

CD66b Regulates Adhesion and Activation of Human Eosinophils¹

Juhan Yoon,* Akihiko Terada,[†] and Hirohito Kita^{2*†}

Eosinophils and their products are likely important in the pathophysiology of allergic diseases, such as bronchial asthma, and in host immunity to parasitic organisms. However, the mechanisms for proinflammatory mediator release by eosinophils are poorly understood. CD66b (CEACAM8, CGM6, NCA-95) is a single chain, GPI-anchored, highly glycosylated protein belonging to the carcinoembryonic Ag supergene family. CD66b is an activation marker for human granulocytes; however, its biological functions are largely unknown in eosinophils. We found that CD66b is highly expressed on the surface of human peripheral blood eosinophils isolated from healthy individuals. Engagement of CD66b, but not CD66a, by mAb or a natural ligand, galectin-3, activated a Src kinase family molecule, hemopoietic cell kinase (Hck), and induced cellular adhesion, superoxide production, and degranulation of eosinophils. CD66b molecules were localized in lipid rafts, and disruption of lipid rafts or removal of the GPI anchor inhibited the adhesion and activation of eosinophils. Importantly, CD66b was constitutively and physically associated with a β_2 integrin, CD11b, and cross-linking of CD66b induced a striking clustering of CD11b molecules. Thus, CD66b molecules are involved in regulating adhesion and activation of eosinophils, possibly through their localization in lipid rafts and interaction with other cell surface molecules, such as CD11b. Binding of exogenous or endogenous carbohydrate ligands(s) to CD66b may be important in the release of proinflammatory mediators by human eosinophils. *The Journal of Immunology*, 2007, 179: 8454–8462.

The eosinophil is recognized as a proinflammatory granulocyte implicated in various allergic diseases, such as bronchial asthma and atopic dermatitis, and in immunity to parasites (1). Current evidence suggests that the pathophysiology of these diseases and conditions is closely related to proinflammatory mediators, such as eosinophil major basic protein, eosinophil cationic protein and reactive oxygen species, released when eosinophils are exposed to appropriate stimuli in the tissues (1). Several in vitro studies suggest that eosinophils express receptors for IgA, IgG, cytokines, chemokines, and complement components, and the effector functions of eosinophils are induced by various ligands for these receptors (2–4). However, the triggers and molecular mechanisms of eosinophil activation, in particular those involved in in vivo settings, are not fully understood.

Eosinophils express several Ig superfamily cell surface molecules, such as leukocyte Ig-like receptor/Ig-like transcript (LIR/ILT),³ LIR-1/ILT-2, LIR-2/ILT-4, LIR-3/ILT-5, LIR-7/ILT-1 (5), sialic acid-binding Ig-like lectins (6), IRp60 (7), CD52 (8), and

CD48 (9). Ligation of these molecules with specific mAb either enhances or inhibits eosinophil activation. For example, cross-linking of CD48 induces activation and degranulation of eosinophils (9). In contrast, cross-linking of CD52, CD300a, and Siglec-8 inhibited superoxide production, migration and survival of eosinophils (6–8). Thus, complex networks of signals from both activating and inhibitory cell surface molecules likely regulate eosinophil effector functions, including the release of proinflammatory mediators.

CD66b (CEACAM8, CGM6, NCA-95) is a single chain GPI-anchored glycoprotein and is a member of the Ig superfamily, more specifically the human carcinoembryonic Ag (CEA) family (10). CEAs are widely used as colorectal carcinoma tumor markers but are expressed in normal tissues and during fetal development (10). Indeed, CD66b is exclusively expressed on human granulocytes and is recognized as a granulocyte “activation marker” (11, 12). However, the functions of this molecule are largely unknown in eosinophils (13, 14). Under normal conditions, neutrophils have minimal expression of CD66b, but incubation in vitro with inflammatory agonists, such as fMLP, rapidly increases this expression. Furthermore, expression of CD66b in eosinophils is enhanced in vivo in patients with a helminth infection, specifically with *Schistosoma mansoni*. (15). The eosinophil expression of CD66b is also enhanced in vitro by incubation with activated lymphocytes (16) or by brief incubation with IL-5 (unpublished observations). Interestingly, no homologue for CD66b has been identified in the mouse, suggesting that there may be strong selection pressure (e.g., exposure to microorganisms or parasites) during the evolution of the CEA family molecules. Thus, we hypothesized that CD66b may be critically involved in the activation of human eosinophils.

