE 3.** Cytokine profile of memory CD4<sup>+</sup> T cells recruited by CCL18 in vitro. Peripheral memory CD4<sup>+</sup> T cells were allowed to migrate in a transwell system against culture medium, CXCL12, or CCL18 for 2 h 30 min. Recovered cells were rested overnight and stimulated for 3 d. The production level of IL-10, TGF-β1, IL-4, IFN-γ, and IL-17 was evaluated by ELISA. Results are expressed as mean percentage increase ± SEM as compared with stimulated baseline cells for n = 8–12 subjects. \*p < 0.05, \*\*p < 0.01 compared with medium.



*Human memory CD4<sup>+</sup> T cells attracted by CCL18 suppress T cell proliferation through an IL-10-dependent mechanism*

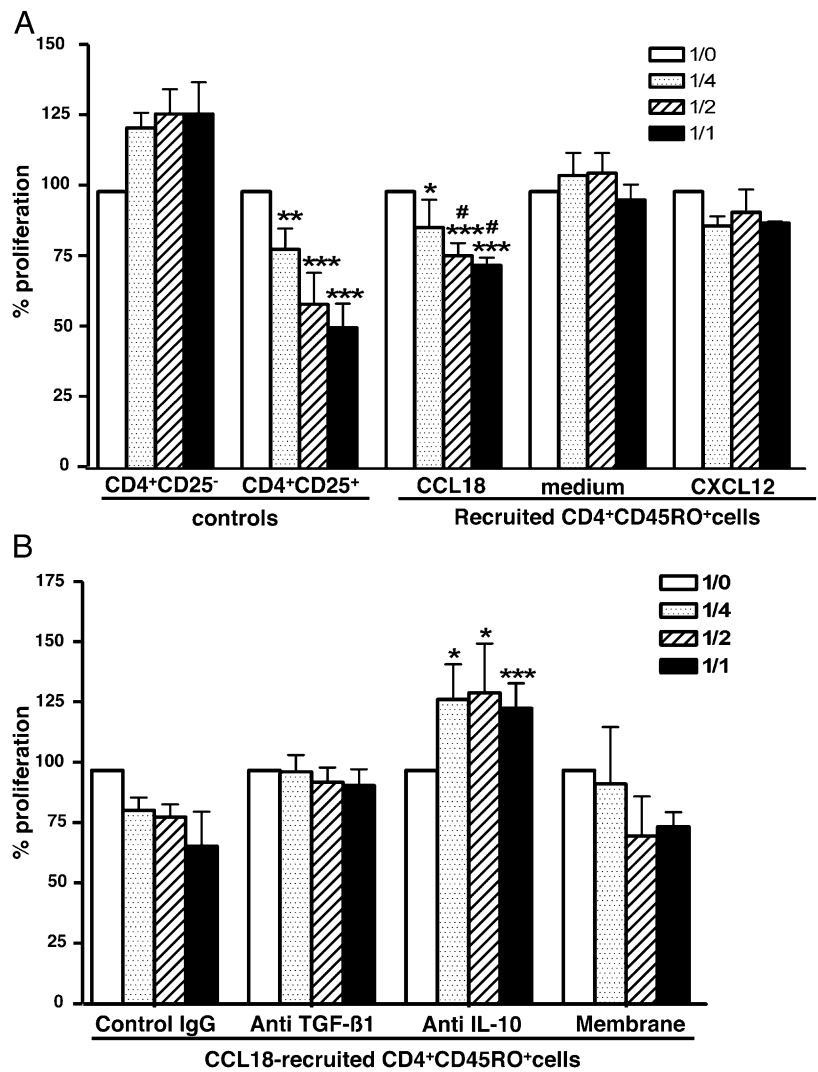
To definitively assess whether the cells recruited by CCL18 are bona fide regulatory cells, we performed functional assays by evaluating the ability of in vitro CCL18-recruited memory CD4<sup>+</sup> T cells to suppress effector CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation. Highly purified CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells sorted by flow cytometry, as well as memory CD4<sup>+</sup> T cells recruited in the presence of medium only, CXCL12, or CCL18 were added at increasing ratios to a fixed number of irradiated PBMCs and autologous CD4<sup>+</sup>CD25<sup>-</sup> effector cells, stimulated with soluble anti-CD3 and CD28 Abs. When evaluated alone for their baseline proliferation, medium- and CXCL12-recruited T cells proliferated to a similar extent as effector T cells, whereas CD4<sup>+</sup>CD25<sup>+</sup> T cells and CCL18-recruited T cells showed a 3- and 2-fold decrease, respectively, in their proliferative response (data not shown). The addition of CD4<sup>+</sup>CD25<sup>-</sup> effector cells as a negative control resulted in increased T cell proliferation, whereas the addition of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (positive control) resulted in a ratio-dependent

