

Increased Interleukin-27 Production by Antigen-Presenting Cells Promotes Regulatory T Cell Differentiation and Contributes to Inducing a Remission in Patients with Eosinophilic Granulomatosis with Polyangiitis

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Key Words

Eosinophilic granulomatosis with polyangiitis ·
Antigen-presenting cells · Cytokine production profile ·
Toll-like receptors · Dendritic cells

Abstract

Background: We investigated the cytokine production profiles of antigen-presenting cells (APCs) and the status of patients with eosinophilic granulomatosis with polyangiitis (EGPA) in order to identify the cytokine profile that contributes to inducing differentiation of CD4⁺ effector Th cells and regulatory T cells. **Methods:** We counted the number of CD4⁺FOXP3⁺ T cells, CD4⁺CD25⁺CTLA-4⁺ T cells, CD4⁺ T cells that predominantly produce IL-10 (Tr1 cells) and IL-17 (Th17 cells) and monocytes and monocyte-derived dendritic cells (mDCs) expressing Toll-like receptor (TLR)2 and TLR4 in the peripheral blood of 47 EGPA patients and 40 bronchial asthma patients (who did not have EGPA) and calculated the percentages of monocytes and mDCs that produced IL-23p19 and IL-27 in response to lipopolysaccharide (LPS) stimulation. **Results:** Lower TLR4 expression was observed on the

monocytes of relapsed EGPA patients and lower expression of both TLR2 and TLR4 on their mDCs than on the cells from EGPA patients in remission or non-EGPA patients. The percentages of monocytes expressing TLR4 were positively correlated with the percentages of regulatory T cells in peripheral blood. In addition, the percentages of monocytes and the percentages of mDCs that produced IL-27 and IL-23p19 in response to LPS stimulation were positively correlated with the percentages of Tr1 cells and Th17 cells in peripheral blood. A positive correlation was also found between the percentages of mDCs that produced IL-27 and the percentages of Tr1 cells. **Conclusion:** Increased dominance of IL-23p19 and IL-27 production by the APCs of EGPA patients may be linked to differentiation of Th17 cells and Tr1 cells.

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Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA; also known as Churg-Strauss syndrome) is a rare disease that is characterized by peripheral and tissue eosinophil-

ia followed by allergic granulomatosis and necrotizing vasculitis [1]. The precise cause of EGPA is still unknown. Naïve T cells must receive signals via their receptors (TCRs), costimulatory molecules and cytokine receptors to induce protective T cell responses [2]. The signals can be provided by professional antigen-presenting cells, such as activated dendritic cells (DCs). DCs are activated when they encounter pathogen-associated molecular patterns (PAMPs) that are recognized by pathogen-recognition receptors, e.g. Toll-like receptors (TLRs). TLR engagement alerts the immune system to danger and leads to the activation of innate immune cells, and TLR triggering induces DC maturation, which is essential to the induction of adaptive immune responses. In a previous study, we demonstrated involvement of Th17 cells and Tr1 cells in the pathogenesis of EGPA and observed higher numbers of Th17 cells and lower numbers of Tr1 cells in the blood of EGPA patients during relapses than during remission [3–6]. We also observed a lower percentage of CD14⁺ monocytes expressing TLR4 and TLR2 in relapsed EGPA patients than in EGPA patients in remission, although there were no differences between relapsed patients and patients in remission with regard to the expression of either TLR3 or TLR6. Monocytes express DC-SIGN/CD209 when TLR4 is activated by exposure to microbes, and the monocytes translocate into the T cell zone of secondary lymph nodes, where they present antigens to naïve T cells [7]. A decrease in expression of TLR4 is likely to be involved in changing the cytokine production profile of antigen-presenting cells (APCs) in response to lipopolysaccharide (LPS) and the status of EGPA patients. The production of both IL-23p19 and IL-27 in APCs contributes to inducing differentiation of Th17 cells and regulatory T cells. Interleukin 23 (IL-23) is a heterodimeric cytokine composed of two disulfide-linked subunits: a p19 subunit that is unique to IL-23 and a p40 subunit that is shared with IL-12. IL-23 is secreted by activated macrophages and DCs. Production of IL-23p19 by APCs is triggered by host immune stimuli like interferons and by bacterial products, such as LPS, that signal through TLR. Whereas IL-12 derives the differentiation of Th1 cells, IL-23p19 favors the development of a T cell subset with a unique expression profile, characterized by prominent production of IL-17, and therefore referred to as Th17 cells [8].

IL-27, on the other hand, is known to antagonize Th17 development, induce IL-10 production and suppress IL-6-induced T cell proliferation [9–11]. The production of these immunomodulatory cytokines is likely to be involved in changes in the status of EGPA patients. The

purpose of this study was to determine whether these immunomodulatory cytokines IL-12, IL-23p19 and IL-27 were involved in the change in status of the EGPA patients.

The results of this study showed a lower expression of TLR2 and especially of TLR4 on the surface of the monocytes and monocyte-derived DCs (mDCs) of the relapsed EGPA patients, and a higher percentage of monocytes and mDCs producing IL-23p19 and IL-27, which may be linked to an increased percentage of Th17 cells and Tr1 cells in the blood.

