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CD48 Is an Allergen and IL-3-Induced Activation Molecule on Eosinophils[1]

Ariel Munitz,* Ido Bachelet,* Ron Eliashar,† Marat Khodoun,‡ Fred D. Finkelman,‡ Marc E. Rothenberg,§ and Francesca Levi-Schaffer[2]*¶

Eosinophils are involved in a variety of allergic, parasitic, malignant, and idiopathic disorders by releasing a variety of factors including specific granule proteins, lipid mediators, and proinflammatory and immunoregulatory cytokines and chemokines. In addition, they interact with various cell types in the inflamed tissue. Yet, the mechanism of eosinophil activation is still poorly understood. Recently, we described the expression and function of the CD2-subfamily of receptors and especially 2B4 on human eosinophils. In this study we focus on CD48, the high-affinity ligand of 2B4. CD48 is a GPI-anchored protein involved in cellular activation, costimulation, and adhesion, but has not been studied on eosinophils. We demonstrate that human eosinophils from atopic asthmatics display enhanced levels of CD48 expression and that IL-3 up-regulates CD48 expression. Furthermore, cross-linking CD48 on human eosinophils triggers release of eosinophil granule proteins. Assessment of CD48 expression in a murine model of experimental asthma revealed that CD48 is induced by allergen challenge and partially regulated by IL-3. Additionally, anti-IL-3 reduces CD48 expression and the degree of airway inflammation. Thus, CD48 is an IL-3-induced activating receptor on eosinophils, likely involved in promoting allergic inflammation. *The Journal of Immunology,* **2006, 177: 77–83.**

Eosinophils are bone marrow-derived cells that are normally found in selected mucosal surfaces such as the gastrointestinal tract. However, blood and tissue eosinophilia may also be present in allergic, parasitic, malignant, and idiopathic disorders (1–3). Complex networks of activating and inhibitory signals are likely to directly regulate the immunological or inflammatory activities coordinated by eosinophils. For example, eosinophils express receptors for IgA, IgG, cytokines, chemokines, and complement components (3). Recently, they have been found to display several additional Ig superfamily cell surface receptors that are able to regulate their activation such as leukocyte Ig-like receptor/Ig-like transcript (LIR-3/ILT-5)[3], LIR-1/ILT-2, LIR-2/ILT-4, LIR-7/ILT-1 (4), sialic acid-binding Ig-like lectins (5, 6), and IRp60 (CD300a) (7).

Activation of eosinophils results in the secretion of specific granule proteins, synthesis and release of lipid mediators, proinflammatory and immunoregulatory cytokines and chemokines (1–3). Interestingly, although eosinophils can release a variety of mediators in response to diverse stimuli, the mechanism(s) of their activation is poorly understood. Therefore, a central question concerning eosinophils is to understand how these cells are activated, particularly in disease states.

Receptor definition and surface phenotyping can be a useful tool to understand the complexity of cellular activation. This approach led to the identification of NK cell-regulating molecules and better understanding of their biology, development, and function (8). Recently, we examined the role of CD2 subfamily receptors on eosinophils. These include CD2, CD48 (Blast-1 and BCM-1), CD58 (LFA-3), CD84 (Ly9B), CD150 (signaling lymphocytic activation molecule), CD229 (Ly9), 2B4 (CD244), BCM-like membrane protein, SF2001 (CD2F-10), NTB-A (SF2000 and Ly108), and CS1 (CD1-like receptor-activating cytotoxic effects) (9). We demonstrated that 2B4, the high-affinity ligand for CD48, activates human eosinophils to release IL-4 and IFN-γ, and to develop cytotoxic activity against tumor cell lines in vitro (10). Inasmuch as eosinophils express both CD48 and 2B4, we have focused our current study on CD48.

CD48 is a GPI-anchored protein that exists both in a membrane-associated and a soluble form (11, 12). It is likely to have broad immunological importance because it is expressed on almost all leukocyte populations. In addition to being a high-affinity ligand for 2B4, it is a low-affinity ligand of CD2 and thus can provide costimulatory/stimulatory signals to CD2 or 2B4-expressing cells (9). Moreover, cells can be activated by signals transduced through CD48 itself (13).

Interestingly, CD48 expression is increased in several infectious diseases, including varicella, measles, rubella, mononucleosis, streptococcus tonsillitis, sepsis, and appendicitis (14).

In this study, we show that signaling through CD48 results in eosinophil activation, and that anti-IL-3 treatment reduces CD48 expression and eosinophilic inflammation in mice. These results suggest that IL-3 and CD48 are important regulators of eosinophil effector function in allergic settings.

*Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel; †Department of Otolaryngology-Head and Neck Surgery, The Hebrew University School of Medicine, Hadassah Medical Center, Jerusalem, Israel; ‡Department of Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267; §Division of Allergy and Immunology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45267; and ¶David R. Bloom Center for Pharmacology, The Hebrew University of Jerusalem, Jerusalem, Israel

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[2] Address correspondence and reprint requests to Dr. Francesca Levi-Schaffer, Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel. E-mail address: fls@cc.huji.ac.il

[3] Abbreviations used in this paper; LIRs/ILTs, leukocyte Ig-like receptor/Ig-like transcript; FEV_1, forced expiratory volume 1; EPO, eosinophil peroxidase; EDN, eosinophil-derived neurotoxin; BALF, bronchoalveolar lavage fluid; MFI, mean fluorescence intensity; β_C, common β-chain; rh, recombinant human.

