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The Pan-B Cell Marker CD22 Is Expressed on Gastrointestinal Eosinophils and Negatively Regulates Tissue Eosinophilia

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CD22 is currently recognized as a B cell-specific Siglec and has been exploited therapeutically with humanized anti-CD22 mAb having been used against B cell leukemia. In this study, tissue-specific eosinophil mRNA microarray analysis identified that CD22 transcript levels of murine gastrointestinal (GI) eosinophils are 10-fold higher than those of lung eosinophils. To confirm the mRNA data at the protein level, we developed a FACS-based protocol designed to phenotype live GI eosinophils isolated from the murine lamina propria. Indeed, we found that jejunum eosinophils expressed remarkably high levels of surface CD22, similar to levels found in B cells across multiple mouse strains. In contrast, CD22 was undetectable on eosinophils from the colon, blood, thymus, spleen, uterus, peritoneal cavity, and allergen-challenged lung. Eosinophils isolated from newborn mice did not express CD22 but subsequently upregulated CD22 expression to adult levels within the first 10 d after birth. The GI lamina propria from CD22 gene-targeted mice harbored more eosinophils than wild type control mice, whereas the GI eosinophil turnover rate was unaltered in the absence of CD22. Our findings identify a novel expression pattern and tissue eosinophilia-regulating function for the “B cell-specific” inhibitory molecule CD22 on GI eosinophils. *The Journal of Immunology*, 2012, 188: 000–000.

Eosinophils are multifunctional effector leukocytes involved in a variety of allergic disorders including asthma and eosinophilic gastrointestinal (GI) diseases (1, 2). Bridging the gap between innate and adaptive immunity, eosinophils have a pivotal role in Th2 inflammatory states. After exiting the bone marrow, circulating eosinophils home to the GI tract and become GI resident cells, a process orchestrated by eotaxin, its receptor CCR3 (3, 4), and the $\alpha 4 \beta 7$ integrin systems (5). In both mice and humans, the GI tract harbors a large number of eosinophils in the lamina propria layer underneath the epithelium, forming the largest reservoir of eosinophils during homeostasis (6–8). It has been reported that GI eosinophils have a slower turnover rate compared with lung and blood eosinophils because of specific survival signaling (8). In contrast with circulating eosinophils, GI eosinophils have a more activated phenotype, measured by their degree of degranulation and expression of di-

verse cytokines (9, 10). It has been suggested that GI eosinophils are involved in mucosal antibacterial defense by actively releasing their toxic mediators and mitochondrial DNA (11). GI eosinophils are also causatively related to the pathogenesis of a variety of GI tract disorders, such as eosinophilic GI diseases (12), ulcerative colitis (13), Crohn’s disease (14), and intestinal tumors (15).

CD22 is a member of the sialoadhesin subclass of the Ig superfamily and is currently recognized as a B cell-specific surface glycoprotein expressed on both normal and malignant B cells throughout each stage of B cell development, except for plasma cells (for review, see Ref. 16). CD22 is generally an inhibitory coreceptor, serving as a molecular damper that negatively regulates BCR signaling, and is therefore critical for B cell activation, proliferation, and survival (17–21). After BCR cross-linking, the phosphorylated ITIMs in the cytoplasmic moiety of CD22 recruit effector molecules such as SHP and SHIP-1, which negatively regulate the strength of BCR and CD19 downstream signaling (22). In addition to its cytoplasmic motifs, CD22 possesses seven Ig-like domains in its extracellular domain and may regulate cell–cell adhesion (23, 24). As a sialic acid-binding, Ig-like lectin (Siglec), CD22 binds to its natural ligand 2, 6-linked sialic acid, which is present on the glycoproteins widely expressed on the surface of hematopoietic and nonhematopoietic cells (25). Given its B cell-specific expression pattern, CD22 has become one of the attractive target molecules for diagnosis and therapy of B-lineage acute leukemia (26), as well as other B-tropic autoimmune disorders such as systemic lupus erythematosus (27) and Sjögren’s syndrome (28).

Eosinophils have long been thought to participate in GI parasitic infections and allergic lung diseases; nonetheless, the transcriptional signature difference between GI and lung eosinophils has not been explored at a genome-wide level. To elucidate the physiological and pathological functions of eosinophils in a tissue-specific fashion, we examined differential gene expression of eosinophils isolated from the GI tract and lungs during homeostasis. Tissue-specific mRNA microarray analysis with mRNA extracted from

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The sequences presented in this article have been submitted to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession code GSE33807.

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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; FDR, false discovery rate; GI, gastrointestinal; MBP, major basic protein; Tg, transgenic; WT, wild type.

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flow-sorted GI and lung eosinophils indicated that, among the 513 genes with significantly altered expression between GI and lung eosinophils, expression of the pan-B cell marker CD22 is upregulated 10-fold in GI compared with lung eosinophils. This transcriptional difference was verified by quantitative RT-PCR and flow cytometry at the protein level. We then used CD22 gene-deficient mice to examine a possible function of this inhibitory coreceptor in GI eosinophils and found that CD22 negatively regulates GI lamina propria eosinophilia. To our knowledge, this is the first report demonstrating that CD22 is highly expressed in eosinophils and functionally involved in regulating GI eosinophil levels.

Materials and Methods

Mice

C57BL/6N (CD22^{+/+}) mice were initially obtained from Charles River (Wilmington, MA), and CD22 gene-deficient mice (backcrossed to C57BL/6J for >20 generations before arrival) were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c mice, germ-free Swiss Webster mice, and non-germ-free Swiss Webster mice were obtained from Taconic Farms (Hudson, NY). CD2-IL-5 transgenic (Tg) mice were obtained from Dr. Colin Sanderson (Institute for Child Health Research, Perth, Australia) and then backcrossed onto the BALB/c background for at least 20 generations. In all experiments, we used 2- to 3-mo-old, age- and sex-matched mice, which were housed in specific pathogen-free conditions at Cincinnati Children's Hospital Medical Center under Institutional Animal Care and Use Committee-approved protocols. All mice were housed in a room with an ambient temperature of 22°C and a 12-h light cycle.

