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Human Eosinophils and Human High Affinity IgE Receptor Transgenic Mouse Eosinophils Express Low Levels of High Affinity IgE Receptor, but Release IL-10 upon Receptor Activation

Hiroyuki Kayaba,^{1,2} David Dombrowicz,¹ Gaetane Woerly,¹ Jean-Paul Papin, Sylvie Loiseau, and Monique Capron³

FcεRI expressed by human eosinophils is involved in IgE-mediated cytotoxicity reactions toward the parasite *Schistosoma mansoni* in vitro. However, because receptor expression is low on these cells, its functional role is still controversial. In this study, we have measured surface and intracellular expression of FcεRI by blood eosinophils from hypereosinophilic patients and normal donors. The number of unoccupied receptors corresponded to ~4,500 Ab binding sites per cell, whereas 50,000 Ab binding sites per cell were detected intracellularly. Eosinophils from patients displayed significantly more unoccupied receptors than cells from normal donors. This number correlated to both serum IgE concentrations and to membrane-bound IgE. The lack of FcεRI expression by mouse eosinophils has hampered further studies. To overcome this fact and experimentally confirm our findings on human eosinophils, we engineered IL-5 × hFcεRIα double-transgenic mice, whose bone marrow, blood, spleen, and peritoneal eosinophils expressed FcεRI levels similar to levels of human eosinophils, after 4 days culture with IgE in the presence of IL-5. Both human and mouse eosinophils were able to secrete IL-10 upon FcεRI engagement. Thus, comparative analysis of cells from patients and from a relevant animal model allowed us to clearly demonstrate that FcεRI-mediated eosinophil activation leads to IL-10 secretion. Through FcεRI expression, these cells are able to contribute to both the regulation of the immune response and to its effector mechanisms. *The Journal of Immunology*, 2001, 167: 995–1003.

The high affinity IgE receptor, FcεRI, has been considered for a long time to be expressed only on mast cells and basophils and to be responsible for triggering immediate hypersensitivity reactions (1). More recently, FcεRI expression was demonstrated on other human cell types, including eosinophils (2), platelets (3), epidermal Langerhans cells (4, 5), dendritic cells (6) and monocytes/macrophages (7), allowing the receptor to play a role in anti-parasitic effector function in vitro (2, 3) or in Ag presentation (6, 8). Despite the low amounts of FcεRI expressed by human eosinophils, this cell population is able to mediate Ab-driven cellular cytotoxicity (ADCC) reactions toward *Schistosoma mansoni* larvae in vitro, and to release eosinophil peroxidase, a pharmacologically active mediator, upon cross-linking of FcεRI with anti-FcεRIα-chain mAb (2).

By contrast, in mouse, FcεRI expression is restricted to mast cells and basophils (9, 10), suggesting a species polymorphism linked to the cellular distribution of FcεRI. The lack of FcεRI (and CD23) expression on wild-type mouse eosinophils (9) provided

the beginning of an explanation to the long lasting debate about the role of IgE and eosinophils in mouse immunity to schistosomiasis.

Furthermore, two recent papers (11, 12) reopened the controversy about the role of FcεRI expressed by human eosinophils. On one hand, Seminario et al. (11) were unable to detect FcεRI at the surface of eosinophils, while demonstrating high amounts of FcεRIα inside the cell and released in medium, as a soluble receptor. On the other hand, Kita et al. (12) detected low levels of surface expression but failed to measure any biological effect (degranulation, superoxide anion production, or leukotriene C4 release) upon receptor activation by IgE and anti-IgE. To reassess the levels of FcεRI expression, we took advantage of a recent method for quantification of surface or intracellular binding sites by flow cytometry. We determined the number of unoccupied FcεRIα molecules expressed by purified eosinophils from a large series of patients with eosinophilia, as well as from normal donors.

We also intended to confirm our results on an experimental model that was more relevant to the human situation than WT mice. Therefore, transgenic (Tg)⁴ mice expressing human FcεRIα (hFcεRIα) under the control of its own promoter elements were first produced (13). These animals expressed a “humanized” receptor with a cellular distribution similar to humans, including eosinophils (after infection by *S. mansoni*). Because naive mice have a very low number of eosinophils compared with humans and rats, hFcεRIα Tg mice were crossed with IL-5 Tg mice exhibiting massive eosinophilia in different organs (14). Eosinophils from these hFcεRIα × IL-5 double-Tg animals expressed a low number

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⁴ Abbreviations used in this paper: Tg, transgenic; hFcεRIα, human FcεRIα; mIgG1, mouse IgG1; rhIL-5, recombinant human IL-5; SA-PE, PE-conjugated streptavidin; ABC, Ab binding capacities; MFI, median fluorescence intensity; hIgE, human myeloma IgE; cIgE, chimeric hIgE; ADCC, Ab-driven cellular cytotoxicity;

of surface FcεRI and contained high amounts of intracellular hFcεRIα. Nevertheless, receptor activation was sufficient to trigger IgE-dependent adherence of eosinophils to *S. mansoni* larvae and, as for human eosinophils, a significant IL-10 release.

Taken together, our results demonstrate that low levels of unoccupied FcεRI at the surface of eosinophils endow these cells with both effector and regulatory function in immune response.

Materials and Methods

Eosinophil donors

A total of 24 different hypereosinophilic patients and 6 normal donors were selected for this study, after informed consent. Hypereosinophilia was associated with skin diseases, hypereosinophilic syndromes, allergy, and hematological disorders. The characteristics of eosinophil donors, eosinophil preparations and serum IgE levels are summarized in Table I.

Animals

Animals were bred and housed in a specific pathogen-free facility. IL-5 × hFcεRIα Tg mice were obtained by crossing IL-5 Tg animals (14), in which IL-5 expression in T cells is driven by the human CD2 promoter, with hFcεRIα Tg animals (13), where the transgene is expressed under the control of its own promoter elements. Six- to 12-wk-old F₂ IL-5 × hFcεRIα Tg animals and their IL-5 Tg littermate controls were used for the experiments. Expression of the IL-5 transgene was assessed by monitoring blood eosinophilia, whereas presence of the hFcεRIα transgene was analyzed by Southern blot as previously described (13).

S. mansoni cycle and infections

A Guadeloupean strain of *S. mansoni* was maintained using *Biomphalaria glabrata* snail as the invertebrate intermediate host and, in the mice, as vertebrate definitive host. For in vivo experiments, animals were infected percutaneously with 50 cercariae after shaving the abdominal skin, and were sacrificed 46 days later for cell collection. For in vitro experiments

schistosomula were collected in MEM after the application of cercariae to isolated pieces of Swiss mouse abdominal skin for 3 h.