Materials and Methods

Patients

Between September 2010 and January 2012, we recruited 47 EGPA patients (33 in remission and 14 in a relapse) and 40 bronchial asthma patients who did not have EGPA to participate in this study (table 1). EGPA was diagnosed on the basis of the American College of Rheumatology criteria. Remission was defined as the absence of any clinical signs or symptoms of active vasculitis for at least 6 months. A relapse was defined as a recurrence of vasculitis symptoms with or without an increase in eosinophils as a proportion of peripheral leukocytes following an initial remission after that required resumption of immunosuppressive therapy or an increased dose of immunosuppressive agents.

Immunological Analysis

Peripheral blood mononuclear cells (PBMCs) obtained from the subjects were costimulated for 5 h with PMA and ionomycin, and intracellular cytokines were measured after fixing and permeabilizing the cells. Plastic adherent monocytes in PBMCs from all subjects were cultured with GM-CSF and IL-4 for 6 days to generate immature DCs, and at the end of day 6 LPS was added to the cultures to induce maturation into mDCs. The amount of cytokines IL-12, IL-23p19 and IL-27 produced in the monocytes and mDCs was measured according to the same protocol that was used for intracellular cytokine staining. Expression of TLRs, including TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9, on the surface of monocytes and mDCs was calculated. We used PBMCs obtained from each subject to calculate TLR expression, cytokine production and the percentages of regulatory T cells.

Reagents

The following monoclonal antibodies (mAbs) were used for flow cytometric analysis: fluorescein isothiocyanate (FITC)-conjugated mouse IgG1, mouse IgG2a, anti-CD4, anti-CD14, anti-CD25, phycoerythrin (PE)-conjugated mouse IgG1, mouse IgG2a, rat IgG2a, anti-CD3, anti-CD4, anti-CD14, anti-CD25, anti-TLR2, anti-TLR3, anti-TLR4, anti-TLR6, FITC-conjugated anti-human IFN- γ , anti-human IL-2 and anti-human IL-10. Anti-human IL-21 mouse mAbs were purchased from Biolegend (San Diego, Calif., USA). PE-conjugated anti-human FOXP3, anti-human CTLA-4 mAbs were purchased from eBioscience (Waken B Tech Co. Ltd., Japan). FITC-conjugated anti-human IL-23p19, PE-conjugated anti-human IL-12p35/p70 and anti-human IL-27 mAbs were pur-

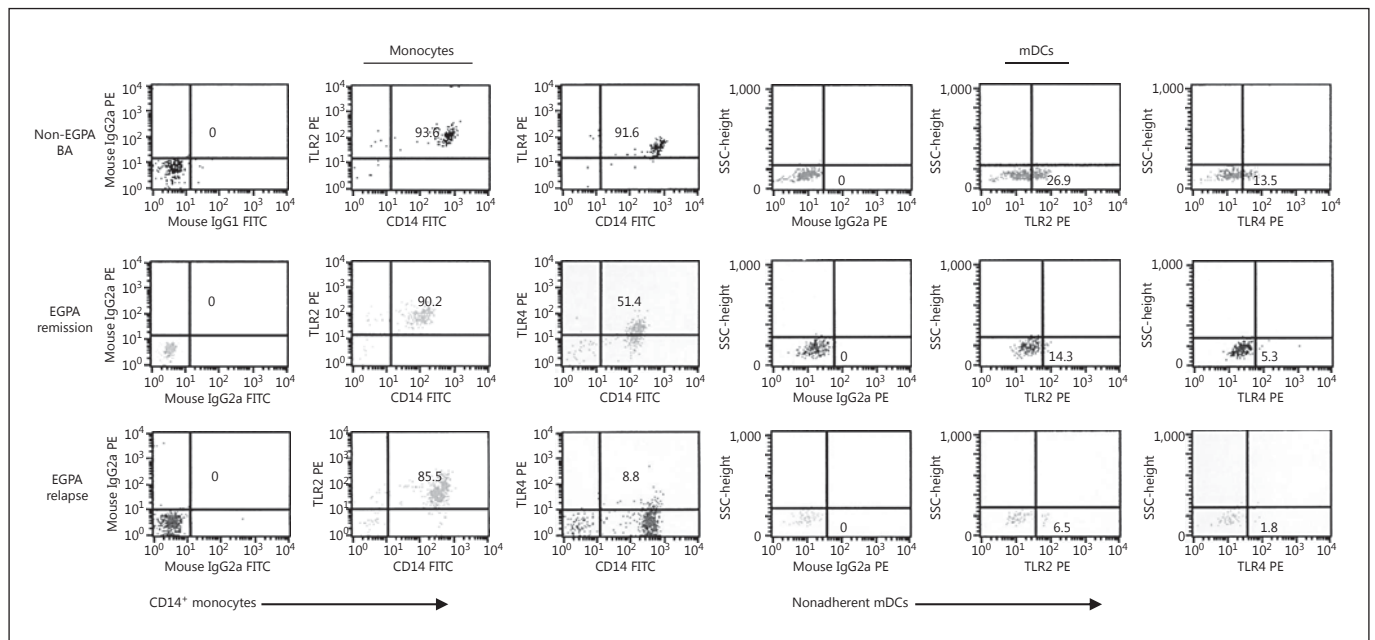


Fig. 1. Representative FACS traces showing an expression of TLR2 and TLR4 on the surface of monocytes and mDCs. BA = Bronchial asthma; SSC = side scatter.

