

Expression of CD28 and CD86 by Human Eosinophils and Role in the Secretion of Type 1 Cytokines (Interleukin 2 and Interferon γ): Inhibition by Immunoglobulin A Complexes

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Summary

Eosinophils are the source of various immunoregulatory cytokines, but the membrane molecules involved in their secretion have not been clearly identified. Here we show that peripheral blood eosinophils from hypereosinophilic patients could express membrane CD86 but not CD80. The T cell costimulatory molecule CD28 is also detected on the eosinophil surface. CD28 ligation but not CD86 ligation resulted in interleukin (IL)-2 and interferon (IFN)- γ secretion by eosinophils, whereas IL-4, IL-5, and IL-10 were not detected. In contrast to T cells requiring two signals for effective stimulation, CD28 ligation alone was sufficient for optimal eosinophil activation. Eosinophil-derived IL-2 and IFN- γ were biologically active, as supernatants from anti-CD28-treated cells were able to induce CTLL-2 proliferation and major histocompatibility complex class II expression on the colon carcinoma cell line Colo 205, respectively. Addition of secretory immunoglobulin (Ig)A-anti-IgA complexes, which could induce the release of IL-10, very significantly inhibited both CD28-mediated IL-2 and IFN- γ release. These results suggest that the release of type 1 (IFN- γ and IL-2) versus type 2 cytokines by eosinophils is not only differential but also dependent on cross-regulatory signals. They confirm that through activation of costimulatory molecules, eosinophils could function as an immunoregulatory cell involved in the release of both type 1 and type 2 cytokines.

Key words: eosinophils • CD28 • CD86 • type 1 cytokines • secretory IgA

Proverbially implicated in parasitic infections and allergic manifestations, eosinophils are in fact associated with most of the inflammatory or infectious disorders. Their major function was thought to be restricted to degranulation and release of highly cytotoxic proteins. However, in addition to being cytotoxic mediators, eosinophils have the capacity to produce a large number of cytokines, including pro- and antiinflammatory cytokines, growth factors, and chemokines (1). The demonstration that eosinophils from patients or donors could synthesize and release IL-5 (2, 3), IL-4 (4, 5), and IL-10 (6, 7) suggested that eosinophils could also participate in the regulation of the Th2 pathway of the immune response. Eosinophilia, frequently associated with increased IgE levels, appears mainly linked to a Th2 response. However, the clinical heterogeneity of hypereosinophilic syndromes, including patients with and without increased IgE levels (8), led us to suggest that eosinophils could be also associated with a Th1 response. Preliminary studies have indicated that eosinophils from hypereosinophilic patients stained positively for IFN- γ (9), while IL-2 and more recently IL-12 were also detected within eosinophils (10, 11), indicating that eosinophils

could play important roles in the modulation of the immune response, in both its type 1 and type 2 pathways.

Until now, very few studies have reported the secretion of these various cytokines by eosinophils, and they were mainly based on the use of nonphysiological stimuli such as calcium ionophores or serum-coated particles (5). The more convincing results on cytokine secretion were obtained after activation of eosinophils with IgA immune complexes, which induce the release of IL-5 (3) and IL-4 (4) but do not seem to be able to induce the secretion of Th1 cytokines.

In addition to Fc receptors involved in the release of granule proteins, eosinophils express a large variety of membrane molecules, including MHC class II (12), CD40 (13), and CD40L (14), which participate in their cross-talk with T and B cells. Previous studies have shown that eosinophils can function as APCs (15, 16). Thus, it was of interest to investigate whether eosinophils could express other costimulatory molecules potentially involved in cytokine release.

It is now well accepted that the major T cell costimulatory pathway involves the CD28 molecule (17). After en-

gement of the TCR with a class II (or I) MHC molecule on the APC, IL-2 production and IL-2 receptor expression are initiated; the second signal provided by the CD28/B7 interaction stabilizes IL-2 mRNA and increases IL-2 secretion, resulting in T cell proliferation and clonal expansion (18, 19). CD28 seems to be expressed only on T cells, and its expression increased after T cell activation. CD28 interacts with molecules of the B7 family present mainly at the surface of murine and human APCs, as well as on activated T and B cells (20). Two members have been identified: B7-1 (CD80) and B7-2 (CD86). Most APCs require stimuli for the induction of CD80 and CD86 expression, but CD86 is induced more rapidly than CD80 (21). It has been suggested that CD80-expressing APCs provided the co-stimulus for type 1 cells and CD86-expressing APCs for type 2 cells (22).

In this work we showed, by flow cytometry, that purified eosinophils from eosinophilic patients express membrane CD86 but not CD80. In addition, the presence of CD28 was also detected on eosinophil membrane. After CD28 ligation, eosinophils were able to secrete IL-2 and IFN- γ , with biological activities, whereas no secretion of IL-4, IL-5, or IL-10 was detected. During the course of these experiments, it was shown that the addition of IgA-anti-IgA complexes was able to inhibit the release of IL-2 and IFN- γ mediated by CD28 ligation. In contrast, IgA complexes induced the release of IL-10, which in turn was inhibited by the addition of anti-CD28 Abs. These results suggest differential pathways leading to the release of type 1 (IFN- γ and IL-2) versus type 2 (IL-10) cytokines by eosinophils. The demonstration that eosinophils could express costimulatory molecules involved in the selective secretion of type 1 cytokines suggests a new function for eosinophils in the regulation of the immune response.