suppression of proliferation (60.25% inhibition at 1:1 ratio; Fig. 5A). Addition of memory CD4<sup>+</sup>CD25<sup>-</sup> T cells, recruited by medium or CXCL12, did not significantly suppress T cell proliferation, as compared with the negative control. In contrast, addition of CCL18-recruited memory CD4<sup>+</sup> T cells significantly suppressed proliferation, as compared with the negative control with 42.7% inhibition at a 1:1 ratio, as well as compared with medium-recruited cells at 1:2 and 1:1 ratio (Fig. 5A). Two main mechanisms have been shown to contribute to Treg-mediated suppression, cell-cell contact, and soluble inhibitory molecules, such as IL-10 and TGF-β (38). To investigate the mechanism involved in the suppressive effect of CCL18-recruited memory CD4<sup>+</sup>CD25<sup>-</sup> T cells, we performed inhibition and cell contact experiments. The addition of a blocking anti-IL-10 mAb completely reversed the suppression of responder T cell proliferation at all ratios (Fig. 5B). The addition of a neutralizing anti-TGF-β mAb induced a small recovery of T cell proliferation, which was, however, not statistically significant (Fig. 5B). To investigate whether part of the suppressive effect was cell-contact dependent,



**FIGURE 4.** Human T cell subpopulations and cytokine-expressing cells recruited by CCL18 in vivo. CCL18 was injected into human skin xenografts immediately after i.p. reconstitution of SCID mice with autologous PBMCs. Skin xenografts were recovered 24 h after intradermal CCL18 or diluent injections. (A) Cryostat sections of human skin biopsies were immunostained with (a) mAbs against human CD45RO<sup>+</sup> memory T cells, CD25<sup>+</sup> and Foxp3<sup>+</sup> cell subpopulations, and (b) with Abs against human cytokines. Results are expressed as mean number of positive cells ± SEM per mm<sup>2</sup> (n = 8). (B) Microphotographs of human skin graft 24 h after diluent (b, c, e) or CCL18 (a, d, f) intradermal injection immunostained with anti-CD4 Ab (a, b), anti-CD25 Ab (c, d) or anti-IL-10 Ab (e, f). Arrows point out positive cells. Original magnification ×250. \*p < 0.05, \*\*p < 0.01.

**FIGURE 5.** Inhibitory effect of CCL18-recruited memory CD4<sup>+</sup> T cells on proliferation of CD4<sup>+</sup>CD25<sup>-</sup> effector cells. **(A)** Memory CD4<sup>+</sup> T cells recruited by CXCL12 or CCL18 were added at suppressor/responder ratios of 1:4, 1:2, and 1:1 to autologous CD4<sup>+</sup>CD25<sup>-</sup> effector cells stimulated with soluble anti-CD3 and anti-CD28 Abs. Irradiated autologous PBMCs were used as APCs. Proliferation was measured by incorporation of [<sup>3</sup>H]thymidine at 48 h. The CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were used as negative and positive controls, respectively. The results are presented as mean percentages  $\pm$  SEM of proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells alone. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 compared with the negative control, #*p* < 0.05 compared with medium-recruited cells. *n* = 6 experiments with triplicate wells for each condition. **(B)** Mechanisms involved in the inhibitory effect of CCL18-recruited cells on the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> effector cells. Isotype, anti-IL-10, or anti-TGF- $\beta$ 1 neutralizing Abs were added to the co-culture system, and a transwell system was used to abrogate the cell contact between the suppressor and responder cells. Results are expressed as described earlier; \**p* < 0.05, \*\*\**p* < 0.01 compared with isotype-treated CCL18-recruited memory T cells. *n* = 4 experiments with triplicate wells for each condition.



