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# 2B4 (CD244) Is Expressed and Functional on Human Eosinophils<sup>1</sup>

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Eosinophils are present in parasitic, allergic, various immunological, and malignant disorders as well as in a variety of idiopathic hypereosinophilic syndromes. However, their exact role in some of these conditions remains elusive. They can be activated both *in vivo* and *in vitro* by various agonists, such as Igs, lipid mediators, and cytokines. By phenotyping the surface of the eosinophils, it may be possible to better define their function(s) in different pathophysiological settings. In the present work we screened eosinophils with a panel of Abs recognizing CD2 subfamily receptors usually present on a number of hemopoietic cells. We have demonstrated that human peripheral blood eosinophils, but not basophils or neutrophils, express NTB-A. In addition eosinophils express 2B4, CD84, CD58, and CD48, but not signaling lymphocytic activation molecule or CD2, on their surface (FACS). Cross-linking of 2B4 on eosinophils elicited a significant release of eosinophil peroxidase (30 min), IFN- $\gamma$ , and IL-4 (18 h). Moreover, activation of eosinophils via 2B4 induced eosinophil-mediated cytotoxicity toward two malignant cell lines, i.e., mouse mastocytoma P815 and EBV-infected 721.221 B cell lines. Cross-linking of 2B4 on the surface of eosinophils or pervanadate treatment elicited ERK and tyrosine phosphorylation, respectively. Furthermore, we showed that eosinophils express slam-associated protein. The demonstration that human eosinophils express a functional 2B4 receptor indicates a broader role for these cells in health and disease. *The Journal of Immunology*, 2005, 174: 110–118.

Eosinophils are bone marrow-derived, blood-circulating granulocytes with a typical bi-lobuled nucleus and cytoplasmic granules. Under physiologic conditions, eosinophils are found mainly in mucosal surfaces such as the gastrointestinal tract (1). However, blood eosinophilia is detected together with tissue eosinophilia in allergic, some parasitic, immunological, and malignant diseases as well as in a variety of idiopathic hypereosinophilic syndromes (2–5). Even though eosinophils have been attributed an effector role in allergic inflammation, such as the tissue damage activity in asthma, there is still no consensus on the exact activity of eosinophils in this condition and others.

Eosinophils express a variety of receptors for different agonists in their pathophysiological environment that can activate them. For example, they express receptors for IgA, IgG, cytokines, chemokines, and complement components (4–6). Activation of eosinophils results in the secretion of specific, crystalloid granule proteins such as major basic protein (MBP),<sup>3</sup> eosinophil peroxidase

(EPO), eosinophil-derived neurotoxin, and eosinophil cationic protein. In addition, eosinophils synthesize and release lipid mediators, such as platelet-activating factor, leukotriene C<sub>4</sub>, PGE<sub>2</sub>, as well as proinflammatory and immunoregulatory cytokines and chemokines (6).

Receptor definition and surface phenotyping of the eosinophils are possible tools to better understand their functions in different pathophysiological settings. This approach has led to the identification of NK cell-regulating molecules that resulted in a marked progress of understanding the biology, development, and function of these cells (7).

A wide range of surface activatory and/or inhibitory molecules, such as leukocyte Ig-like receptor/Ig-like transcript, Siglecs, CD28, and CD86 (8–12), that had been previously thought to be expressed mainly by T and NK cells have recently been found to be expressed by eosinophils. It appears that a complex of activatory and inhibitory networks can regulate the activities of eosinophils.

Our interest has focused on the receptors belonging to the CD2 subfamily of the Ig superfamily. They include CD2, CD48 (Blast-1 and BCM-1), CD58 (LFA-3), CD84 (Ly9B), CD150 (signaling lymphocytic activation molecule (SLAM)), CD229 (Ly9), 2B4 (CD244), BCM-like membrane protein, SF2001 (CD2F-10), NTB-A (SF2000 and Ly108), and CS1 (CD1-like receptor-activating cytotoxic effects) (13, 14).

Of this family, 2B4 is expressed on virtually all NK cells, T cells, a large subset of CD8<sup>+</sup> T cells, and a small percentage of CD4<sup>+</sup> T cells, monocytes, and basophils (15). Cross-linking of 2B4 on NK cells triggers their activation (13), and interaction of 2B4 with its high affinity ligand CD48 (16) results in lysis of tumor cells and of CD48-transfected cells (15). Cross-linking of 2B4 on NK cells involves multiple distinct pathways (17) and requires the unique molecule slam-associated protein (SAP) (17–19). Interestingly, mutations or absence in the SAP gene result in a severe disease, X-linked lymphoproliferative disease (20–22)

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<sup>3</sup> Abbreviations used in this paper: MBP, major basic protein; EM, enriched medium; EPO, eosinophil peroxidase; HBA, HBSS supplemented with 0.1% BSA and 0.02% sodium azide; SAP, slam-associated protein; Siglecs, sialic acid-binding Ig-like lectin; SLAM, signaling lymphocytic activation molecule.

In the present work we have hypothesized that eosinophils could express cell surface receptors belonging to the CD2 subfamily that may regulate their function. We have now shown a restricted expression pattern of the CD2 subfamily receptors on the surface of eosinophils. Eosinophils can be activated by 2B4 to release EPO, IFN- $\gamma$ , and IL-4. Engagement of 2B4 on FcR<sup>+</sup> cells or direct 2B4-CD48 interactions lead to lysis of tumor cells. In addition, we have demonstrated for the first time that eosinophils express SAP.

## Materials and Methods

### Antibodies

Three sources of anti-2B4 mAbs were used: P158 (15), C1.7 (Coulter-Immunotech), and PP35 (23). Anti-CD2 FITC-conjugated mAbs (MT910) were obtained from DakoCytomation. Anti-CD58 (1C3), anti-CD84 FITC-conjugated (2G7), anti-HLA-DR PerCp-Cy5.5-conjugated (G46-6), and mouse IgG2a isotype control mAbs were purchased from BD Pharmingen. Anti-NTB-A mAbs (MA127 and ON56) were obtained as previously described (24). Anti-CD48 (4H9), goat polyclonal anti-SLAM (recognizing the N terminus of SLAM from human origin), rabbit polyclonal, anti-SAP (recognizing aa 1–128 representing the full-length of SAP from human origin), rabbit polyclonal, anti-pERK, anti-ERK, and anti- $\beta$ -actin mAbs were obtained from Santa Cruz Biotechnology. Anti-Fc $\epsilon$ RI PE-conjugated mAbs (AER-37) were obtained from eBioscience. Mouse IgG1, IgG2a, and PE-conjugated mouse IgG2a Abs were obtained from DakoCytomation, FITC-conjugated mouse IgG1 Abs was purchased from Ancell. FITC anti-rabbit and FITC anti-goat Abs were obtained from Pierce. Goat anti-mouse F(ab)' IgG, PE-conjugated goat anti-mouse F(ab)', HRP-conjugated goat anti-rabbit, HRP donkey anti-goat, and HRP-conjugated goat anti-mouse Abs were obtained from Jackson ImmunoResearch Laboratories). Sheep F(ab)' anti-mouse Ab was purchased from ICN Pharmaceuticals (Aurora, OH).