Table 1. Characteristics of subjects

	Non-EGPA asthma group (n = 40)	EGPA relapse group (n = 14)	EGPA remission group (n = 33)
Age, years	50.4±13.0	55.4±12.3	58.0±16.0
Sex, male/female	18/22	5/9	10/23
Median eosinophil count in blood (range), cells/ml	135 (20–38,400)	220 (20–1,650)	200 (0–1,100)
IgE, U/ml	76.2±56.5	457.6±402.1	584.5±1,057.7
Type of asthma (atopic/nonatopic)	34/6	6/8	15/18
Daily dose of OCS (converted to prednisolone equivalents), mg	–	5.5±3.6	6.0±2.0
Patients taking immunosuppressants, %		40.9	14.8
CYC/AZA/CSA/MTX		2/3/5/1	0/2/2/0

Values are means ± SD, unless otherwise indicated. AZA = Azathioprine; CSA = cyclosporine; CYC = cyclophosphamide; MTX = methotrexate; OCS = oral corticosteroids.

chased from R&D Systems (Cosmo Bio Co. Ltd., Tokyo, Japan). Anti-human TLR7 rabbit, anti-human TLR9 mouse mAbs were purchased from Imgenex Co. Ltd. LPS was purchased from Cosmo Bio Co.

Cell Culture, Intracellular Cytokine Staining and FACS Analysis

PBMCs (1×10^6 cells) obtained from each subject who gave written informed consent were incubated at 37°C for 5 h in me-

dium alone or in medium containing 50 ng/ml PMA and 1 µg/ml ionomycin at 37°C in the presence of 10 µg/ml brefeldin A (MP Biomedicals, Inc., Wako, Osaka, Japan) to stimulate them to produce and accumulate cytokines. The stimulated PBMCs were then harvested and stained with FITC-conjugated anti-CD4 mAb, and after suspending then in 0.1 ml of 5.5% paraformaldehyde fixative, they were incubated at 4°C for 15 min. The cells were then washed twice with Hanks' solution containing saponin (0.1%, w/v) and sodium azide (0.09%, w/v, SAP buffer) by centrifuging at 200 g for