we separated CCL18-recruited memory CD4<sup>+</sup> T cells from CD4<sup>+</sup>CD25<sup>-</sup> effector cells by a 0.4- $\mu$ m pore size membrane in a transwell. Under these conditions, CCL18-recruited memory CD4<sup>+</sup> T cells were still able to inhibit the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> effector cells (40.8% inhibition at a ratio of 1:1; Fig. 5B). These data indicate that CCL18 recruits Tregs that, in turn, inhibit CD4<sup>+</sup>CD25<sup>-</sup> effector cell proliferation through an IL-10-, but not a cell contact-dependent mechanism. Collectively, these findings show that CCL18 is able to recruit Tregs, which act through IL-10 production but do not express Foxp3.

## Discussion

In this study, we show for the first time, to our knowledge, that CCL18 recruits a particular subset of Tregs expressing CD25 but not Foxp3, which act through the production of IL-10. Hitherto, several Treg subsets have been reported, including natural and adaptive T cells. CD4<sup>+</sup> T cells constitutively expressing CD25 were first described as natural Tregs conferring self-tolerance. However, the IL-2R  $\alpha$ -chain CD25 is expressed by activated effector T cells as well. Human T cells expressing high levels of CD25, rather than cells expressing low levels of CD25, were shown to support Treg activity (34). The expression of IL-7R  $\alpha$ -chain CD127 is useful to help discriminating CD4<sup>+</sup>CD25<sup>+</sup> Tregs, which downregulate CD127 (CD127<sup>low</sup>), from activated effector CD4<sup>+</sup>CD25<sup>+</sup> T cells, which upregulate CD127 (CD127<sup>high</sup>) (31). In this study, CCL18 induced a clear recruit-

ment of both highly purified memory CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cells, which was, however, less prominent when total, nonselected, memory T cells were used, arguing for a competition within different responding subpopulations. Although CCL18-recruited T cells did not express Foxp3, neither at the transcriptional nor at the protein level, they were bona fide Tregs, as shown by their functional ability to inhibit T cell proliferation. Because chemotaxis in vitro (39) and cell accumulation in response to injected chemokines do not always accurately predict chemokine function in vivo, a physiological setting of human skin leukocyte recruitment was used. The particular phenotype observed in vivo was also observed in vivo in a human setting, indicating that it was not linked to an in vitro artifact. Expression of the transcription factor Foxp3 is a feature of natural Treg (1), which arises during thymic development through high-affinity recognition of self-Ags (40, 41). Therefore, these data suggest that CCL18-recruited Tregs are not natural, but rather adaptive Tregs. CCL18-recruited Tregs were demonstrated to act through IL-10. The production of IL-10 by CD4<sup>+</sup> T cells has been associated with different subsets of Tregs including Foxp3<sup>-</sup> cells, in particular, in humans and among the peripherally induced adaptive Tregs. The best characterized of the latter subset are Tr1 cells, originally described as a progeny of naive CD4<sup>+</sup> T cells activated ex vivo in the presence of IL-10 or by IL-10-conditioned dendritic cells (42, 43). Tr1 cells are characterized by abundant production of IL-10 and exert suppression by a cell-