GI eosinophil isolation

Animals were euthanized by CO₂ asphyxiation, and jejunums were excised 6–16 cm from the pylorus. Duodenum, ileum, and colon were anatomically defined as 6 cm distal to the pylorus, 10 cm proximal to the cecal sphincter, and 5 cm distal to the cecum narrowing, respectively. The intestine segment was opened, washed with PBS, and then subjected to EDTA buffer treatment for 15 min (1× HBSS with 10% FCS, 5 mM EDTA, 40 mM HEPES) at 37°C with agitation. After an HBSS wash, tissues were minced into small pieces and treated with collagenase A (Roche) solution (2.4 mg/ml in complete RPMI supplemented with 10% FCS) for 30 min at 37°C with moderate agitation. The digest was passed through a 19-G needle three times and then two layers of gauze, and the filtered cell suspension was centrifuged at 300 × g for 5 min. After an additional wash with HBSS, the cells were suspended in appropriate volumes for flow cytometry staining or other experiments. The same procedure was applied to other solid tissues harboring eosinophils without modification, including the lung, stomach, and uterus.

Flow cytometry analysis of total GI lamina propria cells

Polychromatic flow cytometry compensation matrix setting was acquired in a pilot study with eosinophils from IL-5 Tg mice. Single-cell suspensions of ~1 million total GI lamina propria cells were stained with markers for eosinophils and target proteins of interest using the six-color FACS system. Abs/dyes (clone number) used in this study were purchased from BioLegend (CD22-AF647 OX-97 and isotype RTK-2071), Invitrogen (LIVE/DEAD Violet dye), BD (CD45-FITC 30-F11, Siglec-F-PE E50-2440, CD11b-PECy7 M1/70, 7-aminoactinomycin D [7AAD]), and R&D Systems (CCR3-PE 83103). Staining was performed on ice for 30 min in staining buffer (0.5% BSA, 0.01% NaN₃ in 1× HBSS) with manufacturer-suggested titers, followed by a wash with the staining buffer. Stained cells were resuspended and subjected to analysis with the BD FACS Canto II flow cytometer. Raw flow cytometry data were analyzed by FlowJo software (Tree Star, Ashland, OR). GI eosinophils were identified as 7AAD⁻CD45⁺Siglec-F⁺CD11b⁺ events. Quantification expression data were presented as the change in mean fluorescence intensity (MFI) (Δ MFI = CD22 Ab MFI – isotype control MFI). For phospho-FACS, freshly isolated GI lamina propria cells were washed with PBS and rested in complete RPMI 1640 for 30 min, and then kinetically subjected to the challenging media. Samples treated with vehicle media served as controls for baseline normalization. At the end of the incubation, cells were immediately fixed by adding an equal volume of 4% formaldehyde and incubated at 37°C for 10 min, followed by ice-cold methanol permeabilization for 30 min. Specific Thr²⁰²/Tyr²⁰⁴ p44/42 Ab (E10; Cell Signaling Technology, #4375) was used together with previously mentioned eosinophil markers to assess

eosinophil MAPK activation. GI eosinophils were identified and gated as CD45–Siglec-F–CD11b triple (+) events. Stimulated MFI values were normalized to vehicle-treated samples for graphic presentation.

Flow imaging analysis of GI eosinophils

Single-cell suspensions stained with different Abs were prepared as described in the earlier mentioned method and fixed with 2% formaldehyde in PBS. The Ab-stained samples were run through the ImageStreamX system (Amnis, Seattle, WA) following the manufacturer's instructions. Compensation matrix was established at the pixel level with single-stain controls. The image output was performed by analyzing the raw image data with the software of Amnis-IDEAS (Amnis).

Genome-wide microarray analysis on sorted eosinophils and quantitative PCR validation

Lung and GI eosinophils were isolated as mentioned in earlier sections, with the pulmonary circulation being perfused before isolation procedures. Live eosinophils were sorted as DAPI⁻CCR3⁺Siglec-F⁺CD45⁺CD4⁻CD8a⁻CD19⁻B220⁻ side scatter high (SSC^{high}) cells from 10 animals using FACS Aria (BD). Total RNA from sorted eosinophils was extracted by a standard TRIzol RNA isolation (Invitrogen) and subsequently column-purified with an RNeasy Mini Kit (Qiagen). mRNA integrity was validated by the Agilent 2100 bio-analyzer (Agilent Technologies, Santa Clara, CA). Eosinophil mRNA was amplified and labeled with the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) and subjected to the GeneChip Mouse Gene ST 1.0 Array chip (Affymetrix), which covers the whole mouse genome with 28,853 probe sets. Microarray expression analysis was performed at Cincinnati Children's Hospital Medical Center's Chip Core facility, and expression data were analyzed by the software of Genespring GX 11 (Agilent Technologies). In brief, the Affymetrix raw expression values were first filtered with the threshold of 400. Differential expression between GI and lung eosinophils was identified by a >2-fold change and a *p* value <0.01 with a false discovery rate (FDR) correction. The same mRNA sample used for microarray analysis was reverse transcribed with the iScript cDNA Synthesis Kit (170-8891; Bio-Rad), and real-time PCR was performed with the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using a pair of primers spanning exons 4 and 5 of the CD22 transcript (forward 5'-GAAAATC-CACCCGATACGTGC-3'; reverse 5'-TTGGAACGGTTTCTCCGAGAC-3'), resulting in a 115-bp amplicon.

OVA-induced GI inflammation model

As originally described by Brandt et al. (29), animals of sensitive strains (littermates of BALB/c-C57BL/6 F4 backcross) were initially sensitized with 100 µg OVA/Alum twice, 14 d apart under aseptic conditions. Beginning on day 28, OVA (50 mg in saline) or saline was administered by oral gavage to the animal every other day for a total of seven times. Mice were sacrificed 24 h after the last OVA challenge. Eosinophils were then isolated from the inflamed GI tract for CD22 expression analysis.

Aspergillus-induced allergic lung model

Protein extract of 100 µg *Aspergillus fumigatus* (GREER, Lenoir, NC) was dissolved in sterile saline and administered into the airway of wild type (WT) BALB/c mice by intranasal inhalation. A total of nine challenges was given following a Monday/Wednesday/Friday regimen with 1 challenge/day for 3 wk, as previously reported (30).