Materials and Methods

Eosinophil Donors. A total of 21 different patients with hyper-eosinophilia associated with skin diseases, allergy, hypereosinophilic syndromes, or myeloproliferative disorders were selected for this study, after informed consent. Five normal donors were also included in the study. The characteristics of eosinophil donors and eosinophil preparations are summarized in Table I.

Reagents. Anti-human CD16 and CD3 magnetic beads and the magnetic cell separation system (MACS[®]) were purchased from Miltenyi Biotec. Percoll was obtained from Amersham Pharmacia Biotech. RPMI 1640 medium, glutamine, penicillin, streptomycin, and FCS were obtained from GIBCO BRL. The FITC-conjugated and nonconjugated anti-CD28 (clone B-T3), anti-CD86, and mouse IgG1 mAbs were purchased from Diaclone. The FITC-conjugated and nonconjugated anti-CD80 were from Immunotech. The anti-CD28 Ab (9.3 mAb) was a gift from Dr. C. June (University of Pennsylvania School of Medicine, Philadelphia, PA). The anti-CD28 (clone CD28.2) and the anti-mouse IgG F(ab')₂ were from Sigma Chemical Co. The FITC-conjugated anti-IFN- γ , anti-IL-2, and anti-IL-10 mAbs and isotype-matched Abs were obtained from Diaclone. The nonconjugated anti-IL-2 was from Genzyme Corp. The anti-IFN- γ , anti-IL-10, and isotype control Abs were from Dia-

Table I. Summary of the Characteristics of Eosinophil Donors

Donors	Diagnosis	Patient group	Eosinophilia*	Percentage of eosinophils [†]
1	Allergy	A	1,780	99
2	Allergy	A	1,200	98
3	Churg-Strauss	A	1,550	90
4	Churg-Strauss	A	13,800	99
5	Allergy	A	2,070	100
6	Eczema	SK	1,900	100
7	Eczema	SK	1,000	95
8	Erythroderma	SK	1,400	97.5
9	Angioma	SK	8,050	99.5
10	Psoriasis	SK	2,100	94
11	Psoriasis	SK	750	96.5
12	HES	HD	1,370	97.5
13	HES	HD	1,610	97
14	Lymphoma	HD	5,900	95
15	HES	HD	3,540	98
16	HES	HD	4,000	98
17	HES	HD	9,900	99.5
18	Allergy	A	1,100	97
19	HES	HD	43,700	99.5
20	Eczema	SK	2,400	98
21	Angioma	SK	3,100	97
22	Normal donor	ND	<300	97.5
23	Normal donor	ND	<300	85
24	Normal donor	ND	<300	96
25	Normal donor	ND	<300	83
26	Normal donor	ND	<300	93.5

A, allergy; HD, hematological disorders; HES, hypereosinophilic syndromes; ND, normal donors; SK, skin diseases.

*Absolute numbers of eosinophils/mm³.

[†]Percent purity of eosinophil preparation after purification on MACS[®].

clone. The recombinant human (rh)IL-2 was purchased from Boehringer Mannheim, and the rhIFN- γ was from Diaclone. Human secretory IgA was obtained from Sigma Chemical Co. Anti-human IgA mAb was from Immunotech. The Quantum Simply Cellular quantification kit and the mouse alkaline phosphatase anti-alkaline phosphatase (APAAP)¹ detection system were purchased from Dako. The goat anti-CD28 (C-20) was from Santa Cruz Biotechnology. The mouse IgG control Ab and the horseradish peroxidase (HRP)-conjugated anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories.

Eosinophil Purification. Eosinophils were isolated from the venous blood of patients by the method described previously (23) with minor modifications, using immunomagnetic beads and the magnetic cell separation system (MACS[®]). Diluted whole blood (1:1) was layered onto a Percoll gradient ($d = 1.082$ g/liter) and

¹Abbreviations used in this paper: APAAP, alkaline phosphatase anti-alkaline phosphatase; HRP, horseradish peroxidase; LDH, lactate dehydrogenase.

centrifuged at 1,800 rpm for 20 min. The granulocyte pellet, mainly neutrophils and eosinophils, was harvested and depleted of erythrocytes by hypotonic saline lysis. In brief, the granulocyte pellet was incubated for 30 min at 4°C with anti-CD16 and anti-CD3 immunomagnetic beads in order to remove neutrophils and contaminating lymphocytes, respectively. Eosinophils were eluted by passage of the cells through the field of a permanent magnet. After isolation, eosinophil preparations were cytocentrifuged, and the cytopspins were stained with May-Grünwald-Giemsa (RAL 555; Rieux). The purity of eosinophil preparations usually reached >97% for patients (Table I).

Flow Cytometric Analysis. Freshly purified eosinophils were resuspended at 3×10^6 /ml in PBS/1% BSA. Aliquots of 50 μ l were incubated with FITC-conjugated anti-CD28, anti-CD80, or anti-CD86 mAb or FITC-conjugated isotype-matched Ab at a final concentration of 5 μ g/ml for 1 h at 4°C in round-bottomed 96-well plates. After two washes in PBS, the cells were resuspended in PBS/0.5% BSA before analysis.