contact-independent, cytokine-dependent mechanism that involves both IL-10 and TGF- $\beta$  (7). These characteristics fitted the general properties of CCL18-recruited Tregs; however, CCL18 did not attract Tr1 clones or anti-CD3/CD46-differentiated T cells. Notably, the Tr1 clones that were used in this study did express Foxp3 (25), a feature that was missing in the cells recruited by CCL18. With respect to anti-CD3/CD46-differentiated T cells, they are also considered as Tr1 type cells linked to their ability to suppress T cell proliferation and produce the suppressive cytokine IL-10 (32), as well as their ability to induce granzyme-mediated cytotoxicity (44). Among other Treg populations not expressing Foxp3, a subset of CD69<sup>+</sup>CD4<sup>+</sup> T cells has been described to suppress T cell proliferation through membrane-bound TGF- $\beta$  in mice (45). However, in contrast with CCL18-recruited Tregs, the latter subpopulation does not express CD25 and does not produce IL-10, ruling out a potential similarity between them. Some CCL18-recruited T cells expressed increased levels of LAP/TGF- $\beta$ 1. The latent form of TGF- $\beta$ 1 has been previously detected on the surface of activated human and mouse Foxp3<sup>+</sup> Tregs and shown to convert Foxp3<sup>-</sup> T cells into Foxp3<sup>+</sup> Tregs (46–48). Recently, this marker has been shown to characterize in humans another subset of CD4<sup>+</sup> Tregs not expressing Foxp3 (49). In the latter study, LAP/TGF- $\beta$ 1-expressing cells were found mainly within the CD4<sup>+</sup>CD25<sup>high</sup> T cells, although at low levels, as compared with the CD4<sup>+</sup>CD25<sup>int</sup> and CD4<sup>+</sup>CD25<sup>low</sup> populations. This CD4<sup>+</sup>LAP/TGF- $\beta$ 1<sup>+</sup>Foxp3<sup>-</sup> subset produced IL-10 and TGF- $\beta$ 1 upon stimulation, and furthermore exhibited TGF- $\beta$ 1- and IL-10-dependent suppressive activity in vitro, thereby exhibiting characteristics similar to those of CCL18-recruited Tregs, and suggesting that CCL18 may recruit part of this subpopulation. We have recently shown that CCL18 is also able to generate adaptive Tregs from CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (22). In contrast with these findings, 72–96 h were required to generate these cells, which expressed Foxp3 and acted through both cytokine- and cell–cell contact-dependent mechanisms. Therefore, CCL18 has a dual, immediate and delayed, activity: it rapidly recruits a particular subset of Tregs, whereas it is also able to induce the conversion of CD4<sup>+</sup>CD25<sup>-</sup> effector cells into Tregs.

The suppression of ongoing immune responses in diverse tissues requires memory Tregs to express a diverse array of adhesion and chemokine receptors (50, 51). The appropriate localization of Tregs into distinct tissues is essential for their in vivo activity. The receptor(s) binding CCL18 remain(s) unidentified today. The differential localization of naive, in lymph nodes, versus memory T cells, in tissues, suggests that at least two different receptors may mediate CCL18 activity. The data presented in this article suggest that some memory Tregs express CCL18 receptor(s), as they are able to bind labeled CCL18 and to migrate into tissues in response to this molecule. Of interest, the chemokine receptor CCR4 is also expressed by Tregs (9–12, 52) and also directs T cells to the lung (53). Although CCR4 is not a receptor for CCL18 (14, 19), it appears to be highly expressed on the cell surface of the cells recruited by CCL18. CCR4 expression on Tregs is functionally active in vivo. Indeed, mice with a complete loss of CCR4 in the Treg compartment experience development of severe inflammatory disease in the lungs related to a lack of Treg accumulation in this organ (54). Altogether, these data suggest that coexpression of CCL18R and CCR4 on memory Tregs may favor their localization to the lung. Moreover, CCL18 is highly expressed by healthy alveolar macrophages and by alternatively activated macrophages, which are well documented to have anti-inflammatory properties. A recent study has shown that Tregs steer monocytes toward alternatively activated monocyte/macrophages that secrete high levels of CCL18 (55). Altogether, these

data suggest that CCL18 could play a crucial role in maintaining lung tolerance by attracting Tregs into the lung and by contributing to Treg–alternatively activated macrophage mutual interactions.