Major basic protein immunostaining and GI eosinophil quantitative morphometric analysis

The jejunum tissue was fixed in 4% paraformaldehyde in PBS, embedded in paraffin, cut into 5-µm transverse sections, and immunostained with anti-major basic protein (anti-MBP) Ab (a kind gift of Dr. James Lee, Mayo Clinic, Scottsdale, AZ) following common immunohistochemistry procedures. For morphometric analysis, all of the MBP-labeled eosinophils on the whole transverse jejunum section were enumerated as the number of total eosinophils for this transverse section. To acquire the specific area of lamina propria on the same section, we used the digital micrograph of the whole transverse section to calculate areas of manually outlined lamina propria by Image-Pro PLUS software (Media Cybernetics). The lamina propria eosinophil density was calculated by dividing the earlier two values into the unit of eosinophil number per square millimeter.

GI eosinophil turnover assay

GI eosinophil turnover assay was adopted from a previous publication (8). In brief, animals were continuously fed with BrdU-containing water so-

lution at a concentration of 80 mg/dl for 6 d, and GI eosinophil isolation was performed as described earlier. BrdU⁺ eosinophils were detected using the BrdU APC Detection Kit from BD Pharmingen (BD catalog no. 552598) in conjunction with the eosinophil markers CD45, Siglec-F, and CD11b (as described earlier). The intranuclear staining was based on the manufacturer's suggested protocols. After flow cytometry analysis, the gated eosinophil population was further gated by CD11c and BrdU with a no BrdU treatment control as an intensity reference. The CD11c^{low} BrdU^{high} subpopulation represented newly migrated GI eosinophils (8).

Statistical analysis

Statistical significance was analyzed using a two-tailed Student *t* test in all instances except for the OVA sensitization study, in which a two-way ANOVA was used. Data are graphed as mean $\pm$ SEM.

Results

GI eosinophils express a unique set of genes with 10-fold upregulation of CD22 transcript compared with lung eosinophils

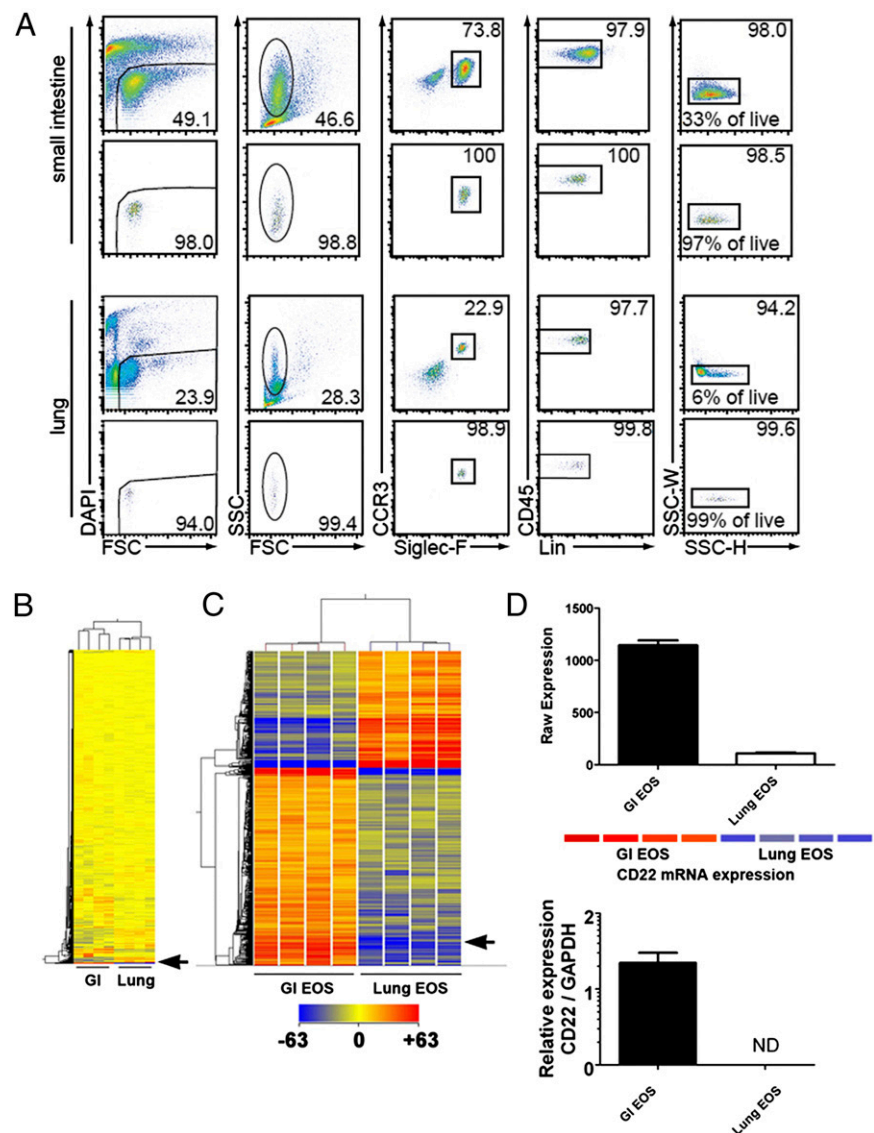
To address the physiological role of eosinophils in the GI tract and lung under homeostatic healthy conditions, we analyzed tissue-specific eosinophil gene expression patterns by genome-wide expression microarray analysis using mRNA isolated from FACS-sorted eosinophils from the small intestine or lung of naive

BALB/c mice. As shown in Fig. 1A, we readily detected GI and lung eosinophil populations by multicolor FACS staining after purification. Live eosinophils were identified as DAPI⁺CCR3⁺Siglec-F⁺CD45⁺CD4⁺CD8a⁺CD19⁺B220⁺SSC^{high} cells. The eosinophil samples were sorted to a purity ranging from 93.4 to 98.7% (7 out of 8 samples had a purity of >96%). Whole-genome expression profile analysis was performed on four normal lung and four normal intestine eosinophil mRNA samples. Among the 28,853 genes probed by the array, we found a cluster of 513 probe sets that was differentially regulated (fold change > 2, *p* < 0.01 post-Benjamini Hochberg FDR) between GI and lung eosinophils, with 319 being upregulated and 194 being downregulated (Fig. 1B, 1C). Among the genes upregulated in GI eosinophils, CD22 was upregulated 10-fold as shown by the Affymetrix raw expression values (Fig. 1D). Conventional quantitative RT-PCR verified this microarray finding, demonstrating that CD22 transcripts were indeed strongly expressed by GI eosinophils as compared with the nearly undetectable levels in lung eosinophils (Fig. 1D).