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Materials and Methods

Reagents and chemicals

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

Human peripheral blood eosinophil donors

Eosinophils were purified from peripheral blood of atopic asthmatics (see below) or age- and sex-matched normal individuals (blood eosinophil levels 5–10%) by MACS-negative immunomagnetic separation as described previously (10). Asthmatic donors were all atopic individuals (total IgE >100 IU/ml blood) requiring intermittent β_2-agonist treatment (forced expiratory volume 1 values ranging from 75–90% of normal). Nonasthmatic gender-matched controls were nonatopic and had forced expiratory volume 1 values >95% of normal.

Informed consent was obtained from all volunteers according to the guidelines established by the Hadassah-Hebrew University Human Experimentation Helsinki Committee. Eosinophil preparations were resuspended in medium containing RPMI 1640, 200 U/ml penicillin, 200 μg/ml streptomycin, and 10% v/v heat-inactivated FCS (RPMI 1640-10%), and were at least 98% pure (Kimura's staining) and at least 98% viable (trypan blue exclusion staining).

Human nasal polyp digestion

Cells were isolated and obtained from nasal polyps of atopic asthmatic patients (7) or age- and sex-matched nonasthmatic individuals according to guidelines established by the Hadassah-Hebrew University Human Experimentation Helsinki Committee. Nasal polyps were washed twice in RPMI 1640-2% FCS, minced to fragments of ~1 mm^3, and subsequently digested by incubation for 60 min at 37°C with an enzyme mixture containing collagenase type-I (6 mg/gram tissue), hyaluronidase (3 mg/gram tissue), and DNase (100 μg/gram tissue). The digested tissue was filtered through a 150-mesh nylon cloth. Collected cells contained >55–90% eosinophils (Kimura's staining) and had a viability of >94% (trypan blue exclusion). Contaminating cells were usually macrophages and, to a lesser extent, lymphocytes. Eosinophils in the cell suspension were identified as SSC^{high} and $CCR3^{+}$ cells using FACS analysis.

Human eosinophil cell culture

For receptor up-regulation experiments, freshly isolated human peripheral blood eosinophils were seeded in 96-plate, U-shaped wells (Nunc) (2×10^5/200 μl) in RPMI 1640-10%, and incubated (37°C, 5% CO_2) for the indicated time points with IL-3 obtained from two different sources (PeproTech and R&D Systems) or with various other cytokines or chemokines (2–200 ng/ml; all purchased from PeproTech. Thereafter, the cells were washed, and CD48 expression was assessed by FACS.

For mediator release assays, 96-well plates (Nunc) were precoated with sheep anti-mouse IgG $F(ab')_2$ in PBS (25 μg/ml, 2 h, 37°C, 5% CO_2). Afterward, plates were washed three times with PBS and incubated with anti-CD48 mAb (BD Pharmingen) or an irrelevant isotype-matched control mAb (DakoCytomation) (1 μg/ml, 2 h at 37°, 5% CO_2) and washed three times. Freshly isolated eosinophils were seeded in these precoated wells (2×10^5/200 μl) in RPMI 1640-10% (as described above) and incubated for 30 min–18 h (37°C, 5% CO_2). At the end of the incubation, cells were centrifuged (300 × *g*, 5 min, 4°C), supernatants collected, aliquoted, and stored at −80°C until assessed for eosinophil granule protein expression and activity.

Eosinophil granule protein determination

Eosinophil peroxidase. EPO release was determined by a colorimetric assay as described previously (10). Briefly, eosinophil culture supernatants (50 μl) were incubated (10–15 min, 37°C, 5% CO_2) with a substrate solution that contained 0.1 mM *O*-phenylenediamine dihydrochloride in 0.05 M Tris buffer (pH 8.0), 0.1% Triton X-100 (37°C, 5% CO_2), and 1 mM hydrogen peroxide (Merck). The reaction was stopped by the addition of 4 mM sulfuric acid (BDH Chemicals), and the absorbance was determined at 492 nm in a spectrophotometer (PowerWave XS; Bio-Tek Instruments).

Eosinophil-derived neurotoxin (EDN). *EDN* in the eosinophil culture supernatants was determined by an ELISA kit according to the manufacturer's instructions (MBL International). Lower detection limit was 1.61 ng/ml.

Animal studies

Experimental asthma. BALB/c female mice (7–8 wk old) were obtained from Harlan Laboratories and housed under specific pathogen-free conditions. Mice were sensitized by i.p. injection with 100 μg of OVA adsorbed onto 1 mg of aluminum hydroxide in 250 μl of saline on days 0 and 14. On days 24 and 27, the mice were lightly anesthetized with inhaled isofluorane and challenged intranasally with 50 μg of OVA or saline. The allergen challenge was performed by applying 50 μl to the nares using a micropipette with the mouse held in a supine position. After instillation, the mice were held upright until alert. Mice were sacrificed by isofluorane inhalation at the indicated time points (0–24 h) following allergen challenge, and bronchoalveolar lavage fluid (BALF) was performed for differential cell counts (15). In addition, lungs were excised, digested as described (16), (differential cell counts).

