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Human Eosinophils Produce Biologically Active IL-12: Implications for Control of T Cell Responses

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The present study assessed the capacity of eosinophils (EOS) to synthesize the cytokine IL-12. Blood-derived, highly purified human EOS from six atopic patients and two nonatopic individuals were treated in culture with IL-4, IL-5, granulocyte-macrophage CSF, IFN-γ, TNF-α, IL-1α, RANTES, and complement 5a, respectively. The expression of both IL-12 protein and mRNAs for the p35 and p40 IL-12 subunits was strongly induced in all donors by the Th2-like cytokines IL-4 and granulocyte-macrophage CSF and was also moderately induced by TNF-α and IL-1α. IL-5 treatment resulted in IL-12 synthesis in four atopic donors and one nonatopic donor, whereas IFN-γ induced IL-12 synthesis in only two atopic donors. In contrast, RANTES exclusively induced mRNA for the p40 subunit without detectable protein release, and complement 5a had no effect on IL-12 mRNA or protein expression. EOS-derived IL-12 was biologically active, because supernatants derived from IL-4-treated EOS superinduced the Con A-induced expression of IFN-γ by a human Th1-like T cell line. This activity was neutralized by anti-IL-12 Abs. In conclusion, EOS secrete biologically active IL-12 after treatment with selected cytokines, which mainly represent the Th2-like type. Consequently, EOS may promote a switch from Th2-like to Th1-like immune responses in atopic and parasitic diseases. *The Journal of Immunology, 1998, 161: 415–420.

Eosinophilic granulocytes play an important role in host defense against parasites (1), some drug reactions, and allergic diseases including atopic dermatitis, allergic asthma, and allergic rhinitis (2, 3). Attraction into inflamed tissue and the subsequent activation of eosinophils (EOS)2 is regulated by a wide set of biologic response modifiers, including chemokines (4, 5) and cytokines, among which those of the Th2-like type (e.g., IL-3, IL-4, IL-5, and granulocyte-macrophage CSF (GM-CSF)) are of particular importance (6–12). EOS contain large amounts of various granule proteins and degranulate and release stored proteins upon activation (13). In addition, activated EOS are able to generate reactive oxygen intermediates (14, 15). For some time, these functions were thought to constitute the only contribution of EOS to inflammatory responses. Recently, EOS have been shown to synthesize and release cytokines, including IL-4 (16) and IL-5 (17). Within the model of Th1- and Th2-like immune responses, IL-4 and IL-5 belong to the Th2-like cytokines. Therefore, EOS are not only activated by Th2-like cytokines but are also able to support Th2-like inflammatory responses and consequently perpetuate allergic diseases. Parasite defense as well as allergic diseases, especially atopic dermatitis, are also characterized by the activation of IFN-γ-producing NK cells (18, 19) and Th1-like T cells (20–22), respectively. The differentiation of these lymphocyte subsets is driven primarily by the cytokine IL-12 (23, 24). EOS infiltration is a hallmark for parasitosis (25, 26) as well as for atopic eczema (27), and EOS might therefore contribute to the generation and differentiation of those IFN-γ-producing T cells that are involved in these diseases. Therefore, we assessed the capacity of EOS to produce biologically active IL-12.

Materials and Methods

Patients

Venous blood samples were obtained from patients and healthy volunteers after written informed consent. The atopy syndrome of patients (“atopics”) was based on 1) a personal history of allergic asthma, allergic rhinoconjunctivitis, and/or atopic eczema, 2) enhanced serum IgE levels, and 3) the presence of specific IgE directed against house dust mite protein. Healthy volunteers (“nonatopics”) did not fulfill any of these criteria.