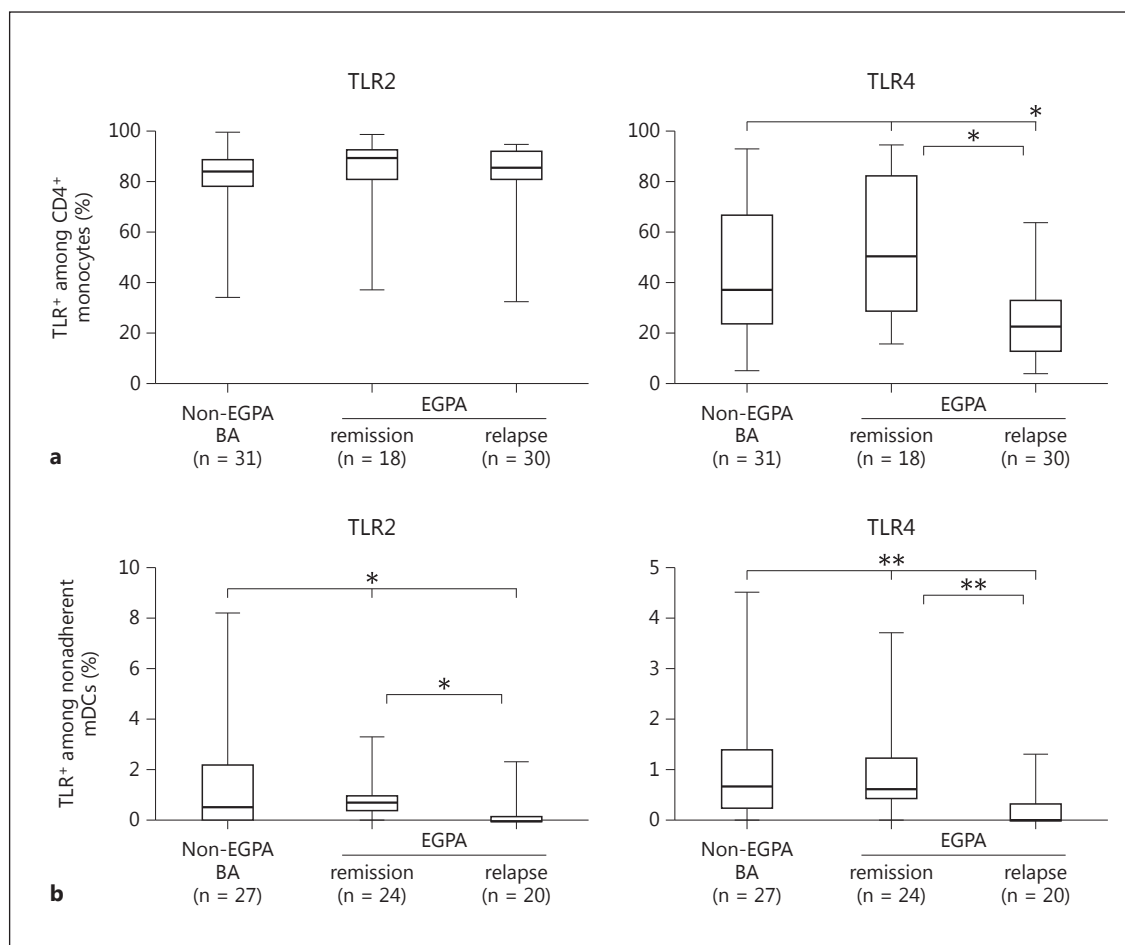


Fig. 2. Relationship between percentages of monocytes and mDCs expressing TLRs. **a** Percentage of CD14⁺ monocytes expressing TLR2 and TLR4 in the non-EGPA asthma patient group and the EGPA group. **b** Percentage of mDCs expressing TLR2 and TLR4 after maturation in response to LPS stimulation. The line in each box represents the median value. * $p < 0.01$, ** $p < 0.001$. BA = Bronchial asthma.

7 min, and the pellet was resuspended in 0.1 ml of SAP buffer. After adding mAb (2 μ l/ 10^5 cells) for each mAb against each of the cytokines, the cells were incubated for 35 min at room temperature in the dark. The cells were then washed twice with 2 ml of SAP buffer and resuspended in PBS. PBMCs (5×10^5 cells) were incubated for 2 h in medium containing a 10-ng/ml concentration of IFN- γ and then stimulated for 20 h with LPS (100 ng/ml) to produce cytokines. The PBMCs were then stained with FITC-conjugated anti-CD14 mAb and fixed. The amount of cytokine production in the CD14⁺ monocytes was measured according to the protocol used for intracellular cytokine staining. PBMCs were cultured for 2 h at 37°C in 10% FCS-containing RPMI 1640 medium for 2 h at 37°C in a 12-well microplate (Iwaki glass, Co., Ltd., Tokyo, Japan). The nonadherent cells were aspirated, and the adherent monocyte-rich cells were cultured for 6 days in medium containing a 50-ng/ml concentration of GM-CSF and 10-ng/ml concentration of IL-4. Every 2 days, half of the culture medium was replaced with fresh medium containing the same concentration of

both cytokines. At the end of 6 days, LPS was added to medium to a final concentration of 1 μ g/ml and the cells were incubated overnight to mature DCs. The amount of cytokine production and TLR3, TLR7 and TLR9 expression in the mature DCs were measured according to the protocol used for intracellular cytokine staining. They were analyzed with a FACSCalibur™ cell sorter, and the data were analyzed with CellQuest Analysis software. The cells were gated electronically according to their light-scattering properties to exclude dead and contaminant cells.

Statistics

Nonparametric descriptive and analytical statistics were used to analyze the data. A Mann-Whitney U test was used for comparisons between data from the 3 patient groups. Correlation coefficients were calculated by Spearman's rank correlation test. p values < 0.05 were considered statistically significant in all analyses.