For neutralizing experiments, anti-IL-3 Ab clone MP2-8F8 was grown as ascites in pristane-primed mice and purified by a combination of ammonium sulfate fractionation and DEAE-cellulose ion exchange chromatography (17). Anti-IL-3 (2 mg/mouse in 300 μl of saline) or an appropriate isotype-matched control was administered i.p. on day 23 (24 h before allergen challenge) and on days 24 and 27 (1 h before allergen challenge). Mice were sacrificed 18 h after the last allergen challenge. BALF was performed for differential cell counts, and eosinophils were assessed for CD48 expression. In addition, lungs were excised, fixed in 4% paraformaldehyde, paraffin embedded, and stained by H&E. Calculation of total lung inflammation was performed by assessing alveolar space and perivascular and peribronchial infiltrate using the following key: 0, no inflammation; 1, light inflammation; 2, moderate inflammation; 3, severe inflammation.

Allergic peritonitis. BALB/c female mice (8–10 wk old) were sensitized s.c. on days 0 and 7 with 100 μg of OVA adsorbed onto 1.6 mg of aluminum hydroxide in 300 μl of saline. On day 11, the mice were challenged i.p. with 3 μg of OVA in 200 μl of saline and sacrificed at the indicated time points (6–48 h). Thereafter, the peritoneal cavity was washed with 5 ml of Tyrode's gelatin buffer for differential cell counts.

For experiments involving IL-5 transgenic mice, CD2-driven IL-5 transgenic mice were obtained as described previously (18).

All experiments involving animals and primary animal cells were approved by the Institutional Animal Experimentation Ethics Committee

IL-3 administration

IL-3 (PeproTech) was administered intranasally or systemically in lightly anesthetized (isofluorane) BALB/c female mice (7–8 wk old). Briefly, recombinant murine IL-3 (2–4 μg in 50 μl of saline for intranasal administration and 8–10 μg in 100 μl of saline) was delivered in conjunction with anti-IL-3 mAb (4–20 μg) (IL-3C). This forms an IL-3/anti-IL-3 mAb complex (IL-3C) that slowly releases IL-3 with an in vivo half-life of ~24 h, as compared with a half-life of several minutes for free IL-3 (19). The mice received IL-3C every other day for 21 days. Mice were sacrificed 24 h after the last administration of IL-3C. Spleen, lung, and BALF cells were assessed for CD48 expression by FACS (see below).

IL-3 determination

IL-3 in the BALF of saline and OVA-treated mice was measured using DuoSet (R&D Systems) according to manufacturer's instructions. Lower detection limit of the assay was 3.9 pg/ml.

Flow cytometry

The expression of CD48 on human peripheral blood and nasal polyp eosinophils was assessed as previously described (7, 10) using an Ab purchased from Santa Cruz Biotechnology (clone 4H9).

For identification of CD48 expression on murine cells, differential cell staining was performed by four-color flow cytometry using anti-CD3 APC, anti-c-*kit* Pe-Cy5, anti-FcεRI FITC, anti-CD4 Pe-Cy5, anti-CD48 PE, anti-CD49d PE, anti-Ly49b PE (eBioscience), anti-CCR3 FITC (R&D, Systems), and anti-CD45R APC (Miltenyi Biotec). The different cell types were identified by their surface Ags and physical parameters (SSC vs FSC) as described previously (15, 20). Briefly, eosinophils were characterized as SSC^{high}, $CCR3^{high}$, $CD49d^{high}$, c-kit^{low}, $Fc\varepsilon RI^{low}$, $Ly49b^{-}$, $CD3^{-}$; basophils as SSC^{int}, $CCR3^{low}$, c-kit^{-}, $Fc\varepsilon RI^{high}$, $Ly49b^{high}$, $CD3^{-}$. For each preparation, at least ten thousand cells were collected, and data analysis was performed using CellQuest software (BD Biosciences).

Statistical analysis

Statistical significance was calculated using parametric analysis (ANOVA, followed by paired Student's *t* test assuming equal variance), *p* values <0.05 were considered significant.

Results

Peripheral blood eosinophils and nasal polyp eosinophils of atopic asthmatics express increased levels of CD48

Our previous studies demonstrated that human eosinophils express significant levels of CD48 (Ref. 10 and Fig. 1*A*). Because CD48 has been reported to be elevated in several disease states, we aimed to determine whether CD48 expression on eosinophils is elevated in atopic asthmatic donors compared with nonasthmatic controls. As assessed by FACS analysis, peripheral blood eosinophils from atopic asthmatic donors expressed higher levels of CD48 (mean fluorescence intensity (MFI) 16.87 ± 6.16; $n = 7$; $p < 0.01$) compared with eosinophils from nonasthmatic donors (MFI 7.07 ± 2.63) (Fig. 1*B*). Nasal polyposis has been linked to bronchial asthma, and the percentage of infiltrating eosinophils in the polyps can reach as high as 60% (21). We found that nasal polyp eosinophils obtained from asthmatic donors demonstrated significantly higher CD48 levels (MFI 10.11 ± 4.26; $n = 11$; $p < 0.01$) than nasal polyp eosinophils from nonasthmatic individuals (MFI 4.68 ± 2.57) (Fig. 1*C*).