Reagents

Anti-human CD16- and CD3-coated magnetic beads, anti-mouse CD45R (B220)-, CD8α (Ly-2)-, and CD90 (Thy1.2)-coated magnetic beads and the MACS system were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden). RPMI 1640 and MEM, glutamine, penicillin, streptomycin, G418, and FCS were obtained from Life Technologies (Paisley, U.K.). BSA, paraformaldehyde, and saponin were obtained from Sigma (St. Louis, MO). The blocking anti-FcεRIα (15.1, a mouse IgG1 (mIgG1)) mAb (5) and rabbit polyclonal anti-FcεRIα antiserum (997) (15) were a kind gift from Dr. J.-P. Kinet (Harvard Medical School, Boston, MA). Both 15-1 and mIgG1 isotype control (Diacclone, Besançon, France) were FITC-labeled in our laboratory. Recombinant human IL-5 (rhIL-5) was obtained from Diacclone. Mouse anti-FcεRIα mAb CRA-1 (IgG2b nonblocking) and CRA-2 (IgG1 blocking) (16) were kind gift from Dr. C. Ra (Juntendo University, Tokyo, Japan), and 22E7 (IgG1 nonblocking) (17) was provided by Dr. A. Tscopoulos (Institut Pasteur de Lille, Lille, France). Mouse anti-rat FcεRIα mAb (3A92) was kindly provided by Dr. T. Flemming (Beth Israel Deaconess Medical Center, Boston, MA). Human myeloma IgE (hIgE) was purchased from Bennett Laboratories (Laguna Niguel, CA). Hybridoma supernatant containing chimeric hIgE (cIgE) molecules (composed of the Fc portion of hIgE and Fab portion of anti-4-hydroxy-3-nitrophenacetyl mIgE) was prepared in our laboratory (18). Biotinylated rat anti-mouse λ1 and λ2 L chain, rat anti-mouse FcγRII/RIII (2.4G2) (19) and mouse anti-human IgE were purchased from PharMingen (San Diego, CA). PE-conjugated donkey anti-mouse IgG (H + L) F(ab')₂ and FITC-conjugated goat anti-mouse IgG (Fcγ specific) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-conjugated streptavidin (SA-PE) was obtained from Molecular Probes (Eugene, OR). PE-conjugated rat anti-mIL-10 was purchased from Caltag Laboratories (Burlingame, CA). PE-conjugated rat IgG1 was obtained from Immunotech (Coulter, Miami, FL). The anti-mIgG F(ab')₂ and FITC-labeled anti-hIgE were obtained

Table I. Characteristics of eosinophil donors

Donors	Diagnosis	Group of Patient	IgE (U/ml)	Eosinophilia ^a	% Eosinophils ^b
1	Normal donor	N	ND	<300	85
2	Normal donor	N	ND	<300	96.5
3	Normal donor	N	ND	<300	83
4	Normal donor	N	ND	<300	93.5
5	HES	HES	43.5	1,810	99.5
6	HES	HES	2330	700	98
7	HES	HES	150	900	95
8	HES	HES	900	1,500	100
9	HES	HES	1984	1,900	100
10	Allergy	A	172	1,200	98
11	Churg & Strauss	A	16.4	1,550	90
12	Churg & Strauss	A	2493	13,800	99
13	Allergy	A	275	2,070	100
14	Allergy	A	854	1,700	93.5
15	Allergy	A	4.4	3,400	99
16	Allergy	A	218	ND	99
17	Angioma	SK	84.3	8,050	99.5
18	Psoriasis	SK	59	750	96.5
19	Eczema	SK	1282	2,400	98.5
20	Pemphigoid	SK	251	2,200	95
21	Eczema	SK	412	1,500	90
22	Lymphoma	HD	530	9,900	99.5
23	Lymphoma	HD	465	5,900	99
24	Myeloproliferation	HD	ND	52,000	96
25	Tumor	HD	ND	5,100	100
26	Lymphoma	HD	14,367	43,700	99.5
27	Eczema	SK	939	2,900	93
28	HES	HES	1299	4,000	98
29	Normal donor	N	26.1	100	95
30	Normal donor	N	10.6	200	95

^a Absolute numbers of eosinophils per mm³.

^b Percent purity of eosinophil preparations after purification with MACS. N, Normal donors; HES, hypereosinophilic syndrome; A, allergy; SK, skin diseases; HD, hematological disorders.

from Sigma. Quantum Simply Cellular quantification kit and Quifkit were purchased from Dako (Glostrup, Denmark).

Cell purification

Human eosinophils were isolated from the venous blood of patients, using immunomagnetic beads and the MACS system, as previously described with minor modifications (20). Diluted whole blood (1:1) was layered onto a Percoll gradient (density = 1.082 g/l) and centrifuged at 1800 rpm for 20 min. The granulocyte pellet, containing mainly neutrophils and eosinophils, was harvested and depleted of erythrocytes by hypotonic saline lysis. Briefly, the granulocyte pellet was incubated for 30 min at 4°C with anti-CD16- and anti-CD3-coated immunomagnetic beads to remove neutrophils and contaminating lymphocytes, respectively. Purified eosinophils were obtained by passage of the cells through the field of a permanent magnet. After isolation, eosinophil preparations were cytocentrifuged and cytopspins were stained with May Grünwald Giemsa (RAL 555, Rieux, France). The purity of eosinophil preparations was usually above 97%.

Mouse peripheral blood cells were obtained by retro-orbital puncture. Peritoneal cells were obtained by flushing the peritoneal cavity with 10 ml of ice-cold PBS. Splenocytes were obtained by gentle dissociation of the spleen in ice-cold PBS. Bone marrow cells were isolated from femur and tibia of mice by flushing the bone marrow cavities with ice-cold PBS. Aggregates were removed from cell suspensions by filtration on a nylon filter and erythrocytes were lysed using hypotonic saline. After washing, the cells were resuspended in PBS for the experiments. For activation experiments, splenic eosinophils were purified using a MACS (21). Non-fractionated cell suspension (1×10^8 cells/ml) was incubated for 15 min with CD90 (Thy1.2), CD45R (B220), and CD8 α (Ly-2) magnetic beads. Purified eosinophils were obtained 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). Purity of splenic eosinophil preparations was ranging between 90 and 99%.

Cell culture

Culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (complete medium). Purified human eosinophils, purified mouse splenic eosinophils, and unfractionated mouse cells were cultured for 4 days in complete medium with the addition of 2.5 ng/ml rIL-5 with 0–10 μ g/ml of cIgE. Unfractionated mouse cells were used for flow cytometry analyses for surface and intracellular expression of Fc ϵ RI. Rat basophilic leukemia cells were kept in complete medium. Chinese hamster ovary (CHO) cells stably transfected with the three chains of the human Fc ϵ RI (13) were kept cultured in complete medium containing 1 mg/ml G418.

In vivo receptor up-regulation

IL-5 \times hFc ϵ RI α Tg mice were injected i.v. 4 times at 24 h interval with 100 μ g cIgE as previously reported for mouse IgE (22). Animals were sacrificed 24 h after the last injection. Peritoneal and splenic cells were obtained as described above and analyzed for Fc ϵ RI expression. Blood samples were taken to determine IgE serum concentration at the time of sacrifice.