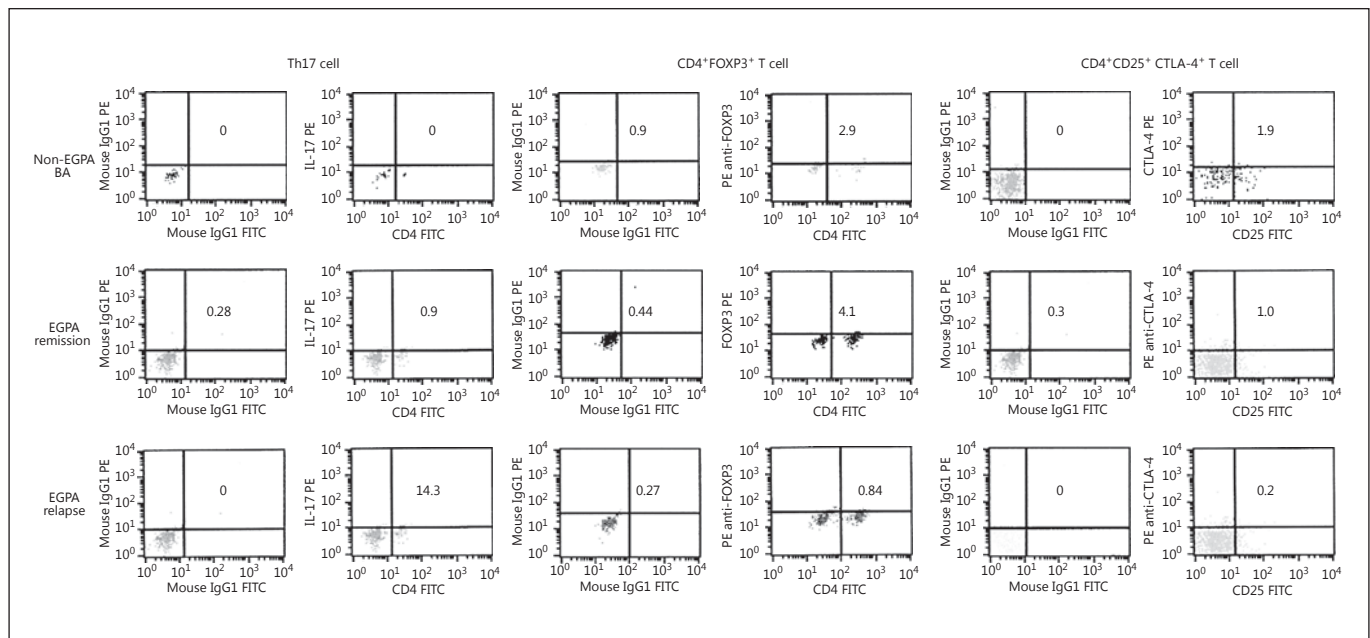


Fig. 3. Representative FACS traces showing expression of FOXP3 and CTLA-4 and production of IL-17 from CD4⁺ T cells. BA = Bronchial asthma.

Results

The Percentages of Monocytes and Percentages of mDCs Expressing TLR4 Are Associated with the Disease Status of EGPA Patients

We examined TLR expression on monocytes (fig. 1). A lower expression level of TLR4 was observed on the surface of monocytes from the relapse group versus the remission group (mean $\pm$ SD 24.5 ± 15.5 vs. $53.6 \pm 27.8\%$, $p < 0.01$) and non-EGPA asthma group ($49.6 \pm 23.1\%$, $p < 0.01$) (fig. 2a). There were no differences between the percentages of cells expressing other TLRs. Next, we measured TLR expression on mDCs. A lower expression level of both TLR2 (0.11 ± 0.1 vs. $0.35 \pm 0.21\%$, $p < 0.01$) and TLR4 (0.24 ± 0.14 vs. $0.58 \pm 0.35\%$, $p < 0.001$) was observed on the surface of mDCs from the relapse group versus the remission group and non-EGPA asthma group (0.11 ± 0.1 vs. $1.3 \pm 0.31\%$, $p < 0.01$ for TLR2 and 0.24 ± 0.14 vs. $0.65 \pm 0.32\%$, $p < 0.001$ for TLR4). There were no differences between the percentages of cells expressing other TLRs (fig. 2b).

Relationship between the Percentages of Peripheral Blood Monocytes Expressing TLR4 and the Percentages of Regulatory T Cells and Th17 Cells

We investigated the relationship between the percentage of monocytes expressing TLR4 and the percentages of

regulatory T cells and Th17 cells in the peripheral blood of all of the EGPA patients as a whole (fig. 3). The percentage of TLR4 expression was positively correlated with the percentage of regulatory T cells that expressed FOXP3 or CTLA-4, whereas the percentage of TLR4 expression was inversely correlated with the percentage of Th17 cells in the peripheral blood (fig. 4).

The Ability of Monocytes and mDCs to Produce IL-23p19 and IL-27 Reflects the Disease Status of EGPA Patients

To investigate the relationship between the cytokine production profile and disease status of EGPA patients, we investigated the ability of monocytes to produce IL-12p35/p70, IL-23p19 and IL-27 in response to LPS stimulation (fig. 5). A higher percentage of monocytes producing IL-23p19 was observed in the blood of the relapsed EGPA patients (median $\pm$ SD $3.6 \pm 2.7\%$) than in the blood of the non-EGPA asthma patients ($2.1 \pm 1.5\%$, $p < 0.05$) and the EGPA patients in remission ($0.39 \pm 1.5\%$, $p < 0.05$). On the other hand, a higher percentage of monocytes producing IL-27 was observed in the EGPA patients in remission ($1.1 \pm 1.0\%$) than in the non-EGPA asthma patients ($0.11 \pm 0.39\%$, $p < 0.05$) or relapsed EGPA patients ($0.29 \pm 0.5\%$, $p < 0.01$) (fig. 6a), but there were no differences in the percentages of