The expression of CD48 on human eosinophils is up-regulated by IL-3

The observation that human eosinophils from asthmatic donors display elevated levels of CD48 suggests that its expression may be regulated by a mediator involved in asthma pathogenesis. To clarify which mediator may regulate CD48, freshly isolated eosinophils were incubated with cytokines, growth factors, and chemokines, including IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, IFN-γ, GM-CSF, stem cell factor, TGF-β, eotaxin-1, RANTES, and MIP-1α that are found in the asthmatic milieu. Although IL-3, IL-5, and GM-CSF share a common β-chain (β_C) that transduces their signal, only IL-3 up-regulated CD48 expression (Fig. 2*A*). IL-3 elicited its effect in a concentration-dependent fashion, with a maximal effect at 20 ng/ml (1.51 ± 0.13-fold increase, 2.11 ± 0.13-fold increase, and 1.91 ± 0.06-fold increase, respectively, following stimulation with 2, 20, or 200 ng/ml IL-3; $n = 5$; $p < 0.001$). To verify that the effect of IL-3 is not due to a specific IL-3 batch, eosinophils were incubated with IL-3 from two commercial sources. Notably, these two different sources of IL-3 gave similar results (data not shown). Furthermore, eosinophils that were cultured in the presence of recombinant human (rh)IL-3 and anti-IL-3 mAb displayed reduced levels of CD48 in comparison to eosinophils treated with IL-3 or anti-IL-3 alone ($p < 0.05$; data not shown). Kinetic analysis revealed that IL-3-induced up-regulation peaked at 24 h (2.14 ± 0.15-fold increase; $n = 3$; $p < 0.01$) (Fig. 2*B*).

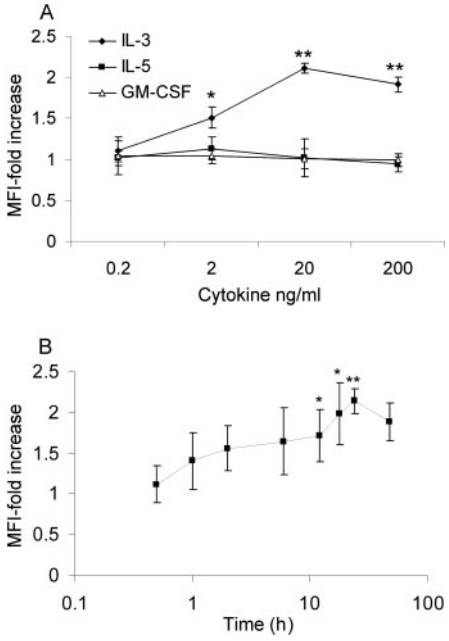

FIGURE 2. The expression of CD48 on human peripheral blood eosinophils is regulated by IL-3. Human peripheral blood eosinophils were incubated with the indicated concentrations of rhIL-3, rhIL-5, or rhGM-CSF (*A*) for 18 h. Thereafter, cells were washed twice, stained with anti-CD48 mAb followed by goat anti-mouse FITC, and analyzed by FACS. Kinetic analysis (*B*) was performed by incubating human peripheral blood eosinophils with 20 ng/ml rhIL-3 for the indicated time points. Thereafter, cells were assessed for CD48 expression as described above. Data are presented as MFI, each dot represents one donor. *, $p < 0.05$; **, $p < 0.005$; $n = 5$.

CD48 activates human eosinophils to release EPO

Expression of CD48 on the eosinophil surface suggests that eosinophil responses may be regulated by this receptor. Because CD48 triggers lymphocyte activation (22), we hypothesized that it

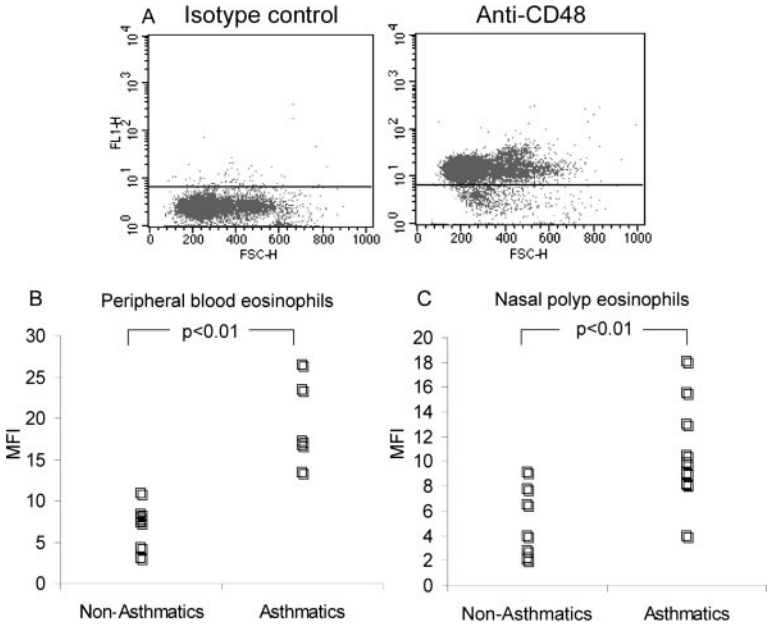

FIGURE 1. Peripheral blood eosinophils and nasal polyp eosinophils of atopic asthmatics display enhanced levels of CD48 expression. *A*, A representative dot-plot analysis of CD48 expression (anti-CD48) on the surface of human peripheral blood eosinophils is shown. Horizontal bar indicates the background staining obtained by isotype-matched control Ab (Isotype control). Human peripheral blood eosinophils (*B*) and human nasal polyp eosinophils (*C*) from atopic asthmatic ($n = 7$ and 8, respectively) or normal ($n = 6$ and 9, respectively) individuals were stained with anti-CD48 mAb followed by goat anti-mouse FITC and analyzed by FACS. For identification of nasal polyp eosinophils, the cells were additionally stained with rat anti-mouse CCR3 PE. $CCR3^+/SSC^{high}$ cells were identified as eosinophils and analyzed for CD48 expression. Data are presented as MFI, each dot represents one donor. *, $p < 0.05$; **, $p < 0.005$.