### Reagents and chemicals

RPMI 1640 supplemented with L-glutamine, heat-inactivated FCS, and penicillin-streptomycin solutions were obtained from Biological Industries. All chemicals used in this study were purchased from Sigma-Aldrich and were of the best available grade.

### Eosinophil and neutrophil purification

Eosinophils were purified from the peripheral blood of mildly atopic individuals (blood eosinophil levels, 5–10%) as previously described (25). Written informed consent was obtained from all volunteers according to the guidelines established by the Hadassah-Hebrew University Human Experimentation Helsinki Committee. Briefly, venous blood (50–100 ml) was collected in heparinized syringes and left to sediment in 6% dextran (Amersham Biosciences). Leukocytes were centrifuged on Ficoll-Hypaque (density, 1.077; 25 min, 700  $\times$  g, 22°C; Amersham Biosciences). Neutrophils and contaminating lymphocytes were tagged in the granulocyte-enriched pellet with micromagnetic beads bound to anti-CD16 and anti-CD3 Abs (Miltenyi Biotec). Eosinophils were purified by passing the cell suspension through a magnetic column (MACS). They were collected at a purity of at least 98% by Kimura staining (no CD56<sup>+</sup> cells were observed in the contaminating fractions, by FACS analysis; data not shown) and at a viability of at least 98% by trypan blue staining. For neutrophil isolation, the magnetic column was washed three times with RPMI 1640 containing 0.05 M EDTA and heat-inactivated FCS (2%, v/v). Neutrophils were collected at a purity of >96% and a viability of >98%.

### Enrichment of human peripheral blood basophils

Peripheral blood was collected and centrifuged on Ficoll-Hypaque as described above. Basophils were purified using the basophil isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Basophils were collected at a purity of >80% as determined by FACS analysis (HLA-DR Fc $\epsilon$ RI<sup>+</sup>).

### Flow cytometry

For flow cytometry (FACS) analysis, cells ( $1 \times 10^5$ ) were incubated in 15% human serum (to block FcRs) in a final volume of 100  $\mu$ l of HBSS supplemented with 0.1% BSA and 0.02% sodium azide (HBA) for 30 min on ice. Eosinophils and neutrophils were cultured with different Abs recognizing CD2 subfamily epitopes, followed by goat anti-mouse FITC Abs (1/200) when needed. For detection of CD2 subfamily receptors on human basophils, freshly isolated basophils were incubated for 30 min on ice with

anti-HLA-DR PerCp-Cy5.5-conjugated (1/10), anti-Fc $\epsilon$ RI PE-conjugated Abs (1/5), and an anti-CD2 subfamily receptor recognizing Ab, followed by goat anti-mouse FITC Abs when needed.

For intracellular staining, eosinophils were fixed in 4% paraformaldehyde in HBA for 15 min at room temperature, then permeabilized in HBA containing saponin (0.1%), BSA (1 mg/ml), and human serum (10%) for 30 min on ice. Anti-SAP or irrelevant control Abs (2  $\mu$ g/ml) were added to these fixed permeabilized cells (30 min on ice), then incubated with FITC anti-rabbit Abs (1/600, 30 min on ice).

After staining, the cells were analyzed on a FACSCalibur system (BD Biosciences). For each staining, at least 10,000 events were collected, and data analysis was performed using CellQuest software (BD Biosciences).

### Cell culture and activation

Ninety-six well plates (Nunc) were precoated with sheep anti-mouse F(ab)' in PBS (25  $\mu$ g/ml, 2 h, 37°C, 5% CO<sub>2</sub>). Afterward, plates were washed three times with PBS and incubated with C1.7 (anti-2B4) or irrelevant isotype-matched control Ab (1–5  $\mu$ g/ml, 2 h at 37°C, 5% CO<sub>2</sub>) and washed again three times. Freshly isolated eosinophils were seeded in these precoated wells ( $2 \times 10^5$ /200  $\mu$ l) in medium containing RPMI 1640, 200 U/ml penicillin, 200  $\mu$ g/ml streptomycin, and 5% (v/v) heat-inactivated FCS (enriched medium (EM)) and incubated for 30 min to 18 h (37°C, 5% CO<sub>2</sub>). At the end of the incubation, cells were centrifuged (250  $\times$  g, 5 min, 4°C), and supernatants were collected, aliquoted, and stored at –80°C until assessed for EPO, IFN- $\gamma$ , and IL-4.

### EPO determination

EPO release was determined by a colorimetric assay as previously described (26). Briefly, eosinophil culture supernatants (50  $\mu$ l) were incubated (10–15 min, 37°C, 5% CO<sub>2</sub>) with a substrate solution consisting 0.1 mM O-phenylenediamine dihydrochloride in 0.05 M Tris buffer (pH 8.0) containing 0.1% Triton X-100 (37°C, 5% CO<sub>2</sub>) and 1 mM hydrogen peroxide (Merck). The reaction was stopped by the addition of 100  $\mu$ l of 4 mM sulfuric acid (BDH), and absorbance was determined at 492 nm in a spectrophotometer (PowerWave XS; Bio-Tek Instruments).

### Cytokine determination assay

IFN- $\gamma$  and IL-4 in the eosinophil culture supernatants were quantified using commercially available specific ELISA kits according to the manufacturer's instructions. IFN- $\gamma$  was detected using DuoSet (R&D Systems); IL-4 was detected by Eli-pair (Diacclone). The lower limits for assay sensitivity are 7 and 1.1 pg/ml for IFN- $\gamma$  and IL-4, respectively.

### Redirected cytotoxicity assays

The cytotoxicity of human peripheral blood eosinophils was assessed using the FcR<sup>+</sup> mouse mastocytoma P815 cell line (target cells) as previously described (27) with slight modifications. Briefly, target cells were labeled with [<sup>35</sup>S]methionine (Amersham Biosciences) overnight and thereafter seeded on 96-well, U-shaped plates (Nunc;  $5 \times 10^3$ /100  $\mu$ l; in EM), washed three times (250  $\times$  g, 5 min, 4°C), and incubated in the presence or the absence of C1.7 Ab or matching isotype control (1  $\mu$ g/ml) for 1 h on ice. Freshly isolated eosinophils or YTS NK cells as positive controls (effector cells) were added (100  $\mu$ l/well, in EM) at different E:T cell ratios (50–6.25:1 and 10:1, respectively). After 18-h incubation (37°C, 5% CO<sub>2</sub>), the cells were centrifuged (250  $\times$  g, 5 min), and the supernatants were collected for the cytolytic activity assay. The percentage of specific lysis was calculated by the following equation:  $(a - b)/(c - b)$ , where  $a$  indicates the radioactivity of target cells mixed with effector cells,  $b$  is the radioactivity in the supernatant of the target cells alone, and  $c$  is the radioactivity in the supernatant of target cells alone after lysis with 0.1 M NaOH.