As specific membrane microdomains that are enriched in sterols and sphingolipids, lipid rafts play several important roles in immune cells. For example, they contribute to BCR signaling and Ag uptake (17, 18), to ligand-mediated signaling from T cell receptors (19, 20) and from high affinity IgE receptors (21), and to MHC class II-mediated Ag presentation (22, 23). GPI-anchored proteins

*Department of Immunology, and [†]Department of Medicine, Division of Allergic Diseases, Mayo Graduate School, Mayo Clinic College of Medicine, Rochester, MN 55905

Received for publication March 15, 2007. Accepted for publication October 3, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grant AI34486 and the Mayo Foundation.

² Address correspondence and reprint requests to Dr. Kita Hirohito, Departments of Immunology and Medicine, Division of Allergic Diseases, Mayo Clinic College of Medicine, 200 First Street Southwest, Rochester, MN 55905. E-mail address: kita.hirohito@mayo.edu

³ Abbreviations used in this paper: LIR/ILT, leukocyte Ig-like receptor/Ig-like transcript; CEA, carcinoembryonic antigen; M β CD, methyl β -cyclodextrin; MBP, myelin basic protein; PI-PLC, phosphatidylinositol-specific phospholipase C; EDN, eosinophil-derived neurotoxin.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/\$2.00

are often localized in these lipid rafts, where they possibly play roles in regulating molecular interactions, signal transduction, and cell activation (24–27). To the best of our knowledge, the presence and function of lipid rafts have not been previously reported in eosinophils.

We now show that perturbation of CD66b by mAb or by a natural ligand, galectin-3, results in strong cellular adhesion and degranulation of human eosinophils. Furthermore, lipid rafts and β_2 integrins are likely involved in eosinophil activation induced by CD66b. Our observations suggest that CD66b may be an important positive regulator of eosinophil effector functions during inflammation, host immunity, and allergic diseases.

Materials and Methods

Reagents

Mouse anti-human CD66b mAb (clones 80H30 and GM-2H6, both IgG1 isotype) were obtained from Serotec and Abcam, respectively. Mouse anti-human CD66a mAb (clones 29H2 and GM8G5, both IgG1 isotype; clone 283340, IgG2b isotype) were purchased from Novocastra Laboratories, Alexis Biochemicals, and R&D Systems, respectively. Control mouse IgG1, polyclonal Ab for the Src kinase family (rabbit anti-Hck, Fgr, Lck, Lyn), control rabbit IgG, anti-CD11b mAb (clone 2LPM19C, IgG1) and peroxidase-conjugated rabbit anti-mouse IgG Abs were obtained from Santa Cruz Biotechnology. F(ab')₂ of anti-human CD66b mAb (80H30) and control mouse IgG1 were generated by using an Ab F(ab')₂ fragmentation kit (Pierce Biotechnology). FITC-conjugated anti-CD11b mAb (clone ICRF44, IgG1) was from Ancell Corporation, and FITC-conjugated control mouse IgG1 and anti-CD11a (clone G43-25B, IgG2a) were purchased from BD Biosciences. Control mouse IgG2b was from R&D Systems. FITC- and peroxidase-conjugated cholera toxin B subunit, peroxidase-conjugated goat anti-rabbit IgG, human serum albumin, methyl β -cyclodextrin (M β CD), F(ab')₂ of goat anti-mouse IgG (Fc specific), FITC-conjugated F(ab')₂ of sheep anti-mouse IgG, and myelin basic protein (MBP, exogenous substrate of Src kinase family in vitro kinase assay) were obtained from Sigma-Aldrich. Human galectin-3 was purchased from RDI; human rIL-5 and phosphatidylinositol-specific phospholipase C (PI-PLC) were obtained from Calbiochem. A Src kinase-specific inhibitor (PPI) was purchased from A. G. Scientific. Anti-phosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology.

Cell isolation

Human eosinophils were isolated from 15 healthy volunteers using Percoll density gradient centrifugation and the MACS system for negative selection with anti-CD16 microbeads (Miltenyi Biotec) as described earlier (28). The purity of eosinophils was >96%, and cells were used immediately. The Mayo Clinic Rochester Institutional Review Board approved the protocol to obtain blood from volunteers; all provided informed consent.

