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# Involvement of CCL18 in Allergic Asthma<sup>1</sup>

Patricia de Nadaï,\* Anne-Sophie Charbonnier,\* Cécile Chenivresse,\* Stéphanie Sénéchal,\* Clément Fournier,<sup>†</sup> Jules Gilet,\* Han Vorng,\* Ying Chang,\*<sup>‡</sup> Philippe Gosset,\* Benoît Wallaert,\*<sup>†</sup> André-Bernard Tonnel,\*<sup>†</sup> Philippe Lassalle,\* and Anne Tsicopoulos<sup>2,\*†</sup>

Allergic asthma is associated with a pulmonary recruitment of Th type 2 cells, basophils, and eosinophils, mainly linked to chemokine production. CCL18 is a chemokine preferentially expressed in the lung, secreted by APCs, induced by Th2-type cytokines, and only present in humans. Therefore, CCL18 may be involved in allergic asthma. PBMC from asthmatics allergic to house dust mite cultured in the presence of *Dermatophagoides pteronyssinus* 1 (Der p 1) allergen secreted CCL18, 48 and 72 h after stimulation, whereas those from healthy donors did not. Part of CCL18 was directly derived from Der p 1-stimulated plasmacytoid dendritic cells, whereas the other part was linked to monocyte activation by IL-4 and IL-13 produced by Der p 1-stimulated T cells. In bronchoalveolar lavages from untreated asthmatic allergic patients, CCL18 was highly increased compared with controls. Functionally, CCL18 preferentially attracted in vitro-polarized Th2 cells and basophils, but not eosinophils and Th1 cells, and induced basophil histamine and intracellular calcium release. These data show a new function for CCL18, i.e., the recruitment of Th2 cells and basophils, and suggest that CCL18 may play a predominant role in allergic asthma. *The Journal of Immunology*, 2006, 176: 6286–6293.

Allergic asthma is a Th2 lymphocyte-mediated inflammatory airway disorder characterized by airway eosinophil and basophil infiltration, increased mucus production, bronchial remodeling, leading to airway obstruction, and high levels of specific IgE in response to commonly inhaled environmental allergens (1).

Cell recruitment to inflammatory sites is orchestrated by chemokines. These molecules are small m.w. cytokines with a strong chemotactic activity. They are classified into CXC, CC, C, and CX3C subfamilies according to the position of conserved cysteine residues. These molecules are involved in physiological cell trafficking, leukocyte homing, cell activation, and tissue inflammation.

The effects of chemokines are mediated by a subset of transmembrane, G protein-coupled receptors, whose differential expression on cells leads to chemokine function specificity. Some chemokines have been shown to preferentially attract Th2 cells, such as eotaxine/CCL11 and MDC/CCL22, through binding to CCR3 and CCR4 (2), respectively, whereas IP-10/CXCL10 and MIG/CXCL9 preferentially attract Th1 cells (3) through CXCR3. Interestingly, the Th2-attracting chemokines CCL11 and CCL22 are induced by IL-4 (4), whereas the Th1-attracting chemokines CXCL10 and CXCL9 are induced by IFN- $\gamma$  (5, 6).

Recently, different groups have described a new chemokine designated as CCL18/PARC (pulmonary and activated regulated chemokine) (7), CCL18/DC<sup>3</sup>-CK1 (dendritic cell-derived ChemoKine 1) (8), CCL18/AMAC-1 (alternative macrophage activation-associated CC chemokine-1) (9), and CCL18/MIP-4 (macrophage inflammatory protein 1) (10). CCL18 is a 7.8-kDa chemokine, which is strongly expressed in healthy human lung and less in thymus and lymph nodes (7). CCL18 receptor and its murine homologue are unknown. CCL18 is mainly secreted by APCs, in particular by monocytes, alveolar macrophages, monocyte-derived DCs (MD-DC) (8, 9, 11), and eosinophils (12). The Th2 cytokines IL-4, IL-13, and IL-10 up-regulate CCL18 expression in alveolar macrophages, DC (9), and monocytes, whereas IFN- $\gamma$  inhibits its production (9). Functionally, CCL18 attracts naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells (8, 10), B lymphocytes (13), and immature DC (14), and is thought to be involved in primary immune responses. CCL18 has been reported to be highly expressed in a number of different diseases, such as atheromatosis (15), chronic hepatitis C (16), septic and rheumatoid arthritis (17), cutaneous contact hypersensitivity (18), hypersensitivity pneumonitis (19), ovarian carcinoma (20), giant cell arteritis (21), vernal keratoconjunctivitis (22), and, more recently, atopic dermatitis (23–25). However, it has not been possible to assess CCL18 functionality in murine models because *CCL18* gene probably does not exist in mice. Indeed, this gene has been generated by fusion of duplicated *MIP-1 $\alpha$*  genes, likely to have occurred after the diversification of rodents and primates (26).

Because CCL18 is preferentially expressed in the lung and induced by Th2 cytokines, we hypothesized that it might be involved in allergic asthma. We show in this study that CCL18 is inducible by allergen and attracts Th2 cells and basophils, suggesting an important role in asthma.

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; MD-DC, monocyte-derived DC; AA, allergic asthmatic; Dpt, *Dermatophagoides pteronyssinus*; RAST, radioallergosorbent test; NA, nonallergic; BAL, bronchoalveolar lavage; Der p 1, *Dermatophagoides pteronyssinus* 1; Cl, control isotype; mDC, myeloid DC; pDC, plasmacytoid DC; ICC, immunocytochemistry.

## Materials and Methods

### Subjects

Venous blood was collected from 26 allergic asthmatic (AA) patients sensitive to house dust mite. All patients had a clinical history of asthma and exhibited positive skin prick tests toward *Dermatophagoides pteronyssinus* (Dpt) allergen, positive radioallergosorbent tests (RAST class 3), and elevated serum IgE levels. None had received oral or inhaled corticosteroids within the 2 mo before the sample collection. Patients were under  $\beta_2$  agonists as required. Venous blood was also obtained from 12 nonallergic (NA) subjects, with negative skin prick tests, total IgE levels  $<100$  KU/L, and negative RAST against Dpt.

Bronchoalveolar lavages (BAL) were obtained from five untreated AA patients, seven inhaled steroid-treated AA patients, and eight NA control subjects. None of the subjects were smokers or had oral steroids. The study was approved by the hospital ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale no. 9307).

