

The chemokine CCL18 generates adaptive regulatory T cells from memory CD4⁺ T cells of healthy but not allergic subjects

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ABSTRACT The purpose of this study was to assess the direct effect of CCL18, a chemokine elevated in allergic diseases and induced by Th2 cytokines, on the polarization of human CD4⁺ T cells. Purified human T cells from healthy subjects were pretreated or not with CCL18, and evaluated for cytokine production. CCL18-pretreated memory but not naive CD4⁺ T cells exhibited an increased production of IL-10 (12.3±2.6 vs. 5.6±0.9 ng/ml for medium) and TGF-β1 but not IL-4, IFN-γ, and IL-17 compared with control cells. Pretreatment of highly purified CD4⁺CD25[−] memory T cells with CCL18 led to their conversion to CD4⁺CD25[−]Foxp3⁺ regulatory T cells able to inhibit the proliferation of CD4⁺CD25[−] effector T cells by both cytokine and cell contact-dependent mechanisms. However, this regulatory effect of CCL18 was lost when T cells originated from allergic subjects in relation with a decreased binding of CCL18 to these cells [0.7±0.3 mean fluorescence intensity (MFI)] as compared to those from healthy subjects (6.0±1.7 MFI). This study is the first to define a chemokine that generates adaptive regulatory T cells from CD4⁺CD25[−] memory T cells. This mechanism appears defective in allergic patients and may underlie the decreased tolerance observed in allergic diseases.—Chang, Y., de Nadai, P., Azzaoui, I., Morales, O., Delhem, N., Vorng, H., Tomavo, S., Ait Yahia, S., Zhang, G., Wallaert, B., Chenivresse, C., Tsicopoulos, A. The chemokine CCL18 generates adaptive regulatory T cells from memory CD4⁺ T cells of healthy but not allergic subjects. *FASEB J.* 24, 000–000 (2010). www.fasebj.org

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CHEMOKINES ARE A SUPERFAMILY of small proteins that bind to G-protein-coupled receptors on target cells and act as mediators of directional migration of immune cells to sites of inflammation and injury (1–3). The importance of chemokines has grown in recent years, as it has become increasingly recognized that they are key

players in many disease processes, including inflammation, autoimmune diseases, infectious diseases, and cancer (4). Many evidences suggest that their function is not solely restricted to chemotaxis, since chemokines have been implicated in many other immune functions such as dendritic cell maturation (5–7), B-cell antibody class switching (8), T-cell activation (9–12), and differentiation. For the latter, it has been shown that some chemokines, such as MIP-1α, favor Th1 differentiation, whereas MCP-1 favors Th2 differentiation (13).

CCL18, previously named PARC (pulmonary and activated regulated chemokine) (14), DC-CK1 (dendritic cell-derived chemokine 1) (15), and AMAC-1 (alternative macrophage activation-associated CC chemokine-1) (16), belongs to the CC-chemokine family and is strongly expressed in human lung and less in thymus and lymph nodes (16). CCL18 is mainly secreted by antigen-presenting cells (APCs), including monocytes, alveolar macrophages, and monocyte-derived DCs (MDDCs) (15–17). CCL18 expression in alveolar macrophages, DCs, and monocytes is up-regulated by the Th2 cytokines IL-4 and IL-13, but also by IL-10, and inhibited by IFN-γ (16). Hitherto, CCL18 receptors were still unknown, and it has not been possible to assess CCL18 functionality in murine models, because the CCL18 gene probably does not exist in mice. Indeed, this gene has been generated by fusion of duplicated MIP-1α genes, likely to have occurred after the diversification of rodents and primates (18).

CCL18 is mainly chemotactic for naive T cells (15), but can also attract skin-homing memory T cells (19), Th2 cells (20), basophils (20), B lymphocytes (21), and

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immature DCs (22). High expression of CCL18 has been reported in allergic diseases and various inflammatory disorders (23). In the laboratory, we have shown an association between CCL18 and allergic asthma (20) with increased CCL18 production in bronchoalveolar lavages and sera from asthmatic allergic patients, compared with healthy subjects. It was also reported that CCL18 serum level correlated with the disease severity of atopic dermatitis (24), was present in skin biopsies of atopic dermatitis patients but not in normal or psoriatic skin, and mediated skin homing of a subpopulation of human memory T cells (19). Few studies have investigated the function of CCL18 besides its chemotactic effect. Recently, Wimmer *et al.* (25) reported that CCL18 enhanced the proliferation of monocytes and increased cytokine production, thereby promoting hematopoiesis in long-term bone marrow cultures.

In the present study, we investigated the nonchemotactic effects of CCL18 on human peripheral CD4⁺ T cells, with the hypothesis that CCL18 may favor a Th2 cytokine profile. This study shows unexpectedly and for the first time to our knowledge, that a chemokine can convert memory CD4⁺CD25⁻ T cells from healthy subjects into T cells expressing a regulatory phenotype, suggesting that some chemokines may directly participate in the suppression of inflammatory responses. Conversely, this property was lost in allergic subjects in association with a decreased binding of CCL18 to its putative receptor.

MATERIALS AND METHODS

Subjects

Venous blood was obtained from 24 healthy nonallergic (NA) subjects, with negative skin-prick tests, total IgE levels <100 kU/L, and negative RAST. Venous blood was also collected from 8 allergic patients. These patients had a clinical history of allergy and exhibited positive skin-prick tests toward usual aeroallergens, positive radioallergosorbent tests (RAST class 3), and elevated serum IgE levels. The study was approved by the Centre Hospitalier Régional et Universitaire de Lille ethics review committee, and all donors signed an informed consent form.

Isolation of naive and memory CD4⁺ T cells and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density gradient centrifugation over Ficoll/Hypaque (Pharmacia, Uppsala, Sweden). Human memory CD4⁺CD45RO⁺ and naive CD4⁺CD45RA⁺ cells were isolated from PBMCs by negative selection over a MACS column using memory and naive CD4⁺ T-cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) with a purity of 96–98%. The RPMI 1640 medium used for culture was supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. Memory and naive CD4⁺ cells (10⁶ cells/ml) were incubated with recombinant human CCL18 (R&D Systems, Minneapolis, USA) at concentrations ranging from 10⁻⁷ to 10⁻¹⁰ M, and 10⁻⁷ M

recombinant human SDF1α/CXCL12 (PeproTech, Rocky Hill, NJ, USA) and medium as controls. In some experiments, neutralizing anti-CCL18 antibody (Ab) or neutralizing pan-anti-TGF-β Ab (R&D Systems), isotype control, or pertussis toxin (Invitrogen, Camarillo, CA, USA) were added to the culture conditions. Preliminary kinetics experiments (between 2.5 and 24 h) showed that the optimal incubation time for maximal cytokine production was 2.5 h. After 2.5 h incubation at 37°C in 5% CO₂, the cells were washed and stimulated or not (10⁵ cells/well) with 10 µg/ml coated anti-CD3 mAb (OKT3 clone) and 2 µg/ml soluble anti-CD28 mAb (BD Pharmingen, Franklin Lakes, NJ, USA) in 96-well, flat-bottom microculture plates (BD Pharmingen). After 3 d of stimulation, the culture supernatants and cells were harvested for further experiments. In some experiments, after the 3 d of stimulation, cells were rested and followed daily in terms of cytokine production and regulatory phenotype (see below). Cell proliferation was evaluated using the BrdU ELISA kit (Roche, Mannheim, Germany), according to the manufacturer's protocol.