Flow cytometric analysis of surface Fc ϵ RI

Freshly purified human eosinophils were resuspended at 4×10^6 /ml in PBS-1% BSA. Aliquots of 50 μ l were incubated with FITC-conjugated anti-Fc ϵ RI α (15.1), FITC-conjugated isotype-matched Ab at a final concentration of 2.5 μ g/ml for 1 h at 4°C in round bottom 96-well plates. Staining specificity was controlled by preincubating the cells with hIgE for 15 min on ice before the addition of the FITC-15.1 mAb. After two washes in PBS, cells were resuspended in PBS-0.5% BSA before analysis. Membrane-bound IgE was detected using FITC-conjugated anti-hIgE (1:200). For cells cultured for 4 days in the presence of cIgE, staining was performed using PE-conjugated anti-mouse IgG (H + L) F(ab')₂ (1:200) after additional saturation with cIgE.

Mouse cells were resuspended at 2×10^6 /ml in PBS containing 0.1% BSA and 0.05% sodium azide. One hundred-microliter aliquots were used per sample. Unless otherwise specified, all incubation steps were performed on ice for 30 min. Surface expression of hFc ϵ RI was analyzed after saturation of Fc γ RII/RIII receptors with 150 μ g/ml 2.4G2. Except for the determination of occupied receptors following injection of cIgE, cells were first incubated with cIgE, then after washing, with a biotinylated anti-mouse λ 1 and λ 2 L chain (1:100) followed by SA-PE (1:200). Anti-mouse λ 1 and λ 2 L chain was omitted in control samples. For quantification, cIgE

was detected using PE-conjugated anti-mouse IgG (H + L) F(ab')₂ (1:200). For eosinophils from *S. mansoni*-infected mice, murine IgE already bound to hFc ϵ RI at cell surface was detected with biotinylated anti-mouse IgE followed by SA-PE (1:200). Biotin-conjugated anti-mouse IgE was omitted for control samples. Eosinophils were identified on the basis of their forward and side scatters. Ten thousand events were usually acquired per sample. Thresholds were set on control stainings (included for every sample at every time point).

Samples were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

Flow cytometric analysis of intracellular Fc ϵ RI

Human eosinophils were fixed with 2% paraformaldehyde in PBS for 10 min. After washing in PBS, cells were resuspended at 4×10^6 /ml in PBS containing 1% BSA and 0.1% saponin (permeabilization buffer) for 10 min at room temperature. The samples were then incubated for 30 min with 15.1 mAb or isotype-matched Ab at a final concentration of 25 μ g/ml (saturating concentration) in permeabilization buffer. After washing with permeabilization buffer, cells were incubated for 10 min with 5 μ l normal goat serum to block nonspecific binding, then FITC-conjugated anti-mouse IgG F(ab')₂ was added for 20 min. Samples were washed twice in permeabilization buffer, once in PBS, and were resuspended in PBS-0.5% BSA for analysis.

For mouse eosinophils, permeabilization was performed as described for human eosinophils. Detection of intracellular Fc ϵ RI was performed, after saturation of surface Fc ϵ RI with 150 μ g/ml cIgE and 150 μ g/ml 2.4G2, using anti-hFc ϵ RI α (15-1) mAb (10 μ g/ml) and FITC-conjugated anti-mouse IgG (Fc γ specific) (1:100). Anti-hFc ϵ RI α was replaced by an isotype-matched Ab in control samples.

Measurement of membrane and intracellular unoccupied Fc ϵ RI

For human eosinophils, the number of unoccupied receptors at the cell surface and intracellularly were determined with the Quantum Simply Cellular quantification kit (direct staining) and the Quifkit (indirect staining), respectively, according to the manufacturer's instructions. For murine eosinophils, both surface and intracellular unoccupied receptors were determined using the Quifkit. Quantum kit is based on goat anti-mouse IgG coated-microbeads with different Ab binding capacities (ABC). FITC-conjugated 15-1 and FITC-conjugated isotype-matched Ab were incubated with the beads. A calibration curve was obtained for each Ab by plotting the median fluorescence intensity (MFI) values against the ABC reported for the beads. For each experimental sample, ABC values were deducted by interpolation of the MFI on the calibration curve. The number of 15-1 specific binding sites, thus grossly reflecting the number of Fc ϵ RI molecules, was calculated by subtracting the ABC value for the isotype control Ab from the ABC value for 15-1. Based on a similar principle, Quifkit beads are coated with different amounts of mouse anti-human CD5 Ab. Beads were incubated with the following relevant secondary Abs: PE-conjugated anti-mouse-IgG (H + L) F(ab')₂ (surface expression on mouse eosinophils, RBL, and CHO transfected with hFc ϵ RI α β γ), FITC-conjugated anti-mouse IgG (Fc γ specific) (intracellular expression and surface expression following cIgE injection for mouse eosinophils) or FITC-conjugated anti-mouse IgG F(ab')₂ (intracellular expression in human eosinophils). Calibration curves were obtained as for the Quantum kit. For each experimental sample, ABC values were obtained for the incubation of the secondary Abs following incubation with the relevant primary Ab or with its isotype-matched control. The specific ABC was calculated by subtraction of the ABC value for the isotype control from the ABC value for the primary Ab.

Detection of mouse IL-10 by intracellular flow cytometry

After fixation and permeabilization (as described above), mouse eosinophils were incubated first with 5 μ l normal rat serum followed by the addition of 5 μ g/ml PE-conjugated rat anti-mouse IL-10 or PE-conjugated rat IgG1. After 30 min, cells were washed twice in permeabilization buffer, once in PBS and were resuspended in PBS-0.5% BSA for analysis.

Eosinophil activation

Highly purified human eosinophils (2×10^6 /ml in a 24-well plate) were incubated first with cIgE for 1 h at 37°C followed by the addition of anti-hIgE at 10 μ g/ml. Alternatively cells were stimulated with 10 μ g/ml 15.1 mAb, followed by the addition of 10 μ g/ml anti-mIgG F(ab')₂. Supernatants were collected after 18 h and analyzed for cytokine release.

Purified murine splenic eosinophils (2×10^6 cells/ml in a 24-well plate) were incubated for 4 days with 5 μ g/ml cIgE or with 5 μ g/ml control ascites, in the presence of 2.5 ng/ml rIL-5. Fc ϵ RI activation was achieved

by the addition of 10 $\mu\text{g/ml}$ anti-human IgE to the culture. Supernatants were collected after 18 h and analyzed for cytokine release.

Cytokine quantification

IL-10 was assayed in eosinophil supernatants using specific Elisa kit (Diaclone, and R&D Systems, Minneapolis, MN, for human and mouse, respectively) according to the manufacturer's instructions. The lower detection limit was 5 pg/ml for hIL-10 and <4 pg/ml for mIL-10.

Statistical analyses

Statistical significance was determined using Student's *t* test for unpaired groups with a 95% confidence level. Correlation between IgE levels in the serum from the patients and FcεRI surface expression was established using Spearman's rank coefficient. Analyses were performed using Statview software.