### PBMC isolation

PBMC were prepared from blood collected on heparin and purified on a Ficoll-Hypaque gradient (Amersham Biosciences) as described previously (27). PBMC ( $2 \times 10^6$  cells/well) were cultured in 12-well, flat-bottom microculture plates (Nunc) with complete RPMI 1640 in the presence or absence of *Dermatophagoides pteronyssinus* 1 (Der p 1) (the major allergen of Dpt) (1, 10, 100, and 500 ng/ml) (Indoor Biotechnologies) for 2, 6, 12, 24, 48, and 72 h. LPS content of Der p 1 was  $<0.1$  EU/ml as assessed by the *Limulus* amoebocyte test. In some cases, neutralizing Abs to IL-4, IL-13 (10  $\mu$ g/ml; R&D Systems), and IL-10 (1  $\mu$ g/ml; R&D Systems) or control isotype (CI) Abs (IgG2a and IgG2b; R&D Systems) were added to the culture before allergen stimulation. Culture supernatants were collected, aliquoted, and stored at  $-80^\circ\text{C}$  until use.

### Peripheral blood DC preparations

Myeloid DC (mDC) and plasmacytoid DC (pDC) were purified using the blood DC isolation kit and the BDCA-4 cell isolation kit (Miltenyi Biotec) as described previously (28). mDC (Lin-1<sup>-</sup>CD123<sup>-</sup>HLA-DR<sup>+</sup>CD11c<sup>+</sup>) and pDC (Lin-1<sup>-</sup>CD123<sup>+</sup>HLA-DR<sup>+</sup>CD11c<sup>-</sup>) purity was determined by immunostaining with FITC-labeled Lin-1, PE-labeled CD123, Cychrome-labeled HLA-DR, and APC-labeled CD11c (BD Biosciences) and was  $\sim 70\%$  for mDC and 90% for pDC.

Purified mDC or pDC were cultured in complete RPMI 1640. Cells were seeded at a density of  $0.5 \times 10^6$ /ml. Cell suspensions were cultured in the absence or presence of 500 ng/ml Der p 1 or 10 ng/ml IL-4 for 24 h only because of DC viability. Supernatants were recovered and frozen until use.

### Eosinophil, monocyte, and basophil isolation

After the Ficoll-Hypaque gradient, the granulocyte pellet was harvested and depleted of erythrocytes by hypotonic saline lysis. Eosinophils were depleted in neutrophils and contaminating lymphocytes, respectively, by anti-CD16 and anti-CD3 immunomagnetic beads (Miltenyi Biotec) with a purity of 90–95%. Blood monocytes were purified from PBMC by positive selection over a MACS column using anti-CD14-coupled microbeads (Miltenyi Biotec) and were cultured at  $10^6$  cells/ml for 24, 48, and 72 h in the same conditions as for DCs. For basophil isolation, total leukocytes depleted of platelets were layered, after dextran sedimentation of erythrocytes on a Ficoll-Hypaque gradient. Basophils were purified using a basophil isolation kit (Miltenyi Biotec) with a purity of 80–90%.

### Generation of polarized human Th1 and Th2 cells

Because the percentage of Th2 cells in the blood is very low, we chose to use T cells polarized *in vitro* toward a Th1 or a Th2 cytokine profile as described previously (29). CD4<sup>+</sup>CD45RA<sup>+</sup> naive T cells were activated with coated anti-CD3 (10 ng/ml; BD Pharmingen) in the presence of 2 ng/ml IL-12 (R&D Systems) and 200 ng/ml anti-IL-4 (R&D Systems) for induction of Th1 cells, or 10 ng/ml IL-4 (R&D Systems) and 2  $\mu$ g/ml anti-IL-12 (R&D Systems) for induction of Th2 cells. After 3 days, 10 ng/ml IL-2 (Tebu) was added to the cultures. At day 10, cells were restimulated with coated anti-CD3 (2  $\mu$ g/ml) and soluble anti-CD28 (2  $\mu$ g/ml) (BD Pharmingen) for 5 h. Correct polarization was checked by dosage of IL-4 and IFN- $\gamma$  by ELISA.

### Isolation of peripheral blood leukocytes and histamine release

To avoid alterations in the basophil response during purification procedures, mediator release of basophils was studied in total leukocyte, because

histamine is only derived from basophils (30). Venous blood was collected on EDTA, and leukocytes were prepared by dextran as described previously (31). The percentage of basophils was between 1.07 and 2.5% as determined by alcian blue staining. CCL18 at concentrations ranging from  $10^{-9}$  to  $10^{-6}$  M were incubated with the leukocyte suspension ( $3 \times 10^6$  cells/100  $\mu$ l). at  $37^\circ\text{C}$  for 45 min. Supernatants were collected, and histamine release was quantified by using a specific enzymeimmunoassay according to the manufacturer's instructions (Immunotech). Boiling cells for 10 min was used to determine total basophil histamine content. The results were expressed as percentage of total histamine content. Spontaneous histamine release from the cells was  $<5\%$  and was subtracted from the calculated histamine release.

### Analysis of basophil intracellular calcium by flow cytometry

Purified PBMC were prepared as described above for basophils. The protocol was adapted from previously described techniques (32, 33). PBMC were resuspended in RPMI 1640 (without phenol red or sodium bicarbonate, 25 mM HEPES; Invitrogen Life Technologies). Aliquots of  $10^6$  cells were loaded for 20 min at  $37^\circ\text{C}$  with 2  $\mu$ M fluo4AM (Molecular Probes) and an equivalent amount of pluronic F127 (Molecular Probes). The samples were also treated with 7-aminoactinomycin D (Molecular Probes) to permit dead cell discrimination. Cells were then washed once with RPMI 1640, resuspended in the same medium, and kept at  $+20^\circ\text{C}$  in the dark for 30 min. Contaminating cells were discriminated from basophils by staining them with PE-labeled Abs directed against CD3, CD14, CD19, CD56, and HLA-DR (BD Biosciences). Before analysis, samples were placed in a  $37^\circ\text{C}$  water bath. Data were acquired with a flow cytometer (FACSCalibur; BD Biosciences) with excitation at 488 nm. Cells were gated by forward and side scatter properties, and a live gate was determined by 7-aminoactinomycin D and cell surface markers exclusion. Calcium mobilization was determined by a two-parameter density plot collecting linear emission at 530 nm for the gated population over time. Calcium ionophore A23187 was used as a positive control at 10  $\mu$ M and CCL18 at  $10^{-7}$  M.

### F-actin polymerization

The content of F-actin was analyzed by flow cytometry with NBD-phalloidin staining. For eosinophils, 50  $\mu$ l of aliquots of stimulated cell suspensions ( $3 \times 10^6$ /ml) were withdrawn at 5, 10, 20, 30, 60, 120, and 300 s and fixed in a 3.7% formaldehyde buffer with 100  $\mu$ g/ml lysophosphatidylcholine. For Th1 and Th2 cells, the cell suspension was at  $1 \times 10^6$  cells/ml, and the cells were fixed with 0.1% paraformaldehyde. After 30 min, the cells were stained with 0.33  $\mu$ mol/L NBD-phalloidin and permeabilized with 0.1% saponin for T cells. The mean fluorescence intensity was measured by flow cytometry. Results were expressed as the ratio of the mean fluorescence intensity of the chemokine- and buffer-stimulated samples.