Isolation of human eosinophilic granulocytes

Human granulocytes were isolated from the heparin-anticoagulated venous blood of six atopic donors and two nonatopic donors as described previously (28). For further purification of the EOS, the granulocytes were resuspended at a concentration of 10^6/ml in HEPES-buffered HBSS (pH 7.4) containing 1 mg/ml BSA (HBSS/BSA). EOS were purified using a modification of a method described previously (29). For this purpose immunomagnetic beads (Dynabeads M-450, Dynal, Oslo, Norway) were coated with anti-CD16 Ab. In brief, 2 ml beads (4 × 10^6 beads/ml) was mixed with 50 μl anti-CD16 Ab (1 mg/ml) and incubated for 24 h at 4°C in Minosorp tubes (Nunc, Roskilde, Denmark) on a rotary mixer. Coated beads were washed three times in HBSS/BSA and retrieved using a Dynal magnetic particle concentrator (MPC-6; Dynal). The anti-CD16 Ab-coated beads were stored at a concentration of 2 × 10^6 beads/ml HBSS/BSA under sterile conditions at 4°C for a maximum of 1 wk. Each total of 1 ml of granulocytes was centrifuged in Minosorp tubes for 7 min at 820 × g at 4°C; the supernatant was aspirated, and 500 μl of the anti-CD16 Ab-coated beads were added subsequently to the pellet. The mixture was incubated for 1 h at 4°C on a rotary mixer. Thereafter, HBSS/BSA was added, and the cells which were coupled to the beads were removed magnetically using the magnetic particle concentrator. The supernatant was aspirated, and residual beads were removed by a second magnetic separation step. The resulting supernatant was washed as described above, and the resulting EOS were resuspended in HBSS/BSA. If necessary, the magnetic purification procedure was repeated once more. The EOS were quantitated with
on-line UV spectrophotometer (A260 nm), allowing an exact quantification of amplification products. Relative amounts of cDNA from each sample to be inserted into the PCR were calculated to yield similar amounts of β-actin transcript products; these calculations were confirmed by repeating RT-PCR for β-actin with the calculated amount of cDNA.

For investigation of cytokine mRNA expression, five times more cDNA was inserted than for β-actin PCR. PCR was conducted with 28 cycles, which was within the linear amplification range for all cytokine cDNAs. The following primer pairs specific for the p35 and p40 subunits of IL-12 and for β-actin were used (5’-3’): IL-12 p35 subunit: AACCGAGGAATTCCTCATG and TCTGTCGAATGCTACGGCGG; IL-12 p40 subunit: AAGGAGGCAAGTTCTAAGGC and TTTGCGGCAATGACGTGG; and β-actin: GTGGGCGCCCAAGGCCACCA and CCTCTTAAATGTCAGCCAGCATTTG.

Additionally, a PCR for β-actin was performed in parallel to each cytokine PCR as described above. Furthermore, the PCR for each cytokine sample was conducted at least two times. Products were quantified by ion-exchange chromatography as described above. To ensure the identity of products, their chromatogram peaks were collected, digested with an appropriate endonuclease, and fragments were visualized on agarose gel by ethidium bromide staining.

**Detection of IL-12 protein**

For the detection of the IL-12 present in the 24-h supernatants of eosinophils (500,000 cells/ml culture medium), a highly sensitive hIL-12 ELISA was employed (Laboserv, Giessen, Germany). This ELISA is able to detect the IL-12 heterodimer as well as the p70 homodimer with a detection limit of 5 pg/ml.

**Determination of biologic activity of EOS-derived IL-12**

For the detection of the IL-12-specific biologic activity, cells from a previously described human T cell line of Th1-like phenotype (22) were incubated in the presence of EOS conditioned medium (CM) with an addition of neutralizing anti-IL-12 Abs (Genzyme) or the respective isotype control Ab. As positive controls, T cells were incubated in the presence of 20 ng/ml rhIL-12 (Genzyme). After a 48-h culture in the presence of Con A (10 μg/ml, Sigma, Munich, Germany), the IFN-γ mRNA expression of T cells was determined by RT-PCR (see above) as described previously (22) using the following primer pair (5’-3’): GCATCGTTTTGGGTTCTCTTGGC and CACGATCTGACCTCTTTGTC.