Flow cytometry sorting of T-cell subsets

PBMCs were isolated from peripheral blood, as described above, and CD4⁺ or CD4⁺CD45RO⁺ T cells were purified by magnetic separation (Miltenyi Biotec). Cells were further stained with anti-CD4-FITC (BD Pharmingen) and anti-CD25-PE (BD Pharmingen) for 30 min on ice, washed in PBS/2% BSA, filtered, and then sorted by flow cytometry (FACS Aria; BD Biosciences, San Jose, CA, USA). CD4⁺CD25⁻, CD4⁺CD25^{high}, CD4⁺CD45RO⁺CD25⁻ T-cell purity was >98%.

Quantification of the cytokine profile

Concentration of IL-4, IL-10, IFN-γ (Diaclone Research, Besançon, France), IL-17, and TGF-β1 (R&D Systems) in cell culture supernatants was measured by means of ELISA, according to the manufacturer's recommendations, and expressed in nanograms per milliliter. The level of sensitivity was 1.1 pg/ml for IL-4; 12.5 pg/ml for IL-10 and IFN-γ, 15.6 pg/ml for IL-17 and TGF-β1. Results were expressed as means ± SE.

Flow cytometry phenotype analysis

The phenotypes related to regulatory T cells were evaluated by flow cytometry using a standardized protocol as described previously (26). For the extracellular markers, cells were stained with anti-CD25-APC (BD Pharmingen), anti-CD103-FITC (Beckman Coulter, Fullerton, CA, USA), anti-CTLA-4-FITC (R&D Systems) and biotinylated latency-associated peptide of TGF-β1 (R&D Systems) followed by streptavidin-PE (R&D Systems). For the intracellular marker, cells were labeled by using anti-human Foxp3-FITC staining set (eBioscience, San Diego, CA, USA), according to the manufacturer's recommendations. For CCL18 receptor expression, cells were labeled with biotin conjugated CCL18 (Sigma, Steinheim, Germany) followed by streptavidin-APC (R&D Systems). As a control, we used a low-molecular-weight protein derived from *Plasmodium falciparum*. The complete open reading frame of a gene encoding the *P. falciparum* TAF10 homologue (27) 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 (Amersham) according to manufacturer's instructions). The purified *P. falciparum* TAF10 was subjected to SDS-PAGE followed by Coomassie blue staining and then labeled using a biotinylation kit (Sigma). In some experiments, naive and memory T cells were incubated with recombinant human cytokines (IL-4, IL-13, IL-10, and IFN- γ ; all used at 10 ng/ml and purchased from R&D Systems) for 24 h, and assessed for CCL18 receptor expression by flow cytometry as described above.

Real-time quantitative RT-PCR

After removal of the supernatants, the memory CD4⁺ T cells or CD4⁺CD45RO⁺CD25⁻ T cells were resuspended in TRI Reagent (Ambion, Austin, TX, USA), 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, USA). 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 $\delta\delta$ Ct method. Primer pairs were as follows: Foxp3, 5'-GAAACAGCACATTCACAGAGTTC-3' and 5'-ATGGCCCAGCGGATGAG-3'; β -actin, 5'-TCCTCACCCTGAAGTACCCCA-3', and 5'-AGCCACACGCAGCTCAT-TGT-3'.

Proliferation suppression assays

Flow cytometry sorted CD4⁺CD25⁻ T cells (5×10^4 /well) were stimulated in triplicate wells with soluble anti-CD3 mAb (10 ng/ml) and anti-CD28 mAb (10 ng/ml) (BD Pharmingen) in 96-well round-bottom plates (Corning Costar, Corning, NY, USA), in the absence or presence of CCL18-pretreated CD4⁺CD45RO⁺CD25⁻ T cells at suppressor/responder ratios of 1:4, 1:2 and 1:1. Irradiated (5000 rad) autologous PBMCs were used as APCs (5×10^4 /well). Purified sorted CD4⁺CD25⁺ cells were used as positive control of T-cell suppression and medium- and CXCL12-pretreated CD4⁺CD45RO⁺CD25⁻ T cells as negative controls of T-cell suppression. Proliferation was measured in triplicate after [methyl-³H]-thymidine (³H-TdR; 1 μ Ci/well; GE Healthcare, Little Chalfont, UK) 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). Incorporated radioactive thymidine was detected by scintillation counting using a 1450 Trilux β -counter (Wallac), and estimated in count per minute (cpm). Results were expressed as mean $\pm$ SE percentage of proliferation of CD4⁺CD25⁻ effector cells alone. In some experiments, 1 μ g/ml anti-IL-10 (R&D Systems), anti-TGF β 1 (R&D Systems), or isotype antibody was added to the cultures. To investigate a contact-dependent mechanism, CD4⁺CD25⁻ T cells were separated from CCL18-pretreated CD4⁺CD45RO⁺CD25⁻ T cells by using a 96-transwell plate with 0.4- μ m polycarbonate membrane (Corning, Avon, France).

Statistical analysis

Statistical analysis for production of cytokines was first performed within the subgroups by using the ANOVA test and,

when significant, followed by the *post hoc* Bonferroni-Dunn test. The paired *t* test was performed for phenotype analysis and suppression assays. Values of $P < 0.05$ were considered statistically significant. Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

CCL18 increases IL-10 and TGF- β 1 production of memory CD4⁺ T cells from healthy subjects

Naive and memory CD4⁺ T cells from healthy subjects were incubated with 10^{-7} to 10^{-10} M CCL18 for 2.5 h, washed, and stimulated or not for 3 d to determine whether CCL18 could modify T-cell cytokine production. At baseline, no cytokine production was observed in the absence of anti-CD3/CD28 stimulation except for TGF- β 1. In CCL18-pretreated naive CD4⁺ T cells, no cytokine modification was observed (Fig. 1A), whereas a marked change of a selective cytokine profile occurred in stimulated memory CD4⁺ T cells pretreated with CCL18. IL-10 production significantly increased in stimulated memory CD4⁺ T cells pretreated with 10^{-9} and 10^{-10} M CCL18 (Fig. 1B; 12.3 ± 2.6 vs. 5.6 ± 0.9 ng/ml for medium) as did TGF- β 1 in both stimulated and nonstimulated cells treated by 10^{-10} M CCL18 (3.0 ± 0.6 vs. 1.4 ± 0.2 ng/ml for medium and 2.3 ± 0.5 vs. 1.0 ± 0.1 ng/ml for medium, respectively). The effect was lost at 10^{-12} M CCL18 (data not shown). The increased production of IL-10 and TGF- β 1 was not related to increased cell numbers, as verified by evaluating the cell proliferation after 3 d of stimulation, which did not differ between the different conditions (data not shown). The increased production of IL-10 and TGF- β 1 was CCL18 specific, as evidenced by its suppression when a neutralizing anti-CCL18 antibody was introduced in the assay (data not shown). In contrast, the secretion of IL-4, IFN- γ , and IL-17 was not affected by CCL18 pretreatment. This change of cytokine profile suggested that CCL18 pretreatment might favor the development of regulatory T cells able to secrete antiinflammatory cytokines, such as IL-10 and/or TGF- β 1 (28).