### Detection of intracellular CCL18 in peripheral blood dendritic subsets

PBMC ( $2 \times 10^6$  cells/ml) from AA patients were cultured for 48 h in the presence or absence of 10 ng/ml recombinant human IL-4 (R&D Systems), 500 ng/ml Der p 1 (Indoor Biotechnologies), or TLR7 agonist imiquimod at 10 nM (InvivoGen) and treated with brefeldin A (10  $\mu$ g/ml; Sigma-Aldrich) for the last 3 h. PBMC were labeled with FITC-labeled Lin-1, Cychrome-labeled HLA-DR, and APC-labeled CD11c (BD Biosciences). Surface-stained cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences). Intracellular CCL18 was stained with biotinylated goat anti-human CCL18 (R&D Systems) followed by streptavidin-PE (Immunotech). Biotinylated normal goat IgG was used as CI (R&D Systems). pDC were identified as CD11c<sup>-</sup>, HLA-DR<sup>+</sup>, Lin-1<sup>-</sup> and mDC as CD11c<sup>+</sup>, HLA-DR<sup>+</sup>, Lin-1<sup>-</sup>. Data were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

### Semiquantitative RT-PCR

After removal of the supernatants, PBMC were resuspended in TRIzol reagent (Invitrogen Life Technologies), and total cellular RNA was extracted according to the manufacturer's procedure. RNA concentrations were measured with use of a spectrophotometer. RNA integrity was determined by visualizing the 18S and 28S ribosomal RNA bands after gel electrophoresis in 0.8% agarose after Gelstar nucleic acid staining (FMC BioProducts). cDNA synthesis was performed as described previously (27). The sequences of the primers were for human GAPDH: forward, 5'-GTCTTACCACCACCATGGAG-3' and reverse, 5'-CCAAAGTTGTCA

TGGATGACC-3'; and for CCL18: forward, 5'-CCTGTGCACAAGTTG GTACCAAC-3' and reverse, 5'-TGGCTCAGGCTCCTGTTCCCT-3' (Eurogentec). For amplification of GAPDH 25 cycles at 55°C were performed and 27 cycles at 62°C for CCL18 using *Taq* DNA polymerase (Invitrogen Life Technologies). Amplified PCR products were observed by gel electrophoresis in 1.5% agarose after Gelstar nucleic acid staining.

#### BAL analysis

BAL were filtered through a sterile surgical gauze and centrifuged at 1700 rpm for 10 min at +4°C. BAL fluid was collected and used for ELISA. Cells were enumerated and resuspended in PBS, and cytopspins were fixed in 4% paraformaldehyde/PBS for 30 min and dehydrated in alcohol. Immunocytochemistry (ICC) was performed using a modified alkaline phosphatase anti-alkaline phosphatase method as described previously (34) with anti-CCL18 mAb (R&D Systems) at 2.5 µg/ml.

#### Quantification of chemokine and cytokine protein levels

Concentrations of CCL18 (R&D Systems) IL-4, IL-10, IL-13, and IFN-γ (Diaclone Research) were measured by ELISA according to the manufacturer's recommendations and expressed in picograms per milliliter. The level of sensitivity was 31.2 pg/ml for CCL18, 1.1 pg/ml for IL-4, 3.12 pg/ml for IL-13, 12.5 pg/ml for IL-10, and 12.5 pg/ml for IFN-γ. Results were expressed as mean ± SEM.

#### Chemotaxis assay

For all the cell types evaluated, CCL18 (at a concentration of 10<sup>-9</sup>–10<sup>-6</sup> M) and control RPMI 1640 were added in a 48-well microchemotaxis chamber (NeuroProbe) with 5-µm pore polycarbonate filters (Nucleopore). Differentiated human Th1 and Th2 cells were resuspended in RPMI 1640 at a concentration of 2 × 10<sup>6</sup> cells/ml and for incubated for 2 h at 37°C in 5% CO<sub>2</sub>. T cells that do not stick to the filter and fall into the inferior chamber were counted in it. Eosinophils and basophils were resuspended at a concentration of 10<sup>6</sup> cells/ml and incubated for 45 min and 1.5 h, respectively, at 37°C in 5% CO<sub>2</sub>. Cells that had migrated stick to the filter and thus were counted on it under a microscope at a magnification of 500-fold. The positive controls were CXCL10, CCL22, and CCL11 (Tebu) for Th1 and Th2 cells and basophils and eosinophils, respectively. The negative controls were CCL22, CXCL10, and CCL1 for Th1 and Th2 cells and basophils and eosinophils, respectively. In some cases, neutralizing Abs to CCR3 (R&D Systems) or desensitization with CCL22 were used. Each condition was performed in triplicate. Results were expressed as percentage of migration of the positive control (considered as 100%). Chemotaxis was distinguished from chemokinesis as described previously (27).

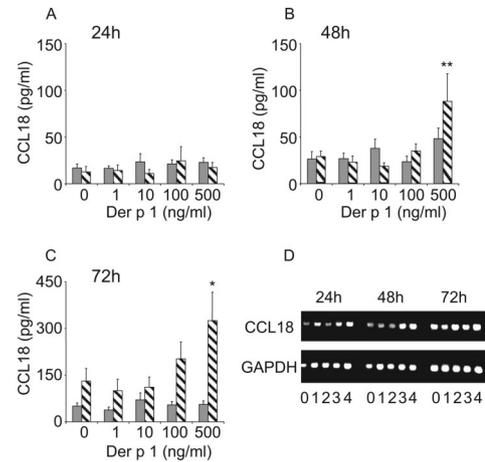
#### Statistical evaluation

Statistical analysis was performed using a one-way ANOVA for multiple comparisons followed by a post hoc Bonferroni test. Single comparisons were performed using Wilcoxon's test for paired values and Mann-Whitney for unpaired data. Values of *p* < 0.05 were regarded as statistically significant. Statistical analysis was performed using the Statview 5 software (Abacus Concepts) on Macintosh.