**Results**

The supernatants of unstimulated EOS contained low amounts of IL-12 protein as detected by ELISA. The same result was obtained when EOS were cultured for 24 h in the presence of LPS, rhRANTES, or rhTNF-α, respectively. In marked contrast, EOS stimulated with rhGM-CSF or rhIL-4 released high amounts of IL-12 protein into the culture medium regardless of whether cells were obtained from atopics or nonatopics (Table I).
always associated with IL-12 mRNA expression for both IL-12 subunits, the IL-12 p35 and IL-12 p40 protein (Fig. 1, A–C). In addition, treating EOS with rhRANTES led to the selective up-regulation of IL-12 p40 mRNA (Fig. 1, A and C) without an increase of IL-12 protein in the culture medium, although the ELISA used was able to cross-react with the IL-12 p40 subunit alone. The expression of mRNA was maximal at 4 h after stimulation, but not yet associated with IL-12 protein secretion at this time point. IL-12 mRNA signals were weak and only inconsistently observed in 24-h-stimulated EOS (data not shown). To exclude the possibility that IL-12 mRNA signals in EOS preparations are due to monocytes contaminating the preparation in a manner analogous to the EOS experiments, purified monocytes were stimulated with rhIL-4, rhIL-5, rhGM-CSF, and rhIFN-γ. The RT-PCR results (Fig. 2) demonstrated constitutively expressed mRNA for both IL-12 subunits. Expression of the p40 subunit remained essentially unaltered after any of the stimulations. In contrast, treating monocytes with either IL-4 or IL-5 led to a strong down-regulation of IL-12 p35 mRNA, whereas GM-CSF and IFN-γ stimulations were not able to influence IL-12 p35 mRNA expression significantly.

The bioactivity of EOS-secreted IL-12 was assessed according to its capacity to enhance IFN-γ expression in human Th1 cells (Fig. 3, A and B). In this assay, rhIL-12 is able to superinduce the Con A-induced expression of IFN-γ mRNA. Supernatants derived from rhIL-4-treated EOS were able to enhance IFN-γ mRNA expression in a range that was comparable with the effect of rhIL-12. This effect was completely blocked by the addition of neutralizing anti-IL-12 Abs but not by a respective isotype control Ab. In contrast, the addition of supernatants from rhIFN-γ-stimulated EOS, which did not secrete ELISA-detectable IL-12, did not affect IFN-γ mRNA in Th1 cells.

FIGURE 1. A indicates the representative RT-PCR results for the IL-12 p35, IL-12 p40, and β-actin expression of EOS from donor 2 (see Table I). The 4-h stimulation of EOS, the isolation of RNA, and the RT-PCR conducted with the respective primer pairs are described in Materials and Methods. The amplification products were visualized using an ethidium bromide-stained agarose gel. Lanes 1 through 8 depict results from the following stimulations: 1, unstimulated control; 2, IL-4; 3, IL-5; 4, GM-CSF; 5, RANTES; 6, unstimulated control; 7, C5a; 8, IL-1α; and 9, TNF-α. B and C are a summary of the semiquantitative RT-PCR conducted for IL-12 p35 (B) and IL-12 p40 (C) from donors 1 through 8 (see Table I). The purification and stimulation of EOS, the isolation of RNA, the semiquantitative RT-PCR conducted with primer pairs for IL-12 p35 and β-actin as a housekeeping gene, as well as the quantification of amplification products by ion-exchange chromatography were conducted as described in Materials and Methods. Data are given as the fold expression of unstimulated EOS (control = 1) and represent the mean ± SD from all donors assessed. High SDs for IL-5- and IFN-γ-stimulated EOS result from interindividual differences in responsiveness toward these cytokines (see results in Table I for comparison).
Discussion