CCL18 increases the expression of markers associated with regulatory cells on memory CD4⁺ T cells through TGF- β production

To further evaluate whether CCL18 may be involved in the development of regulatory T cells, the phenotypes associated with regulatory T cells were assessed. Even though there is no clear specific cell surface marker of regulatory cells, some markers have been shown to be preferentially expressed on human regulatory T cells, in particular, CD103 (α E β 7 integrin) (29), CTLA4 (30), and LAP/TGF β 1 (31). CCL18 pretreatment at

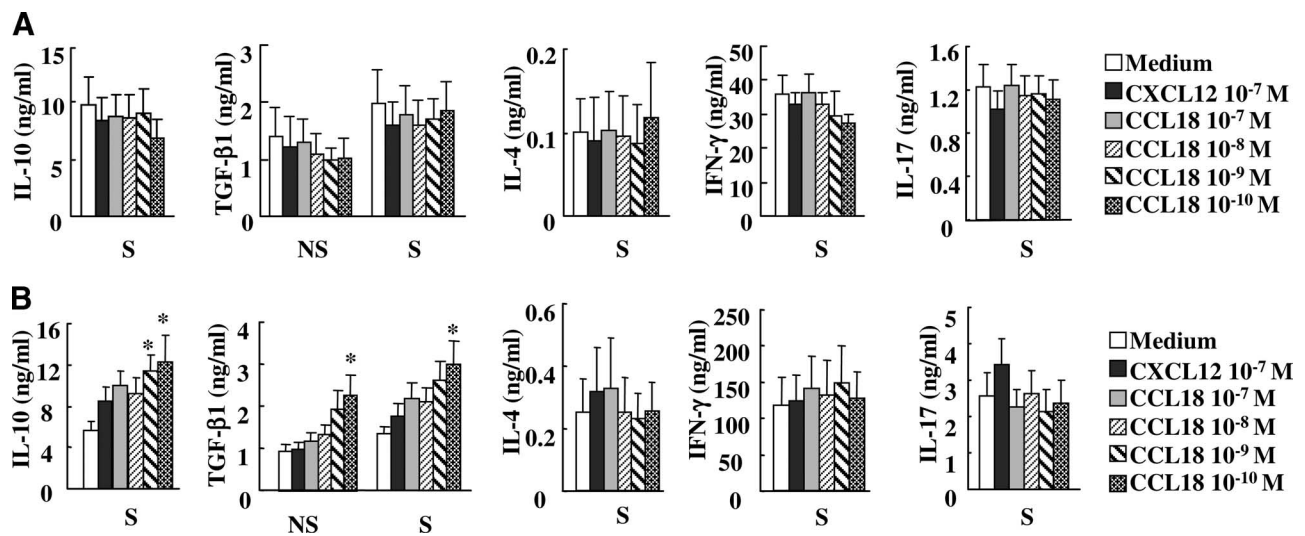


Figure 1. Cytokine profiles of pretreated naive and memory $CD4^+$ T cells from healthy subjects. Peripheral naive (A) or memory (B) $CD4^+$ T cells were pretreated with medium, SDF-1 α /CXCL12, or CCL18 for 2.5 h, then washed and stimulated or not for 3 d. Production level of IL-10, TGF- β 1, IL-4, IFN- γ , and IL-17 was evaluated by ELISA. Results are expressed as means $\pm$ SE for $n = 12$ subjects. * $P < 0.05$ vs. control group. S, stimulated; NS, nonstimulated.

10^{-10} M significantly increased the expression of the surface molecules CD103 and CTLA-4 on stimulated memory $CD4^+$ T cells (Fig. 2A), as well as the expression of CTLA-4 and LAP/TGF- β 1 on nonstimulated memory $CD4^+$ T cells (Fig. 2B) as evaluated by flow cytometry. As a more specific readout of regulatory T cells, the specific regulatory T-cell transcription factor Foxp3 (32) was assessed intracellularly. The percentage

of $CD25^+Foxp3^+$ cells was significantly increased after CCL18 pretreatment, in both stimulated (Fig. 2A; 18.1 ± 5.6 vs. $12.8 \pm 4.1\%$; $n = 6$) ($P < 0.05$) and nonstimulated memory $CD4^+$ T cells pretreated with CCL18 (Fig. 2B; 4.3 ± 0.8 vs. $2.1 \pm 0.5\%$; $n = 6$; $P < 0.05$). The kinetics of Foxp3 mRNA expression in memory $CD4^+$ T cells was thus examined after 10^{-10} M CCL18 pretreatment. As shown in Fig. 2C, Foxp3 mRNA expression

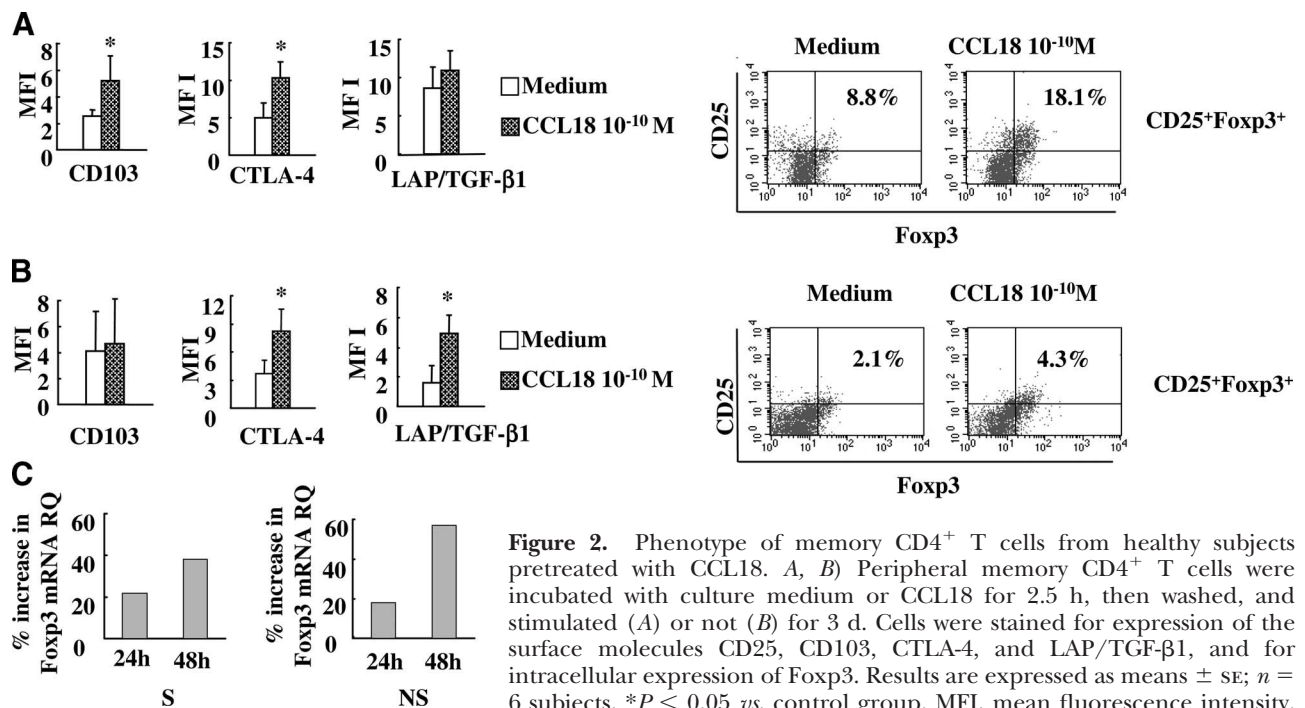


Figure 2. Phenotype of memory $CD4^+$ T cells from healthy subjects pretreated with CCL18. A, B) Peripheral memory $CD4^+$ T cells were incubated with culture medium or CCL18 for 2.5 h, then washed, and stimulated (A) or not (B) for 3 d. Cells were stained for expression of the surface molecules CD25, CD103, CTLA-4, and LAP/TGF- β 1, and for intracellular expression of Foxp3. Results are expressed as means $\pm$ SE; $n = 6$ subjects. * $P < 0.05$ vs. control group. MFI, mean fluorescence intensity. For the positive $CD25^+Foxp3^+$ cells, a representative result of 6 experiments is shown. C) Kinetics of Foxp3 mRNA expression of memory $CD4^+$ T cells incubated with CCL18. Peripheral memory $CD4^+$ T cells were incubated with culture medium or CCL18 for 2.5 h, then washed and stimulated (S) or not (NS) for 24 and 48 h. Total RNA was extracted, and the relative quantity (RQ) of Foxp3 was determined by real-time RT-PCR. A representative result of 4 experiments showing the percentage increase in Foxp3 mRNA relative quantity in CCL18- vs. medium-treated cells is shown.