## Results

### Der p 1 stimulation of PBMC from AA patients up-regulates CCL18 secretion and mRNA expression

To investigate the expression of CCL18 after allergen exposure, PBMC from house dust mite AA and NA subjects were stimulated with different concentrations of Der p 1. CCL18 secretion was assessed at 2, 6, 12, 24, 48, and 72 h after stimulation. At baseline, a spontaneous production of CCL18 protein was obtained that increased with time and was higher in AA patients than in NA subjects at 72 h. At early time points of culture, 2, 6, 12 (data not shown), and 24 h after allergen stimulation (Fig. 1A), no modification was observed in CCL18 secretion as compared with medium alone, in PBMC from either AA or NA donors. After 48 and 72 h of stimulation (Fig. 1, B and C), a significant increase in CCL18 production was observed at a concentration of 500 ng/ml Der p 1, in PBMC from AA patients, but not from NA subjects. This increase was not observed with an irrelevant allergen stimulation such as grass pollen (data not shown) in PBMC from AA



**FIGURE 1.** Der p 1 stimulation of PBMC from AA patients up-regulates CCL18 secretion and mRNA expression. PBMC were isolated from healthy (NA, □) or AA subjects (AA, ▨) and cultured for 24 (A), 48 (B), and 72 (C) h with or without Der p 1 at different concentrations. CCL18 was evaluated in supernatants by ELISA. Results are expressed as mean pg/ml ± SEM (*n* = 7 for NA, and *n* = 10 for AA). \*, *p* < 0.05; \*\*, *p* < 0.01. D, RT-PCR analysis of CCL18 and housekeeping gene GAPDH mRNA expression by PBMC incubated with medium (0) or with Der p 1 at different concentrations (1, 1 ng/ml; 2, 10 ng/ml; 3, 100 ng/ml; 4, 500 ng/ml). One representative experiment of three is shown.

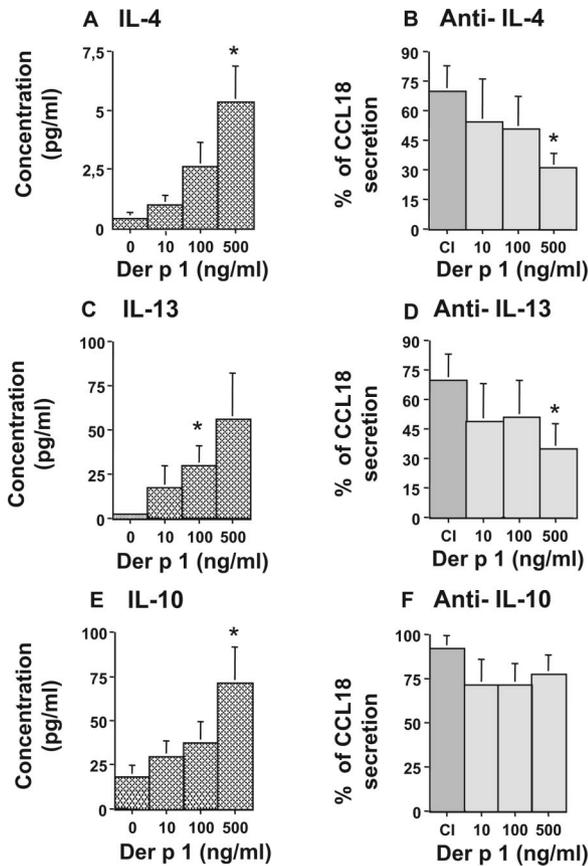
donors. Similar results were obtained at the mRNA level (Fig. 1D). Altogether, these data show that allergen stimulation is a trigger of CCL18 production.

### Der p 1-induced CCL18 up-regulation by PBMC is dependent upon IL-4 and IL-13

Because CCL18 secretion is known to be induced by Th2 cytokines, we evaluated the implication of IL-4, IL-13, and IL-10 in allergen-induced CCL18 secretion. Der p 1-induced IL-4 and IL-13 production started at 48 and 72 h, respectively (Fig. 2, A and C). Anti-IL-4 and anti-IL-13 Abs inhibited CCL18 secretion, by 69 and 65%, respectively, for a concentration of Der p 1 of 500 ng/ml at 72 h (Fig. 2, B and D). In contrast, neutralizing anti-IL-10 Ab had no effect on Der p 1-induced CCL18 secretion (Fig. 2E), although IL-10 protein was induced after 72 h of stimulation (Fig. 2E). Among PBMC, only monocytes and DCs are known to produce CCL18. When purified monocytes were directly stimulated with Der p 1, they only produced background levels of CCL18, whereas they produced high levels when stimulated with IL-4 (Table I). When total PBMC were depleted in either monocytes or CD3<sup>+</sup> T cells, Der p 1-induced CCL18 production was completely abolished (data not shown), showing that both were necessary. Altogether, these data suggest that in AA patients the activation of allergen-specific T cells may lead to the secretion of Th2 cytokines, which in turn may stimulate monocytes to produce CCL18.

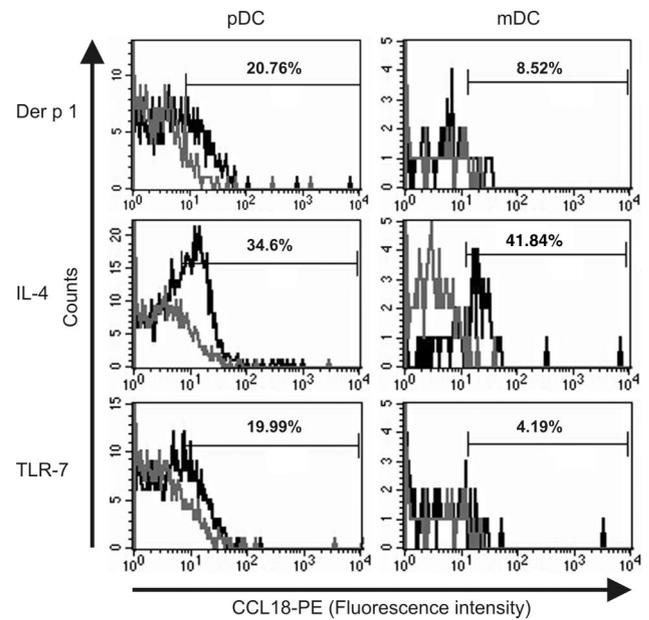
### CCL18 is produced by Der p 1-stimulated pDCs

DC are known to produce CCL18, and in particular MD-DC (4–7, 9); however, these cells are usually derived using either IL-4 or IL-13, introducing a bias. To investigate whether peripheral blood DC may constitute a source of CCL18 under allergen stimulation, the two main subsets, pDC and mDC, were purified. pDC stimulated with Der p 1 preferentially secreted CCL18 at 24 h as compared with mDC (Table I). Because mDC were only 70% pure, data were checked using intracellular CCL18 staining of PBMC DC subsets by flow cytometry. The