GI eosinophils express CD22 protein at levels comparable with B cells

We next examined CD22 protein expression on GI eosinophils. Isolated total jejunum lamina propria cells from BALB/c mice were

FIGURE 1. CD22 mRNA expression in murine GI eosinophils (EOS). **A**, Live eosinophils were isolated under homeostatic conditions from the GI tract and lung by FACS from the gating criteria set for DAPI⁺CCR3⁺Siglec-F⁺CD45⁺CD4⁺CD8a⁺CD19⁺B220⁺SSC^{high} cells. The percentage of each gate relative to the upper gate was printed on each plot. **B** and **C**, mRNA isolated from GI and lung eosinophils was subjected to genome-wide expression microarray analysis. Among the 28,853 unique probe sets screened, 513 genes showed a >2-fold change with *p* < 0.01 (post-FDR). Each lane represents eosinophil RNA isolated from a separate mouse (*n* = 4 per group). The position of CD22 on the heat diagram is shown with the arrow. **D**, CD22 mRNA expression as determined by gene chip analysis Raw Expression value (top) and by real-time PCR (bottom) is given. **D**, An expression heat map is also illustrated for visualization of upregulated bands in red (top panel). In the quantitative PCR graph, CD22 expression in lung eosinophils was not detectable (ND) (bottom panel). FSC, forward scatter; Lin, CD4⁺CD8a⁺CD19⁺B220⁺; SSC, side scatter.



stained with eosinophil markers and anti-CD22 or isotype control Ab. Live eosinophils were identified as $CD45^+7AAD^-SSC^{high}CCR3^+CD11b^+$ events. As shown in Fig. 2A, murine jejunum eosinophils expressed similar levels of surface CD22 to jejunum B cells. Isotype control staining for each cell type did not reveal significant staining. We also performed B220 staining on the two earlier-mentioned populations, and the results indicated that the gated GI eosinophil population did not contain any B cell contamination. Moreover, cell sorting with the same gating strategy demonstrated that $>95\%$ of events in the eosinophil gate studied were eosinophils by morphological analysis (Fig. 2B). As a control, we examined CD22 expression on eosinophils from multiple organs/tissues that harbor eosinophils under physiological conditions. As shown in Fig. 2C, CD22 protein was specifically expressed on GI lamina propria eosinophils in the upper GI tract, with the jejunum having the highest level.

Given the unexpected expression of CD22 by GI eosinophils, we aimed to further substantiate this finding. First, the specificity of the FACS-based analysis of tissue-derived eosinophils was ensured by analysis of CD22 gene-deficient mice, which did indeed reveal a lack of CD22 expression on GI eosinophils as assessed by Amnis IS-100 flow imaging. As shown in Fig. 3B, WT GI eosinophils ($CD45^+Siglec-F^+CD11b^+$) demonstrated high levels of CD22 on the cell membrane, whereas CD22 signals in $CD22^{-/-}$ GI eosinophils (C57BL/6J) and isotype controls (BALB/c) were absent. Taken together, our results demonstrate that GI eosinophils express CD22 protein on their cell membranes.

CD22 is upregulated on GI eosinophils in a postpartum fashion and is independent of intestinal flora

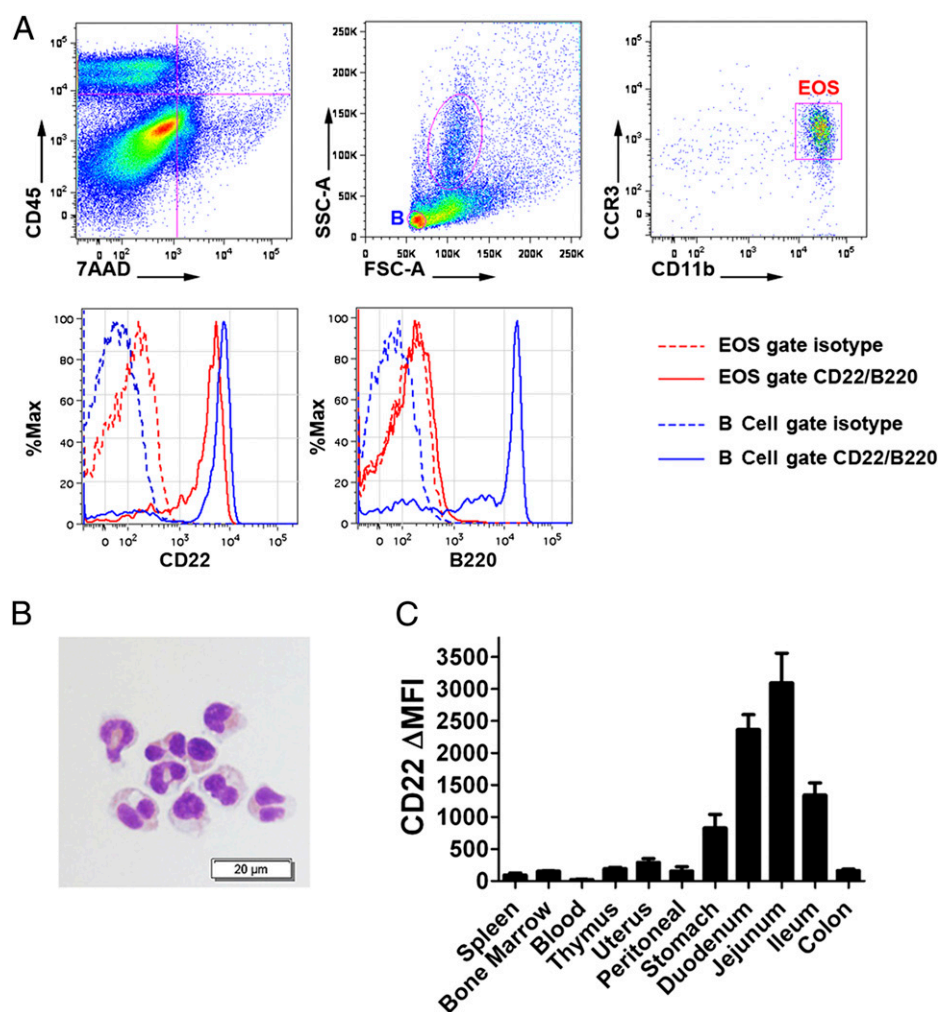
We next investigated what factors regulate GI eosinophil CD22 expression. In the case of B cells, LPS and IL-4 treatment has been shown to upregulate its surface CD22 expression (31). Therefore, we treated total splenocytes with LPS (25 μ g/ml) and IL-4 (100 ng/ml) in the presence of IL-5 (10 ng/ml) for 24 h. Eosinophils and B cells were identified by Siglec-F/CD11b double staining and CD19 staining, respectively. Although LPS and IL-4 upregulated the CD22 expression of spleen B cells by $\sim 30\%$, they failed to enhance the CD22 expression on spleen eosinophils (Fig. 4A).