Results

FcεRI expression by human eosinophils

FcεRI expression on freshly purified human eosinophils was first analyzed by flow cytometry using an anti-hFcεRI α Ab. This highly specific monoclonal Ab, which interacts with the IgE binding site and thus only detects unoccupied receptors, has been successfully used to detect hFcεRI α on human eosinophils (2, 12), epidermal Langerhans cells (4), monocytes (7), and platelets (3). A fluorescein-labeled conjugate (FITC-15.1 mAb) was used. Specific binding of this anti-receptor Ab was detected (Fig. 1A). This binding was significantly inhibited upon incubation of cells with saturating amounts of hIgE before the staining (Fig. 1A). Detection of hFcεRI α on human eosinophils was further confirmed using two other mAbs, CRA-1 (nonblocking) and CRA-2 (blocking) (16), and a rabbit polyclonal antiserum (997) (15) (data not shown). Because the various Abs provided us with similar results and because specific binding of 15-1 could easily be assessed by preincubation with IgE, this later was used for the rest of the experiments. The number of unoccupied receptors present at the cell surface was then determined by quantitative flow cytometry on eosinophils from 22 hypereosinophilic patients and 4 healthy donors. In a preliminary experiment, we validated our quantification method using two cell lines expressing FcεRI. Using a mouse anti-rat FcεRI α (3A92), we determined that rat basophilic leukemia cells expressed $\sim 2.2 \times 10^5$ Ab binding sites at their surface, a number in agreement with a previously published study (23) (Fig. 1B). Likewise quantification of Ab binding sites on CHO cells stably transfected with the three chains of the human FcεRI with 15-1, 22E7 (a nonblocking anti-hFcεRI α), and CRA-1 was giving nearly identical results ($\sim 1.5 \times 10^5$ Ab binding sites) (Fig. 1B). Eosinophils expressed an average of 4548 ± 1090 Ab binding sites at their surface. Although surface expression of FcεRI was detected in every sample analyzed, expression levels were heterogeneous. Sorting according to the pathology revealed that the number of unoccupied receptors was significantly higher on eosinophils from patients with hematological disorders and skin diseases (~ 1.6 - and 1.2 -fold, respectively) when compared with normal donors (Fig. 1C). By contrast, FcεRI expression on eosinophils from patients with allergy or hypereosinophilic syndromes was similar to that found on cells from normal donors (Fig. 1C). Nevertheless, as previously reported by others (12), a correlation ($r = 0.625$) was found between IgE levels in the serum from patients and FcεRI surface expression ($n = 20$) (Fig. 1D). If a correlation between serum IgE levels and membrane-bound IgE (to both FcεRI and FcεRII/CD23) was fully anticipated (Fig. 1E), we also found a highly significant correlation ($r = 0.912$) between receptor-bound IgE and unoccupied FcεRI (Fig. 1F). This thus reflects that the number of unoccupied receptors (available for further IgE binding) does increase with IgE serum concentrations.

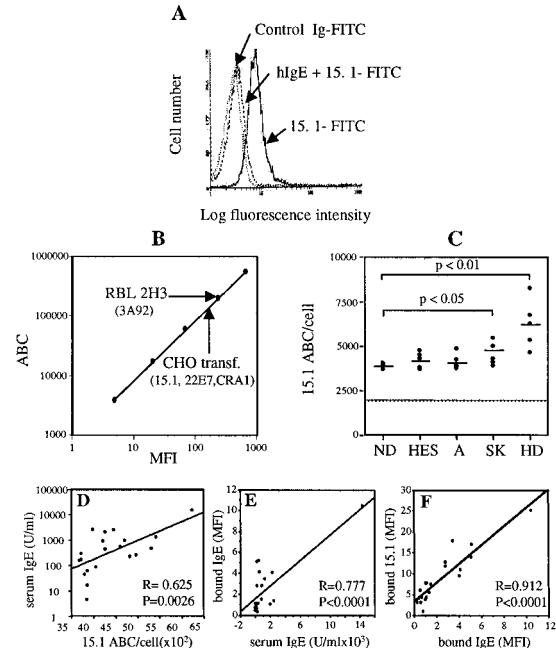


FIGURE 1. FcεRI expression by human eosinophils. **A**, Representative flow cytometric analysis of FcεRI expression. Cells were stained with FITC-conjugated 15.1 mAb (thick line) or isotype-matched control Ab (dotted line). Binding specificity was demonstrated by preincubating the cells with an excess of hIgE before the staining (hatched line). A total of 10,000 events was acquired. **B**, Quantification of FcεRI expression on RBL cells and on hFcεRI $\alpha\beta\gamma$ transfected CHO cells using anti-FcεRI α Abs 3A92 (RBL) and 15-1, 22E7, and CRA-1 (CHO). Data are presented as number of Ab binding sites per cell. **C**, Expression of FcεRI according to the pathology. Cells were stained as in **A**. The number of Ab binding sites per cell was determined. Data are presented as the number of Ab binding sites per cell from 22 eosinophilic patients (Table I, donors 5–26) and 4 normal donors (N, Table I, donors 1–4). Patients were subdivided into four groups according to etiology of the disease: HES, hypereosinophilic syndromes ($n = 5$); A, allergy ($n = 7$); SK, skin diseases ($n = 5$); HD, hematological disorders ($n = 5$). Values of *p* from Student's *t* test. **D**, Correlation between number of surface unoccupied FcεRI and IgE serum concentration. For each patient ($n = 20$) (donors 5–23 and 26), the number of surface binding sites per cell was plotted against the IgE serum concentration (units per milliliter). Curve fitting was performed using Statview software. Spearman's rank coefficient is indicated. **E**, Correlation between IgE serum concentrations and membrane-bound IgE. For each patient ($n = 20$; donors 5–23 and 26), serum concentration (units per milliliter) was plotted against binding of FITC-labeled anti-hIgE (MFI). Curve fitting was performed as in **C**. **F**, Correlation between membrane-bound IgE and unoccupied membrane FcεRI. For each patient ($n = 20$; donors 5–23 and 26), binding of FITC-labeled anti-hIgE (MFI) was plotted against binding of FITC-labeled 15-1 (MFI). Curve fitting was performed as in **C**.