**FIGURE 2.** Der p 1-induced CCL18 up-regulation by PBMC is dependent upon IL-4 and IL-13. Secretion of IL-4 (A), IL-13 (C), and IL-10 (E) by Der p 1-stimulated PBMC from AA patients ( $n = 7-10$ ) at 72 h of culture. Results are expressed in picograms per milliliter. Effect of neutralizing Abs to IL-4 (B), IL-13 (D), and IL-10 (F) compared with CIs on Der p 1-induced CCL18 secretion by PBMC from AA patients at 72 h of culture ( $n = 7-9$ ). Neutralizing Abs were added at a final concentration of 10  $\mu\text{g/ml}$  for anti-IL-4 and anti-IL-13 and of 1  $\mu\text{g/ml}$  for anti-IL-10. Results are expressed as percentage of secretion  $\pm$  SEM; \*,  $p < 0.05$ .

mean percentage of positive pDC and mDC was  $8.85 \pm 4.2$  and  $2.45 \pm 1.5$  for Der p 1 stimulation,  $20.16 \pm 6.3$  and  $14.75 \pm 6.9$  for IL-4 stimulation, and  $7.02 \pm 4.4$  and  $1.63 \pm 0.9$  for TLR7 stimulation, respectively. No difference was observed in CCL18 staining between unstimulated and CI (data not shown). One representative experiment of five is shown in Fig. 3. These data indicate that there are at least two pathways of allergen-induced



**FIGURE 3.** CCL18 intracellular staining by peripheral blood pDC and mDC stimulated by Der p 1 or IL-4 in AA patients. Percentage of pDC and mDC expressing CCL18 after 48-h stimulation by Der p 1, IL-4, or TLR7 agonist after subtraction of the CI. Stimulated cells (black lines) vs the CI (gray lines). PBMC were stained with CD11c-Cy, HLA-DR-APC, and Lin-1-FITC, permeabilized, and stained with biotinylated anti-CCL18 and streptavidin-PE. pDC were identified as  $\text{CD11c}^+$ ,  $\text{HLA-DR}^+$ ,  $\text{Lin-1}^-$  and mDC as  $\text{CD11c}^+$ ,  $\text{HLA-DR}^+$ ,  $\text{Lin-1}^-$ . One representative experiment of five is shown.

CCL18 production: one through monocyte stimulation by T cell-derived Th2 cytokines, and one through direct pDC stimulation.

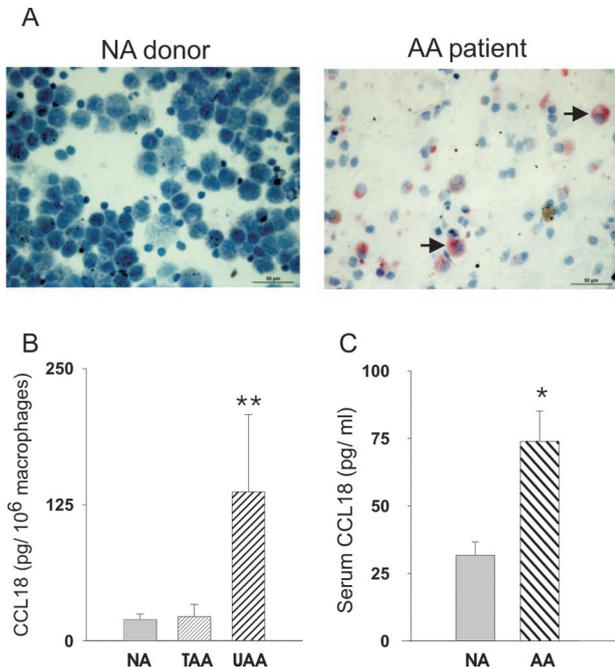
*CCL18 is up-regulated in BAL and sera from AA patients*

To assess the relevance of these data in asthmatic patients, CCL18 expression was evaluated using ICC, on BAL cells from AA patients and controls. CCL18 appeared to be expressed by cells exhibiting the characteristic morphology of alveolar macrophages in both groups, although the staining intensity was very weak in normal controls as compared with AA patients (Fig. 4A). To quantify more precisely CCL18 expression, BAL fluids from untreated AA patients, inhaled steroid-treated AA patients, and NA donors were analyzed by ELISA. Results showed that CCL18 was up-regulated in BAL fluid from untreated AA patients ( $179.2 \pm 65.5 \text{ pg}/10^6$  macrophages, range 8

Table I. CCL18 is secreted by Der p 1-stimulated circulating pDC from AA patients<sup>a</sup>

Donors	Cells	Medium	Der p 1	IL-4
AA	pDC	6.5 $\pm$ 3.0	199.9 $\pm$ 182*	48.2 $\pm$ 28.9
	mDC	7.8 $\pm$ 3.3	17.4 $\pm$ 6.7	50.6 $\pm$ 34.9
	Monocytes 24 h	1.8 $\pm$ 1.8	2.1 $\pm$ 2.1	60.9 $\pm$ 25.5*
	Monocytes 48 h	22.1 $\pm$ 14.2	6.2 $\pm$ 3.6	217.2 $\pm$ 101.4*
	Monocytes 72 h	18.8 $\pm$ 7.6	19.4 $\pm$ 5.2	586.2 $\pm$ 199.6*
NA	pDC	2.95 $\pm$ 1.9	3.1 $\pm$ 2.0	30.9 $\pm$ 17.8
	mDC	6.9 $\pm$ 3.8	3.1 $\pm$ 2.0	31.1 $\pm$ 12.5
	Monocytes 24 h	2.7 $\pm$ 1.7	0	113.7 $\pm$ 36.3*
	Monocytes 48 h	6.7 $\pm$ 2.4	0	597.6 $\pm$ 315.4*
	Monocytes 72 h	33.9 $\pm$ 10.5	35.6 $\pm$ 14.2	3366.8 $\pm$ 1527*

<sup>a</sup>pDC and mDC were isolated from six NA and six AA donors, and monocytes were isolated from seven NA and seven AA donors. DC were cultured for 24 h, monocytes for 24, 48, and 72 h with or without Der p 1 (500 ng/ml) or IL-4 (10 ng/ml). CCL18 concentration was evaluated by ELISA. Results are expressed as mean  $\text{pg/ml} \pm$  SEM; \*  $p < 0.05$  compared to medium.