For intracellular staining, eosinophils were fixed with 2% paraformaldehyde in PBS for 10 min. After washing in PBS, the cells were resuspended at 4×10^6 /ml in PBS containing 1% BSA and 0.5% saponin (permeabilization buffer) for 10 min at room temperature. The samples were then preincubated for 10 min with 5 μ l normal mouse serum in order to block nonspecific binding, and were incubated for an additional 30 min with FITC-conjugated anticytokine mAb or isotype-matched Ab at a final concentration of 5 μ g/ml in permeabilization buffer. After washing in permeabilization buffer, followed by washing in PBS, the cells were resuspended in PBS/0.5% BSA. Samples were analyzed on a FACSCalibur™ using CellQuest software (Becton Dickinson). 10^4 events were usually acquired per sample. Thresholds were set on control stains (included for every sample at every time point).

The antigen density at the cell surface was quantified with the Quantum Simply Cellular quantification kit, in which the median values of fluorescence intensity were converted into Ab binding capacity (ABC) units using calibrating microbeads with specific Ab binding capacity. In parallel to staining of the samples, the goat anti-mouse Ig-coated microbeads were incubated with FITC-conjugated anti-CD28, anti-CD86, or FITC-conjugated isotype-matched Ab. The lower quantification limit for CD28 and CD86 expression was 7,300 ABC units.

Immunocytochemistry. Cytopspins of freshly purified eosinophils were fixed in acetone at -20°C for 10 min. After air drying, slides were stored at -20°C until use. The APAAP method was used for immunostaining. In brief, after saturation with 10% normal rabbit serum diluted in Tris-buffered saline (TBS) containing 1% BSA, cytopspins were incubated with anti-human cytokine or isotype control mAbs at 30 μ g/ml in TBS/1% BSA overnight at 4°C. The slides were washed twice for 15 min in TBS, then incubated with rabbit anti-mouse Igs (1:25) in TBS/1% BSA for 1 h at room temperature. After washing as above, they were incubated with APAAP complex (1:40) for 1 h, and the reaction was developed with New Fuchsin substrate (Dako). The slides were counterstained with Mayer's hematoxylin and mounted with Fluoroprep (BioMérieux). For IFN- γ and IL-10 detection, the intensity of the reaction was increased by performing a second round of APAAP reaction.

Immunoprecipitation and Immunoblotting. Freshly purified eosinophils (2.5×10^7) and Jurkat cells (2×10^7) were lysed on ice for 30 min with 0.5% Triton X-100 in borate-buffered saline (BBS) buffer, pH 8.0, containing 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin, 1 μ g/ml leupeptin. The lysates were centri-

fuged at 12,000 *g* at 4°C. Supernatants were sequentially incubated with 30 μ l of protein G-Sepharose beads (1 h, 4°C; Sigma Chemical Co.), 30 μ l protein G-Sepharose-bound mouse IgG (1 h, 4°C), and then 30 μ l protein G-Sepharose-bound anti-CD28 (9.3 mAb) for 2 h at 4°C. The beads were washed five times with lysis buffer, and immune complexes were resuspended in reducing Laemmli sample buffer. The samples were heated to 95°C for 5 min and then run on 8% SDS-PAGE. The separated proteins were transferred onto nitrocellulose membrane. The membrane was incubated in a blocking buffer containing 5% BSA and 0.1% Tween 20 in PBS for 2 h, followed by incubation with a goat anti-CD28 at a 1:500 dilution in PBS/1% BSA/0.1% Tween 20 for 2 h. After washing in PBS/0.1% Tween 20, the blot was incubated for 1 h with HRP-conjugated anti-goat Ab at a 1:5,000 dilution in PBS/0.1% Tween 20. Immunoblot signals were detected using Renaissance Western Plus reagent from NEN Life Sciences.

Cell Activation. Culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin. Cross-linking experiments were performed in 24-well culture plates. Wells were first coated with 40 μ g/ml anti-mouse IgG F(ab')₂ for 2 h at 37°C in 5% CO₂. After washing, wells were incubated with 10 μ g/ml mouse anti-CD28 (B-T3 mAb), anti-CD86 mAb, or isotype-matched control mAb for 2 h at 37°C in 5% CO₂. The wells were then washed twice with PBS, and 2×10^6 eosinophils in 1 ml culture medium were added per well. After 18 h culture, supernatants were collected and analyzed for cytokine secretion.

For stimulation with IgA and anti-IgA, highly purified eosinophils were first incubated with secretory IgA at a final concentration of 15 μ g/ml. After 1 h incubation at 37°C, cells were transferred into either 24-well plates or anti-CD28-coated plates and stimulated with 20 μ g/ml anti-IgA mAb at 37°C in 5% CO₂. After 18 h culture, cell viability was determined by trypan blue exclusion, and supernatants were collected and analyzed for cytokine secretion.

Lactate Dehydrogenase Assay. Eosinophil cytolysis was followed by measuring the cytoplasmic marker lactate dehydrogenase (LDH) in the supernatants of eosinophils incubated for 18 h with medium alone, immobilized anti-CD28 mAb, IgA immune complexes, or with Triton X-100 as positive control. LDH was evaluated by colorimetric assay (Boehringer Mannheim) (24).

Cytokine Measurements. IL-2, IFN- γ , and IL-10 were assayed in eosinophil supernatants using specific ELISA kits (Diacclone) according to the manufacturer's instructions. The lower detection limit was 10 pg/ml for IL-2, 5 pg/ml for IFN- γ , and 5 pg/ml for IL-10.