However, there is evidence that CCL18 is also implicated in allergic diseases. We and others have shown that CCL18 is up-regulated by allergen stimulation and overexpressed in a number of allergic diseases including allergic asthma, atopic dermatitis, and vernal keratoconjunctivitis (14, 56, 57). Furthermore, CCL18 exhibits chemotactic activity toward Th2 cells that are the cornerstone of the allergic reaction (14). This ability was confirmed in this study, where, besides Treg recruitment, Th2 type cells were also attracted, as shown by the IL-4 production of the recruited cells in vitro and by the binding of CCL18 to IL-4-producing cells. Although the ability of CCL18 to attract both Th2 and Tregs might appear contradictory, a differential attraction cannot be excluded. CCL18-mediated Th2 versus Treg cell recruitment may be affected by a competitive effect linked to a different chemokine receptor, by microenvironmental factors such as endothelial integrins and glycosaminoglycans, by a differential recruitment according to the chemokine concentration, or by homodimerization or heterodimerization of CCL18 and/or its receptor. Interestingly, these two cell subsets have been proposed to derive from the same cell population because they both overexpress a number of similar gene transcripts, including the chemokine receptors CCR4 and CCR8, which might explain conserved chemokine receptor expression on the two subsets (58). Accordingly, it has recently been shown that Th2 memory cells can be converted into Tregs able to suppress allergic asthma (59). In contrast, some reports show the coexistence of these two subsets at sites of allergic inflammation (60).

The overlap of CCL18 receptor expression on Tregs and Th2 subsets may allow their colocalization in the lung to act as a regulatory feedback mechanism in allergic inflammatory conditions. Under basal conditions, as observed in our study, the global effect of CCL18-recruited cells is a suppression of T cell proliferation, suggesting a regulatory function of this chemokine in healthy humans.

In conclusion, the results from this study suggest that CCL18 activates migration of human Tregs. In this respect, CCL18 is likely to contribute to the maintenance of tolerance and/or suppression of deleterious inflammation by attracting Tregs into tissues, acting, in particular, in the major host–environment interface of the lung, where it is highly and constitutively expressed. Therefore, CCL18 may be an attractive therapeutic target, in particular, in inflammatory pulmonary diseases.

## Acknowledgments

We acknowledge Drs. Claudia Kemper and Stan Tomavo for kind gifts of anti-CD46 Ab and purified *P. falciparum* TAF10 protein, respectively.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Zheng, Y., and A. Y. Rudensky. 2007. Foxp3 in control of the regulatory T cell lineage. *Nat. Immunol.* 8: 457–462.
- Sakaguchi, S., M. Toda, M. Asano, M. Itoh, S. S. Morse, and N. Sakaguchi. 1996. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. *J. Autoimmun.* 9: 211–220.
- Read, S., V. Malmström, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
- Izcue, A., J. L. Coombes, and F. Powrie. 2006. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol. Rev.* 212: 256–271.

5. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* 6: 338–344.
6. Weaver, C. T., L. E. Harrington, P. R. Mangan, M. Gavrieli, and K. M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24: 677–688.
7. Roncarolo, M. G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. LeVings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* 212: 28–50.
8. Bromley, S. K., T. R. Mempel, and A. D. Luster. 2008. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat. Immunol.* 9: 970–980.
9. Soler, D., T. R. Chapman, L. R. Poisson, L. Wang, J. Cote-Sierra, M. Ryan, A. McDonald, S. Badola, E. Fedyk, A. J. Coyle, et al. 2006. CCR8 expression identifies CD4 memory T cells enriched for FOXP3+ regulatory and Th2 effector lymphocytes. *J. Immunol.* 177: 6940–6951.
10. Sebastiani, S., P. Allavena, C. Albanesi, F. Nasorri, G. Bianchi, C. Traidl, S. Sozzani, G. Girolomoni, and A. Cavani. 2001. Chemokine receptor expression and function in CD4+ T lymphocytes with regulatory activity. *J. Immunol.* 166: 996–1002.
11. Colantonio, L., A. Iellem, F. Sinigaglia, and D. D'Ambrosio. 2002. Skin-homing CLA+ T cells and regulatory CD25+ T cells represent major subsets of human peripheral blood memory T cells migrating in response to CCL11/309. *Eur. J. Immunol.* 32: 3506–3514.
12. Iellem, A., M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)/CD25(+) regulatory T cells. *J. Exp. Med.* 194: 847–853.
13. Schutyser, E., S. Struyf, A. Wuyts, W. Put, K. Geboes, B. Grillet, G. Opendakker, and J. Van Damme. 2001. Selective induction of CCL18/PARC by staphylococcal enterotoxins in mononuclear cells and enhanced levels in septic and rheumatoid arthritis. *Eur. J. Immunol.* 31: 3755–3762.
14. de Nadaï, P., A. S. Charbonnier, C. Chenivesse, S. Sénéchal, C. Fournier, J. Gilet, H. Vorng, Y. Chang, P. Gosset, B. Wallaert, et al. 2006. Involvement of CCL18 in allergic asthma. *J. Immunol.* 176: 6286–6293.
15. Kodolija, V., C. Müller, O. Politz, N. Hakij, C. E. Orfanos, and S. Goerd. 1998. 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.* 160: 1411–1418.
16. Schutyser, E., A. Richmond, and J. Van Damme. 2005. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J. Leukoc. Biol.* 78: 14–26.
17. Tasaki, Y., S. Fukuda, M. Iio, R. Miura, T. Imai, S. Sugano, O. Yoshie, A. L. Hughes, and H. Nomiya. 1999. Chemokine PARC (SCYA18) generated by fusion of two MIP-1alpha/LD78alpha-like genes. *Genomics* 55: 353–357.
18. Schraufstatter, I., H. Takamori, L. Sikora, P. Sriramarao, and R. G. DiScipio. 2004. Eosinophils and monocytes produce pulmonary and activation-regulated chemokine, which activates cultured monocytes/macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286: L494–L501.
19. Hieshima, K., T. Imai, M. Baba, K. Shoudai, K. Ishizuka, T. Nakagawa, J. Tsuruta, M. Takeya, Y. Sakaki, K. Takatsuki, et al. 1997. A novel human CC chemokine PARC that is most homologous to macrophage-inflammatory protein-1 alpha/LD78 alpha and chemotactic for T lymphocytes, but not for monocytes. *J. Immunol.* 159: 1140–1149.
20. Adema, G. J., F. Hartgers, R. Verstraten, E. de Vries, G. Marland, S. Menon, J. Foster, Y. Xu, P. Nooyen, T. McClanahan, et al. 1997. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 387: 713–717.
21. Vulcano, M., S. Struyf, P. Scapini, M. Cassatella, S. Bernasconi, R. Bonocchi, A. Calleri, G. Penna, L. Adorini, W. Luini, et al. 2003. Unique regulation of CCL18 production by maturing dendritic cells. *J. Immunol.* 170: 3843–3849.
22. Chang, Y., P. de Nadaï, I. Azzouli, O. Morales, N. Delhem, H. Vorng, S. Tomavo, S. Ait Yahia, G. Zhang, B. Wallaert, et al. 2010. The chemokine CCL18 generates adaptive regulatory T cells from memory CD4+ T cells of healthy but not allergic subjects. *FASEB J.* 24: 5063–5072.
23. Azzouli, I., S. A. Yahia, Y. Chang, H. Vorng, O. Morales, Y. Fan, N. Delhem, C. Ple, A. B. Tonnel, B. Wallaert, and A. Tscopoulos. 2011. CCL18 differentiates dendritic cells in tolerogenic cells able to prime regulatory T cells in healthy subjects. *Blood* 118: 3549–3558.
24. Lécart, S., V. Boulay, N. Raison-Peyron, J. Bousquet, L. Meunier, H. Yssel, and J. Pène. 2001. Phenotypic characterization of human CD4+ regulatory T cells obtained from cutaneous dinitrochlorobenzene-induced delayed type hypersensitivity reactions. *J. Invest. Dermatol.* 117: 318–325.
25. Brun, V., H. Bastian, V. Neveu, and A. Foussat. 2009. Clinical grade production of IL-10 producing regulatory Tr1 lymphocytes for cell therapy of chronic inflammatory diseases. *Int. Immunopharmacol.* 9: 609–613.
26. Chang, Y., S. Sénéchal, P. de Nadaï, C. Chenivesse, J. Gilet, H. Vorng, B. Legendre, A. B. Tonnel, B. Wallaert, P. Lassalle, and A. Tscopoulos. 2006. Diesel exhaust exposure favors TH2 cell recruitment in nonatopic subjects by differentially regulating chemokine production. *J. Allergy Clin. Immunol.* 118: 354–360.