### Direct cytotoxicity assays

The cytotoxicity of human peripheral blood eosinophils was assessed using the MHC-I deficient, EBV-transformed B cell line 721.221 (target cells) that expresses high levels of CD48. To study 2B4-CD48 interactions, target cells were labeled with [<sup>35</sup>S]methionine as described above and incubated in the presence or the absence of anti-CD48 mAb or matching isotype control (5  $\mu$ g/ml) for 1 h on ice. Simultaneously, freshly isolated eosinophils or YTS NK cells (effector cells) were incubated in the presence or the absence of C1.7 or matching isotype control (5  $\mu$ g/ml) for 1 h on ice. Both target and effector cells were washed, resuspended, and cocultured (200  $\mu$ l/well, in EM) at different E:T cell ratios (50–6.25:1 and 10:1, respectively). After 18-h incubation (37°C, 5% CO<sub>2</sub>), the cells were centrifuged (250  $\times$  g, 5 min), and cytolytic activity was assessed as described above.

### Immunoprecipitation

Cell lysates were prepared by adding lysis buffer (1 M Tris, 0.5 M EDTA, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Triton X-100, and 0.5% sodium deoxycholate) to freshly isolated eosinophils, neutrophils, or YTS NK cells ( $5 \times 10^6$ ), followed by vortex mixing. Cell debris was removed from the lysates by centrifugation ( $18,000 \times g$ , 15 min, 4°C). The supernatant was precleared using isotype-matched control Ab and then incubated with anti-2B4 (i.e., C1.7, P158, and PP35; 8 h, 4°C), followed by protein A/G (20  $\mu$ l; 8 h, 4°C; Santa Cruz Biotechnology). The immunoprecipitates were washed four times with lysis buffer, eluted from the Sepharose beads by boiling for 15 min in sample buffer (0.1% bromophenol blue, 1.5% 2-ME, 10% SDS, and 0.5 M Tris, pH 6.8), and analyzed by Western blot.

### Western blot

Cell lysates were prepared from freshly isolated eosinophils, neutrophils, or YTS NK cells ( $5 \times 10^6$ ) in lysis buffer as described above. Samples were analyzed on 12.5% SDS-PAGE under reducing conditions. The gels were electrotransferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated for 1 h at room temperature with rabbit anti-SAP Abs (1/500). After the membranes were washed, proteins were detected by secondary immunopure goat anti-rabbit Abs conjugated with HRP (1/5000), followed by ECL detection (Amersham Biosciences).

In assays determining phosphorylated forms of ERK, freshly isolated eosinophils ( $5 \times 10^6/400 \mu$ l, in EM) were incubated with C1.7 or isotype-matched control Abs (1  $\mu$ g/ml) for 30 min on ice. Cells were washed twice ( $250 \times g$ , 4°C, in EM) and cross-linked with sheep anti-mouse F(ab)' (25  $\mu$ g/ml) for 30 min on ice. Then cells were again washed twice ( $250 \times g$ , 4°C, in EM) and incubated for the indicated times (37°C, 5% CO<sub>2</sub>). For positive control, eosinophils were incubated ( $5 \times 10^6$ /well, in EM) in 12-well plate (Nunc) with PMA (50 ng/ml) for 4 min (37°C, 5% CO<sub>2</sub>). After stimulation, cells were lysed and blotted as described above.

### RNA isolation and RT-PCR amplification

Total mRNA was extracted from freshly isolated eosinophils, neutrophils ( $10 \times 10^6$ ), and YTS NK cells ( $8 \times 10^6$ ) using the RNeasy Mini kit (Qiagen). The first-strand cDNA synthesis reaction was catalyzed by SuperScript II RNA reverse transcriptase (Invitrogen Life Technologies) and oligo(dt)<sub>12-18</sub> primer, according to the manufacturer's instructions. PCR amplifications were conducted using the following primer sequences 5'-GCCATGGACGCAGTGGCT-3' and 5'-TGGGGCTTTCAGGCAGACA TC-3' for SAP (28), generating a 400-bp fragment. The MasterCycler (Eppendorf) was used for the PCR amplifications with the following settings: 35 cycles at 94°C for 30 s, 57°C for 1 min, and 72°C for 1 min. Before the first cycle, denaturation at 94°C for 3 min was performed. At the end of all cycles, a primer extension period of 10 min at 72°C was included. The primer sequences for  $\beta$ -actin, which was used as a control to test the efficiency of cDNA synthesis, were 5'-GTACAGGGATAGCACAGCCT-3' and 5'-TCACCAACTGGGACGACATG-3' generating a fragment of 200bp. Amplified products were electrophoresed on 1.5% agarose gel stained with ethidium bromide and were photographed under UV light.

### Statistical analysis

Statistical significance was calculated using parametric analysis (ANOVA, followed by Tukey-Kramer post hoc test). Values were considered significant at  $p < 0.05$ .

## Results

### Expression of CD2 subfamily receptors on human peripheral blood eosinophils, neutrophils, and basophils

We used a panel of Abs to examine the cell surface expression of CD2 subfamily receptors on freshly isolated human peripheral blood eosinophils, neutrophils, and basophils. Representative flow cytometer dot-plots are shown in Fig. 1A. Eosinophils were found to express several CD2 subfamily receptors, including NTB-A, 2B4, CD84, CD58, and CD48 ( $n = 15$ ). Interestingly, the expression of NTB-A was confined only to eosinophils, because basophils ( $n = 4$ ) and neutrophils ( $n = 15$ ) did not express this receptor. In addition, eosinophils as well as basophils, but not neutrophils, expressed 2B4 ( $n = 4$  and 15, respectively). CD84, CD58, and CD48 were also expressed by basophils and neutrophils ( $n = 4$  and 15, respectively). Because 2B4 and NTB-A display a dif-

ferential expression pattern among the three cell types, we next screened these cells with different mAbs recognizing 2B4 and NTB-A to discern whether this pattern is Ab dependent. All three mAbs recognizing 2B4, i.e., P158, PP35, and C1.7 (13, 15, 23), could similarly identify 2B4 on the surface of the eosinophils (Fig. 1B) and basophils, but not on neutrophils (data not shown). In addition, the two mAbs recognizing NTB-A, i.e., ON56 and MA127 (24), displayed the same expression outline on eosinophils (Fig. 1B), neutrophils, and basophils (data not shown).