Determination of Biological Activities of Eosinophil-derived IL-2 and IFN- γ . The biological activity of IL-2 released in eosinophil supernatants was measured by the conventional CTLL-2 assay. In brief, 10^4 CTLL-2 cells per well were cultured in 96-well microtiter plates with serial dilutions of rhIL-2 or eosinophil supernatants (at a 50% dilution) in a final volume of 100 μ l, for 24 h at 37°C in 5% CO₂. 6 h before harvesting, cells were pulsed with 0.5 μ Ci [³H]thymidine. All assays were performed in duplicate.

To determine the biological activity of IFN- γ released by eosinophils, the capacity of IFN- γ to induce expression of MHC class II (HLA-DR) protein on the human colon carcinoma cell line Colo 205 (American Type Culture Collection) was used (25). In brief, 2×10^4 cells in a final volume of 200 μ l were cultured in 96-well plates in the presence of variable concentrations of rhIFN- γ (0.1–10 U/ml) or eosinophil supernatants (at a dilution of 20%), in the presence or absence of neutralizing anti-

IFN- γ mAb (Diacclone). After 48 h culture at 37°C in 5% CO₂, cells were harvested and analyzed for HLA-DR expression by flow cytometry, using PE-conjugated anti-HLA-DR mAb (Becton Dickinson).

Results and Discussion

Detection of Intracellular Cytokines in Human Eosinophils. Freshly isolated eosinophils from eosinophilic patients were used to examine the intracellular expression of IL-2, IFN- γ , and IL-10. Results from flow cytometric analysis presented in Fig. 1 A, which illustrates one representative experiment, showed that human eosinophils could express IL-2 and IFN- γ , as well as IL-10. The specificity of intracellular staining was controlled by incubation of the cells with FITC-conjugated mAb in the presence of an excess of the corresponding recombinant cytokine. For the three cytokines under study, this procedure led to an almost total inhibition of the signal (data not shown).

To confirm the intracellular expression of the cytokines observed by flow cytometry analysis, cytospin preparations of purified eosinophils were incubated with anticytokine mAb from different clones than those used for flow cytometry. The preparations were then processed for immunohistochemical analysis using the APAAP method, followed by counterstaining with hematoxylin. As shown in Fig. 1 B, eosinophils exhibited positive staining for IL-2, IFN- γ , and IL-10. No staining was observed when cells were incubated with an irrelevant isotype control Ab.

Until now, most studies have examined the cytokine content of human eosinophils by nonquantitative ap-

proaches such as reverse transcription PCR for mRNA expression and immunocytochemistry or electron microscopy for intracellular protein detection (2–5). By using cytofluorometry, we showed clearly that a large proportion of human eosinophils have the capacity to express both type 1 (IL-2 and IFN- γ) and type 2 (IL-10) cytokines.

Detection of CD86 and CD28 on Human Eosinophils. To investigate the expression by eosinophils of the costimulatory molecules CD80, CD86, and CD28, freshly isolated human eosinophils from 17 hypereosinophilic patients and 5 normal donors were stained with FITC-conjugated anti-CD80, anti-CD86, or anti-CD28 (B-T3) mAb or isotype-matched Ab and analyzed by flow cytometry. Results presented in Fig. 2 A show clearly that, among the B7 family, eosinophils could express CD86 (B7-2) but not CD80 (B7-1). Unexpectedly, the presence of the T cell-associated molecule CD28 was also detected on eosinophils. Both CD86 and CD28 were detected in all donors, but their expression was variable among individual patients, ranging from 6 to 63% and from 4 to 25%, respectively (Fig. 2 B).

For both CD86 and CD28, the mean fluorescence intensity (MFI), which reflects the level of binding of the Abs, was low, suggesting a low level of membrane expression. Indeed, using a technique for direct quantification of cell surface molecules, we detected $8,003 \pm 231$ and $9,039 \pm 644$ Ab binding sites per cell for CD28 and CD86, respectively ($n = 4$). Preliminary experiments performed on a limited number of healthy donors indicate that normal eosinophils could also express membrane CD86 and CD28, but at a lower level (Fig. 2 B). Due to the small number in each group of eosinophilic patients, it was difficult to ob-