The present study demonstrates 1) EOS expression of IL-12 mRNA for the IL-12 p35 and p40 subunits, 2) the secretion of IL-12 protein by EOS as measured by ELISA, and 3) the presence of IL-12 bioactivity in the supernatants of stimulated EOS. This finding indicates that human eosinophilic granulocytes are endowed with the capacity to synthesize and secrete biologically active IL-12. The amount of IL-12 protein released is above the levels observed in cultured human keratinocytes (37) and may be comparable with that released by cells from the monocytic lineage. IL-12 protein production was preceded by an up-regulation of mRNA for both IL-12 subunits in every case. This may indicate
that EOS IL-12 protein is synthesized de novo via the gene expression of IL-12 p35 and p40, and that it is not due to the release of preformed protein, as has been described for other EOS-derived cytokines (16, 17, 38). Since EOS preparations were derived from peripheral blood, it may be argued that the IL-12 expression observed might be due to ≤2% contamination with monocytes. However, stimulation experiments using purified peripheral blood monocytes revealed clear differences when compared with the respective EOS experiments. In particular, the constitutive mRNA expression of both IL-12 subunits and the completely different IL-12 mRNA expression pattern following cytokine stimulations of monocytes clearly demonstrate that the results obtained for EOS cannot be explained by a monocyte contamination of EOS preparations.

EOS IL-12 production was dependent upon the donor as well as the treatment of EOS with selected biologic response modifiers. IL-12 release by EOS that were derived from the two nonatopic donors was much lower than from cells that were derived from all of the atopic individuals. This result, although obtained with a limited number of donors, is in line with previous observations that EOS from atopic donors are in a preactivated status, which allows increased cytokine production (e.g., of IL-4) (16). Responsiveness toward IL-5 and IFN-γ was characterized by high interindividual differences, whereas IL-4 and GM-CSF consistently induced IL-12 secretion. However, in responsive EOS preparations, IFN-γ as well as IL-5 induced the secretion of large amounts of IL-12 protein that even exceeded those observed after IL-4 stimulations. This functional heterogeneity of EOS upon cytokine treatment may emerge from different environmental conditions in a given individual, which may be able to prime EOS responses toward distinct cytokines. Taken together, most of the cytokines leading to EOS IL-12 production are Th2-like cytokines (IL-4, GM-CSF, and IL-5), although the effects of the Th1-like cytokine IFN-γ cannot generally be excluded.

By virtue of their capacity to secrete IL-12 upon exposure to Th2-like cytokines, EOS may play a previously unrecognized role in switching an ongoing Th2-dominated immune response to a Th1-response. The present data were derived from vitro experiments, but it is interesting to speculate whether EOS IL-12 secretion might also influence T cell-mediated immune responses in vivo. For example, the sequential activation of Th2-like followed by Th1-like T cell activation was observed in positive inhalant allergen patch test reactions in atopic dermatitis patients (39, 40); such test reactions are used as a model to study the pathogenesis of atopic dermatitis. For this system, it has been reported that the tested skin areas during the initiation phase are characterized by 1) an increased in situ expression of Th2-like cytokines and 2) infiltration with activated EOS. This is followed by the development of an eczematoid skin reaction, which is preceded by an in situ expression of IL-12 (39) and, in contrast to the early phase, is characterized by strong IFN-γ expression. Thus, one could put forward a model in which the initial Th2-dominated environment induces EOS to secrete IL-12 which in turn supports the expression of IFN-γ by Th1-like T cells.

EOS-derived IL-12 may also play a role in parasite defense. It could be speculated that EOS-derived IL-12 supports the development of the Th1-like immune response that is required for successful defense. For example, IL-12 production is crucial for the activation of NK cells and for the survival of the infected animal (41–43) in toxoplasma infection. Thus far, macrophages have been thought to be the major IL-12 source (42). In light of the present observations, it is intriguing to speculate that EOS may contribute to IL-12 production during parasitosis and consequently to the effective elimination of parasites.

However, the described IL-12 production by human EOS and its exact pathophysiologic role needs to be investigated further with regard to the broad range of diseases involving eosinophilic granulocytes.

References