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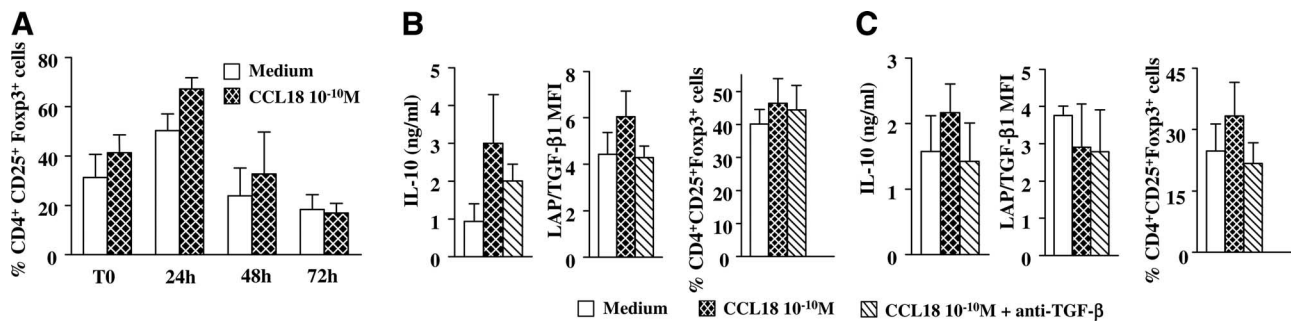


Figure 3. Kinetics of the lasting effect of CCL18 and its dependence on TGF- β . A) Peripheral memory CD4⁺ T cells were incubated with culture medium or CCL18 for 2.5 h; then they were washed and stimulated for 3 d (T0). Cells were then rested for another 24, 48, or 72 h and stained to determine the percentage of CD4⁺CD25⁺Foxp3⁺ cells. B, C) Peripheral memory CD4⁺ T cells were incubated with culture medium or CCL18 for 2.5 h; then they were washed, incubated with neutralizing anti TGF- β , and stimulated for 72 h (B) or 96 h (C). Production of IL-10, expression of LAP/TGF- β 1, and percentage of CD4⁺CD25⁺Foxp3⁺ T cells were assessed. Results are expressed as means $\pm$ SE; n = 3 or 4 subjects.

increased in both stimulated and nonstimulated memory CD4⁺ T cells pretreated with CCL18 at 24 and 48 h as compared with nonpretreated cells. To evaluate whether the Treg phenotype was long lasting, cells were rested after the 72 h of stimulation, and assessed daily for another 72 h. As shown in Fig. 3A, the increased number of CD4⁺CD25⁺Foxp3⁺ cells after CCL18 pretreatment persisted for another 48 h and returned to the control level after 72 h.

As TGF- β 1 was the only cytokine induced by CCL18 in nonstimulated conditions, we evaluated the role of this cytokine in the mechanisms of action of CCL18 on the regulatory phenotype. Memory T cells pretreated with CCL18 were incubated in the presence of neutralizing anti-TGF- β antibody and stimulated for 72 or 96 h. As shown in Fig. 3B, neutralization of TGF- β for 72 h completely inhibited CCL18-induced expression of LAP/TGF β 1 and partly suppressed the production of IL-10, but had no effect on the percentage of CD4⁺CD25⁺Foxp3⁺ cells. However, after 96 h of treatment, neutralization of TGF- β completely suppressed the enhanced IL-10 production and generation of CD4⁺CD25⁺Foxp3⁺ cells, whereas LAP/TGF β 1 was no more induced by CCL18 (Fig. 3C).

These results suggest that CCL18 pretreatment of memory CD4⁺ T cells is able to induce CD25⁺Foxp3⁺ cells consistent with the induction of regulatory T cells, through an initial mechanism involving TGF- β .

CCL18 switches memory CD4⁺CD25⁻ T cells into cells expressing a regulatory phenotype

To evaluate whether CCL18 was amplifying the CD4⁺CD25⁺ subpopulation present in the memory CD4⁺ T cells, or was able to switch CD4⁺CD25⁻ T cells toward a regulatory phenotype, memory CD4⁺ T cells were depleted from CD25⁺ cells by flow cytometry sorting, and incubated with CCL18 for 2 h and 30 min. The same endpoints as for total memory CD4⁺ T cells were examined. As previously observed with total memory CD4⁺ T cells, CCL18-pretreated memory CD4⁺CD25⁻ T cells exhibited significant increased secretion in IL-10 and TGF- β 1 (Fig. 4A), however, at doses of CCL18 100-fold higher than for total CD4⁺ memory T cells. Similar results were also observed in the other assays, including T regulatory phenotype evaluation (Fig. 4B) and Foxp3 mRNA expression (Fig. 4C).

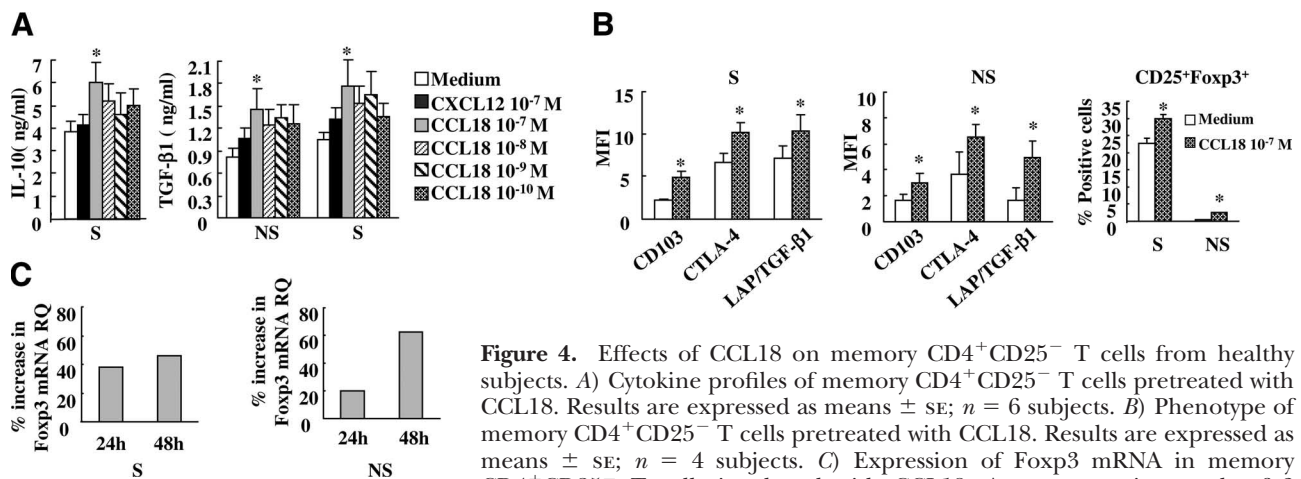


Figure 4. Effects of CCL18 on memory CD4⁺CD25⁻ T cells from healthy subjects. A) Cytokine profiles of memory CD4⁺CD25⁻ T cells pretreated with CCL18. Results are expressed as means $\pm$ SE; n = 6 subjects. B) Phenotype of memory CD4⁺CD25⁻ T cells pretreated with CCL18. Results are expressed as means $\pm$ SE; n = 4 subjects. C) Expression of Foxp3 mRNA in memory CD4⁺CD25⁻ T cells incubated with CCL18. A representative result of 3 experiments showing the percentage increase in Foxp3 mRNA relative quantity in CCL18- vs. medium-treated cells is shown. RQ, relative quantity; S, stimulated; NS, nonstimulated. * P < 0.05 vs. control group.