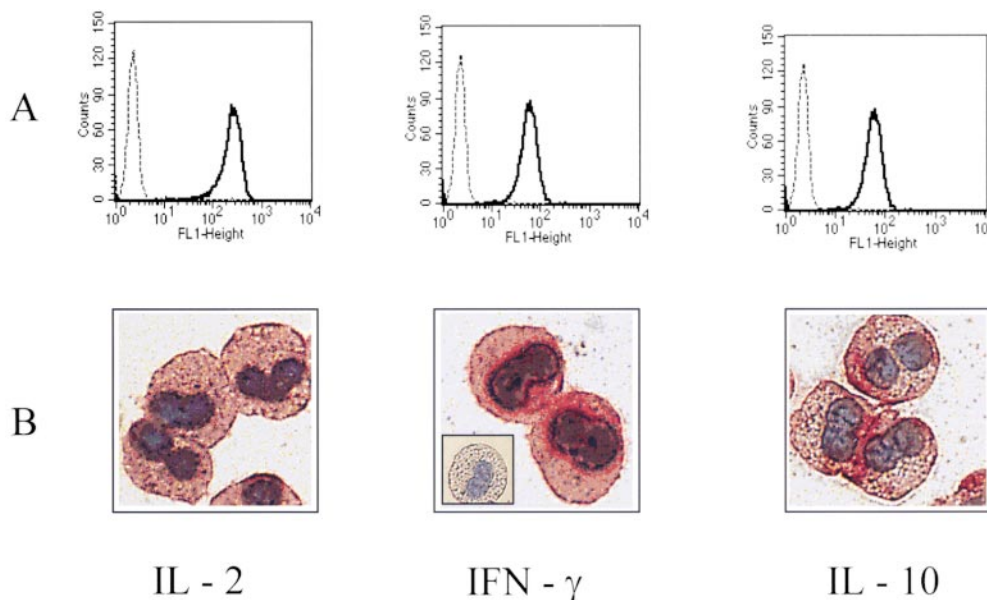


Figure 1. Intracellular production of IL-2, IFN- γ , and IL-10 by human eosinophils. Freshly purified eosinophils (purity >98%) were analyzed by direct immunofluorescence flow cytometry (A) and immunocytochemistry (B). (A) After fixation and permeabilization, the cells were stained with FITC-conjugated anti-IL-2, anti-IFN- γ , or anti-IL-10 (bold line) or isotype-matched Ab (dashed line), as described in Materials and Methods. Cell fluorescence was measured using a FACSCalibur™ equipped with CellQuest software, and thresholds were set according to the isotype-matched controls. A total of 10⁴ cells were usually acquired. (B) Cytospins of eosinophil preparation were incubated with anti-IL-2, anti-IFN- γ , anti-IL-10 mAb or isotype-matched Ab (inset), and the staining was revealed using the APAAP detection system and New Fuchsin coloration. Cells were counterstained with Mayer's hematoxylin (original magnification: $\times 100$).

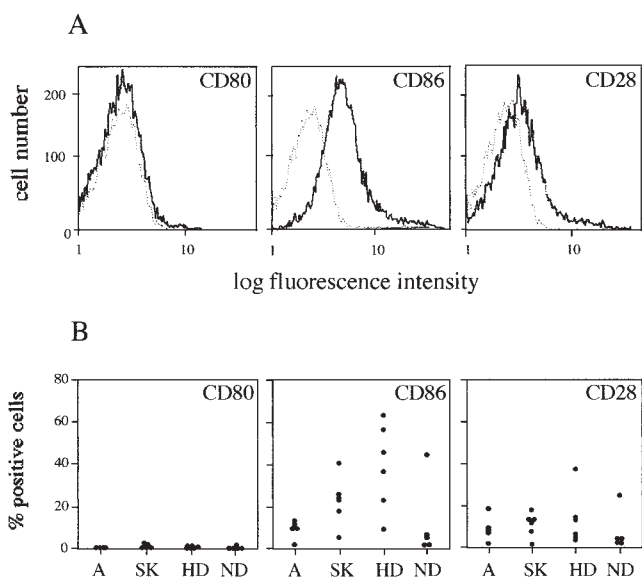


Figure 2. Expression of costimulatory molecules of the CD28/B7 family by human eosinophils. (A) Representative profiles of anti-CD80, anti-CD86, and anti-CD28 mAb binding to freshly purified human eosinophils. Cells were stained with FITC-conjugated anti-CD80, anti-CD86, or anti-CD28 mAb (bold line) or isotype-matched control Ab (dotted line). Samples were analyzed by flow cytometry using a FACSCalibur™ equipped with CellQuest software. A total of 10^4 cells were usually acquired. (B) Variable expression of CD86 and CD28 by individual patients. Data are presented as percentage of positive cells from 17 eosinophilic patients (Table I, donors 1–17) and 5 normal donors (Table I, donors 22–26). The donors were classified as follows: A, allergy; SK, skin diseases; HD, hematological disorders; ND, normal donors. The percentage of positive cells was calculated by subtracting the isotype control from the specific signal.

serve any correlation with the etiology of the disease, as reported for other membrane molecules (26).

To further confirm the expression of CD28 by human eosinophils, immunoprecipitation followed by Western blotting was performed. As shown in Fig. 3, a 50-kD protein was specifically immunoprecipitated with anti-CD28 mAb (9.3 mAb), whereas no band appeared when lysates were immunoprecipitated with mouse IgG-bound Sepharose beads. Under the same conditions, immunoprecipitation using Jurkat cells as positive control showed a much stronger band, indicating that CD28 expression in human eosinophils is lower than in T cells, as already suggested by flow cytometry (Fig. 2). Although CD28 is a homodimeric molecule, comprising a 44-kD subunit, the variation in molecular mass observed between eosinophils and Jurkat cells likely arises as a result of different glycosylation patterns. Such an observation has already been made for a T cell leukemia cell line (27).

Secretion of IL-2 and IFN- γ after CD28 Cross-linking. The demonstration that human peripheral blood eosinophils could express CD86 and CD28 led us to investigate the functional significance of these costimulatory molecules in eosinophil activation. Since it has been reported that CD28 ligation in T cells resulted in IL-2 secretion and pro-

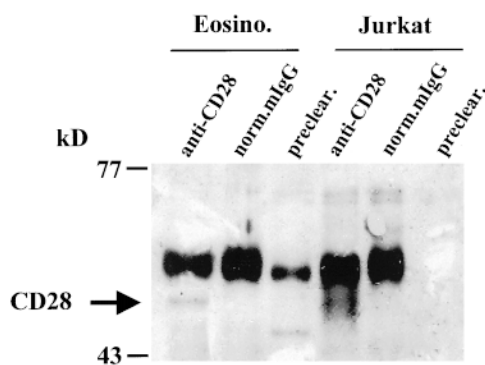


Figure 3. CD28 expression in human eosinophils. Western blot analysis of freshly purified eosinophils (Eosino., 2.5×10^7) and Jurkat T cells (2×10^7) after sequential immunoprecipitation with unconjugated Sepharose beads (preclear.), mouse IgG control Ab (norm.mIgG), and anti-CD28 mAb (anti-CD28 [9.3 mAb]). Material was run on 8% SDS-PAGE and transferred to nitrocellulose. Membrane was probed with anti-CD28 Ab.