27. Gilet, J., Y. Chang, C. Chenivesse, B. Legendre, H. Vorng, C. Duez, B. Wallaert, H. Porte, S. Senechal, and A. Tscopoulos. 2009. Role of CCL17 in the generation of cutaneous inflammatory reactions in Hu-PBMC-SCID mice grafted with human skin. *J. Invest. Dermatol.* 129: 879–890.
28. Callebaut, L., K. Prat, E. Meurice, J. P. Mornon, and S. Tomavo. 2005. Prediction of the general transcription factors associated with RNA polymerase II in *Plasmodium falciparum*: conserved features and differences relative to other eukaryotes. *BMC Genomics* 6: 100.
29. Sénéchal, S., O. Fahy, T. Gentina, H. Vorng, M. Capron, A. F. Walls, A. R. McEuen, M. G. Buckley, Q. Hamid, B. Wallaert, et al. 2002. CCR3-blocking antibody inhibits allergen-induced eosinophil recruitment in human skin xenografts from allergic patients. *Lab. Invest.* 82: 929–939.
30. de Nadaï, P., C. Chenivesse, J. Gilet, H. Porte, H. Vorng, Y. Chang, A. F. Walls, B. Wallaert, A. B. Tonnel, A. Tscopoulos, and H. G. Zerwes. 2006. CCR5 usage by CCL5 induces a selective leukocyte recruitment in human skin xenografts in vivo. *J. Invest. Dermatol.* 126: 2057–2064.
31. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203: 1693–1700.
32. Kemper, C., A. C. Chan, J. M. Green, K. A. Brett, K. M. Murphy, and J. P. Atkinson. 2003. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421: 388–392.
33. Fritzsche, B., N. Oberle, E. Pauly, R. Geffers, J. Buer, J. Poschl, P. Krammer, O. Linderkamp, and E. Suri-Payer. 2006. Naive regulatory T cells: a novel subpopulation defined by resistance toward CD95L-mediated cell death. *Blood* 108: 3371–3378.
34. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. CD4+ CD25high regulatory cells in human peripheral blood. *J. Immunol.* 167: 1245–1253.
35. Nakamura, K., A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF-beta 1 plays an important role in the mechanism of CD4+ CD25+ regulatory T cell activity in both humans and mice. *J. Immunol.* 172: 834–842.
36. Ailakhverdi, Z., D. Fitzpatrick, A. Boisvert, N. Baba, S. Bouguermouh, M. Sarfati, and G. Delespesse. 2006. Expression of CD103 identifies human regulatory T-cell subsets. *J. Allergy Clin. Immunol.* 118: 1342–1349.
37. Birebent, B., R. Lorho, H. Lechartier, S. de Guibert, M. Alizadeh, N. Vu, A. Beauptel, N. Robillard, and G. Semana. 2004. Suppressive properties of human CD4+CD25+ regulatory T cells are dependent on CTLA-4 expression. *Eur. J. Immunol.* 34: 3485–3496.
38. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
39. Proudfoot, A. E. I., T. M. Handel, Z. Johnson, E. K. Lau, P. LiWang, I. Clark-Lewis, F. Borlat, T. N. Wells, and M. H. Kosco-Vilbois. 2003. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. USA* 100: 1885–1890.
40. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Najj, and A. J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
41. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3: 756–763.
42. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
43. Wakkach, A., N. Fournier, V. Brun, J. P. Breitmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18: 605–617.
44. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589–601.
45. Han, Y., Q. Guo, M. Zhang, Z. Chen, and X. Cao. 2009. CD69+ CD4+ CD25- T cells, a new subset of regulatory T cells, suppress T cell proliferation through membrane-bound TGF-beta 1. *J. Immunol.* 182: 111–120.
46. Chen, M. L., B. S. Yan, Y. Bando, V. K. Kuchroo, and H. L. Weiner. 2008. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis. *J. Immunol.* 180: 7327–7337.
47. Tran, D. Q., J. Andersson, D. Hardwick, L. Bebris, G. G. Illei, and E. M. Shevach. 2009. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. *Blood* 113: 5125–5133.
48. Andersson, J., D. Q. Tran, M. Pesu, T. S. Davidson, H. Ramsey, J. J. O'Shea, and E. M. Shevach. 2008. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J. Exp. Med.* 205: 1975–1981.
49. Gandhi, R., M. F. Farez, Y. Wang, D. Kozoriz, F. J. Quintana, and H. L. Weiner. 2010. Cutting edge: human latency-associated peptide+ T cells: a novel regulatory T cell subset. *J. Immunol.* 184: 4620–4624.
50. Huehn, J., and A. Hamann. 2005. Homing to suppress: address codes for Treg migration. *Trends Immunol.* 26: 632–636.
51. Wei, S., I. Kryczek, and W. Zou. 2006. Regulatory T-cell compartmentalization and trafficking. *Blood* 108: 426–431.
52. Baatar, D., P. Olkhanud, K. Sumitomo, D. Taub, R. Gress, and A. Biragyn. 2007. Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL. *J. Immunol.* 178: 4891–4900.
53. Mikhak, Z., M. Fukui, A. Farsidjani, B. D. Medoff, A. M. Tager, and A. D. Luster. 2009. Contribution of CCR4 and CCR8 to antigen-specific T(H)2 cell trafficking in allergic pulmonary inflammation. *J. Allergy Clin. Immunol.* 123: 67–73.e3.
54. Sather, B. D., P. Treuting, N. Perdue, M. Miazgowiec, J. D. Fontenot, A. Y. Rudensky, and D. J. Campbell. 2007. Altering the distribution of Foxp3(+)