could also activate eosinophils. Indeed, cross-linking of CD48 on human eosinophils induced EPO and EDN release (Fig. 3, *A* and *B*, respectively). However, CD48 cross-linking did not induce cytokine release, because IL-4, IL-8, and IFN-γ were not detected in the culture supernatants. Furthermore, cross-linking of CD48 in the presence of IL-3 did not enhance EPO or EDN release or cause enhanced cytokine secretion (data not shown).

IL-3 regulates CD48 expression in mice

We aimed to determine whether IL-3 up-regulated CD48 expression in vivo and therefore turned our attention to the mouse system. Important effector mechanisms are likely to display conserved regulatory pathways between different species. Intranasal administration of IL-3 to BALB/c mice for 21 days significantly increased eosinophil, basophil, and lymphocyte infiltration to the BALF and lungs compared with control saline administration (Fig. 4, *A* and *B*). Furthermore, IL-3 specifically up-regulated CD48 expression on BALF and lung eosinophils and basophils but not on lymphocytes, neutrophils, or macrophages (Fig. 4, *C* and *D*). Consistent with this result, i.v. administration of IL-3C specifically increased eosinophil and basophil numbers and their expression of CD48 in the spleen (Fig. 4, *E* and *F*).

Furthermore, as assessed by an in vivo cytokine capture assay (23), systemic administration of IL-3C increased IL-4 production by 20- to 30-fold (data not shown). Thus, IL-3 activates mediator release in vivo.

To establish whether IL-3 is specifically responsible for CD48 up-regulation in vivo, we were interested to examine the expression of CD48 in response to IL-5 administration. For this experiment, we injected mice with IL-5 complexed to several different anti-mouse IL-5 mAbs. However, no substantial in vivo activity was observed (data not shown). Thus, we examined the expression of CD48 on eosinophils from IL-5 transgenic mice in comparison to wild-type mice. Eosinophils from IL-5 transgenic mice displayed comparable levels of CD48 to wild-type mice (Fig. 4*G*). Therefore, in vivo up-regulation of CD48 expression on mouse eosinophils, like in vitro up-regulation of CD48 on human eosinophils, is induced by IL-3 but not IL-5.

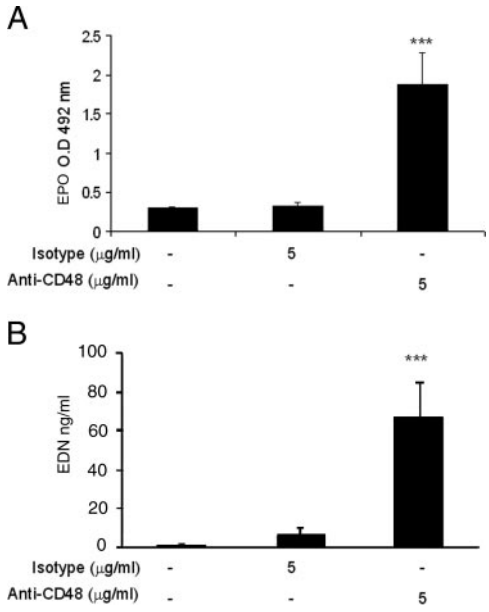

FIGURE 3. CD48 activates human peripheral blood eosinophils to release EPO and EDN. Human peripheral blood eosinophils were cultured in wells precoated with sheep anti-mouse $F(ab')_2$ Ab and either isotype-matched control (isotype) or anti-CD48 mAb (anti-CD48) for 30 min. EPO (*A*) and EDN (*B*) in the culture supernatants was determined by a colorimetric assay. The data represent the mean ± SD of three different experiments performed in triplicate. ***, $p < 0.001$; $n = 3$.

CD48 is up-regulated on murine eosinophils in experimental asthma and experimental allergic peritonitis

Accordingly, we were interested in determining whether CD48 is up-regulated in allergic conditions in mice. To address this, we examined two independent experimental allergy models: in vivo Ag-induced allergic airway inflammation (experimental asthma) and Ag-induced allergic peritonitis. In experimental asthma induced by OVA challenge, expression of CD48 by BALF eosinophils was significantly up-regulated in a time-dependent fashion, whereas saline challenge had no effect (Fig. 5*A*). The kinetics of CD48 expression was similar in the BALF and the lungs, increasing 6 h after the last allergen challenge and peaking at 24 h (data not shown). Eosinophil CD48 expression was also increased in allergic peritonitis, increasing at 8 h and peaking at 48 h (Fig. 5*B*).

Neutralization of IL-3 in experimental asthma reduces CD48 expression

Interestingly, OVA-treated mice displayed low and variable but significantly distinguished levels of IL-3 in comparison to saline-treated mice (Fig. 6*A*).