liferation, cross-linking experiments of purified eosinophils with immobilized anti-CD28 or anti-CD86 mAb were performed, and the release of cytokines was assayed in supernatants. Eosinophils from six individual patients were added to plates coated with anti-CD28 or anti-CD86 mAb, and supernatants were collected after 18 h and analyzed. As shown in Fig. 4, CD28 ligation induced IL-2 and IFN- γ secretion, whereas in the same activation conditions, no release of IL-4, IL-5, or IL-10 was detected (data not shown). Contrary to T cells, stimulation via the CD28 molecule did not appear to require a second signal for optimal eosinophil stimulation, since the addition to anti-CD28 mAb of various stimuli such as LPS, Ca^{2+} ionophores, or PMA did not lead to an increase in cytokine secretion (data not shown). Fig. 4 shows that the secretion of IL-2 and IFN- γ was variable according to individual patients, with a massive production of IL-2 (up to 700 pg/ml) observed for some donors after CD28 stimulation. The levels of IFN- γ were lower than those of IL-2, with a maximum of 180 pg/ml and a minimum of 30 pg/ml. It is interesting to notice that the maximum secretion for IL-2 and IFN- γ was reached for the same eosinophil donors. In contrast to CD28 cross-linking and although CD86 was expressed on these eosinophil preparations, CD86 ligation did not induce the secretion of IL-2 or IFN- γ (Fig. 4) or the release of IL-4, IL-5, or IL-10. Nonstimulated eosinophils or eosinophils cross-linked with isotype-matched Ab produced very low amounts of IL-2 and IFN- γ (<10 pg/ml). In parallel to the anti-CD28 mAb from clone B-T3, we also used anti-CD28 mAb from a different clone. Immobilized 9.3 mAb was also able to induce IL-2 and IFN- γ secretion, although to a lesser extent (data not shown).

To exclude the possibility that some lymphocytes could contaminate the eosinophil preparation and therefore be responsible for the IL-2 and IFN- γ secretion observed, lymphocytes isolated from hyper-eosinophilic patients were stimulated in the same conditions as purified eosinophils with immobilized anti-CD28. No secretion of IL-2 and

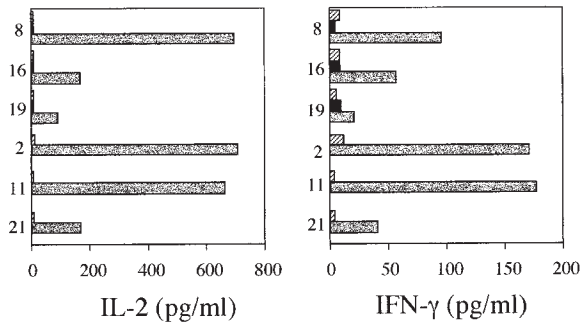


Figure 4. Release of IL-2 and IFN- γ by eosinophils after stimulation with anti-CD28 mAb. For stimulation, wells of a 24-well plate were first coated with 40 μ g/ml anti-mouse IgG F(ab')₂, followed by 10 μ g/ml anti-CD28, anti-CD86, or isotype mouse IgG1 control Ab. After washing, freshly purified eosinophils (2×10^6 in 1 ml) were added per well. After 18 h, supernatants were harvested and analyzed by ELISA. Grey bars, immobilized anti-CD28 mAb-stimulated eosinophils; black bars, immobilized anti-CD86 mAb-stimulated eosinophils; hatched bars, mouse IgG1 control. Results were obtained from six individual patients (Table I, donors 2, 8, 11, 16, 19, and 21).

IFN- γ could be detected, in the absence of additional CD3-TCR triggering.

These findings indicate that activation of the costimulatory molecule CD28 expressed by human eosinophils is able to induce the release of IL-2 and IFN- γ . In contrast, CD86 cross-linking did not induce a similar cytokine release by eosinophils.

Biological Activity of IL-2 and IFN- γ Released by Eosinophils. In spite of the numerous studies published on cytokine secretion by eosinophils, very few have evaluated the biological activity of the cytokines released. Therefore, appropriate bioassays were performed in order to evaluate the functional activity of IL-2 and IFN- γ in eosinophil supernatants. The biological activity of IL-2 in the supernatants of CD28-activated eosinophils was evaluated in a proliferation assay using the IL-2-dependent mouse cytotoxic T cell line CTLL-2. The results presented in Fig. 5 indicate that eosinophil supernatants induced the proliferation of CTLL-2 cells, with large variations according to individual eosinophil donors. Comparison with the results obtained for purified rhIL-2 (Fig. 5 A) indicated that between 100 and 250 pg/ml of biologically active IL-2 could be detected in eosinophil supernatants after CD28 ligation (Fig. 5 B).