A postpartum kinetic study of CD22 expression by GI eosinophils revealed that CD22 expression was not an early event during embryogenesis, but rather was upregulated to adult levels within the first 10 d after birth (Fig. 4B). Because the kinetics of the CD22 postpartum upregulation resemble those of the development of GI commensal flora, we tested whether GI flora have a role in induction of CD22 expression on GI eosinophils. As shown in Fig. 4C, GI eosinophils from 2-mo-old germ-free mice were $CD22^+$; in fact, the CD22 expression level was modestly increased on GI eosinophils in mice lacking intestinal bacteria (Fig. 4D).

GI eosinophil CD22 expression is downregulated by eosinophil activation triggers

To examine whether eosinophil activation signals affect CD22 expression, we compared CD22 expression levels on GI eosinophils from CD2-IL-5 Tg and WT mice. Notably, overexpression

FIGURE 2. CD22 protein expression on eosinophils. **A**, Live GI eosinophils were identified from the total isolated GI lamina propria cells with serial gating for $CD45^+7AAD^-SSC^{high}CCR3^+CD11b^+$ cells (EOS in red). Compared with the B cell-positive control (B in blue), GI eosinophils (red gate/histogram) express similar levels of CD22, which is shown by the difference between the isotype staining (dashed line) and CD22 staining (solid line). There was no contamination from B cells in the EOS gate because all events were B220 $^-$ in the EOS. **B**, FACS with the same EOS gating strategy demonstrated that the events were eosinophils as assessed by morphologic features (circular nuclei, eosinophilic granules) after Diff-Quick staining of cytocentrifuged sorted cells. **C**, CD22 expression on eosinophils from different tissues was assessed by Δ MFI (specific Ab MFI – isotype control MFI) with three to four mice per tissue group. All experiments were independently repeated at least twice. Data are presented as mean $\pm$ SEM.