### 2B4 activates eosinophils to release EPO, IFN- $\gamma$ , and IL-4

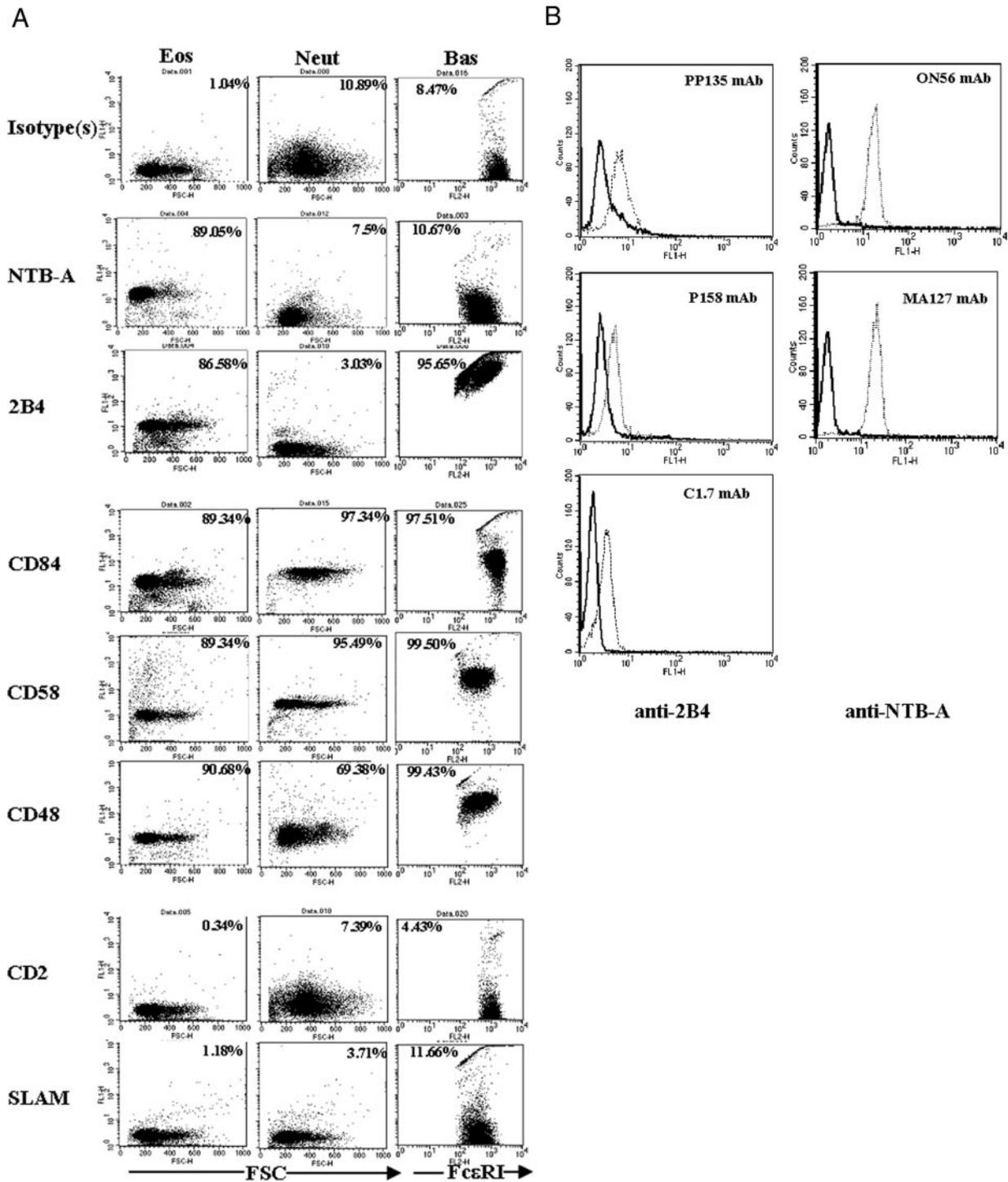
The expression of CD84, NTB-A, and 2B4 on the surface of eosinophils suggests that their responses may be regulated by these receptors. Based on the knowledge that 2B4 triggers NK cell activation (13, 17), we hypothesized that 2B4 may activate human eosinophils as well. As a first marker of eosinophils activation, EPO release was evaluated. From preliminary studies we had already established that cross-linking of the P158 mAb does not elicit EPO release. In these experiments the best activation was achieved by cross-linking of the receptor with the C1.7 mAb. Thus, our next set of experiments was conducted using the C1.7 anti-2B4 mAb. As shown in Fig. 2A, cross-linking of 2B4 induced eosinophils to release EPO. EPO release, as measured by OD units, was significantly higher when 2B4 was cross-linked using the C1.7 mAb (OD,  $0.59 \pm 0.15$ ) in comparison with isotype-matched cross-linking (OD,  $0.15 \pm 0.02$ ;  $p < 0.0001$ ) or unstimulated cells (OD,  $0.17 \pm 0.03$ ;  $p < 0.0001$ ).

Next we determined whether cross-linking of 2B4 on eosinophils could cause cytokine release. We selected IFN- $\gamma$  and IL-4 as typical Th1- and Th2-related cytokines, respectively. As shown in Fig. 2B, cross-linking of 2B4 on eosinophils caused IFN- $\gamma$  release, ranging between 53.7 and 250.9 pg/ml, in comparison with isotype-matched control cross-linking, which ranged between 0 and 45.2 pg/ml ( $p < 0.05$ ), and with unstimulated cells, which ranged between 0 and 8.5 pg/ml ( $p < 0.01$ ). Cross-linking of 2B4 on eosinophils also caused significant IL-4 release (Fig. 2C; 16.5–97.3 pg/ml) in comparison with cross-linking of isotype-matched controls (10.48–20.7 pg/ml;  $p < 0.05$ ) and unstimulated cells (0–19.5 pg/ml;  $p < 0.01$ ).

### 2B4 activation mediates eosinophil cytotoxicity toward P815 and 721.221 cells

The demonstration that 2B4 activates human eosinophils to release cytokines led us to hypothesize that other functions beside cytokine release could be elicited by 2B4 stimulation. Therefore, eosinophil (effector cell (E)) cytotoxicity was tested in a redirected lysis assay toward the P815 mastocytoma cell line (target cell (T)). The C1.7 mAb caused a cytotoxic effect of the eosinophils toward the P815 cells already at an E:T cell ratio of 12.5:1 (Fig. 3A). This effect became significant at an E:T cell ratio of 25:1 ( $18.85 \pm 1.31\%$  C1.7 mAb vs  $2.75 \pm 1.38\%$  isotype-matched control;  $p < 0.01$ ;  $n = 4$ ). Higher E:T cell ratios (50:1 and 100:1) were either the same or less effective than an E:T cell ratio of 25:1 (data not shown).