To determine whether IL-3 is responsible for the elevated expression of CD48 observed in murine experimental asthma, neutralizing Abs to IL-3 or isotype-matched control Abs were administered to OVA-challenged mice. Neutralization of IL-3 in OVA-challenged mice resulted in a 33% decrease ($p < 0.05$) in CD48 expression by BALF eosinophils (Fig. 6*B*), decreased the number of infiltrating BALF eosinophils (Fig. 6*C*), and attenuated lung inflammation (Fig. 6*D*), whereas an isotype-matched control Ab had no effect. In addition, neutralization of IL-3 decreased the levels of IL-4 in the BALF of OVA-challenged mice from 79 ± 2.7 to 61 ± 4.5 pg/ml ($p < 0.05$; $n = 2$) (data not shown).

Discussion

Understanding the role of eosinophils in allergic and inflammatory settings can be achieved by defining the activation pathways that govern their cellular actions. Thus, the molecular mechanisms that control eosinophil activation need further attention (1, 24). We have recently reported the expression and function of CD2-subfamily receptors including CD48, CD58, CD84, NTB-A, and 2B4 on human eosinophils (10). In this study, we expanded our investigations to CD48. To the best of our knowledge this is the first study to evaluate CD48 on eosinophils, although it has been described extensively on other cell types.

CD48 has been reported to be elevated in the serum and on the surface of hemopoietic cells from patients with leukemia and infectious diseases (12, 14). Assessment of both peripheral blood and nasal polyp eosinophils from atopic asthmatics demonstrated that CD48 was indeed elevated in patients with allergic disease compared with nonatopic controls. This is important because bronchial asthma is the most prevalent disease associated with nasal polyposis (21, 25), and the correlation between these two pathologies has been recently summarized in the "one airway-one disease" theory (25). Thus, it is possible that nasal polyp eosinophils from atopic asthmatics have a phenotype similar to that of lung eosinophils from the same donors; as such, CD48 may have a considerable role in eosinophil activation in asthma.

The observation that CD48 expression is enhanced on eosinophils from asthmatics indicates that a factor in the inflammatory environment regulates its expression. Therefore, we evaluated the