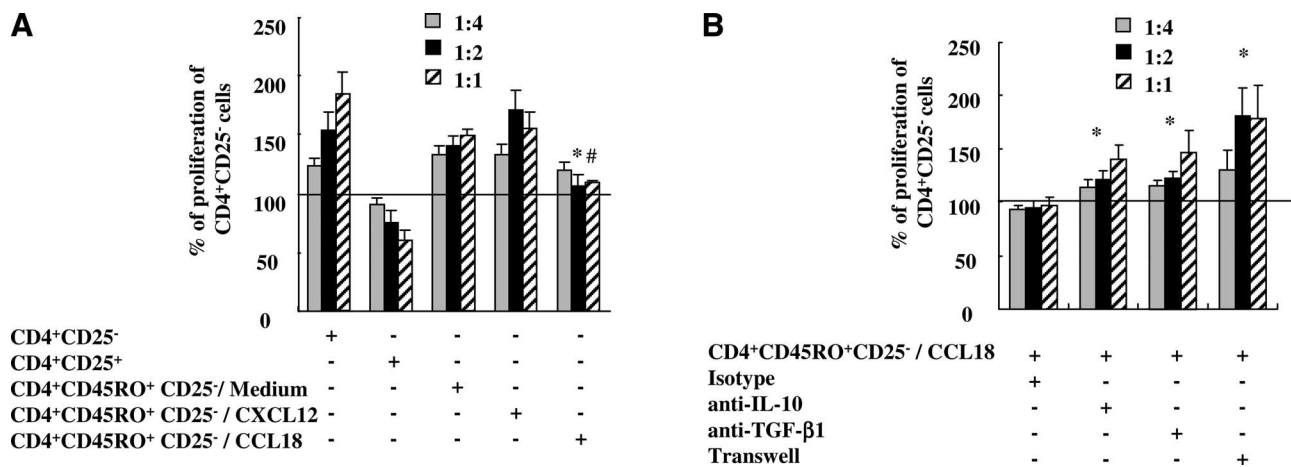


Figure 5. Inhibitory effect of CCL18-pretreated memory CD4⁺CD25⁻ T cells on proliferation of CD4⁺CD25⁻ effector cells. A) Memory CD4⁺CD25⁻ T cells pretreated with SDF-1α/CXCL12 or CCL18 were added at suppressor/responder ratios of 1:4, 1:2, and 1:1 to autologous CD4⁺CD25⁻ effector cells stimulated with soluble anti-CD3 and CD28 antibodies. Irradiated autologous PBMCs were used as APCs. Proliferation was measured by incorporation of ³H-thymidine at 48 h. The CD4⁺CD25⁻; CD4⁺CD25⁺ T cells were used as negative and positive controls, respectively. Results are presented as mean ± SE percentage of proliferation of CD4⁺CD25⁻ cells alone. **P* < 0.05, #*P* < 0.01 vs. medium-pretreated memory CD4⁺CD25⁻ T cells. B) Mechanisms involved in the inhibitory effect of CCL18-pretreated cells on the proliferation of CD4⁺CD25⁻ effector cells. Isotype, anti-IL-10, or anti-TGF-β1 neutralizing antibodies were added to the coculture system; a transwell was used to abrogate cell contact between the suppressor and responder cells. Results are expressed as mean ± SE percentage of proliferation of CD4⁺CD25⁻ T cells cocultured with medium-pretreated memory CD4⁺CD25⁻ T cells. **P* < 0.05 vs. isotype-treated memory T cells; *n* = 3 experiments with triplicate wells for each condition.

CCL18-pretreated memory CD4⁺CD25⁻ T cells inhibit CD4⁺CD25⁻ effector T-cell proliferation by both cytokine and cell contact-dependent mechanisms through activation of G-protein-coupled receptors

To definitely confirm that CCL18 pretreatment switches CD4⁺CD45RO⁺CD25⁻ to regulatory T cells, we next examined the cells for their suppressive function by evaluating T-cell proliferation. Highly purified flow cytometry sorted CD4⁺CD25⁺ cells and memory CD4⁺CD25⁻ T cells pretreated with medium, CXCL12, or CCL18 were added at increasing ratios to a fixed number of irradiated PBMC and autologous CD4⁺CD25⁻ effector cells stimulated with soluble anti-CD3 and CD28 antibodies. The addition of CD4⁺CD25⁻ effector cells resulted in increased T-cell proliferation, whereas the addition of CD4⁺CD25⁺ regulatory T cells resulted in a ratio-dependent suppression of proliferation (69 ± 8% inhibition at a ratio of 1:1; Fig. 5A). Addition of memory CD4⁺CD25⁻ T cells pretreated with medium or SDF-1α/CXCL12 did not significantly suppress T-cell proliferation compared with the negative control (CD4⁺CD25⁻ T cells). In contrast, the addition of CCL18-pretreated memory CD4⁺CD25⁻ T cells significantly suppressed proliferation compared with the negative control (12 ± 7, 35 ± 6, and 45 ± 5% inhibition at 1:4, 1:2, and 1:1 ratio, respectively) and with medium pretreated memory CD4⁺CD25⁻ T (0, 15 ± 8, and 27 ± 6% inhibition at 1:4, 1:2, and 1:1 ratio, respectively; Fig. 5A). Regulatory T cells have been shown to act through two main mechanisms, production of suppressive cytokines, such as IL-10 and TGF-β, and through cell contact inhibition (32). To determine the mechanism involved in the suppressive effect of CCL18-pretreated memory CD4⁺CD25⁻ T cells, inhibition and cell contact experiments were performed.

Blocking anti-IL-10 and anti-TGF-β1 Abs were added to the proliferation assays to evaluate their involvement in the suppressive ability of CCL18-pretreated memory CD4⁺CD25⁻ T cells. As shown in Fig. 5B, blockade of IL-10 or of TGF-β1 partially but significantly restored the proliferation of CCL18-pretreated memory CD4⁺CD25⁻ T cells at all ratios, reducing the inhibition from 32 ± 6% for the control isotype to 14 ± 3% and 13 ± 5% for IL-10 and TGF-β antibodies at a ratio of 1:1. To determine whether part of the suppressive effect was cell contact dependent, CCL18-pretreated memory CD4⁺CD25⁻ T cells were separated from CD4⁺CD25⁻ effector cells by a 0.4-μm pore size membrane in a transwell. Under this condition, the proliferation of CD4⁺CD25⁻ effector cells was almost fully restored, with a residual inhibition of 9 ± 4% at a ratio of 1:1. These results suggest that CCL18-pretreated memory CD4⁺CD25⁻ T cells inhibit CD4⁺CD25⁻ effector cell proliferation by both cytokine and cell contact-dependent mechanisms. The major pathway of action of chemokines is through their binding to specific G-protein-coupled receptors, which leads to cell signaling and activation. However, chemokines can also activate cells through glycoaminoglycan binding (33). To determine the pathway involved, the G-protein inhibitor pertussis toxin was added to the cultures before CCL18 pretreatment. Pertussis toxin totally inhibited the increased production of IL-10 and TGF-β1 induced by CCL18 treatment of memory CD4⁺ T cells (data not shown). Collectively, these data indicate that CCL18 generates CD4⁺CD25⁺Foxp3⁺ Treg cells from CD4⁺CD25⁻ T cells, through a G-protein-coupled re-