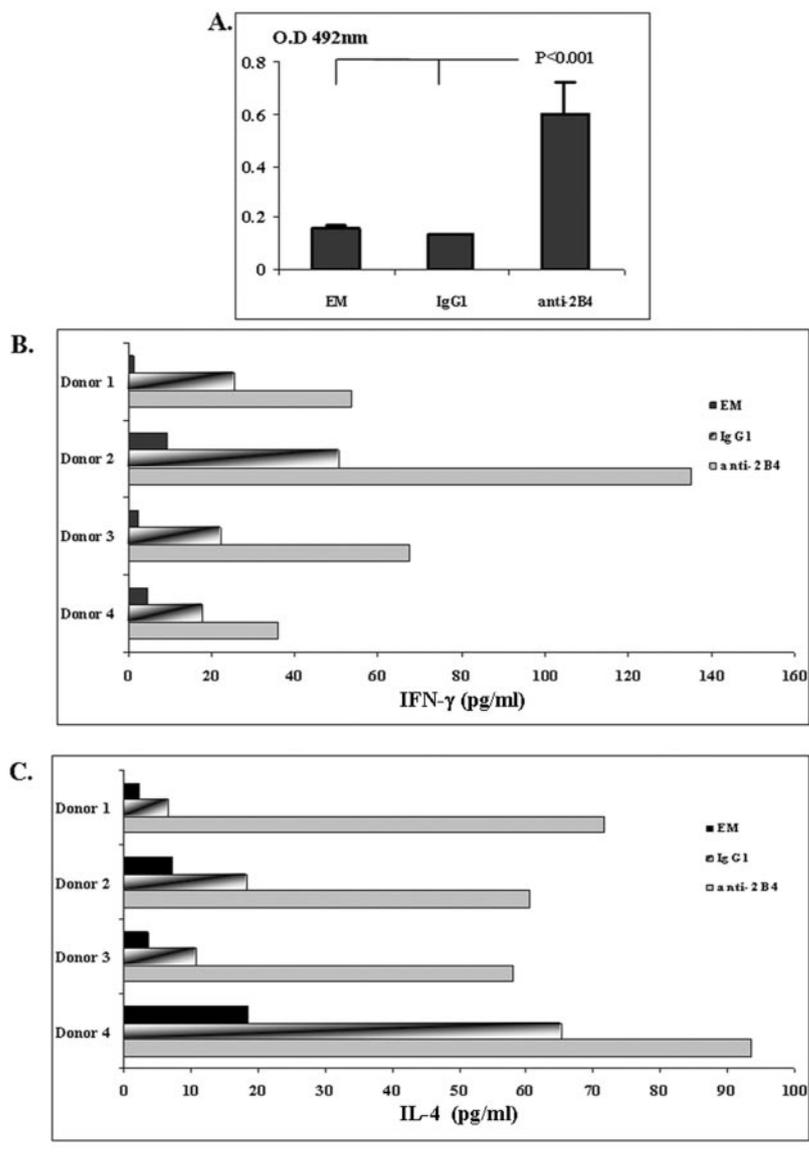
Our next aim was to determine whether eosinophils could display these cytotoxic features toward malignant cells that express CD48, a high affinity ligand of 2B4 (15, 16). For this set of experiments we cocultured eosinophils with the EBV-transfected B cell line 721.221 that expresses high levels of CD48, but no MHC class I. As shown in Fig. 3B, at the highest E:T cell ratio (40:1) neither neutralization of 2B4 on the surface of eosinophils nor of CD48 on the surface of target cells blocked the cytolytic effect of eosinophils ( $39.87 \pm 2.38\%$ ). However, at lower E:T cell ratios



**FIGURE 1.** Expression of CD2 subfamily receptors on human peripheral blood eosinophils, neutrophils, and basophils. *A*, Freshly isolated and purified eosinophils, neutrophils, or basophils were cultured with specific Abs recognizing CD2 subfamily epitopes on the cell surface or appropriate isotype-matched controls, followed by goat anti-mouse FITC when needed. For basophil determination, basophils were stained with anti-HLA-DR, PerCP-Cy5.5 conjugated, and anti-FcεRI, PE-conjugated. HLA-DR<sup>-</sup>/FcεRI<sup>+</sup> cells were electronically gated and assessed for CD2 subfamily epitopes. The data shown are representative results from one of 15 (eosinophils and neutrophils) or four (basophils) different experiments. *B*, Eosinophils were analyzed by FACS using different mAbs recognizing 2B4 (PP35, P158, and C1.7) and NTB-A (ON56 and MA127), followed by FITC anti-mouse Abs. Bold and dotted histogram plots indicate isotype-matched control and anti-2B4 or anti-NTB-A mAbs, respectively. Data are representative of 15 different experiments. The percentage indicates positively stained cells of the total population, as analyzed by CellQuest.

(i.e., 20:1 and 10:1) where eosinophils still caused significant cytotoxicity of 721.221 cells ( $34.47 \pm 2.88$  and  $31.96 \pm 2.71\%$ , respectively), neutralization of CD48 on the surface of the target cells or of 2B4 on the surface of the eosinophils significantly decreased this cytotoxic effect. For example, at an E:T cell ratio of

20:1, blocking 2B4 on eosinophils or CD48 on the target cells decreased the cytotoxic effect to  $12.56 \pm 1.23$  and  $18.37 \pm 1.93\%$ , respectively ( $p < 0.05$ ;  $n = 3$ ). In addition, at an E:T cell ratio of 10:1, blocking 2B4 on eosinophils or CD48 on the target cells decreased cytotoxic effect to  $11.21 \pm 1.45$  and  $7.88 \pm 2.34\%$ ,



**FIGURE 2.** 2B4 activates eosinophils to release EPO, IFN- $\gamma$ , and IL-4. Eosinophils were cultured in untreated (EM) wells or in wells precoated with sheep anti-mouse and either isotype-matched Ig (IgG1) or C1.7 mAb (anti-2B4) for 30 min to 18 h. A, EPO in the culture supernatants was determined by a colorimetric assay (A). The data represent the mean  $\pm$  SEM of five different experiments performed in triplicate.  $p < 0.01$  compared with EM- and IgG1-treated cells. IFN- $\gamma$  (B) and IL-4 (C) were determined in the culture supernatants by ELISAs. The plots represent data obtained from four donors. Experiments were performed in triplicate.

respectively ( $p < 0.01$ ;  $n = 3$ ). The cytolytic effect was blocked completely by these procedures at an E:T cell ratio of 5:1 ( $1.61 \pm 1.1$  and  $1.88 \pm 1.6\%$ ;  $p < 0.001$ ;  $n = 3$ ).