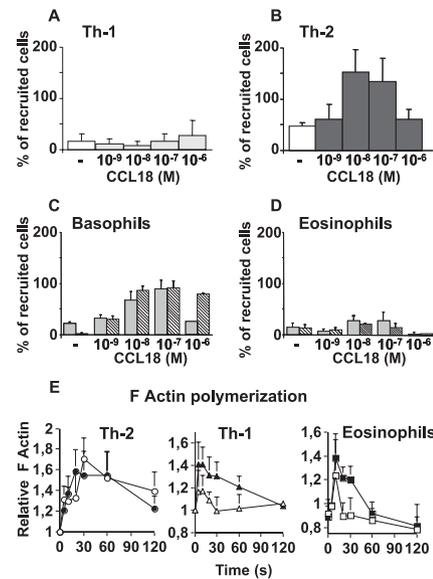


**FIGURE 4.** CCL18 expression in BAL and in sera. *A*, Detection of CCL18 in BAL cells from an AA patient compared with a NA donor using ICC. Scale bar, 50  $\mu$ m. Levels of CCL18 in BAL fluid (*B*) from eight NA donors, seven inhaled steroid-treated AA (TAA) and five untreated AA patients (UAA), and in sera (*C*) from eight NA donors and 26 AA as assessed by ELISA. \*\*,  $p < 0.01$  compared with NA and TAA. \*,  $p < 0.05$  compared with NA.

- 323), whereas inhaled steroid-treated AA patients ( $22.1 \pm 11.2$  pg/10<sup>6</sup> macrophages, range 4–88) exhibited CCL18 levels similar to the controls ( $19.2 \pm 5.2$  pg/10<sup>6</sup> macrophages, range 5–52) (Fig. 4*B*). There was no relationship between CCL18 levels and the severity of asthma or forced expiratory volume in 1 s) (data not shown). The presence of CCL18 was also assessed in sera from AA patients as compared with NA subjects. CCL18 was significantly elevated in AA ( $73.9 \pm 11.2$  pg/ml) compared with NA ( $31.7 \pm 5$  pg/ml) subjects (Fig. 4*C*).

#### CCL18 chemoattracts and activates cells involved in the allergic reaction

To determine the effect of CCL18 on the migration of cells recruited in the allergic reaction, chemotaxis assays were performed on polarized Th1 and Th2 cells, basophils, and eosinophils. CCL18 was unable to recruit polarized Th1 cells (Fig. 5*A*), whereas it dose-dependently attracted polarized Th2 cells (Fig. 5*B*). Basophils from NA and AA donors were also attracted by CCL18, although to a lesser extent than Th2 cells (Fig. 5*C*), whereas eosinophils from either NA or AA donors showed only background level response to CCL18 (Fig. 5*D*). These results suggest that Th2 cells and basophils preferentially express CCL18 receptor. CCL18 receptor is unknown, and previous reports have shown that the receptor is different from CCR1, -2, -3, -4, and -5 (7, 35). Given our results, we verify that the receptor was different from CCR3 and CCR4. Neutralizing Abs (for CCR3) or receptor desensitization (for CCR4) did not abolish CCL18-induced chemotaxis of basophils and Th2 cells (data not shown). To comfort these results, activation of Th1 and Th2 cells and eosinophils was evaluated using another parameter of chemokine activation, i.e., F-actin polymerization. A preferential activation of Th2 cells was observed,



**FIGURE 5.** Effect of CCL18 on the recruitment and activation of cells involved in the allergic reaction. Recruitment of in vitro-polarized Th1 (*A*;  $n = 4$ ) and Th2 (*B*;  $n = 5$ ) cells; basophils from five NA (□) and five AA (▨) donors (*C*); eosinophils from three NA (▤) and three AA (▩) donors (*D*). Negative controls were CCL22 for *A*, CXCL10 for *B* and *C*, and CCL1 for *D*. Results are expressed as mean percentage of migration of the positive control  $\pm$  SEM. *E*, Kinetics of CCL18-induced F-actin polymerization (open symbols) in Th1 and Th2 cells and eosinophils. Positive controls (filled symbols) were CCL22 for Th2 cells, CXCL10 for Th1 cells, and CCL11 for eosinophils. Results are expressed as mean relative F-actin content  $\pm$  SEM ( $n = 5$ ).

although a small transient activation also occurred in Th1 cells and eosinophils but to a lesser extent (Fig. 5*E*).

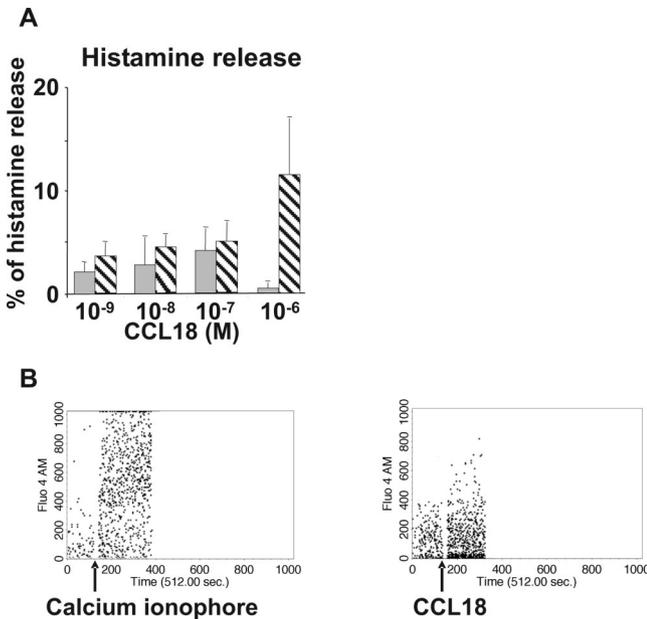
#### CCL18 induces basophil histamine and intracellular calcium release

Besides migration, chemokines can also be potent histamine-releasing factors for basophils. Histamine release was determined in total leukocytes from NA and AA donors after stimulation with CCL18 (Fig. 6*A*). Anti-IgE stimulation induced a higher histamine release in AA patients ( $47.2 \pm 18.4\%$ ) than in NA controls ( $14.3 \pm 6.4\%$ ). CCL18 induced histamine release from leukocytes from both NA and AA donors, with a differential effect at 10<sup>-6</sup> M. To check that CCL18 was indeed able to activate basophils, CCL18-induced intracellular calcium release was investigated. Basophils from PBMC were gated by excluding all other labeled contaminating cells and evaluated for intracellular calcium by flow cytometry after Fluo-4AM loading. CCL18-induced intracellular calcium release (Fig. 6*B*) in ~15% of the basophils.

#### Discussion

Chemokines play a major role in the generation of allergic reactions by recruiting cells involved in the inflammatory reaction, in particular Th2 cells, eosinophils, and basophils. In this study, we have investigated the possible role of CCL18 in allergic asthma. This chemokine has been little explored because of the absence of known homologue in the mouse and the absence of cloned receptor. However, its characteristics led us to suppose that it might have a role in allergic asthma. First, CCL18 is constitutively expressed in the lung (7), and second, it is induced by Th2 type cytokines (7–9).