- regulatory T cells results in tissue-specific inflammatory disease. *J. Exp. Med.* 204: 1335–1347.
55. Tiemessen, M. M., A. L. Jagger, H. G. Evans, M. J. C. van Herwijnen, S. John, and L. S. Taams. 2007. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc. Natl. Acad. Sci. USA* 104: 19446–19451.
56. Günther, C., C. Bello-Fernandez, T. Kopp, J. Kund, N. Carballido-Perrig, S. Hinteregger, S. Fassel, C. Schwärzler, G. Lametschwandner, G. Stingl, et al. 2005. CCL18 is expressed in atopic dermatitis and mediates skin homing of human memory T cells. *J. Immunol.* 174: 1723–1728.
57. El-Asrar, A. M. A., S. Struyf, S. A. Al-Kharashi, L. Missotten, J. Van Damme, and K. Geboes. 2002. Expression of T lymphocyte chemoattractants and activation markers in vernal keratoconjunctivitis. *Br. J. Ophthalmol.* 86: 1175–1180.
58. Umetsu, D. T., J. J. McIntire, O. Akbari, C. Macaubas, and R. H. DeKruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3: 715–720.
59. Kim, B. S., I. K. Kim, Y. J. Park, Y. S. Kim, Y. J. Kim, W. S. Chang, Y. S. Lee, M. N. Kweon, Y. Chung, and C. Y. Kang. 2010. Conversion of Th2 memory cells into Foxp3+ regulatory T cells suppressing Th2-mediated allergic asthma. *Proc. Natl. Acad. Sci. USA* 107: 8742–8747.
60. Curotto de Lafaille, M. A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J. J. Lafaille. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29: 114–126.