Similarly, the bioactivity of IFN- γ secreted by eosinophils was assessed according to its capacity to enhance MHC class II expression on the Colo 205 cell line (25). In parallel with the effect of rhIFN- γ (Fig. 5 C), supernatants derived from eosinophils activated upon CD28 ligation were able to induce MHC class II, as shown by flow cytometric analysis (Fig. 5 D). This effect was significantly inhibited by the addition of neutralizing anti-IFN- γ mAb (Fig. 5 D). In contrast, supernatants from eosinophils incubated with immobilized anti-mouse IgG1 did not induce the release of biologically active IFN- γ .

Cross-regulation of Cytokine Secretion by Secretory IgA and Anti-CD28. Since previous studies have shown that

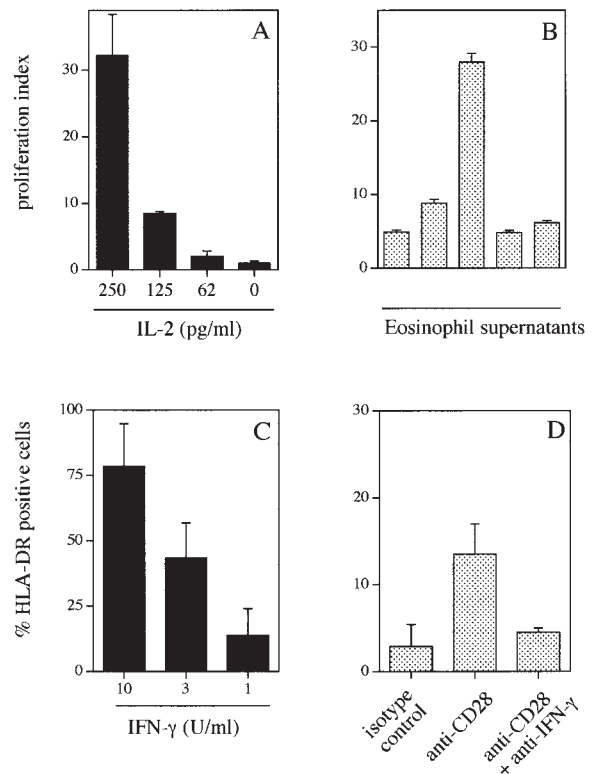


Figure 5. Biological activity of IL-2 and IFN- γ released by CD28-activated eosinophils. (A and B) Detection of the proliferative activity of IL-2 released by eosinophils on an IL-2-dependent cell line. Supernatants collected from eosinophils (2×10^6 /ml) stimulated with immobilized anti-CD28 mAb or isotype control Ab for 18 h were able to sustain the IL-2-dependent growth of the mouse cytotoxic T cell line CTLL-2 (American Type Culture Collection). The CTLL-2 cells were cultured in the presence of rhIL-2 (A) or eosinophil supernatants (50% dilution; B) for 24 h. Proliferation was measured by [³H]thymidine incorporation. Each supernatant obtained from individual patients (Table I, donors 2, 11, and 16) was assayed in duplicate. Results are expressed as proliferation index \pm SD, according to the following formula: proliferation index = dpm (eosinophils stimulated with anti-CD28 mAb)/dpm (eosinophils stimulated with isotype control mAb). An index >3 was considered significant. (C and D) Supernatants collected from eosinophils (2×10^6 /ml) stimulated with cross-linked anti-CD28 mAb or isotype control Ab for 18 h were assayed for IFN- γ -induced MHC class II expression on Colo 205 cells. The Colo 205 cells were cultured in the presence of rhIFN- γ (C) or eosinophil supernatants (20% dilution; D). After 48 h, cells were harvested and stained with a PE-conjugated anti-HLA-DR mAb or an isotype-matched Ab and examined by flow cytometry. Specificity of the bioassay was controlled by adding a neutralizing anti-IFN- γ mAb to the culture. Cell fluorescence was measured using a FACSCalibur™ flow cytometer, and data were analyzed using CellQuest software. Data are presented as mean \pm SD from three different experiments (Table I, donors 2, 8, and 16).

secretory IgA immune complexes could induce eosinophil degranulation, as well as IL-5 and IL-4 secretion (3, 4), we have investigated whether the activation of eosinophils with IgA could influence the CD28-induced cytokine release. As shown in Fig. 6, the addition of secretory IgA and anti-IgA mAb together with anti-CD28 ligation strongly inhibited the secretion of IL-2 ($82 \pm 32\%$ inhibition) and IFN- γ ($74 \pm 23\%$ inhibition) ($n = 3$). These results indi-

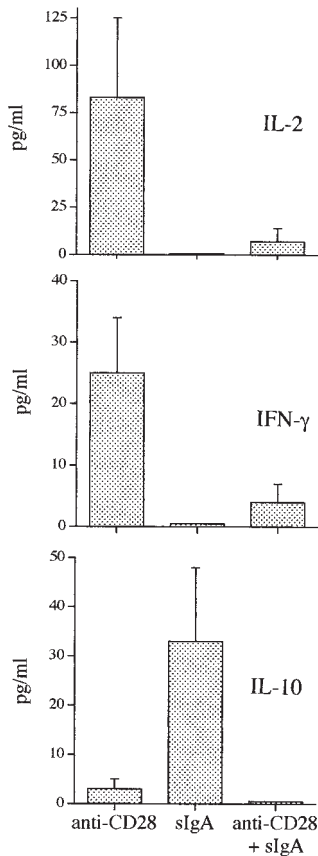


Figure 6. Reciprocal effect of IgA-anti-IgA complexes and CD28 on IL-10, IL-2, and IFN- γ release. Purified eosinophils (2×10^6 /ml) were stimulated with either immobilized anti-CD28 mAb, IgA-anti-IgA, or IgA followed by anti-IgA together with immobilized anti-CD28 as described in Materials and Methods. After 18 h, supernatants were harvested and analyzed by ELISA for cytokine content. Data were obtained from three different patients (Table I, donors 18, 20, and 21) and are presented as mean \pm SEM.

cate that secretory IgA-dependent activation of eosinophils could induce a downregulation of the CD28 activation pathway and prevent type 1 cytokine secretion.