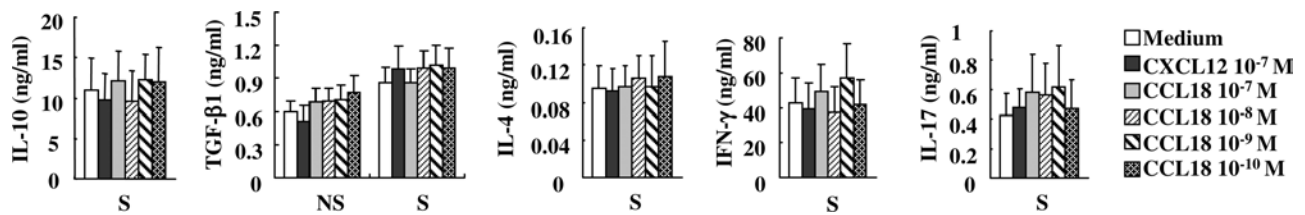


Figure 6. Cytokine profiles of CCL18-pretreated memory CD4⁺ T cells from allergic subjects. Peripheral memory CD4⁺ T cells from allergic subjects were incubated with medium, SDF-1 α /CXCL12, or CCL18 for 2.5 h, then washed and stimulated (S) or not (NS) (21) for 3 d. Production level of IL-10, TGF- β 1, IL-4, IFN- γ , and IL-17 was evaluated by ELISA. Results are expressed as means $\pm$ SE; $n = 5$ subjects.

ceptor, and acts by cytokine- and contact-dependent mechanisms.

CCL18 does not modify the cytokine production of memory CD4⁺ T cells from allergic subjects linked to a decreased binding to its cell surface receptor

We have previously shown in the laboratory that CCL18 secretion is increased in allergic asthmatic subjects at the circulating and bronchoalveolar levels (20). Therefore, we also investigated whether CCL18 might affect the function of memory CD4⁺ T cells from allergic subjects. However, no change was observed in the cytokine profiles of memory CD4⁺ T cells pretreated by CCL18 (Fig. 6). These results suggest that memory CD4⁺ T cells of healthy and allergic subjects may differentially respond to CCL18. Although the precise nature of CCL18 receptor (CCL18R) is still unknown, its expression can be visualized using its labeled ligand. The expression of CCL18R was thus evaluated on the surface of memory CD4⁺ T cells by using biotin-conjugated CCL18. The results showed that memory CD4⁺ T cells from allergic subjects bound less CCL18 [mean percentage and mean fluorescence intensity (MFI) expression: $2.3 \pm 0.8\%$ and 0.7 ± 0.3 ; $n = 8$] than healthy subjects (mean percentage and MFI expression: $5.3 \pm 0.7\%$ and 6.0 ± 1.7 ; $n = 11$; Fig. 7A). Altogether, these data suggest that in allergic subjects, decreased CCL18R binding may explain the lack of regulatory effect of CCL18.

CCL18R is upregulated on naive but not memory T cells from nonallergic subjects by IL-10 and IL-13

To evaluate the potential regulation of CCL18R by cytokines, naive and memory T cells from nonallergic subjects were incubated with Th1, Th2, and Treg cytokines for 24 h, and CCL18R expression was assessed by flow cytometry. IL-10, IL-13, and to a lesser extent IL-4 upregulated CCL18R expression on naive T cells (Fig. 7B) but not on memory T cells (Fig. 7C), whereas IFN- γ had no effect. To try to up-regulate the receptor on T cells from allergic subjects, the same cytokine stimulation was used; however, no induction effect was observed either on naive or memory T cells (data not shown).

DISCUSSION

Chemokines have a fundamental role in providing directional clues for the trafficking of leukocytes to sites of inflammation. However, it has been increasingly evident that chemokines play a broader role in the activation and differentiation of T lymphocytes (9–12, 34). In the present study, we investigated whether CCL18 may have, beside its chemotactic activity, other immunological functions related to T-cell activation. We demonstrated that CCL18 is able to generate adaptive regulatory T cells from CD4⁺CD25[−] T cells of healthy subjects.

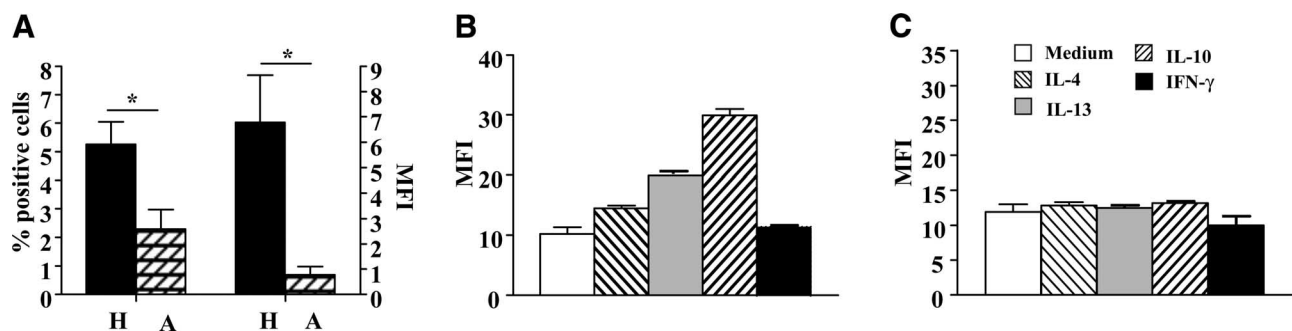


Figure 7. Cell surface expression of CCL18 receptors on CD4⁺ T cells from healthy and allergic subjects. A) Baseline expression of CCL18R evaluated on the surface of memory CD4⁺ T cells from healthy (H) and allergic (A) subjects expressed as mean $\pm$ SE percentage positive cells or MFI of $n = 11$ healthy and $n = 8$ allergic subjects. * $P < 0.02$. B, C). Cytokine-induced expression of CCL18R evaluated on the surface of naive (B) and memory (C) CD4⁺ T cells from healthy subjects, expressed as mean $\pm$ SE MFI of $n = 2$ subjects.

Although CCL18 is chemotactic for both naive and memory T cells (15, 19), only memory CD4⁺ T cells exhibited a change in their functional properties. This difference may be linked to the necessity for CCL18 to have additional signals present on memory T cells for regulatory T-cell conversion. For example, several lymphocyte adhesion molecules are expressed at higher levels or in a higher affinity state on memory T cells than on naive T cells. They have been shown to behave as costimulatory factors that differentially activate naive and memory T cells (35). Alternatively, CCL18 may recognize different chemokine receptors on naive and memory T cells and thus trigger different activation pathways. This hypothesis awaits the identification of the unknown receptors of CCL18.