#### Cross-linking of 2B4 results in ERK phosphorylation

Eosinophil activation involves various pathways (29–31). To investigate whether cross-linking of 2B4 stimulates the MEK1/ERK pathway, freshly isolated eosinophils were stimulated by cross-linking the C1.7 mAb for different time periods (Fig. 4). Cells were then lysed and analyzed by Western blot for ERK phosphorylation (Fig. 4, upper panel). Cross-linking of 2B4 causes the phosphorylation of ERK as early as 2 min after Ab ligation. This process is more evident after 4 min and decreases after 6 min. Cross-linking of an isotype-matched Ab for 4 min did not cause any ERK phosphorylation (IgG1), whereas eosinophils stimulated with PMA displayed an intense ERK phosphorylation pattern (Fig. 4). To verify total amounts of ERK protein, the membrane was stripped and reprobated with anti-ERK Abs (lower panel). Eosinophils were found to express similar protein levels.

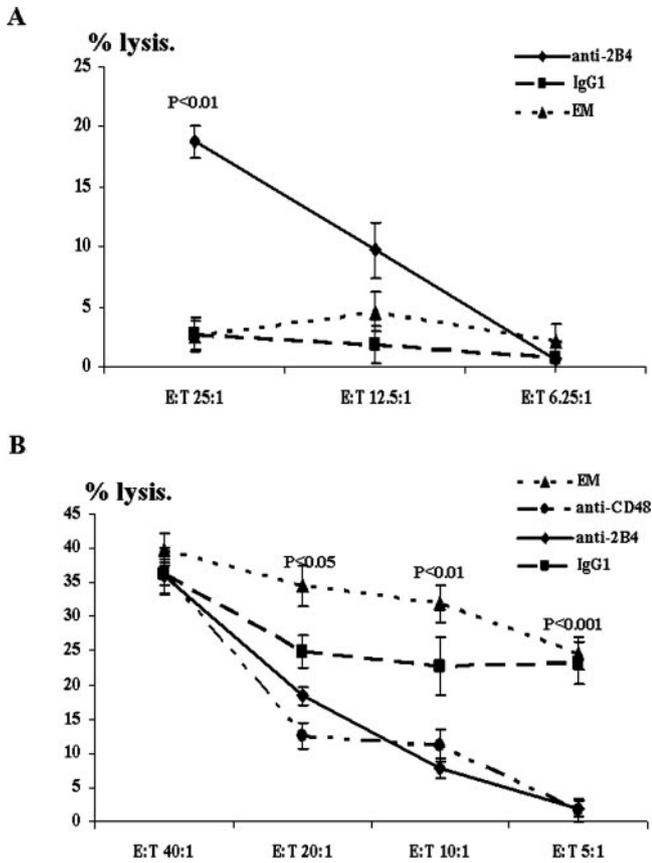
#### Eosinophils express SAP

An additional signaling molecule essential for 2B4 activation is SAP (17–20). Thus, the expression of SAP in freshly isolated eo-

sophils was examined. As shown in Fig. 5A, freshly isolated eosinophils as well as YTS NK cells express SAP at the mRNA level, whereas neutrophils do not ( $n = 3$ ). SAP expression was also analyzed at the protein level using both FACS analysis and Western blot techniques. Intracellular FACS staining revealed that eosinophils express similar levels of SAP as YTS NK cells, whereas their expression in neutrophils is negligible (Fig. 5B;  $n = 3$ ). In addition, Western blot analysis (Fig. 5C) demonstrated that SAP is expressed in YTS NK cells as well as in eosinophils, but not in neutrophils ( $n = 8$ ). Interestingly, eosinophils from one of eight donors did not express SAP (data not shown).

#### Pervanadate treatment causes 2B4 tyrosine phosphorylation in human eosinophils

Recruitment of SAP to 2B4 is dependent on phosphorylation of intracellular tyrosine residues. To determine whether 2B4 in eosinophils undergoes tyrosine phosphorylation, eosinophils were treated with pervanadate, lysed, and immunoprecipitated using anti-2B4 mAbs. Tyrosine phosphorylation was assessed by Western blot. As shown in Fig. 6A, only the PP35 mAb was able to precipitate 2B4 from the eosinophils, whereas the P158 and the C1.7

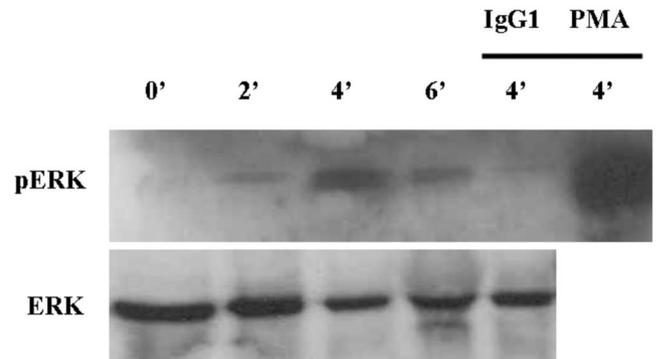


**FIGURE 3.** 2B4 activation mediates eosinophil cytotoxicity toward P815 and 721.221 cells. Eosinophil cytotoxicity was tested against [<sup>35</sup>S]methionine labeled P815 or 721.221 cells in a redirected lysis or direct lysis assay, respectively. *A*, In the redirected lysis assay, the target cells P815 (target) were incubated in the presence of medium alone (EM), IgG1 isotype control (Isotype), or anti-2B4 Abs (anti-2B4), and eosinophils (effectors) were added at different E:T cell ratios. The data are representative results from one of four different experiments performed in duplicate. *p* < 0.001 compared with EM- and IgG1-treated cells. *B*, In the direct lysis assay, 721.221 cells (target) were incubated in the presence or the absence of EM, IgG1 isotype control, anti-CD48, or anti-2B4 Abs, and eosinophils (effectors) were added at different E:T cell ratios. The data are representative results from one of three different experiments performed in duplicate. *p* < 0.05 and *p* < 0.01 compared with IgG1-treated cells.

mAbs were not successful. Furthermore, treatment of the eosinophils and YTS NK cells with pervenadate (Fig. 6*B*) elicited tyrosine phosphorylation of 2B4.

**Discussion**

In the present study we have screened the surface of human peripheral blood eosinophils for the expression of CD2 subfamily receptors and compared it to those of neutrophils and basophils. Our data demonstrate that eosinophils express NTB-A, 2B4, CD84, CD58, and CD48. Interestingly, eosinophils were found to exclusively express NTB-A compared with neutrophils and basophils. In addition, eosinophils and basophils, but not neutrophils, expressed 2B4, indicating that the eosinophils' cell surface markers resemble those of the basophils rather than the neutrophils (8). This finding is particularly interesting because this specific expression pattern of NTB-A (24) could be used to distinguish among eosinophils, neutrophils, and basophils. CD84, CD58, and CD48 were all expressed in eosinophils neutrophils, and basophils, whereas CD2 and SLAM were not expressed on any of these cell types.



**FIGURE 4.** Cross-linking of 2B4 results in ERK phosphorylation. Eosinophils were incubated with anti-2B4 or IgG1 isotype-matched control Abs, followed by sheep anti-mouse F(ab)' for the indicated time points. Cell lysates were analyzed by Western blot with anti-phospho-ERK Abs (upper panel). The same membrane was stripped and reprobed with anti-ERK Abs (lower panel) to check total protein amounts. The data shown are representative results from one of three different experiments.