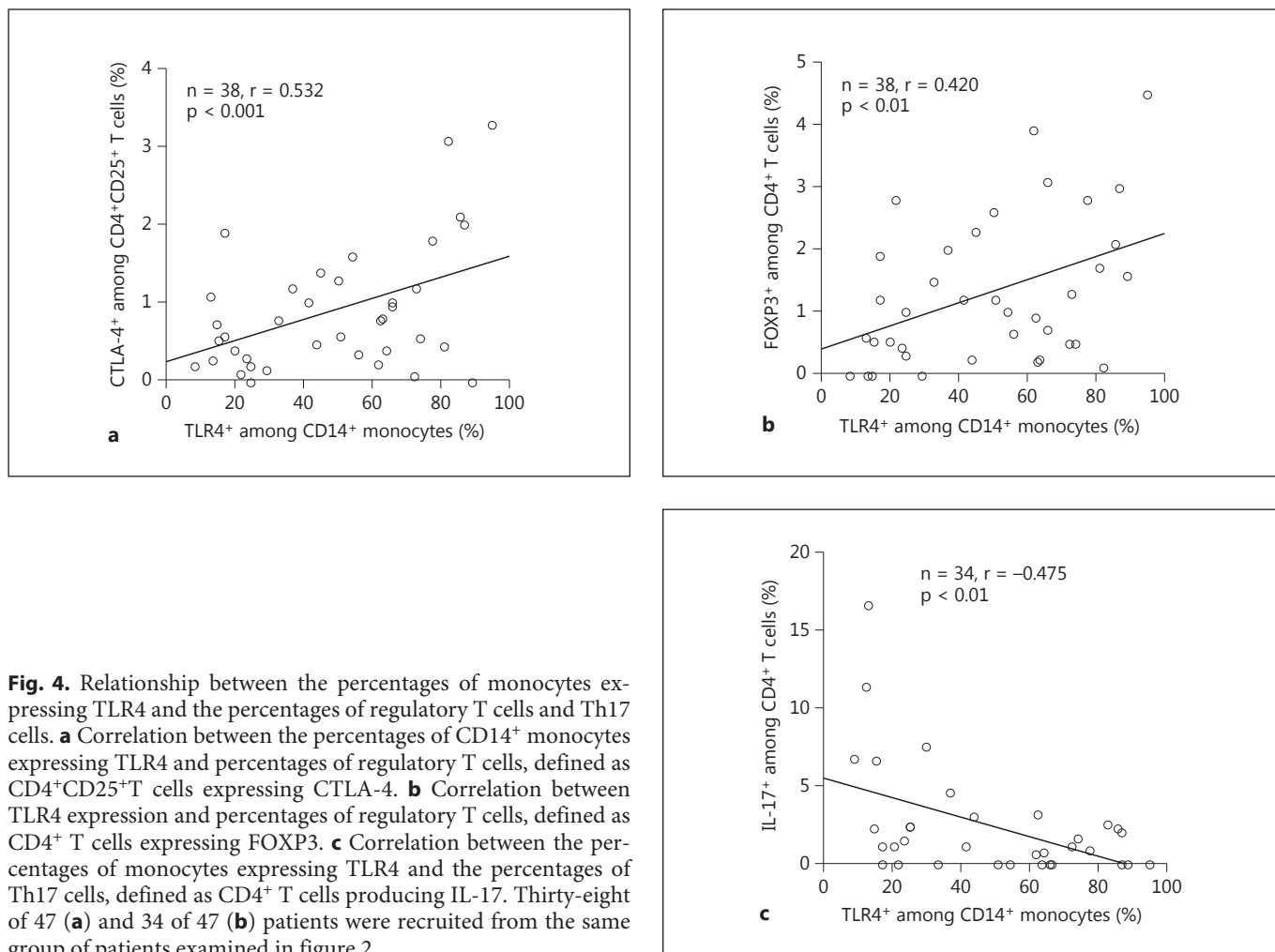
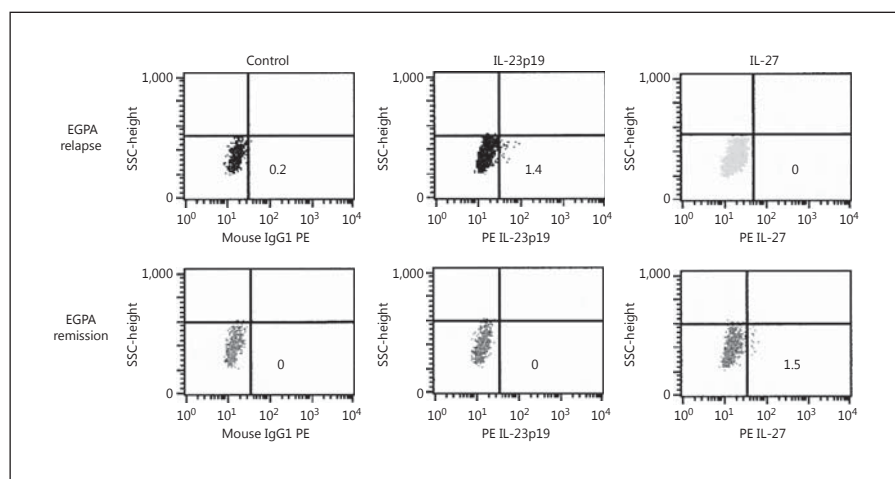


Fig. 5. Representative FACS traces showing a production of IL-23p19 and IL-27 from the mDCs in response to LPS. The numbers show the percentages of mDCs producing cytokines from EGPA patients in remission and relapse. SSC = Side scatter.