As a first step, we evaluated its production in response to allergen stimulation by PBMC from both allergic and NA subjects. A



**FIGURE 6.** Effect of CCL18 on basophil activation. *A*, CCL18-induced histamine release by basophils from three NA (□) and three AA (▨) donors. Results are expressed as mean percentage of total histamine release  $\pm$  SEM. *B*, Flow cytometric analysis of intracellular calcium release by basophils after activation by calcium ionophore (10  $\mu$ M) or CCL18 (10<sup>-7</sup> M). One representative experiment of three is shown.

spontaneous production of the protein and mRNA for CCL18 was obtained that increased with time and was higher in allergic patients than in NA subjects at 72 h. Experiments using purified monocytes showed that they were the source of this baseline production, the increase overtime being probably linked to adherence of monocytes to plastic culture plates. Above this baseline level, the stimulation of PBMC by Der p 1 allergen induced at late time points an increase in this level, but only in allergic patients, showing that this additional production was related to the allergic status of the donor and not to the allergenic protein itself. This production was also allergen-specific because stimulation using an unrelated allergen did not trigger CCL18 release in allergic patients. However, there was no defect in the intrinsic capacity of allergic subjects to produce CCL18, as evaluated by LPS stimulation of PBMC, which induced CCL18 production in both allergic and nonatopic donors (data not shown). The long delay between the stimulus and the appearance of CCL18 has been already found in other studies both in vitro (9) and in vivo (18). To investigate the mechanism of action of Der p 1-induced CCL18 release, neutralizing experiments were performed to assess the role of the Th2 cytokines known to induce CCL18. IL-4, IL-13, and IL-10 were found to be induced by Der p 1 stimulation; however, only IL-4 and IL-13 neutralization inhibited Der p 1-induced CCL18 production by PBMC. Moreover, when total PBMC were depleted in either monocytes or CD3<sup>+</sup> T cells, the increase in CCL18 production induced by Der p 1 was abolished, suggesting that both monocytes as a source of CCL18, and T cells as a source of IL-4 and IL-13 were necessary for this production. Finally, purified monocytes responded to IL-4 but not to Der p 1. Altogether, these data suggest that in allergic patients, the presentation of Der p 1 by APCs to T cells may lead to the secretion of Th2 cytokines, which in turn may stimulate monocytes to produce CCL18. MD-DC are well-known sources of CCL18 (8, 9, 11, 14, 36, 37); however, these cells are usually derived using either IL-4 or IL-13, introducing a bias. To investigate whether peripheral blood DC may

constitute a source of CCL18 under allergen stimulation, purification of the two main subsets, pDC and mDC, was performed. In this study, Der p 1- and TLR7 agonist-stimulated peripheral blood pDC preferentially produced CCL18 as compared with mDC, whereas IL-4 induced CCL18 production in both subsets. In the study by Vulcano et al. (14), CCL18 was produced by IL-10-stimulated mDC but not pDC. Differential receptor expression on DC subsets may explain these differences of CCL18 secretion. The atopic status of the patients is also likely to play a role, because Der p 1-induced CCL18 production by pDC was only observed in allergic patients and not in healthy controls. When related to the number of pDC in total PBMC, Der p 1-induced CCL18 production accounted for only 10–15% of the total CCL18 production. The level of CCL18 production by pDC may, however, be more important in effector mucosal sites, where high numbers of pDC are recruited in allergic patients after allergen challenge (38). Recent data suggest that pDC may also have a tolerogenic function in an experimental murine model of asthma (39). However, we and others (28, 40) have previously shown that pDC isolated from allergic patients and stimulated with the allergen induce a Th2 polarization. Therefore, the exact function of pDC in human allergic asthma has still to be determined.

The in vivo relevance of these in vitro results was next evaluated in BAL from AAs. CCL18 was found quantitatively and qualitatively increased but only in BAL from asthmatic patients not treated with inhaled steroids. Treatment with inhaled steroids has been shown to decrease the numbers of DCs in the bronchial mucosa of atopic asthmatics (41), and dexamethasone has been shown to inhibit CCL18 expression from cultured BAL cells (42) as well as from immature DC (14). Taken together, these different data suggest that steroid treatment may inhibit CCL18 lung expression potentially by acting on macrophages and DCs. A recent gene array study performed in humans in the skin lesions of atopic dermatitis, a Th2-associated disease, showed that *CCL18* was the second most highly induced gene as compared with psoriasis, a Th1-associated disease (23). These data were confirmed in two other works, showing in particular that topical exposure to the relevant allergen resulted in a significant induction of CCL18 in atopic dermatitis patients (24, 25). All of these data show that CCL18 is overexpressed in Th2 type diseases.

We next determined the effect of CCL18 on the recruitment of the cells involved in allergic inflammation. Interestingly, CCL18 recruited polarized Th2 cells but not Th1 cells. These results are in contrast with a recent work that examined the recruitment of atopic dermatitis skin-derived Th1 and Th2 clones by CCL18 (25). In that work, they found that both subpopulations were attracted and that their recruitment was closely associated with the expression of CCR4, a receptor preferentially expressed on skin homing memory T cells (43) expressed by both their Th1 and Th2 clones. In our study, polarized Th1 cells were derived from peripheral blood T cells, did not express CCR4, and were not attracted by CCL22. Although CCR4 might be coexpressed with CCL18 receptor, its specific desensitization by CCL22 did not inhibit Th2 cell recruitment by CCL18. Eosinophils from either allergic or NA patients were not attracted by CCL18, as reported by others (12, 35). However, using the more sensitive technique of actin polymerization as an activation marker, we observed a small and transient activation of Th1 cells and eosinophils, showing that they may express a low level of CCL18 receptor. In contrast, basophils exhibited a dose-dependent chemotactic response to CCL18. This result is surprising because hitherto, eosinophils and basophils have shown a similar repertoire of chemokine receptors. To ascertain this result, basophils were evaluated in other functional assays. Basophils

from both healthy and allergic subjects released histamine and intracellular calcium after CCL18 stimulation, confirming the chemotaxis assay. Altogether, these data suggest that CCL18 receptor is preferentially expressed on Th2 cells and basophils. These data also suggest that CCL18 may be involved in secondary responses involving Th2 cells and not only in primary immune responses as first thought from its ability to recruit naive T cells.