Because only NTB-A and 2B4 displayed a differential expression pattern among the three cell types, we tested various mAbs recognizing these epitopes to discern whether our findings were Ab dependent. All Abs examined demonstrated similar expression patterns of NTB-A and 2B4.

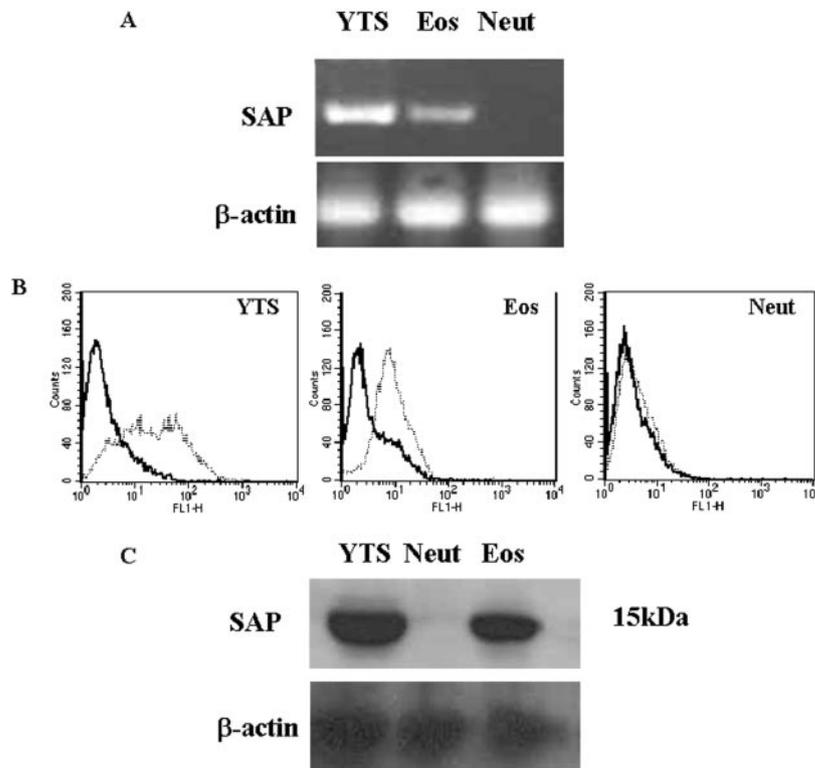
The expression of CD2 subfamily receptors, either specifically or nonspecifically on human eosinophils may contribute to our understanding of the role of eosinophils in different pathophysiological settings. The CD2 subfamily receptors interact with each other in a homotypic or a heterotypic fashion. For example, in humans CD58 is a high affinity ligand for CD2, whereas CD48 is a low affinity ligand for CD2 (32), but a high affinity ligand for 2B4 (15, 33). CD84 and SLAM interact with themselves (34, 35). Hence, our results suggest that eosinophils may interact either with themselves or with other cell types, such as T cells, via these CD2 subfamily receptors. Indeed, it has been shown that a cross-talk between eosinophils and T cells may occur in asthma (36–39). Eosinophils express costimulatory molecules, such as CD28 and CD86 (12, 37), and are able to present Ag (36, 37). Furthermore, it has been well established that murine asthma is dependent on eosinophil-T cell interactions (38, 39). Thus, it is likely that this cross-talk could be mediated by these CD2 subfamily receptors as well as by additional receptors.

The unique expression pattern of 2B4, the many studies that demonstrated its function, and the fact that 2B4 has a well-characterized high affinity ligand (15, 33) led us to investigate its role in eosinophils. The expression of 2B4 on eosinophils suggests that their responses after recruitment to the site of inflammation may be regulated by this receptor. Indeed, we found that 2B4 expression on eosinophils does not change after their culture with various cytokines (e.g., IL-5, IL-3, GM-CSF, IL-2, IFN- $\gamma$ , IL-4, IL-13, and TGF- $\beta$ ) and that nasal polyp tissue eosinophils also expressed this receptor (A. Munitz, I. Bachelet, R. Elyashar, and F. Levi-Schaffer, unpublished observations).

It is well known that 2B4 activates NK cells (13, 17, 40, 41). Thus, we tested whether eosinophils could also be activated via 2B4. Cross-linking of 2B4 caused eosinophils to release EPO. EPO is a member of the family of haloperoxidases that catalyze the peroxidative oxidation of halides and pseudohalides (6). Once EPO is released, it can elicit several effects, some of which are protective and others destructive (6).

Cross-linking of 2B4 on NK cells has been shown to trigger cytokine release, primarily IFN- $\gamma$ , and cross-linking of 2B4 triggered IFN- $\gamma$  release from eosinophils as well. Recent studies have

**FIGURE 5.** Eosinophils express SAP. *A*, For RT-PCR analysis, total RNA was extracted from freshly isolated eosinophils (Eos), neutrophils (Neut), or YTS NK cells (YTS). First-strand cDNA synthesis was catalyzed, and PCR amplification was conducted. Amplified products were electrophoresed on agarose gel, stained with ethidium bromide, and photographed under UV light. *B*, For FACS studies, fixed, permeabilized cells were incubated with anti-SAP or control Abs, followed by FITC-labeled goat anti-rabbit. Data are representative results from one of three different experiments. *C*, For Western blot analysis, isolated eosinophils were lysed and analyzed on SDS-PAGE gel under reducing conditions. After the transfer procedure, the membrane was incubated with rabbit anti-SAP Abs, washed, and detected by secondary immunopure goat anti-rabbit Abs conjugated with HRP, followed by ECL detection. The numbers on the *right* of the panels indicate the positions of the m.w. markers.



shown that eosinophils may have a differential cytokine release profile that is stimuli dependent. For example, it has been shown that eosinophils release IL-12, but not IL-4, in response to leukocyte Ig-like receptor-7 activation (9). In addition, CD28 activates eosinophils to release IL-2 and IFN- $\gamma$ , but not IL-10 (12). Therefore, we investigated whether 2B4 activation may cause eosinophils to also release a Th2-related cytokine, such as IL-4. Our results show that 2B4 can activate eosinophils to release IL-4 as well as IFN- $\gamma$ .

The demonstration that 2B4 activation on eosinophils can elicit both EPO and IL-4 release as well as IFN- $\gamma$  raises the possibility that the function of 2B4 on eosinophils has a broad immunological importance and can contribute to eosinophil effector functions in both Th1- and Th2-like responses. In addition, 2B4 functions on NK cells as a coreceptor to Nkp46, Nkp44, and Nkp30 (42). Even though the expression of these activating receptors is confined to NK cells, we cannot rule out the possibility that 2B4 functions as a coreceptor on eosinophils as well, orchestrating a response with an as yet undefined activatory receptor(s).