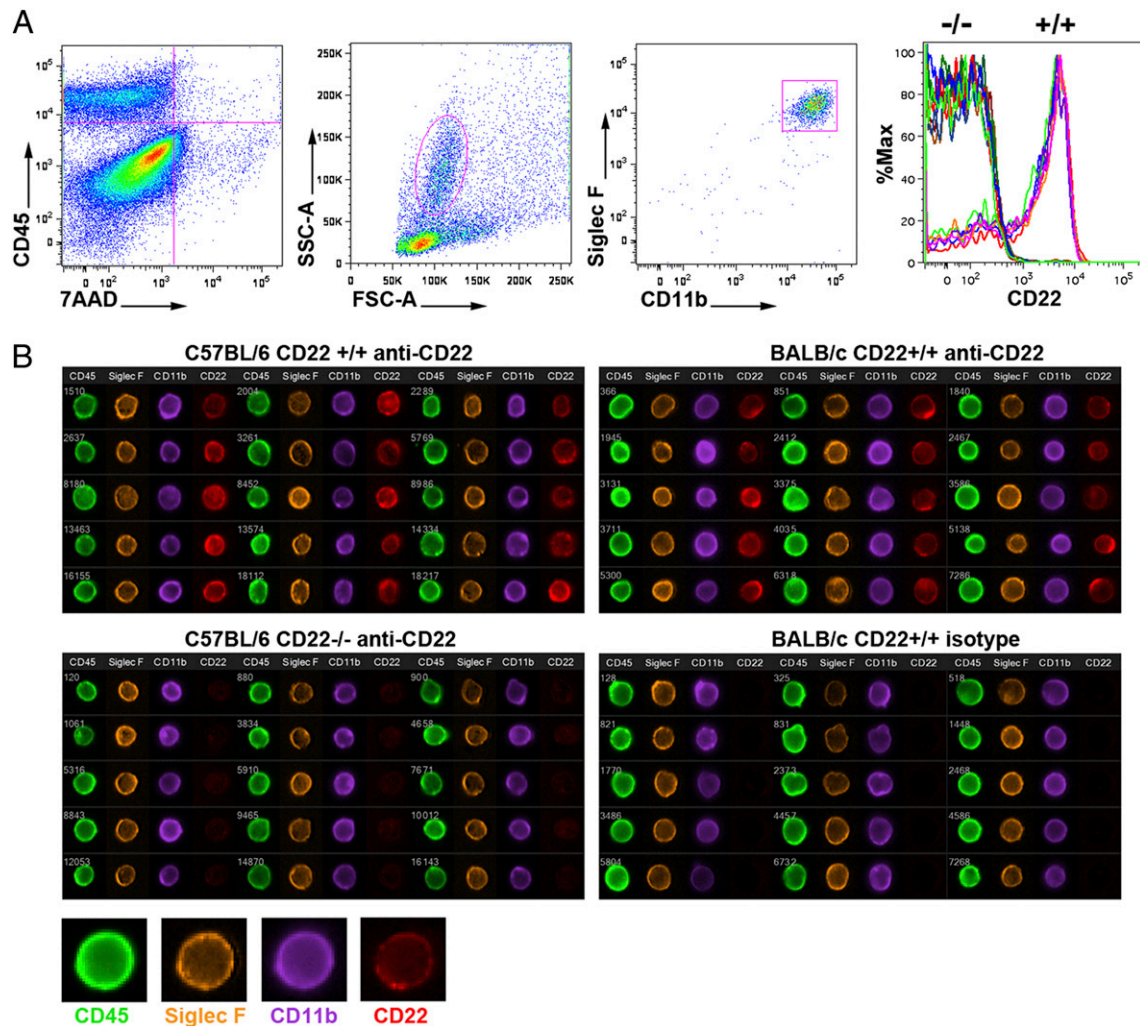


FIGURE 3. GI eosinophil CD22 cell membrane expression WT and CD22-deficient mice by FACS and flow imaging analyses. *A*, GI eosinophils were isolated from CD22^{+/+} and CD22^{-/-} mice (C57BL/6), stained for CD22 and eosinophils markers, and gated as CD45⁺7AAD⁻SSC^{high}CD11b⁺ cells. Serial gating plots for eosinophil CD22 expression are shown. CD22 histograms from four mice of each genotype were overlapped to show consistency. This experiment was independently repeated three times. *B*, Flow imaging was used to characterize the expression pattern of CD22 on GI eosinophils. A single GI eosinophil was presented on four different channels (CD45, Siglec-F, CD11b, and CD22). *Left panels*, GI eosinophil CD22 expression in CD22^{+/+} and CD22^{-/-} C57BL/6J mice. *Right panels*, GI eosinophil CD22 expression in CD22^{+/+} BALB/c mice with anti-CD22 staining and isotype control staining as a reference.

of IL-5 was associated with downregulation of CD22 expression (Fig. 4E). To further confirm that CD22 is downregulated during eosinophil activation, CD22^{+/+} and CD22^{-/-} mice were sensitized with OVA/Alum, orally challenged with saline or OVA to induce GI inflammation (29), and assessed for CD22 expression of GI eosinophils. OVA treatment downregulated GI eosinophil CD22 expression (Fig. 4F). As a control, we measured CD22 expression on lung airway eosinophils after experimental asthma induction (30); CD22 expression was not increased on airway eosinophils in a setting of severe inflammation in the lung (Fig. 4G).

CD22 negatively regulates GI lamina propria eosinophil levels

We hypothesized that CD22 would affect the eosinophil concentration in the GI tract because CD22 may negatively regulate eosinophil function and survival. Notably, flow cytometry analysis indicated that the percentage of eosinophils among total lamina propria cells was significantly increased in CD22^{-/-} mice compared with WT controls ($2.25 \pm 0.14\%$ [$n = 6$] versus $2.83 \pm 0.12\%$ [$n = 8$], mean $\pm$ SEM, $p < 0.01$). The number of eosinophils among 100,000 total lamina propria cells isolated from jejunum tissue was significantly increased in the CD22-deficient

mice (Fig. 5A). In addition, with an independent methodology, we performed MBP staining on CD22^{+/+} and CD22^{-/-} jejunum sections, and the subsequent morphometric quantification analysis revealed significantly increased eosinophilia in the GI lamina propria from CD22^{-/-} mice (Fig. 5B, 5C). We next examined whether the augmented GI eosinophilia present in CD22^{-/-} mice was a result of reduced eosinophil turnover. BrdU incorporation indicated that eosinophil turnover rates were not influenced by CD22 expression, as shown by the comparable percentage of newly synthesized/migrated BrdU⁺ eosinophils in the lamina propria of WT and CD22^{-/-} mice (Fig. 5D; $24.5 \pm 1.2\%$ [$n = 8$] versus $23.8 \pm 1.2\%$ [$n = 8$], mean $\pm$ SEM). To access the functional role of CD22 on GI eosinophil-specific signaling strength, we used the phospho-FACS to quantify the MAPK phosphorylation by the eosinophil priming/survival cytokine IL-5 and eosinophil chemokine eotaxin-2. IL-5 exposure (10 ng/ml) induced rapid and robust p44/42 MAPK phosphorylation in GI eosinophil within 10 min after stimulation, which diminished and returned to baseline by 30 min (Fig. 5E). GI eosinophils isolated from CD22^{+/+} and CD22^{-/-} mice exhibited comparable IL-5-induced MAPK activation. With the same method, we also tested the signaling