Because eosinophils from normal donors, whose IgE levels are very low, weakly express FcεRI at their surface, we investigated whether culture in the presence of IgE was able to increase receptor expression, as previously reported for mast cells (24) and basophils (25). After 4 days of culture (with 2.5 ng/ml IL-5) in the absence of IgE, FcεRI was expressed at higher levels than on freshly purified cells from normal donors (Fig. 2, thin-line histogram). Furthermore, a dose-dependent increase of FcεRI expressed at the surface of eosinophils was observed when cells were cultured in the presence of cIgE (Fig. 2, inset). Thus, it demonstrates that the correlation between IgE concentrations and FcεRI expression also holds true *in vitro*, at least within a certain range. In

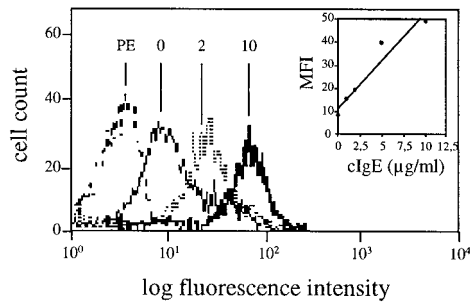


FIGURE 2. IgE-dependent increase of surface FcεRI on eosinophils from normal donors in vitro. Cells were cultured for 4 days with various concentration of cIgE and then incubated with cIgE and receptor expression was determined with biotin-anti-mouse λ1 and λ2 L chain followed by SA-PE. Representative flow cytometric profiles are shown for 0.2 and 10 μg/ml cIgE. Biotin-anti-mouse λ1 and λ2 L chain was omitted on control samples. *Inset*, Relationship between cIgE concentration in the culture medium and fluorescence intensity (MFI), reflecting receptor expression (Table I, donors 29–30).

contrast with eosinophils from normal donors, such a dose-dependent increase was not found for eosinophils from patients that displayed only a moderate increase at the lowest dose of IgE before reaching a plateau. Additionally, eosinophils from patients displayed slightly increased receptor expression after 4 days of culture in the absence of cIgE (data not shown).

Surface and intracellular unoccupied hFcεRIα were then compared on the same samples (*n* = 5). An average of 46,202 ± 8,974 Ab binding sites were detected in the cytoplasm, whereas 4,260 ± 310 Ab binding sites were detected at the cell surface (Fig. 3). Thus far, the pool of cytoplasmic hFcεRIα molecules exceeds the number of receptors at the cell surface of human eosinophils.

Expression of FcεRI on eosinophils from IL-5 × hFcεRI double-Tg mice

Because eosinophils from patients displayed heterogeneous FcεRI expression, we sought to obtain a relevant animal model, which would allow us to study FcεRI expression and function on eosinophils in reproducible conditions and without the inconvenience of material availability. Therefore, we crossed hypereosinophilic IL-5 Tg mice with hFcεRIα Tg animals expressing a humanized FcεRI

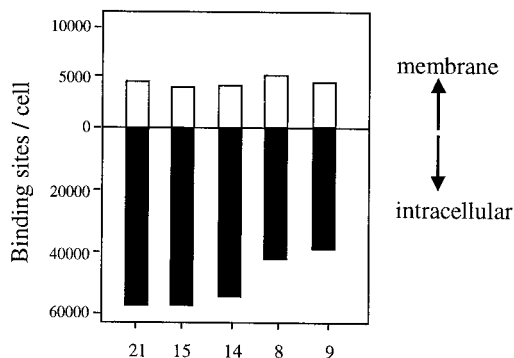


FIGURE 3. Quantitative analysis of surface and intracellular FcεRI expression by human eosinophils. Surface expression of FcεRI was detected as for Fig. 1A. Detection of intracellular receptors was performed after fixation and permeabilization of the cells, by staining with 15.1 mAb and FITC-conjugated anti-mouse IgG F(ab')₂. The number of intracellular Ab binding sites per cell was determined. Data are presented as number of Ab binding sites per cell from five hypereosinophilic patients (donor's number referring to Table I).

with the same cellular distribution as humans. IL-5 × hFcεRIα Tg mice displayed massive eosinophilia in several organs: bone marrow (50.3 ± 6.3% eosinophils), peripheral blood (51.7 ± 11.5% eosinophils), spleen (42.2 ± 7.6% eosinophils), and peritoneal cavity (50.8 ± 11.2% eosinophils).

As for human eosinophils, expression of hFcεRIα on eosinophils from these animals was investigated. Expectedly, flow cytometric analysis allowed us to detect low expression levels (822 Ab binding sites) for humanized FcεRI (hFcεRI) at the surface of freshly isolated splenic (Fig. 4A) and peritoneal (data not shown) eosinophils from IL-5 × hFcεRIα Tg animals, whereas no receptor expression was detected on eosinophils from IL-5 Tg mice (data not shown).

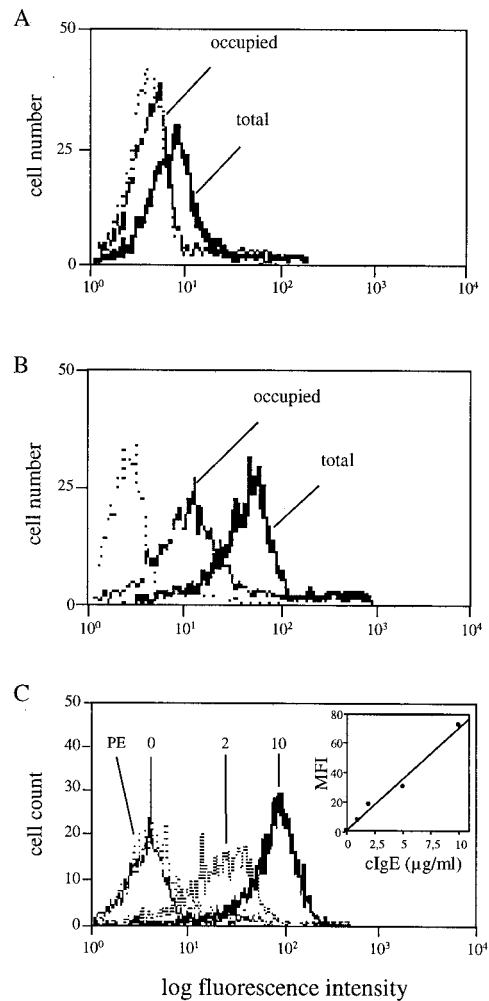


FIGURE 4. FcεRI expression by eosinophils from IL-5 × hFcεRI Tg mice. A and B, FcεRI expression on freshly isolated splenic eosinophils from IL-5 × hFcεRI Tg mice injected (B) or not (A) 4 times with 100 μg cIgE 24 h apart and sacrificed 24 h after the last injection. Cells were incubated (total receptors) or not (unoccupied receptors) with cIgE, then with using PE-conjugated anti-mouse IgG (H + L) F(ab')₂ except for control samples. C, Dose-dependent up-regulation by cIgE of FcεRI expression on splenic eosinophils in vitro. Cells were cultured for 4 days with various concentration of cIgE. Cells were then incubated with cIgE and receptor expression was determined with biotin-anti-mouse λ1 and λ2 L chain followed by SA-PE. Flow cytometric profiles are represented for 0.2 and 10 μg/ml cIgE. Biotin-anti-mouse λ1 and λ2 L chain was omitted on control samples. *Inset*, Relationship between cIgE concentration in the culture medium and fluorescence intensity (MFI), reflecting receptor expression.