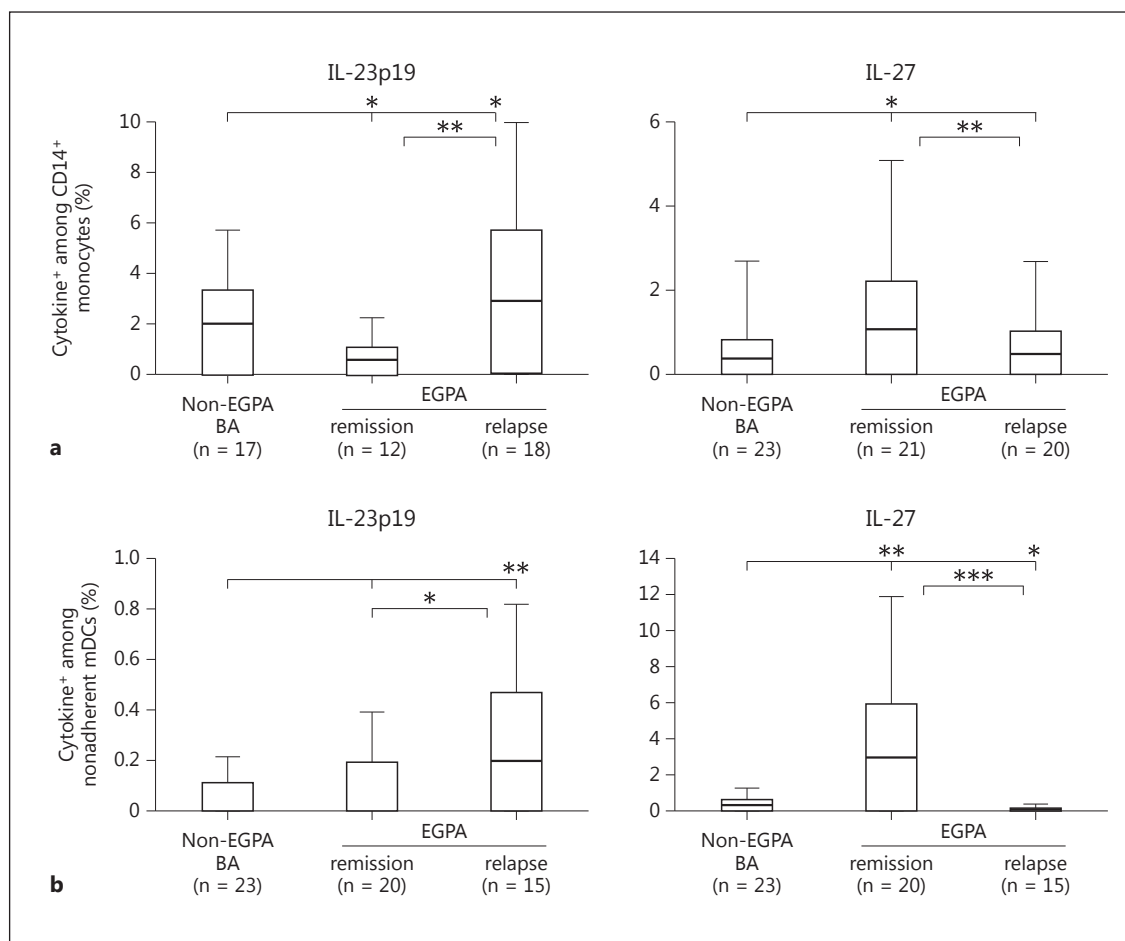


Fig. 6. Levels of cytokine production by monocytes and mDCs and EGPA status. **a** Percentage of CD14⁺ monocytes producing IL-23p19 and IL-27. **b** Percentage of mDCs producing IL-23p19 and IL-27. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. BA = Bronchial asthma.

monocytes that produced IL-12p35/p70. Next, we measured the ability of mDCs to produce the same cytokines. A higher proportion of mDCs producing IL-23p19 was observed in the relapsed EGPA patients ($0.2 \pm 0.21\%$) than in the EGPA patients in remission ($0.07 \pm 0.07\%$, $p < 0.05$), but there were no differences between the percentage of mDCs producing IL-23p19 in the non-EGPA asthma patients ($0.10 \pm 0.24\%$). On the other hand, a higher percentage of mDCs producing IL-27 was observed in the EGPA patients in remission ($2.3 \pm 0.32\%$) than among the non-EGPA patients ($0.3 \pm 0.02\%$, $p < 0.01$) or relapsed EGPA patients ($0.02 \pm 0.01\%$, $p < 0.001$; fig. 6b).

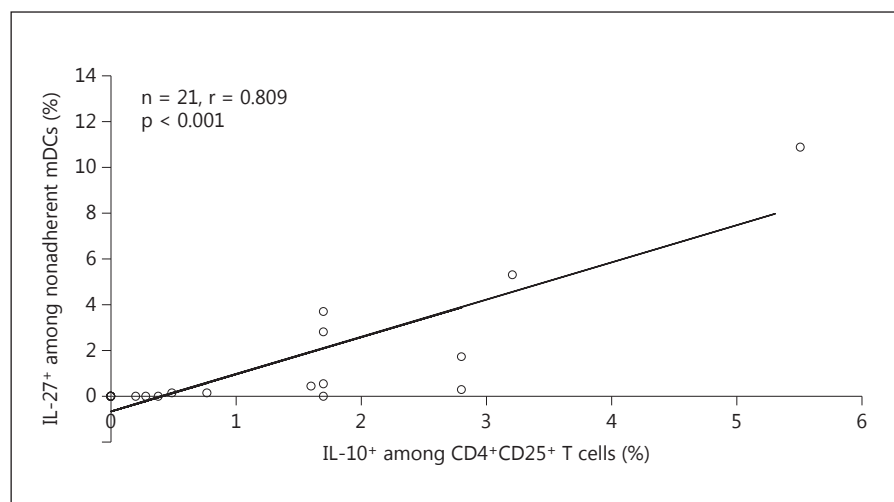
Next, we investigated the correlation between the percentage of mDCs producing IL-27 and the percentage of Tr1 cells in the peripheral blood of the patients. The re-

sults showed that the percentage of mDCs producing IL-27 was positively correlated with the Tr1 cells in the peripheral blood (fig. 7).