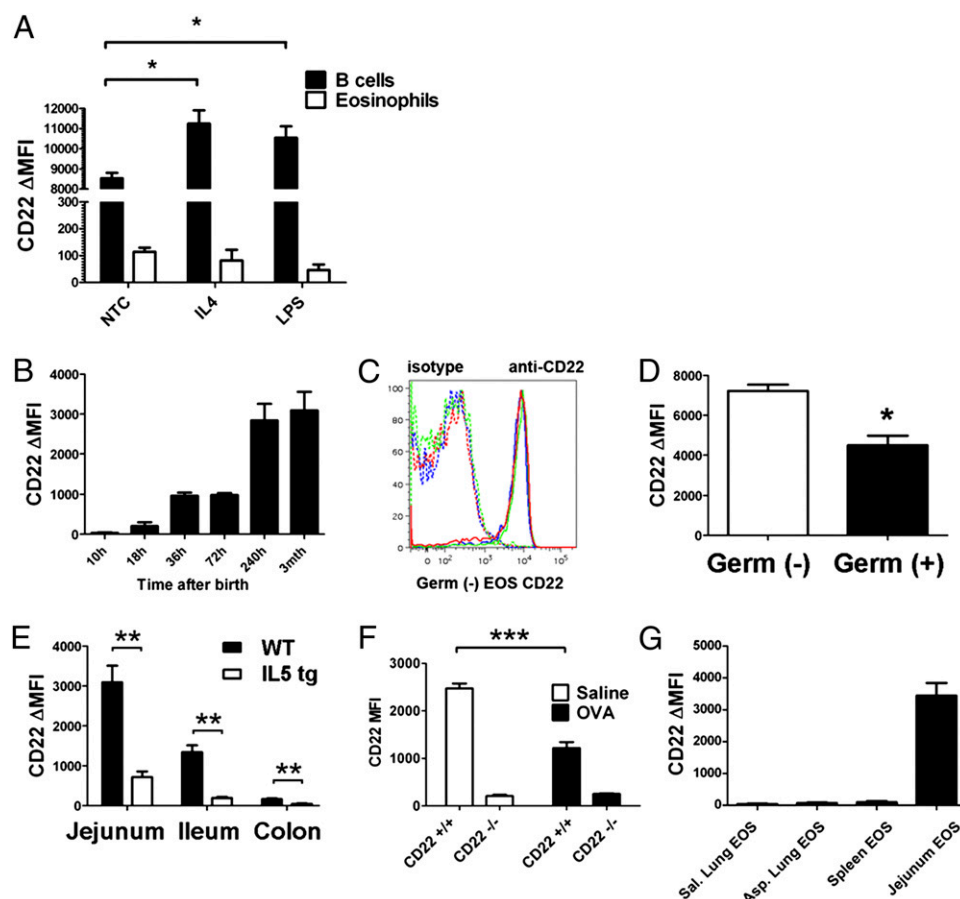


FIGURE 4. Regulation of GI eosinophil CD22 expression as a function of perinatal age, GI bacterial colonization, and cytokine exposure. **A**, Whole splenocytes were cultured 24 h in IL-5 (10 ng/ml) with either IL-4 (100 ng/ml) or LPS (25 μ g/ml). The total cell population was stained for CD19 and eosinophil markers Siglec-F and CD11b, together with anti-CD22 or isotype control. CD22 expression on eosinophils and B cells was quantified by Δ MFI. **B**, CD22 expression was quantified by Δ MFI on GI eosinophils isolated from the intestine of mice at different time points after birth using the same staining strategy. **C** and **D**, GI eosinophils were isolated from 2-mo-old germ-free (germ [-]) and non-germ-free (germ [+]) Swiss Webster mice and stained for CD22 and eosinophil markers as mentioned earlier. **E**, CD22 expression was assessed in GI eosinophils isolated from BALB/c WT and CD2-IL-5 Tg mice. **F**, GI eosinophil CD22 expression was assessed after OVA-induced GI inflammation in CD22-deficient (CD22^{-/-}) and WT (CD22^{+/+}) mice. Animals were i.p. sensitized to OVA and intragastrically challenged with OVA. **G**, BALB/c mice were challenged with *Aspergillus fumigatus* (Asp) or saline (Sal) intranasally to induce Th2-induced airway eosinophilia, and lung eosinophil CD22 expression was assessed in parallel to spleen and GI eosinophils. * p < 0.05, ** p < 0.01, *** p < 0.001, two-tailed t test or ANOVA. EOS, eosinophils; NTC, nontreated controls.

strength of IL-5-induced p38 MAPK activation and eotaxin-2-induced p44/42 activation, and no phenotypic difference was observed between CD22^{+/+} and CD22^{-/-} GI eosinophils (data not shown).

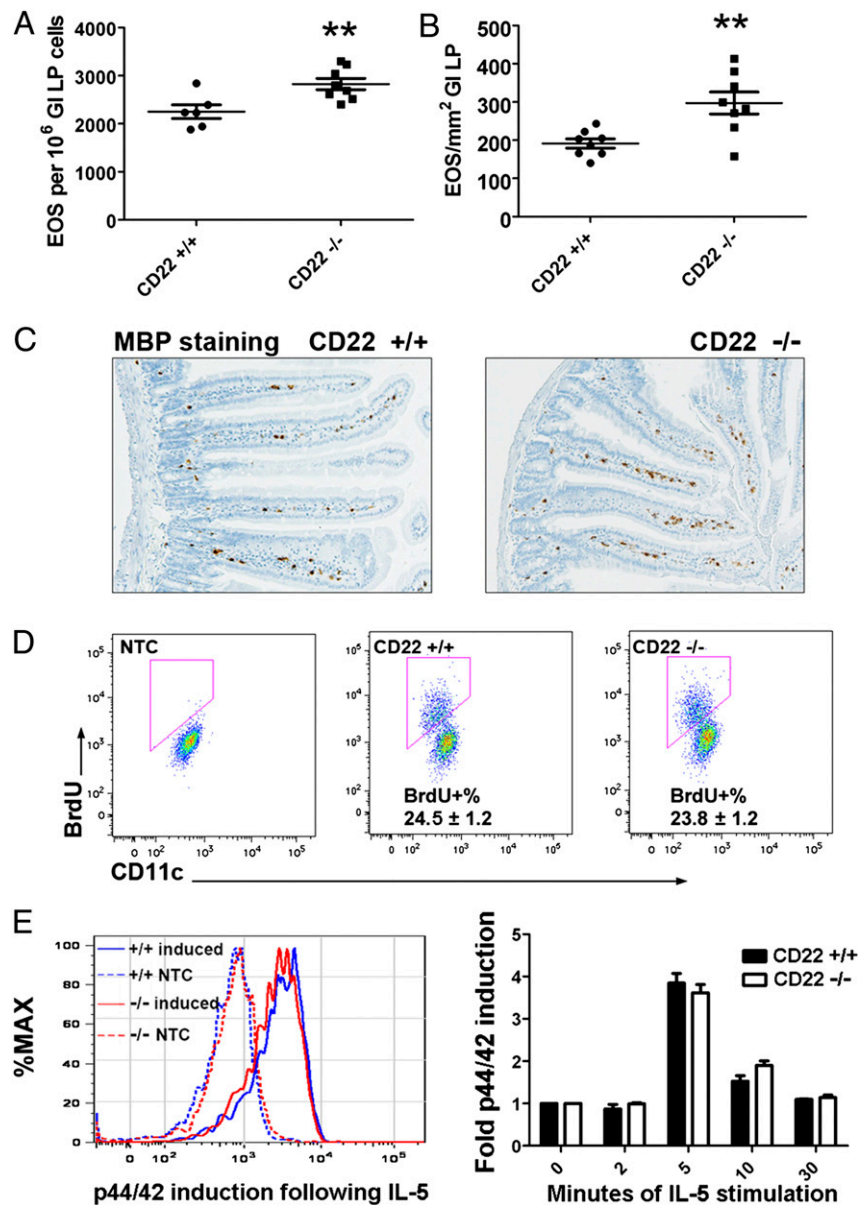
Discussion

GI lamina propria eosinophils are a unique population of immunocytes that consists of the first line of mucosal defense in the GI mucosal barrier. Their expression profiles and specific functions remain to be elucidated, largely because of the difficulty in isolating these cells for subsequent expression and functional studies. In this article, we describe an isolation system that enabled us to examine these understudied cells from the lamina propria of the GI tract, uterus, and stomach by permitting the isolation of viable and functionally intact eosinophils. With this system, we were able to show that eosinophils residing in different target organs express unique tissue-specific transcripts, presumably to suit disparate functions. Notably, although tissue-specific eosinophil transcriptomes have been identified, verification of individual genes as we have done for CD22 is likely critical because contaminating cells in the expression analysis may have had a contributory role to the identified transcriptome. To our knowledge, this study is the first to