To ascertain that the secretion or lack of secretion of cytokines observed was not due to eosinophil death during incubation, the release of LDH, a sensitive marker of cell lysis (24), was measured in the supernatants of eosinophils incubated with medium alone, immobilized anti-CD28, or IgA-anti-IgA for 18 h. Depending on the patient, LDH release ranged from 13.1 ± 1.5 to $32.2 \pm 4\%$ compared with a positive control represented by Triton X-100-lysed cells (data not shown). The trypan blue exclusion method confirmed $\sim 96\%$ cell viability at the end of the culture, in all conditions. These results indicate that cytokine secretion after CD28 and/or IgA complex ligation is not due to a massive cytolysis.

To further elucidate the mechanism of inhibition of the release of type 1 cytokines by IgA complexes, we evaluated the role of IL-10, which is the more potent cytokine involved in the negative regulation of type 1 cytokines. Activation of eosinophils with IgA-anti-IgA induced IL-10 secretion, in contrast to CD28 ligation (Fig. 6). These results favor the view that IgA complexes, by inducing IL-10 release, could have an inhibitory effect on CD28-mediated release of IFN- γ and IL-2. Moreover, the addition of immobilized anti-CD28 at the time of IgA cross-linking led to a significant inhibition of IL-10 secretion ($89 \pm 19\%$ in-

hibition; Fig. 6). These results indicate that IFN- γ and IL-2 on one hand, and IL-10 on the other, could be released by human eosinophils upon different signaling pathways.

In conclusion, our results showed that human eosinophils can express both CD86, classically expressed by APCs, and its CD28 ligand, a potent signaling molecule expressed by T cells. The detection on eosinophils of T cell-associated molecules has already been shown for CD4 (28). The coexpression, on the same cell population, of two molecules acting as ligands for each other has also been reported, for example by eosinophils in the case of CD40-CD40L (13, 14) and by T cells which could, in addition to CD28, express B7 (29). Further experiments are needed to ascertain whether such pairs of molecules are really expressed by the same individual cell, and if this coexpression might be of some significance for cell activation in vivo. However, these results suggest that eosinophils might provide costimulation of T cells (specially type 2 cells) via CD86, and conversely that they might respond to stimulation via CD28 engagement.

In this study, only stimulation of the CD28 molecule, not CD86 ligation, triggered cytokine release by highly purified human eosinophils. In contrast to CD28, able to provide a strong costimulatory signal on T cells, the main function of CD86 is to bind to CD28 and not to act as a signaling molecule in APCs, although the structure of the cytoplasmic tail (three potential phosphorylation sites) indicates a potential signaling role for this molecule (17). The precise function of CD86 has yet to be investigated in other pathways of eosinophil activation.

Although CD28 mainly participates in T cell activation as a second signal, it has been recently shown that stimulation through CD28 could induce early signaling events, such as cytokine synthesis, in resting T cells, without further requirement for TCR engagement (30). Experiments are now in progress to investigate the patterns of signaling events induced by CD28 activation of eosinophils.

An important outcome of this study is the release of type 1 cytokines by eosinophils after CD28 ligation, suggesting that interactions between CD28-expressing eosinophils and B7-expressing cells could induce previously unsuspected functions of eosinophils in the immune response. Until now, IL-2 was believed to be produced mainly by T lymphocytes. However, two groups recently reported the production of IL-2 by human eosinophils, its storage in the crystalloid granule, and IL-2 secretion after GM-CSF and A23187 stimulation (10, 31). The fact that eosinophils can produce IL-2 after CD28 ligation suggests that these cells could induce lymphocyte activation and consequently sustain the inflammatory processes, particularly in chronic diseases associated with eosinophils, such as asthma, skin diseases, or inflammatory bowel diseases. The release of biologically active IFN- γ by eosinophils, which has never been reported, led us to speculate that eosinophils might exert antiviral properties, and prompted consideration of strategies aiming toward the demonstration of this new effector function of eosinophils in innate as well as acquired immunity.

Although at this stage it is not possible to draw definitive

conclusions about the mechanism of inhibition of the CD28-dependent activation induced by secretory IgA-anti-IgA complexes, our results indicating that activation with secretory IgA but not with anti-CD28 could induce the release of IL-10, a potent inhibitory cytokine of the Th1 pathway, suggest the existence of cross-regulatory signals involved in the release of type 1 versus type 2 cytokines by eosinophils. Further experiments are needed to elucidate the precise role of IL-10 in this mechanism and the possible intervention of IL-10 receptor expressed by

eosinophils. Interestingly, the inhibitory role of IL-10 on eosinophil activation as well as its role in the decrease of type 1 cytokine production by T cells have already been reported (for a review, see reference 32). Thus, it will be of interest to investigate whether this inhibitory function of secretory IgA on the CD28-mediated release of type 1 cytokines, which would favor the Th2-mediated response, could be also detected for other cell populations, such as T cells, and whether it could play a role in the low dose oral tolerance phenomenon or other clinically relevant disease situations.

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