Nibbs et al. (35) have shown that CCL18 exerts an antagonist effect on CCR3, which may therefore limit further eosinophil influx induced by CCR3 ligands. Nonetheless, a recent study (44) showed that the ablation of eosinophil infiltrate using anti-IL-5 Ab does not ameliorate asthma, suggesting that cellular infiltration may not be the only cause of the clinical features of the disease. Others mechanisms involving smooth muscle cells and bronchial remodeling may be of importance in asthma pathogenesis. In this context, the implication of CCL18 in collagen type I production and proliferation of human pulmonary fibroblasts (45) and its late induction by the allergen may contribute to the remodeling observed in chronic asthma. Moreover, because CCL18 attracts Th2 cells and basophils and induces histamine release, it may amplify the chronicity of the AA reaction and represent a new target for asthma therapy. Altogether, this study shows a new function for CCL18, which is the preferential recruitment of Th2 cells and basophils in the context of allergic asthma. Its high expression in bronchi from asthmatics suggests a predominant role in the pathophysiology of allergic diseases.

## Disclosures

The authors have no financial conflict of interest.

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# CORRECTIONS

Caprio-Young, J. C., J. J. Bell, H.-H. Lee, J. Ellis, D. Nast, G. Sayler, B. Min, and H. Zaghouni. 2006. Neonatally primed lymph node, but not splenic T cells, display a Gly-Gly motif within the TCR  $\beta$ -chain complementarity-determining region 3 that controls affinity and may affect lymphoid organ retention. *J. Immunol.* 176: 357–364.

**In Results**, in the penultimate sentence of the second paragraph under the heading *A Gly-Gly motif is conserved within the CDR3 of lymph node T cell hybridomas*, reference to Figure 7a and 7b are reversed. The corrected sentence is shown below.

The lymph node TCR contains a rigid loop with an extended planar surface (Fig. 7b), whereas the splenic Th1 TCR presents a round shape with a less extended surface (Fig. 7a).

Taieb, J., N. Chaput, N. Scharz, S. Roux, S. Novault, C. Ménard, F. Ghiringhelli, M. Terme, A. F. Carpentier, G. Darrasse-Jèse, F. Lemonnier, and L. Zitvogel. 2006. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. *J. Immunol.* 176: 2722–2729.

The tenth author's last name is incorrect. The correct name is Guillaume Darrasse-Jèse.

Swiecki, M. K., M. W. Lisanby, F. Shu, C. L. Turnbough, Jr., and J. F. Kearney. 2006. Monoclonal antibodies for *Bacillus anthracis* spore detection and functional analyses of spore germination and outgrowth. *J. Immunol.* 176: 6076–6084.

In Table II, the data reported for GA2–3<sup>b</sup> in column six ( $\Delta rmlD$  spores) should be negative (–) not 2-log shift (++) . The corrected table is shown below.

Table II. *mAbs raised against irradiated B. anthracis spores or purified B. anthracis exosporium*

mAbs	Western		FACS		
	Anti- <i>E. coli</i> Bc1A	Anti-Deglyco, Bc1A	WT Sterne spores	$\Delta bc1A$ spores	$\Delta rmlD$ spores
AB2	ND <sup>a</sup>	ND	+	–	++
AF10 <sup>b</sup>	+	+	+	–	++
AH8 <sup>8</sup>	+	–	+	–	++
BD8 <sup>b</sup>	+	+	+	ND	ND
BE12 <sup>b</sup>	+	+	+	–	++
BF1-4 <sup>b</sup>	+	+	+	–	++
BF12	ND	ND	+	–	++
BG11 <sup>b</sup>	+	–	+	ND	ND
CA3*	+	–	+	–	++
DE3-1 <sup>b</sup>	+	+	+	–	++
DE12	–	–	+	–	++
FD3-4 <sup>b</sup>	+	+	+	ND	ND
EF12 <sup>b</sup>	+	+	+	ND	ND
AA2-1	ND	ND	+	–	++
BA10-1 <sup>b</sup>	+	+	+	–	++
DH4-1 <sup>b</sup>	–	–	+	++	++
EA2-1	ND	ND	+	–	++
EA4-10 <sup>b</sup>	+	+	+	–	++
EA4-10-4	+	+	+	–	++
EG4-4 <sup>b</sup>	+	+	+	–	++
FH6-1 <sup>b</sup>	–	–	+	++	++
GA2-3 <sup>b</sup>	–	–	+	–	–
GB4-4 <sup>b</sup>	–	–	+	ND	ND
GB4-6-2	ND	ND	+	–	++
HB2-2	ND	ND	+	–	++
IC801 <sup>b</sup>	–	–	+	–	++
JB5-1 <sup>b</sup>	+	+	+	–	++
JC8-5 <sup>sb</sup>	+	+	+	–	++

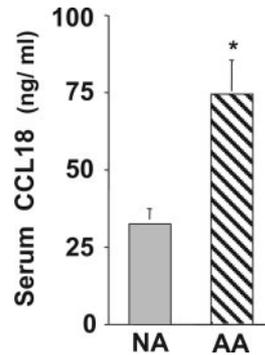
<sup>a</sup> ND, Not determined, –, negative by Western or FACS; +, positive by Western or 1-log shift by FACS; ++, 2-log shift by FACS ABS-EF12, mAbs raised against spores; AA2-I-JC8-5, mAbs and raised against exosporium.

<sup>b</sup> Included in Ref. 9.

de Nadaï, P., A.-S. Charbonnier, C. Chenivesse, S. Sénéchal, C. Fournier, J. Gilet, H. Vorng, Y. Chang, P. Gosset, B. Wallaert, A.-B. Tonnel, P. Lassalle, and A. Tscopoulos. 2006. Involvement of CCL18 in allergic asthma. *J. Immunol.* 176: 6286–6293.

In **Results**, in the last sentence of the paragraph under the heading *CCL18 is up-regulated in BAL and sera from AA patients*, and in Figure 4C, the concentration of serum CCL18 is expressed incorrectly as “pg/ml” instead of “ng/ml.” The corrected sentence and figure are shown below.

CCL18 was significantly elevated in AA ( $73.9 \pm 11.2$  ng/ml) compared with NA ( $31.7 \pm 5$  ng/ml) subjects (Fig. 4C).



Zhang, H.-G., C. Liu, K. Su, S. Yu, L. Zhang, S. Zhang, J. Wang, X. Cao, W. Grizzle, and R. P. Kimberly. 2006. A membrane form of TNF- $\alpha$  presented by exosomes delays T cell activation-induced cell death. *J. Immunol.* 176: 7385–7393.

The third author’s first name is incorrect. The correct name is Kaihong Su.

Vaknin-Dembinsky, A., K. Balashov, and H. L. Weiner. 2006. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J. Immunol.* 176: 7768–7774.

During production, the figure from an unrelated article was inadvertently inserted as the image for Figure 8. The correct figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