Immunoprecipitation and Western blotting

For CD66b detection, eosinophils (10×10^6 cells) were lysed in lysis buffer (25 mM HEPES (pH 7.4), 1% Nonidet P-40, 125 mM NaCl, 100 μ M Na₃VO₄, 1 mM PMSF, and protease inhibitor mixture); the lysate was centrifuged at $15,000 \times g$ for 20 min at 4°C; the supernatant was incubated with anti-CD66b mAb (80H30, 3 μ g/ml) or control mouse IgG1 overnight at 4°C; protein A-Sepharose (40 μ l) was added; and the mixture was incubated for 3 h at 4°C. After several washings with lysis buffer, a 5-fold excess of sample buffer was added, and the mixture was boiled at 95°C for 10 min. After centrifugation at $15,000 \times g$, supernatants were subjected to 10% SDS-PAGE. Proteins in the gel were transferred onto nitrocellulose paper; after blocking with 5% skim milk in PBST (0.2% Tween 20 and PBS) for 1 h at room temperature, the paper was incubated with the appropriate primary and secondary Abs, and the ECL system was used to detect CD66b.

To detect activated Hck and other Src-family molecules, eosinophils (5×10^6 cells/sample) were incubated with 10 μ g/ml anti-CD66b mAb (80H30) on ice for 30 min. Then the primary Ab was cross-linked with 50 μ g/ml F(ab')₂ of goat anti-mouse IgG for intervals between 0 and 30 min at 37°C. Reactions were stopped with cold PBS; cells were centrifuged at $1,000 \times g$ for 5 min at 4°C and immediately lysed with lysis buffer for 1 h on ice. Lysate was centrifuged at $15,000 \times g$ for 15 min at 4°C. Supernatants were incubated with anti-Hck polyclonal Ab (3 μ g/ml) overnight at 4°C. Precipitation with protein A-Sepharose (40 μ l) and Western blot analysis were performed, as described above, except 5% BSA was used instead

of skim milk, and the appropriate primary and secondary Abs were used in the ECL system to detect phosphorylated Hck.

FACS analysis

Isolated eosinophils were washed with PAB (PBS, 3% BSA and 0.1% sodium azide) buffer twice and incubated with mouse anti-human CD66b mAb (80H30), anti-human CD66a mAb (283340), control mouse IgG1, or control mouse IgG2b for 45 min on ice. After washing with PAB, cells were incubated with FITC-conjugated F(ab')₂ of sheep anti-mouse IgG for 30 min on ice. Cells were washed twice with PAB, fixed with 1% formaldehyde for 20 min and analyzed using a FACScan (BD Biosciences), with Becton Dickinson lysis II software. In some experiments, eosinophils (5×10^6 cells) were pretreated with PI-PLC (1 U) for 1 h at 37°C to remove the GPI-anchored protein(s) from the cell surface (29). Cells were fixed with 1% formaldehyde for 20 min and washed with PAB before incubation with anti-CD66b mAb.

Eosinophil superoxide generation and eosinophil-derived neurotoxin (EDN) release assays

Superoxide generation was measured by superoxide dismutase-inhibitable reduction of cytochrome c, as described earlier (2). In brief, for the experiments with immobilized CD66a mAb and CD66b mAb, plates were first coated with 50 μ g/ml F(ab')₂ of goat anti-mouse IgG. After washing, the plates were incubated with anti-CD66a mAb (29H2 and GM8G5, 1 μ g/ml), anti-CD66b mAb (80H30 and GM-2H6, 0.05 to 1 μ g/ml) or control mouse IgG1 in 1% HSA (Sigma-Aldrich) in PBS for 2 h at 37°C and washed twice with 0.9% NaCl. Eosinophils (10^5 cells/well in HBSS with 10 mM HEPES and 0.01% gelatin) were incubated in anti-CD66a, anti-CD66b or isotype control Ab-coated plates, and superoxide production was measured (2). For the experiments with F(ab')₂ of mAb, plates were directly coated with 1 μ g/ml F(ab')₂ of anti-CD66b mAb (80H30) or control mouse IgG1, and blocked with 1% HSA. In certain experiments, we used IL-5 (10 ng/ml), PMA (1 ng/ml) and galectin-3 (10 μ g/ml) to stimulate eosinophils. To quantify eosinophil degranulation, the concentrations of EDN in the cell-free supernatants were measured by specific RIA, as described earlier (2). To examine the effect of cholesterol depletion on eosinophil activation via CD66b, eosinophils were incubated with the cholesterol depletion reagent, M β CD (10 mM), for 1 h at 37°C, 5% CO₂ before exposure to each stimulus. To examine whether the Src kinase family is involved in eosinophil activation, cells were incubated with a Src kinase specific inhibitor (PPI, 10 μ M) for 30 min at 37°C, 5% CO₂. To remove GPI-anchored CD66b from the cell surface, purified eosinophils were incubated with PI-PLC (1U) for 1 h at 37°C, 5% CO₂. After each preincubation, cell viability, using trypan blue exclusion, was >99%.