demonstrate that CD22 is expressed on GI eosinophils, as shown by mRNA and protein analysis and confirmed by absent staining in CD22-deficient mice; this phenomenon can be observed across different mouse genetic backgrounds, namely BALB/c and C57BL/6J, with different propensities for Th1/Th2 polarization. GI eosinophil CD22 expression is an unexpected finding given that CD22 is known as an inhibitory receptor specifically expressed on B lymphocytes.

Although eosinophils are known to express the receptor for LPS and IL-4 (32, 33), which induce CD22 on B cells (31), these factors did not alter CD22 expression on GI eosinophils, suggesting that the CD22 coreceptor coupling and downstream signaling pathways in GI eosinophils are different from those in B cells. The postpartum CD22 expression kinetics data, in combination with the differential eosinophil CD22 expression in multiple tissues, suggest that the intestinal milieu developed postnatally (but not enteric commensal flora alone) contribute to the expression of CD22 on GI eosinophils. As shown by the tissue distribution and lung inflammation data, such a mechanism is not present in eosinophils from other tissues during homeostatic or inflammatory conditions. Notably, a unique feature of GI eosinophils is their activated phenotype. Multiple lines of evidence

FIGURE 5. Effect of CD22 gene deficiency on GI lamina propria eosinophil levels. **A**, GI eosinophils (EOS) were isolated by collagenase digestion and then subjected to five-color flow cytometry analysis. The numbers of eosinophils were enumerated among 10^6 total lamina propria (GI LP) cells collected. The graph shown is a representative experiment independently repeated three times. $**p < 0.01$, two-tailed *t* test. **B**, The jejunum section was stained with anti-MBP, and GI lamina propria MBP (+) eosinophil density was assayed by morphometric analysis. EOS/mm² GI LP, $**p < 0.01$, two-tailed *t* test. **C**, Representative photomicrographs (original magnification $\times 40$) of the jejunum section MBP staining, CD22^{+/+} versus CD22^{-/-} mice. **D**, Animals ingested BrdU-containing water (80 mg/dl) for 6 d and were euthanized and analyzed for the percentage of BrdU⁺ GI eosinophils. The GI eosinophil populations shown on CD11c-BrdU double plots were sequentially gated out as CD45⁺, Siglec-F⁺, and CD11b⁺ events. NTC, nontreated controls, *n* = 8 for each group, independently repeated twice. **E**, By phospho-FACS, freshly isolated GI lamina propria cells were stimulated with IL-5 (10 ng/ml) for indicated time points. The cells were then fixed, permeabilized, and stained with p44/42 (Thr²⁰²/Tyr²⁰⁴) phospho-Ab together with eosinophil markers to assess eosinophil-specific MAPK activation by IL-5. MAPK (p44/42) phosphorylation status of CD45⁺-Siglec-F⁺-CD11b⁺ triple (+) eosinophils was represented in the histogram and quantified in the bar chart, CD22^{+/+} versus CD22^{-/-} GI eosinophils. NTC, nontreated controls, *n* = 3 for each group, independently repeated twice.



have shown that GI eosinophils are highly activated compared with blood eosinophils even in homeostatic mucosa (9, 10). Considering that CD22 is generally deemed to be an inhibitory coreceptor acting through its intracellular ITIM motif on B lymphocytes, it is likely that CD22 serves as a molecular damper in GI eosinophil responses, perhaps to prevent overactivation or to maintain their physiological state. By examining multiple factors associated with GI inflammation, specifically bacterial colonization, systemic IL-5 transgene overexpression, and OVA-induced GI inflammation in mice, we demonstrated that expression of CD22 on GI eosinophils is downregulated after inflammation induced by innate (e.g., bacterial colonization) and adaptive (e.g., OVA) stimuli, and by the molecular signaling induced by relevant cytokine cascades (e.g., IL-5 overexpression). The GI tract forms an environment wherein residential hematopoietic cells are subjected to multiple lines of biological, chemical, and physical challenges. In this regard, downregulating an inhibitory receptor may initiate pro-inflammatory activity of GI eosinophils.

Collectively, we have identified that the canonical B cell marker CD22 is robustly expressed on murine GI eosinophils, which form a major eosinophil reservoir and may have key roles in GI mucosal

defense. It is interesting to note that CD22 has recently been found to be expressed by other non-B cell populations including neurons (34), human basophils (35, 36), cultured human mast cells (37), and T cells (38). Notably, even within B cells, CD22 was recently shown to mediate receptor signaling events other than the BCR pathway (39–41), suggesting that CD22 could functionally regulate certain innate functions beyond the scope of BCR signaling. Although the GI tract has the largest reservoir of eosinophils in mice and humans, there is a paucity of information regarding their homeostatic regulation, except for data concerning the key role of eotaxin-1 and its receptor CCR3, as well as the inhibitory receptor PIR-B (42). In this article, we have identified a novel checkpoint for controlling the baseline level of GI eosinophils involving a receptor not previously identified on eosinophils. Although the molecular control of GI eosinophil-specific expression has not been elucidated, it is interesting that CD22 expression in eosinophils is specific to the GI tract, implying a tissue-specific role. Indeed, early studies have already revealed a nonredundant role for CD22 in regulating the baseline level of GI eosinophil levels. It will certainly be interesting to examine the role of CD22 and its potential

utility as a pharmacological target in eosinophilic inflammatory responses.

Microarray data accession

Eosinophil-specific RNA microarray data have been deposited to the Gene Expression Omnibus public repository under accession code GSE33807 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33807>).

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Disclosures

The authors have no financial conflicts of interest.

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