Because it has been demonstrated that IgE was able to up-regulate FcεRI expression on murine mast cells (22) and basophils (25) *in vivo*, we investigated whether such a phenomenon was also occurring on eosinophils. IL-5 × hFcεRIα Tg mice were injected 4 times with 100 μg cIgE and sacrificed 24 h after the last injection. The number of occupied Ab binding sites and, upon saturation with cIgE, total Ab binding sites present at the surface of freshly isolated splenic and peritoneal eosinophils was determined by flow cytometry. We found that splenic and peritoneal eosinophils had 1950 and 2248 occupied Ab binding sites, respectively, and 5013 and 9094 total Ab binding sites, respectively. A typical histogram is represented for splenic eosinophils on Fig. 4B. Taking this number into account, we deduced that, following the injection of 400 μg of cIgE, ~3000 and 6850 unoccupied Ab binding sites, respectively, were present at the surface of splenic and peritoneal eosinophils. Using a blocking (15-1) or a nonblocking (22E7) anti-hFcεRIα Ab to directly determine the number of unoccupied Ab binding sites, as performed on human cells, we obtained similar results (3205 with 15-1 and 3019 with 22E7) for splenic eosinophils and lower values (4660 and 4075) for peritoneal eosinophils. Taken together, these data show that IgE-mediated FcεRI up-regulation does occur at the surface of eosinophils from IL-5 × hFcεRIα Tg mice *in vivo*, whether the unoccupied or total number of receptors are considered.

As we found for eosinophils from normal donors, expression of hFcεRI after culture with IgE showed a similar dose-dependent increase when compared with eosinophils cultured in the absence of IgE (Fig. 4C and *inset*). However, in contrast with human eosinophils, the mere culture in the absence of IgE did not increase receptor expression (Fig. 4C, thin-line histogram).

Number of Ab binding sites at the surface and of unoccupied intracellular hFcεRIα molecules were then determined for bone marrow, blood, and splenic and peritoneal eosinophils. Resting eosinophils isolated from the 4 organs expressed <1000 Ab binding sites at their surface. After 4 days culture with 5 μg/ml cIgE, the number of Ab binding sites ranged from 4410 to 7804 at the cell surface, according to the origin of eosinophils, whereas culture in the absence of IgE barely affected the number of receptors (Fig. 5A). Thus, in the presence of IgE, eosinophils from IL-5 × hFcεRIα Tg mouse express similar levels of surface FcεRI as human eosinophils.

The number of unoccupied intracellular hFcεRIα molecules in eosinophils freshly isolated from different organs was at least 10 times higher than surface expression by the corresponding cells (Fig. 5, B compared to A). Upon culture in the presence of IL-5 but without IgE, splenic and peritoneal eosinophils had increased numbers of unoccupied intracellular hFcεRIα (Fig. 5B). Except for bone marrow eosinophils, the number of unoccupied intracellular hFcεRIα further increased upon culture with both cIgE and IL-5 (Fig. 5B). Peritoneal eosinophils had significantly more unoccupied intracellular FcεRIα than blood ($p < 0.02$) or splenic ($p < 0.01$) eosinophils (Fig. 5B). As for human blood eosinophils, bone marrow, blood, and splenic and peritoneal eosinophils, IL-5 × hFcεRIα Tg mice harbor, under these experimental conditions, a large intracellular pool of unoccupied hFcεRIα and express low number of surface FcεRI.

Expression of FcεRI by *S. mansoni*-infected mouse eosinophils

Elevated IgE levels are a hallmark of helminthic infections in both human and rodents (26). Furthermore, human (2) and rat (27) eosinophils (but not WT mouse eosinophils) have been shown to participate to FcεRI-dependent ADCC toward *S. mansoni* larvae *in vitro*. To investigate whether up-regulation of eosinophil-expressed FcεRI by IgE was also taking place *in vivo*, during the

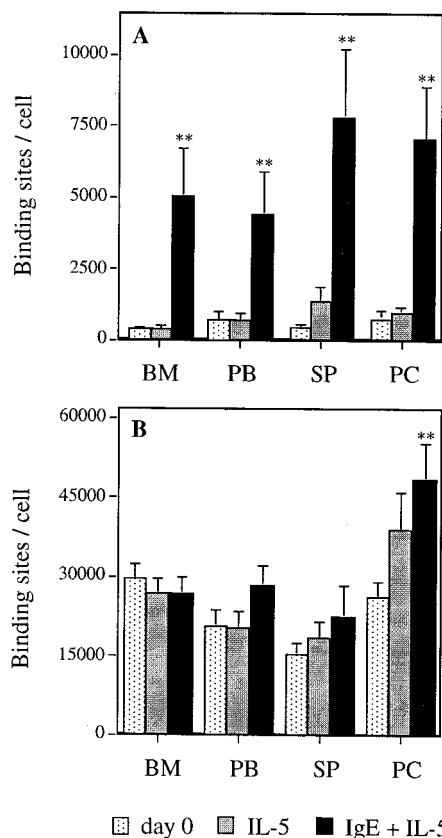


FIGURE 5. Quantitative analysis of surface and intracellular FcεRI expression by murine eosinophils. Eosinophils from bone marrow (BM), peripheral blood (PB), spleen (SP), and peritoneal cavity (PC) were analyzed immediately after sacrifice (□) or after 4 days culture in the presence of 2.5 ng/ml rhIL-5 without (▨) or with (■) 5 μg/ml cIgE. *A*, Surface expression of FcεRI was detected after receptor saturation with cIgE using PE-conjugated anti-mouse IgG (H + L) F(ab')₂. *B*, Intracellular FcεRI was revealed by staining with 15.1 mAb followed by FITC-conjugated anti-mouse IgG (Fcγ specific). The number of Ab binding sites per cell was determined. Data are presented as mean ± SEM from four to seven different experiments. **, $p < 0.01$ compared with corresponding cells cultured in the absence of cIgE (Student's *t* test).

course of schistosomiasis, we determined the number of Ab binding sites on hFcεRIα × IL-5 Tg mouse eosinophils after 46 days infection with *S. mansoni*, at a time when IgE levels begin to increase. Eosinophils isolated from the different organs from infected animals displayed a significant increase in FcεRI surface expression when compared with cells from noninfected mice (Fig. 6). The maximum increment was observed for peritoneal and splenic eosinophils. Interestingly, this increased membrane expression of FcεRI was associated to decrease in the intracellular pool of unoccupied hFcεRIα in bone marrow, blood, and peritoneal eosinophils (significant only for peritoneal eosinophils). These experiments show that infection by *S. mansoni* is able to up-regulate surface expression of FcεRI on eosinophils *in vivo*, increased IgE levels are likely to play an important part in this phenomenon.