Different populations of regulatory T (Treg) cells, including natural thymic derived and adaptive Treg cells, have been described. Natural CD4⁺CD25⁺ Treg cells have been shown to preferentially express some membrane-associated molecules, in particular, CD103 (α E β 7 integrin) (36), CTLA-4 (37) and membrane-bound TGF- β 1 (38), which are believed to play a role in their contact-dependent suppressive effect *in vitro*, (39, 40), whereas IL-10 and TGF- β are needed *in vivo* for mediating suppression or conditioning a suppressive milieu (41). They express the forkhead box P3 (FOXP3) transcription factor, which plays important roles in the genetic program governing regulatory T-cell development and function (42–44). On the other hand, adaptive regulatory T cells mediate their inhibitory activities by producing mainly immunosuppressive cytokines, such as TGF- β and IL-10 (45, 46). However, they might still require direct cell-cell contact to initiate the suppressive cascade (32, 47). In the present study, we demonstrate that CCL18 generates adaptive regulatory T cells using highly purified flow cytometry-sorted memory CD4⁺CD25⁺ T cells. Notably, we show that the cells exhibited suppressive membrane cell markers, Foxp3 expression, and IL-10 and TGF- β cytokine production, as well as a suppressive effect of T-cell proliferation, after CCL18 treatment. However, we cannot rule out an additional activating effect of CCL18 on natural Treg cells, since the optimal dose needed to convert CD4⁺CD25⁺ T cells into Treg cells was higher than that needed when CD4⁺CD25⁺ T cells were not depleted. Human Treg cells comprise a dynamic population, which is generated continuously from either preexisting CD45RO⁺ regulatory T cells or from CD4⁺CD25⁺ memory T cells that proliferate rapidly and then enter the regulatory T-cell pool (48–50). A few factors, including TGF- β 1 (51), thombospondin/CD47 interaction (52), and nitric oxide (53) have been shown to be able to generate regulatory T cells from CD4⁺CD25⁺ T cells. In our experiments, TGF- β was induced by CCL18, even without CD3/CD28 costimulation, and the generated Treg phenotype was similar to TGF- β -induced adaptive Tregs, as described in the literature, with CTLA4, CD25, and Foxp3 being expressed (54). Therefore, the role of TGF- β as an initial trigger was evaluated in

neutralizing experiments, which showed that indeed, TGF- β was involved in the generation of Tregs by CCL18, with a particular kinetics of events, since the first abolished event was the induction of LAP/TGF- β 1, followed by the abolition of IL-10 induction and the induction of Foxp3⁺ cells.

We and others have previously shown that CCL18 level is increased in allergic diseases, such as asthma and atopic dermatitis (19, 20). Therefore, we also investigated CCL18's effect on memory CD4⁺ T cells obtained from allergic subjects. However, no conversion toward a regulatory phenotype was observed, which was consistent with the decreased CCL18R staining on the cell surface of memory CD4⁺ T cells from allergic subjects. This may be related to a desensitization phenomenon linked to the elevated circulating levels of CCL18 found in allergic subjects. In this context, it would be of interest to manage to up-regulate CCL18R on memory T cells from allergic donors; however, the cytokines that we tested were not able to modulate its expression in either allergic or nonallergic subjects, although it had an enhancing effect on naive T cells from nonallergic subjects. The exact mechanisms regulating CCL18R expression in allergic subjects need further investigation. However, these data suggest that the decreased feedback regulation by CCL18 in allergic subjects may partly participate to the lack of resolution of allergic inflammatory reactions and may account for the elevated levels of CCL18 in allergic diseases though persistent Th2 cytokine production. These data also suggest that CCL18 may contribute to the homeostasis of regulatory T cells in the periphery and have an anti-inflammatory potential. This is in agreement with a previous study that reported that CCL18 was especially chemotactic for immature DC and may be part of an inhibitory pathway devoted to limiting the generation of specific immune responses at peripheral sites (22). This might particularly hold true for the lung, where CCL18 is constitutively expressed (14).

In summary, we show for the first time, to our knowledge, that a chemokine can generate adaptive regulatory T cells and may provide a negative feedback mechanism of inappropriate inflammatory responses. These findings suggest an important mechanism by which T cells may be regulated at the site of inflammation. This mechanism appears to be deficient in allergic patients, and may partly participate in the lack of resolution of the inflammatory allergic reaction in these patients. FJ

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REFERENCES

1. Le, Y., Zhou, Y., Iribarren, P., and Wang, J. (2004) Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell. Mol. Immunol.* 1, 95–104