Discussion

To investigate the relationship between the TLR expression and status of EGPA patients, we measured the percentage of both monocytes and mDCs expressing TLR2 and TLR4. In the previous study, we had observed a lower percentage of monocytes expressing only TLR4 in patients with relapsed EGPA than that in the patients in remission, but there was a lower expression of both TLR2 and TLR4 on the surface of the monocytes and the percentage of monocytes expressing TLR2 was relatively

Fig. 7. Correlation between the percentages of mDCs producing IL-27 and the percentages of CD4⁺CD25⁺ T cells producing IL-10. Pearson's correlation test revealed a significant linear correlation between the proportions of mDCs that produced IL-27 and the proportions of CD4⁺CD25⁺ T cells that produced IL-10. Twenty-one of 35 individuals were recruited from the same group of patients examined in figure 5.



higher than that expressing TLR4. Our current observations show few changes; this study involved more cases, but no difference in the percentages of monocytes expressing TLR2 was observed, although lower expression of both TLR2 and TLR4 on the surface of mDCs was observed in the relapsed EGPA patients than in the non-EGPA asthma patients or EGPA patients in remission. A decrease in the level of TLR expression on the surface of APCs controls activation of adoptive immune responses [12]. The mechanisms responsible for the decrease in TLR2 and TLR4 on the surface of mDCs are unclear. Since there was no difference between the doses of drugs prescribed for the 2 groups of EGPA patients, oral corticosteroid therapy is less likely to cause a decrease in TLR expression. Repeated ligation of TLR4 with lipopeptides, LPS and some homologues to monocytes and DCs may be linked to a decrease in signal transduction that maintains immune homeostasis in relapsed EGPA patients. Lower expression of these TLRs may also lead not only to a decrease in sensing invading microbes by pattern recognition receptors (PRRs) but to a decrease in the maintenance of immunological tolerance. Alternatively, lower expression of both TLRs, especially TLR4, may be linked to a decrease in the expression of B- and T-lymphocyte attenuator (BTLA) on DCs. BTLA is a coinhibitory molecule with similarities to CTLA-4 and programmed cell death 1 (PD-1) and is expressed on DCs and macrophages as well as on B cells and T cells. BTLA plays a role in the regulation of T cell activation and in autoimmune disease models. Kobayashi et al. [13] recently reported finding that BTLA inhibits TLR4 signaling in DCs. Their findings may suggest that a lower percentage of DCs expressing

TLR4 is linked to the decrease in BTLA. It is necessary to examine the association between the degrees of expression of TLR4 and of coinhibitory molecules such as BTLA on mDCs.

The production of IL-12, IL-23p19 and IL-27 in DCs contributes to inducing a differentiation of Th1 cells, Th17 cells and regulatory T cells (Tr1 cells). To investigate the association between the cytokine production profiles of DCs and the status of EGPA patients, we measured the ability of both monocytes and DCs to produce these cytokines. In a previous study, we demonstrated the involvement of Th17 cells and Tr1 cells in the pathogenesis of EGPA and found that higher numbers of Th17 cells and lower numbers of Tr1 cells were present in the blood of EGPA patients during relapses than during remission. Higher percentages of both monocytes and mDCs producing IL-23p19 were calculated from the relapsed EGPA patients than in either the non-EGPA asthma patients or EGPA patients in remission. On the other hand, a higher percentage of both monocytes and mDCs producing IL-27 were calculated in the EGPA patients in remission. IL-27 is known to antagonize Th17 cell development by inducing IL-10 production. The percentages of monocytes producing IL-27 were inversely correlated with the percentages of Th17 cells. These results suggested that the percentages of Tr1 cells and Th17 cells reflect the status of EGPA. The percentage of IL-23p19-producing mDCs in the blood of the relapsed EGPA patients was approximately twice as high as in the blood of the patients in remission. On the other hand, the percentage of IL-27-producing mDCs in the blood of the patients in remission was approximately 6 times as high as the percentage in the

blood of the relapsed EGPA patients. Interestingly, the loss of the ability of mDCs to produce IL-27 was observed in the relapsed EGPA patients. A change in the percentage of mDCs expressing both TLRs may be strongly reflected by their ability to produce IL-27 rather than IL-23p19.

The results of this study showed lower expression of TLR2 and especially of TLR4 on the surface of the monocytes and mDCs of the relapsed EGPA patients, and a

higher percentage of monocytes and a higher percentage of mDCs producing IL-23p19 and IL-27 may be linked to an increased percentage of Th17 cells and Tr1 cells in the blood.

Disclosure Statement

The authors declare that no financial conflict of interest exists in relation to the content of the article.

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