Functional role of FcεRI

Having established that both human and Tg mouse eosinophils expressed comparable numbers of FcεRI, we investigated the contribution of FcεRI to both effector and regulatory function of eosinophils. Because IgA immune complexes have been recently shown to induce the release of IL-10 by human eosinophils (20),

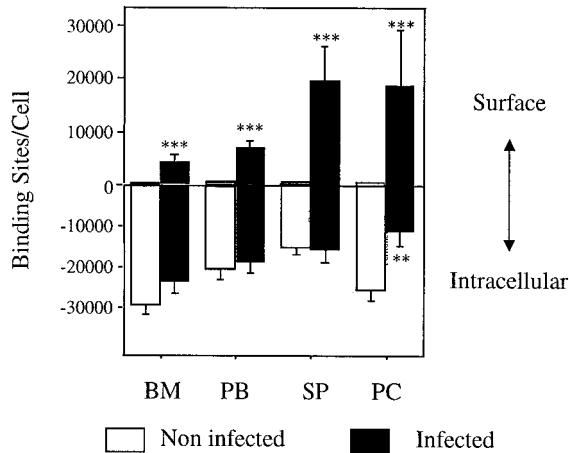


FIGURE 6. FcεRI expression by eosinophils from *S. mansoni*-infected mice. Bone marrow (BM), peripheral (PB), and splenic (SP) and peritoneal (PC) cells were prepared from *S. mansoni*-infected mice (■) and control mice (□). Eosinophils were stained for membrane and intracellular FcεRI detection as in Fig. 4 and the number of Ab binding sites per cell was determined as previously described. Results are expressed as mean \pm SEM from three to six different experiments. **, $p < 0.01$; ***, $p < 0.001$ compared with the corresponding cells from noninfected animals (Student's *t* test).

it was worth investigating whether IgE and FcεRI would also mediate IL-10 release by human or Tg mouse eosinophils. Upon receptor engagement with cIgE and secondary Ab, human eosinophils were able to release 62 ± 41 pg/ml IL-10 (above control), whereas receptor cross-linking with anti-FcεRI α mAb (15.1 mAb) and anti-mIgG released up to 200 pg/ml IL-10 (Fig. 7A). We then verified that, in our experimental model, mouse eosinophils contained IL-10 as previously demonstrated upon *S. mansoni* infection (28). Intracellular staining, revealed that eosinophils from IL-5 \times hFcεRI α Tg mice contained high amounts of IL-10 (Fig. 7, inset). After culture in the presence of cIgE, receptor cross-linking with anti-hIgE led to a release of 234 ± 126 pg/ml mIL-10 (above control) (Fig. 7B). These results demonstrate that both human and IL-5 \times hFcεRI α Tg mouse eosinophils are able to secrete an immunoregulatory cytokine upon FcεRI-dependent activation.

Discussion

FcεRI expression by human eosinophils and its involvement in IgE-mediated ADCC reactions toward *S. mansoni* larvae had been first reported in 1994 (2). Using quantitative and sensitive methods, we have shown here that levels of unoccupied receptors at the surface of human eosinophils correspond to ~ 4500 Ab binding sites. The use of an anti-receptor Ab interacting with the IgE binding site without prior treatment with lactic acid (which would have damaged cellular integrity) prevented us from detecting IgE-occupied FcεRI. Nevertheless, in agreement with a previous study, we were able to find a correlation between receptor expression level and serum IgE concentrations (12). The correlations found between serum IgE levels, membrane-bound IgE, and unoccupied FcεRI reflect that, even in patients with high IgE levels, these receptors are not saturated, probably due to IgE-mediated receptor up-regulation. Otherwise these correlations would have been observed only for patients with (very) low IgE levels. We were also able to demonstrate that the number of unoccupied receptors was significantly increased in some pathologies (hematological disorders and skin diseases) but not in others (allergies and hypereosinophilic syndromes) when compared with normal donors. Hematological disorders (tumors, myeloproliferative lymphoma, or

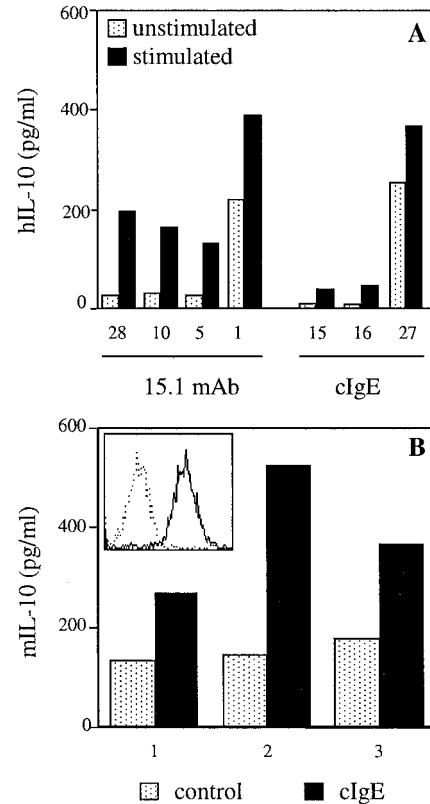


FIGURE 7. FcεRI-mediated release of IL-10 by human and mouse eosinophils. *A*, Freshly purified human eosinophils were incubated with either 10 μ g/ml 15.1 mAb or mIgG1 for 1 h, then with 10 μ g/ml anti-mIgG F(ab')₂. Alternatively, cells were incubated with 10 μ g/ml cIgE for 1 h, followed by the addition of 10 μ g/ml anti-hIgE. Supernatants were harvested 18 h later and IL-10 concentration was determined by ELISA. Results from 7 donors are shown (donor's number referring to Table I). *B*, Purified splenic mouse eosinophils were cultured for 4 days in the presence of 2.5 ng/ml rhIL-5 with 5 μ g/ml cIgE (■) or with 5 μ g/ml control ascites (□). Anti-hIgE (10 μ g/ml) was then added. Supernatants were harvested 18 h later and IL-10 concentration was determined by ELISA. Results from 3 different experiments are shown. *Inset*, Intracellular detection of IL-10 in mouse peripheral blood eosinophils. After fixation and permeabilization, cells were stained with PE conjugated anti-mIL-10 (thick line) or isotype-matched Ab (dotted line).

eosinophilic leukemia) included in this study are characterized by high levels of cell proliferation; thus, it is possible that undifferentiated, proliferating eosinophils or eosinophil precursors would be more sensitive to receptor up-regulation. An increased IgE receptor expression would provide eosinophils with a means to exert anti-tumoral activity through IgE-mediated ADCC. It has been shown on one hand that eosinophils could play a role in IL-4 anti-tumoral activity (29) and, on the other hand, that FcεRI participated in anti-tumoral activity (30, 31). Nevertheless, even in hematological disorders, surface expression of unoccupied FcεRI by eosinophils is low, when compared with basophils, which express up to 6×10^5 receptors at their surface (32). The absence of Fc β , which has recently been shown to increase surface expression of the receptor (33) in eosinophils might account at least in part for these differences between cell types.