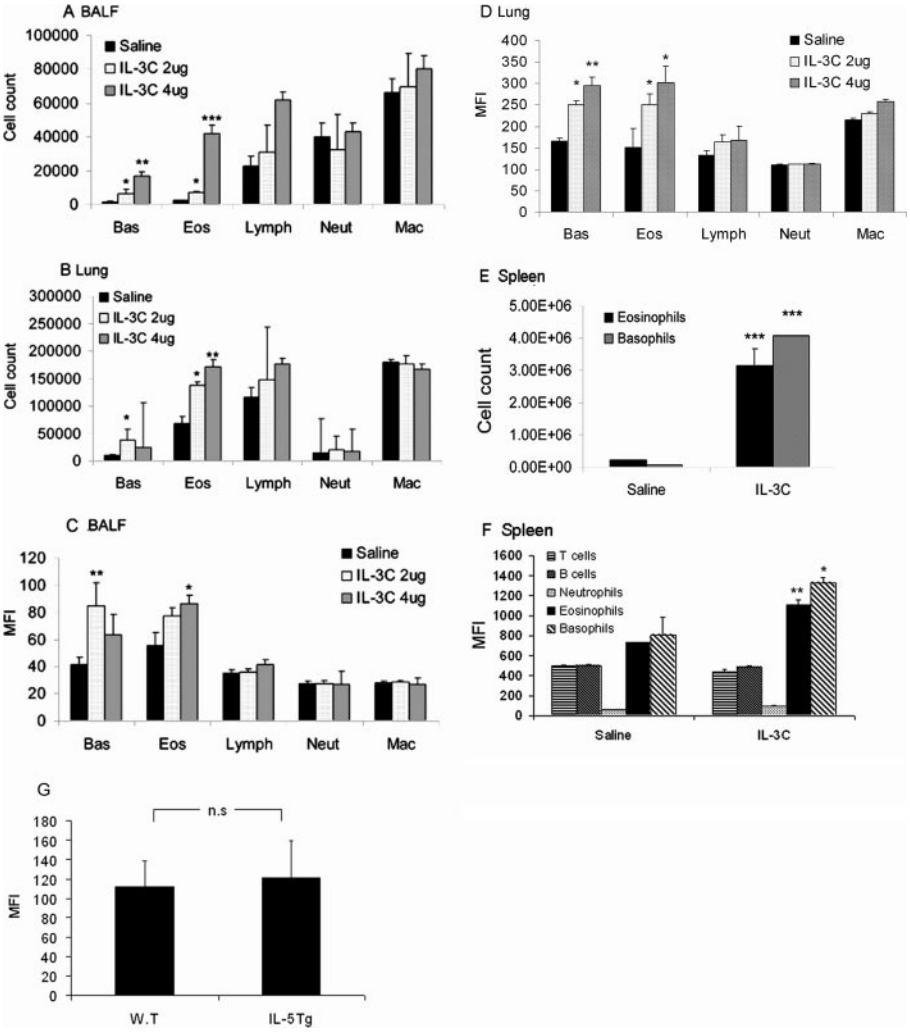

FIGURE 4. IL-3 regulates CD48 expression in vivo. IL-3 mixed with anti-IL-3 mAb at a 2:1 molar ratio (IL-3C) was administered intranasally (*A–D*) or systemically (*E* and *F*) every other day for 21 days to normal BALB/c mice. Twenty-four hours after the last IL-3C administration, mice were sacrificed, BALF was performed, and lungs and spleen were excised for differential cell counts as assessed by FACS analysis (*A*, *B*, and *E*). For assessment of CD48 expression (*C*, *D*, and *F*), BALF and lung cells were stained additionally with anti-CD48 mAb and evaluated by FACS. Eosinophils from the spleen of IL-5 transgenic or wild-type mice (*G*) were stained with anti-CD48 mAb and evaluated by FACS. For *A*, *B*, and *E*, data are presented as total cell number ± SD; for *C–F*, data are presented as MFI ± SD, from four to six mice per group. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; $n = 3$.

expression of CD48 on human peripheral blood eosinophil after incubation with various cytokines and chemokines. The survival cytokines IL-3, IL-5, and GM-CSF (26) share a β_C-chain that is responsible for activating their signaling pathways. Hence, it was expected that IL-3, IL-5, and GM-CSF would influence eosinophils similarly. Nevertheless, our results demonstrate that only IL-3 up-regulated CD48 expression on eosinophils, indicating that IL-3 can elicit a β_C-chain-independent signaling cascade in eosinophils. Importantly, administration of IL-3 enhanced the expression levels of CD48 in vivo on murine BALF and lung eosinophils as well as basophils.

To confirm that the ability of IL-3 to regulate CD48 in vivo is independent of IL-5, GM-CSF, and the IL-3 β_C chain, we assessed CD48 expression on eosinophils from IL-5 transgenic mice. Eosinophils from these mice displayed CD48 levels similar to those of wild-type mice. Our findings regarding the ability of IL-3 to transduce independent signaling cascades are consistent with previous observations that IL-3, unlike the other β_c-related cytokines, up-regulates human eosinophil CD86 (27) and down-regulates CCR3 (28). In addition, IL-3 is the most efficient cytokine that up-regulates CD69 on the surface of basophils in comparison with IL-5 and GM-CSF (29). In fact, Mire-Sluis et al. (30) demonstrated the induction of independent signaling cascades by IL-3-, IL-5-, and GM-CSF-specific α-chains. Moreover, exposure to IL-5 stimulates eosinophils to drastically decrease IL-5Rα-chain expression and increase the expression of IL-3Rα-chain (31). Therefore, eosinophils that are primed by IL-5 become further responsive to IL-3. Altogether, these results may indicate that IL-3 has unforeseen roles in eosinophil biology, and the mechanism by which IL-3 specifically signals deserves further attention.

To determine whether IL-3 up-regulates CD48 in allergic disorders, we studied CD48 expression on eosinophils in murine experimental asthma and allergic peritonitis. Our data demonstrate that CD48 expression increased in a time-dependent fashion after allergen challenge. IL-3 neutralization in OVA-challenged mice reduced eosinophil CD48 expression, but not to the baseline level that is observed in saline-treated mice. Thus, although IL-3 is the only cytokine we have identified, it is unlikely to be the only factor responsible for up-regulation of CD48 in vivo. On B and T lymphocytes CD48 is regulated by IFNs and, most importantly, IL-4 (32, 33). Thus, IL-3 and IL-4 may act in concert to influence CD48 expression on various cell types. Alternatively, higher concentrations of anti-IL-3 may be required for a more dramatic effect.

Interestingly, cross-linking of CD48 on human eosinophils triggered EPO release but no cytokine release even in the presence of IL-3. We speculate that under certain circumstances, IL-3 can potentiate the responses elicited by CD48. For example, IL-3 enhances the ability of eosinophils to internalize *Escherichia coli* via CD48 (A. Munitz, I. Bachelet, and F. Levi-Schaffer, unpublished observation). Furthermore, IL-3 has been shown to prime and augment eosinophil-LTC_4 generation in response to calcium ionophore and enhance cytotoxicity toward Ab-coated helminths (34).

The exact downstream signaling mechanism of CD48 is an intriguing question (35). CD48 is located in rafts that are rich in

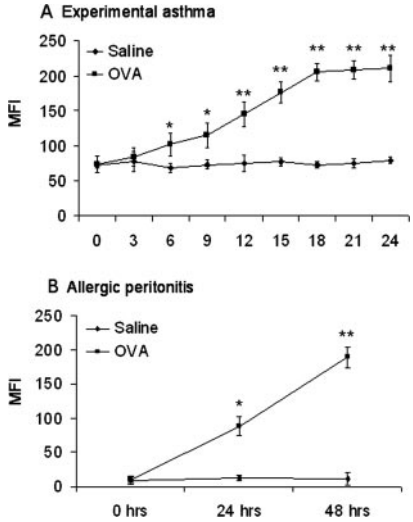

FIGURE 5. CD48 is up-regulated on murine eosinophils in experimental asthma and in allergic peritonitis. OVA/alum-sensitized mice were challenged with OVA. Mice were sacrificed at the indicated time points after the last allergen challenge, and BALF (*A*) or peritoneal lavage (*B*) was performed. The cells were stained with PE-labeled anti-CD48 and FITC-labeled anti-CCR3. $CCR3^{+}/SSC^{high}$ cells were gated and analyzed for CD48 expression. Data are expressed as MFI ± SD from four to six mice per group. *, $p < 0.05$; **, $p < 0.005$; $n = 3$.