Another aspect of 2B4 activation on NK cells is enhancement of their cytotoxic effects toward several malignant cells (15, 17, 43). Eosinophils have been documented to be elevated in peripheral blood and/or to infiltrate the tissue in some malignant disorders (5, 44, 45). In addition, eosinophils have been shown to display direct and indirect antitumor effects both *in vitro* and *in vivo* (45–49). Therefore, we were interested to determine whether 2B4 can trigger eosinophil cytotoxicity toward malignant cells, as it does in NK cells.

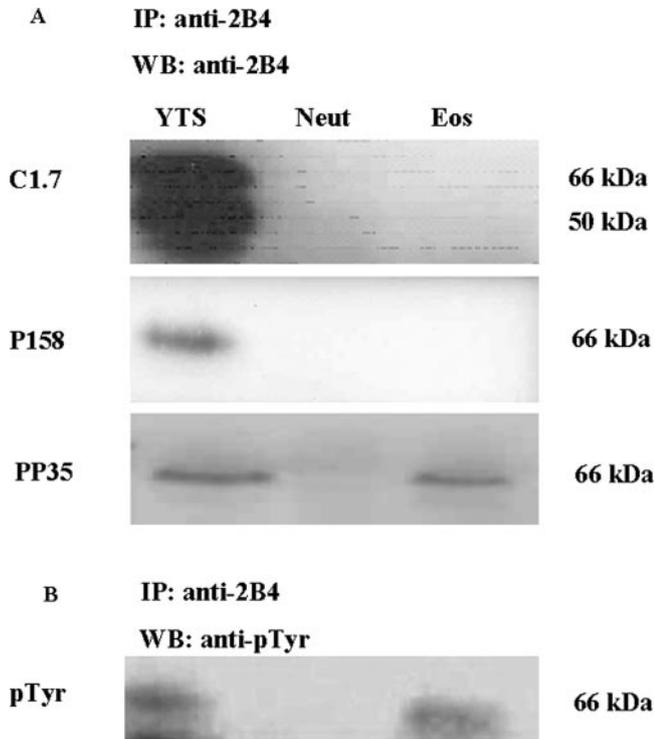
We could show that activation of eosinophils via 2B4 causes them to display cytotoxic effects toward the FcR<sup>+</sup> P815 mouse mastocytoma cell line as well as toward the CD48<sup>+</sup> 721.221 B cell lymphoma. It is important to note that the vast majority of receptor studies involving NK stimulation were performed with NK cells activated by IL-2 (50). However, in this work, freshly isolated eosinophils, without any previous *in vitro* priming were tested and found to be active. Interestingly, eosinophils were not able to lyse

melanoma cells transfected with CD48 (1106mel) and soluble CD48 did not activate eosinophil mediator release (data not shown). Importantly, a recent study by Lee et al. (51) has demonstrated that 2B4 acts as an inhibitory receptor, rather than an activating one, on CD48 ligation. Although the study was conducted on murine 2B4, it is feasible that human 2B4 can exert versatile signaling cascades as well (5, 51). Thus, we cannot exclude a possibility that additional cell-cell interactions regulate 2B4-CD48 interactions. Taken together, our results indicate that eosinophils may participate in the immune response against tumors in direct cell-cell and receptor-ligand interactions also via 2B4-CD48 interactions.

Examination of the signal transduction pathway of 2B4-stimulated NK cells reveals that activation of 2B4 entails complex interactions involving LAT, Ras, Raf, ERK, and p38 (52–54). Cross-linking of 2B4 on eosinophils resulted in ERK phosphorylation as early as 2 min after the ligation and peaked at 4 min. Nevertheless, the p38 MAPK pathway was not activated (data not shown). Thus, the signaling cascade elicited by 2B4 on eosinophils slightly differs from that in NK cells and may recruit diverse pathways.

CD84, 2B4, NTB-A, Ly-9, and SLAM all display cytoplasmic tyrosine-based motifs similar to ITIMs (13, 14). In 2B4, phosphorylation of these residues and recruitment of SAP, an Src homology 2 domain-containing molecule, results in NK cell activation. SAP is an adaptor molecule that recruits the tyrosine kinase Fyn and probably other Src kinases to SLAM and most likely to related receptors. The expression of SAP is limited to several cell types and has been shown to be expressed mainly in T and NK cells (55). Thus, to determine whether SAP may be functional on human eosinophils, the expression of this unique molecule was examined.

Freshly isolated eosinophils, but not neutrophils, expressed SAP at both mRNA and protein levels. These results are in contradiction with the findings of Nakajima et al. (20), who showed that eosinophils do not express SAP. This could be explained by the different sources of eosinophils, in their case atopic dermatitis patients and in ours mildly atopic subjects (M. Colonna, unpublished observations) or by the different Abs used.



**FIGURE 6.** Pervenate treatment causes 2B4 tyrosine phosphorylation in human eosinophils. Cell lysates of freshly isolated eosinophils, neutrophils, or YTS NK cells were precleared and then incubated with various anti-2B4 Abs, followed by protein A/G. The immunoprecipitates were analyzed by Western blot using either anti-2B4 (A) or anti-phosphotyrosine Abs (B). Proteins were detected by secondary immunopure goat anti-mouse Abs conjugated with HRP, followed by ECL detection. The numbers on the right of the panels indicate the positions of the m.w. markers.

Interestingly, immunoprecipitation of 2B4 from the surface of the eosinophils was only successful when using the PP35 Ab, but not the P158 or the C1.7 Abs. Indeed, these different Abs have been shown to activate different functions on NK and T cells (13). For example, the C1.7 Ab-dependent IFN- $\gamma$  release from NK cells required IL-2, IL-12, or target cells (K562) and had a low efficiency in precipitating 2B4 (13, 19). In addition, the P158 Ab did not induce any cytokine production from NK cells and had no effect on T cell-mediated killing (13, 15). Therefore, our results could also be explained by the differences among these Abs.

Treatment of eosinophils with pervenate resulted in tyrosine phosphorylation of 2B4 even though our studies could not demonstrate a direct interaction between 2B4 and SAP in the eosinophils. Thus, the role of SAP signaling in eosinophils remains to be clarified. Intriguingly, eosinophils express several cell surface receptors that potentially recruit SAP for their function, including Siglec 10 and Siglec 8 (10, 11) as well as CD84, 2B4, and NTB-A. Interestingly, among these receptors Siglec-8 is uniquely expressed on eosinophils, mast cells, and basophils, and NTB-A is restricted to eosinophils among the myeloid lineage. Therefore, SAP may have a greater role in regulating eosinophil functions.

In conclusion, the demonstrations that CD2 subfamily receptors are expressed and that 2B4 is functional on eosinophils are of particular interest. Defining the conditions in which eosinophils can interact with CD48 through 2B4 will make it possible to shed more light on the factors that regulate eosinophil functions in health and disease.

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