Along the same lines, we were also able to increase receptor expression on eosinophils from normal donors in vitro, upon a 4-day culture in the presence of cIgE. Such a dose-dependent increase was not observed on eosinophils from patients, which already expressed more receptors and with high IgE concentrations

in their serum. A proportionally comparable dose-dependent increase of receptor expression on eosinophils from patients would represent a much more important increase in the number of additional receptors expressed over such a short period of time. Aside from the time factor, the limited range of IgE-driven FcεRI up-regulation on eosinophils in vitro might also be attributed in part to their lack of FcRβ protein.

In contrast, we demonstrated that eosinophils contain large amounts of unoccupied intracellular FcεRIα. These differences in the number of unoccupied receptors between surface and intracellular compartments could explain why some studies failed to detect surface expression, but were able to report on the presence of FcεRI inside the cells (11). Three factors could contribute to this excess of intracellular unoccupied FcεRIα. First, high levels of proteolysis could lead to the release of almost all the surface-expressed FcεRIα as a soluble receptor. Second, an extensive storage of receptors in eosinophil granules before their exportation to the surface could take place. Third, an overproduction of FcεRIα compared with FcRγ might occur. FcRγ is necessary for surface expression and/or function of FcεRI, FcγRI (34), and FcαRI (35), all expressed by human eosinophils, and thus might be the key factor limiting surface expression of these three receptors, considered as a whole. Such a competition has been demonstrated between FcεRI and FcγRIII on murine bone marrow-derived mast cells (36).

To obtain a confirmation of our results in a relevant experimental model and because WT mice do not express FcεRI (13), we crossed previously generated hFcεRIα Tg mice expressing the receptor on eosinophils (under the control of hFcεRIα promoter) (13) with hypereosinophilic IL-5 Tg animals (14). These IL-5 × hFcεRIα Tg mice not only provided us with an abundant and reproducible source of material, but also allowed us to study eosinophils from different organs. Freshly isolated cells obtained from naive animals expressed <1000 Ab binding sites at their surface, whereas the receptor was not detectable on cells from IL-5 Tg animals. Such a low surface expression on eosinophils from IL-5 × hFcεRIα Tg animals might be due to the virtual absence of IgE in naive mice. As previously reported for other cell types (22, 25, 37) as well as for rat eosinophils (27), injection of IgE or culture in the presence of IgE led to a significant increase in surface expression, which was then comparable to the levels observed in humans. Although the molecular mechanisms underlying this phenomenon are only partially understood, it is now widely admitted that IgE stabilizes receptors anchored at the membrane, while allowing more receptors to be synthesized and targeted to the membrane, thus concurring to the increased expression. Nevertheless, unoccupied intracellular hFcεRIα was also present in high amounts in mouse eosinophils, when compared with surface expression.

We have shown that eosinophils obtained from various organs expressed different levels of FcεRI at their surface. Bone marrow eosinophils expressed the lower number of receptors, followed by blood and splenic and peritoneal eosinophils. This leads us to envision a pathway along which eosinophils would mature and, among other parameters, increase their expression levels of FcεRI. In humans and most likely in naive animals, eosinophils mature in the bone marrow for 3–4 days, then migrate and stay for several hours to 1 day in the blood stream to finally reach the organs where they remain for 2–7 days in tissues (38). This would likely explain why peritoneal eosinophils display the highest expression and are probably the most differentiated. Because spleen has an open circulatory system and blood cells freely migrate into the splenic stroma without transendothelial migration, splenic eosinophils might have lacked the necessary stimuli provided, during transendothelial migration, through adhesion molecules-mediated cell-

cell contacts and might thus be biologically different from other tissue eosinophils, such as peritoneal ones (39, 40).

During experimental infection with *S. mansoni*, FcεRI surface expression was greatly increased in particular on splenic and peritoneal eosinophils from double-Tg mice. According to our proposed model, it seems logical that the most differentiated types of eosinophils would be found in tissues.

Expectedly, higher levels of surface expression were reached after a 46-day in vivo infection when compared with a 4-day in vivo treatment with IgE and all the more with an in vitro IgE-induced up-regulation experiment. In the former case, IgE is not the only factor that is likely to promote FcεRI expression over such an extended period; IL-4 is another one (24, 41–43). A similar phenomenon had been observed on intestinal rat mast cells, where infection by *Nippostrongylus brasiliensis* was more efficient at increasing FcεRI expression (measured by detection of FcRβ) than the mere injection of IgE (44). A kinetic study of FcεRI expression on eosinophils from IL-5 × hFcεRIα Tg mice along the course of *S. mansoni* infection might provide information about the respective roles of the infection by itself, of IL-4 and of IgE on the increase in receptor expression. Nevertheless, we have shown already that eosinophils from *S. mansoni*-infected hFcεRIα Tg mice displayed IgE-dependent cytotoxicity toward *S. mansoni* larvae, whereas eosinophils from similarly infected WT animals were ineffective. In contrast, we have also shown that hFcεRIα Tg displayed decreased granuloma volume when compared with WT animals (45). In these animals, expression is not restricted to eosinophils, but also extends to APC (monocytes and epidermal Langerhans cells); however, one can argue that FcεRI might not only affect serum IgE levels through Ag presentation, but also eosinophil activation.

Measurements, on the same eosinophil samples, of surface and intracellular FcεRIα, allowed us to get some additional insight about the mechanisms of ligand-induced receptor up-regulation. When IgE is present in the biological fluids (*i.e.*, upon *S. mansoni* infection or in vitro in culture medium), the amount of surface FcεRI increases by remaining for longer period of time at the surface. IgE-receptor complexes are reinternalized more slowly, and are protected from proteolysis and thus released in the medium at a slower rate than unoccupied receptors. Even if the synthesis of new receptors is stimulated at the transcriptional and/or at the transductional level upon helminthic infection, the large intracellular pool of free FcεRIα, existing in cells from naive animals is partially depleted. This phenomenon was more strikingly observed on peritoneal eosinophils. It thus means that, even if basal levels of surface FcεRI expression are low, they can be greatly increased in some situations (parasitic infection, inflammatory diseases, etc.) and play a greater role than previously inferred by some recent studies (11, 12).

We were also able to demonstrate that, upon FcεRI engagement with IgE and anti-hIgE, both human and IL-5 × hFcεRIα Tg mouse eosinophils were able to release IL-10, which is abundant inside cells from both species (20, 28). Aside from their involvement in cytotoxic reactions, eosinophils are thus likely to act not only as effectors but also as modulators of the immune response. IL-10, also released by human eosinophils upon triggering with secretory IgA (20) affects, among other cell types, the Ag presenting capacity of macrophages and decreases type 1 cytokine production by lymphocytes.

In conclusion, we have here demonstrated, using two parallel systems, human and IL-5 × hFcεRIα Tg mice, that eosinophils express low amounts of FcεRI at their surface, while possessing a large intracellular pool of unoccupied FcεRIα. Surface expression

can be up-regulated and allows eosinophils to participate to FcεRI-mediated reactions. The use of a relevant animal model, more faithfully reproducing the human situation, should provide us with more information about the role of this receptor in eosinophil function and in human diseases.

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