glycosphingolipids, cholesterol as well as important signaling molecules such as Src-family protein tyrosine kinases and G-proteins. Therefore, the close proximity of these signaling molecules may explain the capability of signal transduction (13, 36, 37). Indeed, cross-linking of CD48 on T lymphocytes induced mobilization of the intracellular calcium inositol triphosphate concentration (38), and cross-linking of CD48 combined with CD3 induced enhanced IL-2 release, TCR signaling, and cytoskeletal reorganization (39–41). Moreover, cross-linking of CD48 on B cells induced strong homotypic adhesion, increased CD40-mediated activation, and enhanced responses to IL-4 and/or IL-10 stimulation (42, 43). As complex networks of activating and inhibitory signals govern the responses coordinated by eosinophils, increased CD48 expression might shift the resting threshold of eosinophils toward activation.

Taken together, our results suggest that CD48 may serve as a multifaceted molecule that regulates several eosinophil effector functions in disease settings. For example, elevated levels of CD48 on eosinophils and basophils correlated with increased infiltration of these cells to the lung, BALF, and spleen. In addition, CD48 has been reported to function as an adhesion molecule (33), and it can bind directly to heparan sulfate on the surface of epithelial cells (44). Consequently, CD48 may influence homing, transmigration, and tissue retention of eosinophils in allergic settings.

Eosinophils can propagate the inflammatory state by proinflammatory interactions with other cell types (3, 45). CD48 may allow eosinophils to interact with NK and NKT cells (that express 2B4; Ref. 46) and have been shown to participate in allergy, particularly by releasing IL-4 and IL-13 (47). Thus, increased expression of CD48 on lung eosinophils might provide stimulatory signals to NKT cells that promote and sustain a Th2 environment. Furthermore, it has been shown that eosinophils express costimulatory molecules, such as CD28 and CD86 (48), and are capable of Ag presentation (49) and T cell cross-talk in asthma (3, 44, 50). Thus, CD48-CD2 interactions mediated by eosinophils are likely to affect multiple responses.

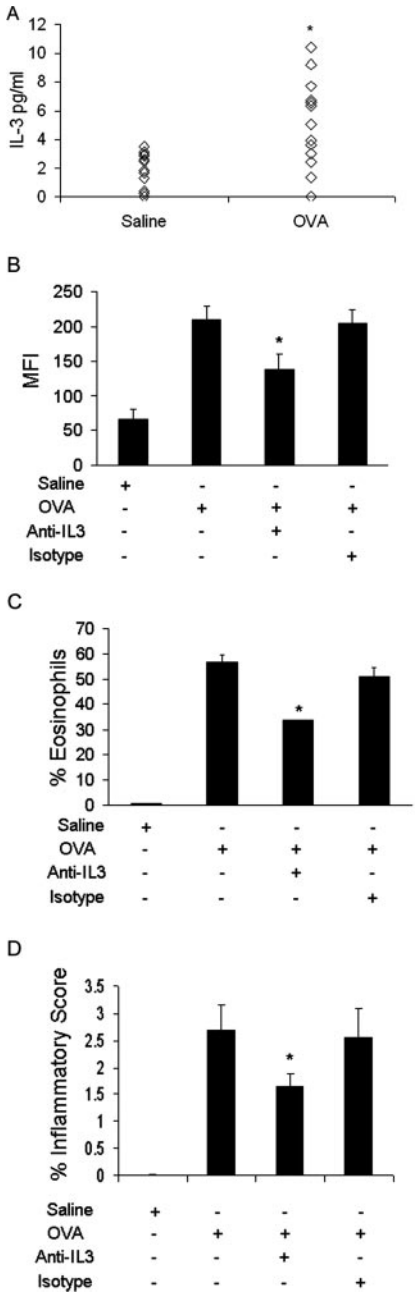

FIGURE 6. Neutralization of IL-3 in murine experimental asthma reduces CD48 expression. IL-3 levels in the BALF of saline- and OVA-treated mice was evaluated using DuoSet ELISA development kit (*A*). OVA/alum-sensitized mice were treated with anti-IL-3 mAb or an isotype-matched control mAb (2 mg/mouse) on day 23 (24 h before allergen challenge) and days 24 and 27 (1 h before allergen challenge). BALF was performed, and lungs were excised, 24 h after the last allergen challenge. BALF cells were stained with PE-labeled anti-CD48 and FITC-labeled anti-CCR3. $CCR3^{+}/SSC^{high}$ cells were gated and analyzed for CD48 expression (*B*) and percentage of eosinophils (*C*). Lungs were fixed, paraffin embedded, stained for H&E, and scored as described (*D*). *A*, Each ◇ represents IL-3 in the BALF of a single mouse. The data are expressed as mean of triplicate wells per mouse. *B* and *C*, Data are expressed as MFI ± SD or percentage of cells, respectively. *D*, Data are expressed as mean inflammatory score ± SD. All data were obtained from four to six mice per group. *, $p < 0.05$; $n = 3$.

Taken together, we propose that CD48 and IL-3 have important roles in eosinophil activation in a variety of conditions not previously described.

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Disclosures

The authors have no financial conflict of interest.

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