2. Sallusto, F., and Baggiolini, M. (2008) Chemokines and leukocyte traffic. *Nat. Immunol.* **9**, 949–952
3. Bromley, S. K., Mempel, T. R., and Luster, A. D. (2008) Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat. Immunol.* **9**, 970–980
4. Zlotnik, A., Yoshie, O., and Nomiya, H. (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol.* **7**, 243
5. Braun, M. C., Lahey, E., and Kelsall, B. L. (2000) Selective suppression of IL-12 production by chemoattractants. *J. Immunol.* **164**, 3009–3017
6. Cappello, P., Fraone, T., Barberis, L., Costa, C., Hirsch, E., Elia, A. R., Caorsi, C., Musso, T., Novelli, F., and Giovarelli, M. (2006) CC-chemokine ligand 16 induces a novel maturation program in human immature monocyte-derived dendritic cells. *J. Immunol.* **177**, 6143–6151
7. Marsland, B. J., Battig, P., Bauer, M., Ruedl, C., Lassing, U., Beerli, R. R., Dietmeier, K., Ivanova, L., Pfister, T., Vogt, L., Nakano, H., Nembrini, C., Saudan, P., Kopf, M., and Bachmann, M. F. (2005) CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. *Immunity* **22**, 493–505
8. Shang, L., Fukata, M., Thirunarayanan, N., Martin, A. P., Arnaboldi, P., Maussang, D., Berin, C., Unkeless, J. C., Mayer, L., Abreu, M. T., and Lira, S. A. (2008) Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria. *Gastroenterology* **135**, 529–538
9. Nanki, T., and Lipsky, P. E. (2000) Cutting edge: stromal cell-derived factor-1 is a costimulator for CD4+ T cell activation. *J. Immunol.* **164**, 5010–5014
10. Taub, D. D., Turcovski-Corralles, S. M., Key, M. L., Longo, D. L., and Murphy, W. J. (1996) Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation in vitro. *J. Immunol.* **156**, 2095–2103
11. Bacon, K. B., Premack, B. A., Gardner, P., and Schall, T. J. (1995) Activation of dual T cell signaling pathways by the chemokine RANTES. *Science* **269**, 1727–1730
12. Molon, B., Gri, G., Bettella, M., Gomez-Mouton, C., Lanzavecchia, A., Martinez, A. C., Manes, S., and Viola, A. (2005) T cell costimulation by chemokine receptors. *Nat. Immunol.* **6**, 465–471
13. Karpus, W. J., Lukacs, N. W., Kennedy, K. J., Smith, W. S., Hurst, S. D., and Barrett, T. A. (1997) Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J. Immunol.* **158**, 4129–4136
14. Hieshima, K., Imai, T., Baba, M., Shoudai, K., Ishizuka, K., Nakagawa, T., Tsuruta, J., Takeya, M., Sakaki, Y., Takatsuki, K., Miura, R., Opendakker, G., Van Damme, J., Yoshie, O., and Nomiya, H. (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
15. Adema, G. J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., Foster, J., Xu, Y., Nooyen, P., McClanahan, T., Bacon, K. B., and Figdor, C. G. (1997) A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* **387**, 713–717
16. Kodelj, V., Muller, C., Politz, O., Hakij, N., Orfanos, C. E., and Goerdts, S. (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
17. Vissers, J. L., Hartgers, F. C., Lindhout, E., Teunissen, M. B., Figdor, C. G., and Adema, G. J. (2001) Quantitative analysis of chemokine expression by dendritic cell subsets in vitro and in vivo. *J. Leukoc. Biol.* **69**, 785–793
18. Tasaki, Y., Fukuda, S., Iio, M., Miura, R., Imai, T., Sugano, S., Yoshie, O., Hughes, A. L., and Nomiya, H. (1999) Chemokine PARC gene (SCYA18) generated by fusion of two MIP-1alpha/LD78alpha-like genes. *Genomics* **55**, 353–357
19. Gunther, C., Bello-Fernandez, C., Kopp, T., Kund, J., Carbalido-Perrig, N., Hinteregger, S., Fassl, S., Schwarzler, C., Lametschwandner, G., Stingl, G., Biedermann, T., and Carbalido, J. M. (2005) CCL18 is expressed in atopic dermatitis and mediates skin homing of human memory T cells. *J. Immunol.* **174**, 1723–1728
20. De Nadai, P., Charbonnier, A. S., Chenivesse, C., Senechal, S., Fournier, C., Gilet, J., Vornig, H., Chang, Y., Gosset, P., Wallaert, B., Tonnel, A. B., Lassalle, P., and Tsicopoulos, A. (2006) Involvement of CCL18 in allergic asthma. *J. Immunol.* **176**, 6286–6293
21. Lindhout, E., Vissers, J. L., Hartgers, F. C., Huijbens, R. J., Scharenborg, N. M., Figdor, C. G., and Adema, G. J. (2001) The dendritic cell-specific CC-chemokine DC-CK1 is expressed by germinal center dendritic cells and attracts CD38-negative mantle zone B lymphocytes. *J. Immunol.* **166**, 3284–3289
22. Vulcano, M., Struyf, S., Scapini, P., Cassatella, M., Bernasconi, S., Bonocchi, R., Calleri, A., Penna, G., Adorini, L., Luini, W., Mantovani, A., Van Damme, J., and Sozzani, S. (2003) Unique regulation of CCL18 production by maturing dendritic cells. *J. Immunol.* **170**, 3843–3849
23. Schutysse, E., Richmond, A., and Van Damme, J. (2005) Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J. Leukoc. Biol.* **78**, 14–26
24. Oflazoglu, E., Simpson, E. L., Takiguchi, R., Grewal, I. S., Hanifin, J. M., and Gerber, H. P. (2008) CD30 expression on CD1a+ and CD8+ cells in atopic dermatitis and correlation with disease severity. *Eur. J. Dermatol.* **18**, 41–49
25. Wimmer, A., Khaldoyanidi, S. K., Judex, M., Seroby, N., Discipio, R. G., and Schraufstatter, I. U. (2006) CCL18/PARC stimulates hematopoiesis in long-term bone marrow cultures indirectly through its effect on monocytes. *Blood* **108**, 3722–3729
26. Gilet, J., Chang, Y., Chenivesse, C., Legendre, B., Vornig, H., Duez, C., Wallaert, B., Porte, H., Senechal, S., and Tsicopoulos, A. (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
27. Callebaut, I., Prat, K., Meurice, E., Mornon, J. P., and Tomavo, S. (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
28. Maloy, K. J., and Powrie, F. (2001) Regulatory T cells in the control of immune pathology. *Nat. Immunol.* **2**, 816–822
29. Allakhverdi, Z., Fitzpatrick, D., Boisvert, A., Baba, N., Bouguermouh, S., Sarfati, M., and Delespesse, G. (2006) Expression of CD103 identifies human regulatory T-cell subsets. *J. Allergy Clin. Immunol.* **118**, 1342–1349
30. Birebent, B., Lorho, R., Lechartier, H., de Guibert, S., Alizadeh, M., Vu, N., Beauplet, A., Robillard, N., and Semana, G. (2004) Suppressive properties of human CD4+CD25+ regulatory T cells are dependent on CTLA-4 expression. *Eur. J. Immunol.* **34**, 3485–3496
31. Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H., and Strober, W. (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
32. Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008) Regulatory T cells and immune tolerance. *Cell* **133**, 775–787
33. Chang, T. L., Gordon, C. J., Rosic-Mrkic, B., Power, C., Proudfoot, A. E., Moore, J. P., and Trkola, A. (2002) Interaction of the CC-chemokine RANTES with glycosaminoglycans activates a p44/p42 mitogen-activated protein kinase-dependent signaling pathway and enhances human immunodeficiency virus type 1 infectivity. *J. Virol.* **76**, 2245–2254
34. Luther, S. A., and Cyster, J. G. (2001) Chemokines as regulators of T cell differentiation. *Nat. Immunol.* **2**, 102–107
35. Semnani, R. T., Nutman, T. B., Hochman, P., Shaw, S., and van Seventer, G. A. (1994) Costimulation by purified intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3 induces distinct proliferation, cytokine and cell surface antigen profiles in human “naive” and “memory” CD4+ T cells. *J. Exp. Med.* **180**, 2125–2135
36. Lehmann, J., Huehn, J., de la Rosa, M., Maszyzna, F., Kretschmer, U., Krenn, V., Brunner, M., Scheffold, A., and Hamann, A. (2002) Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 13031–13036
37. Tang, Q., Boden, E. K., Henriksen, K. J., Bour-Jordan, H., Bi, M., and Bluestone, J. A. (2004) Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* **34**, 2996–3005
38. Nakamura, K., Kitani, A., and Strober, W. (2001) Cell contact-dependent immunosuppression by CD4(+)CD25(+) regula-

- tory T cells is mediated by cell surface-bound transforming growth factor beta. *J. Exp. Med.* **194**, 629–644
39. Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10**, 1969–1980
 40. Thornton, A. M., and Shevach, E. M. (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296
 41. Miyara, M., and Sakaguchi, S. (2007) Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* **13**, 108–116
 42. Yagi, H., Nomura, T., Nakamura, K., Yamazaki, S., Kitawaki, T., Hori, S., Maeda, M., Onodera, M., Uchiyama, T., Fujii, S., and Sakaguchi, S. (2004) Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int. Immunol.* **16**, 1643–1656
 43. Hori, S., Nomura, T., and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061
 44. Wan, Y. Y., and Flavell, R. A. (2007) Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766–770
 45. Hawrylowicz, C. M., and O'Garra, A. (2005) Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* **5**, 271–283
 46. Li, M. O., and Flavell, R. A. (2008) Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* **28**, 468–476
 47. Bluestone, J. A., and Abbas, A. K. (2003) Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* **3**, 253–257
 48. Walker, L. S., Chodos, A., Eggena, M., Dooms, H., and Abbas, A. K. (2003) Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J. Exp. Med.* **198**, 249–258
 49. Vukmanovic-Stejic, M., Zhang, Y., Cook, J. E., Fletcher, J. M., McQuaid, A., Masters, J. E., Rustin, M. H., Taams, L. S., Beverley, P. C., Macallan, D. C., and Akbar, A. N. (2006) Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J. Clin. Invest.* **116**, 2423–2433
 50. Fisson, S., Darrasse-Jeze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R., and Salomon, B. L. (2003) Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J. Exp. Med.* **198**, 737–746
 51. Pyzik, M., and Piccirillo, C. A. (2007) TGF-beta1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets. *J. Leukoc. Biol.* **82**, 335–346
 52. Grimbort, P., Bouguermouh, S., Baba, N., Nakajima, T., Allakhverdi, Z., Braun, D., Saito, H., Rubio, M., Delespesse, G., and Sarfati, M. (2006) Thrombospondin/CD47 interaction: a pathway to generate regulatory T cells from human CD4+ CD25- T cells in response to inflammation. *J. Immunol.* **177**, 3534–3541
 53. Niedbala, W., Cai, B., Liu, H., Pitman, N., Chang, L., and Liew, F. Y. (2007) Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25- T cells via p53, IL-2, and OX40. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15478–15483
 54. Zheng, S. G., Wang, J. H., Stohl, W., Kim, K. S., Gray, J. D., and Horwitz, D. A. (2006) TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J. Immunol.* **176**, 3321–3329

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