Detection of cell morphological changes by CD66b-coated beads

To investigate CD66b cross-linking effects on morphological changes, polystyrene beads or tissue culture plates were coated with F(ab')₂ of goat anti-mouse IgG (50 μ g/ml) at 4°C overnight, washed with 0.9% NaCl, and incubated with anti-CD66b mAb (80H30) or control mouse IgG1 for 1 h at 37°C. Freshly isolated eosinophils were incubated with Ab-coated beads or plates for 30 min or 3 h at 37°C, respectively. After fixation with 3.7% formaldehyde for 20 min at 4°C, the suspensions were washed twice with PBS. Images were taken with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss).

Lipid raft isolation

Eosinophils (2×10^7 cells) were lysed in the TNE buffer (10 mM Tris-HCl, NaCl 150 mM, and EDTA 5 mM) with 1% Triton X-100, and the lysate was passed 5 times through 25 gauge needles. The lysates were mixed with equal volumes of 85% sucrose in TNE buffer with protease inhibitor mixtures and transferred to a centrifuge tube (30). Samples were overlaid with 6 ml of 35% sucrose and 4 ml of 3.5% sucrose in TNE buffer. After centrifugation for 16 h at $288,000 \times g$ in a Beckman Coulter SW41Ti rotor, fractions (1 ml each) were collected from the top of the gradients. Each fraction was precipitated with 20% TCA and washed twice with cold acetone.

In vitro Src family kinase assay

Eosinophils (10^7 cells) were stimulated with immobilized anti-CD66b mAb or control Ab for 10 or 30 min. at 37°C. Then, cells were lysed in 25 mM HEPES (pH 7.4), 1.5% Nonidet P-40, 125 mM NaCl, 100 μ M Na₃VO₄, 1 mM PMSF and protease inhibitor mixture for 30 min on ice. After centrifugation at $15,000 \times g$ for 20 min, the supernatants were incubated with anti-Hck, -Fgr, -lyn and -lck Abs (3 μ g/ml) for 3 h at 4°C,

followed by incubation with protein A-Sepharose overnight at 4°C. After the immunoprecipitated complexes were washed with lysis buffer and the kinase reaction buffer (100 mM Tris-HCl (pH 7.2), 125 mM MgCl₂, 25 mM MnCl₂, 2 mM EGTA, 100 μ M Na₂VO₄, 2 mM DTT), the complexes were suspended in 30 μ l of kinase reaction buffer with 10 μ Ci [³²P]ATP (PerkinElmer Life Science) and substrate MBP (10 μ g/sample) and finally incubated for 20 min at 30°C. Reactions were stopped with 5 $\times$ sample buffer, and samples were subjected to SDS-PAGE. Gels were dried for 1 h at 80°C and then exposed to the bio-MS film (Kodak) for 30 min at -80°C and developed.

Coimmunoprecipitation of CD66b and CD11b

Eosinophils (5–6 $\times$ 10⁶ cells) were activated by incubation with anti-CD66b mAb (80H30) or control mouse IgG1 (10 μ g/ml) and F(ab')₂ of goat anti-mouse IgG (50 μ g/ml) for 15 min at 37°C. Activation was stopped by adding cold PBS. After washing with PBS, cells were lysed with lysis buffer (0.5% Nonidet P-40, 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, and a protease inhibitor mixture) for 30 min on ice. Lysates were precleared with protein A/G-Sepharose and incubated with anti-CD66b mAb or control Ab (2 μ g/ml) overnight at 4°C. Subsequently, protein A/G-Sepharose was added and incubated for 2 h at 4°C. After washing (3 $\times$) with lysis buffer, proteins were eluted by adding sample buffer and boiling for 5 min at 95°C. CD11b was detected by Western blot analysis with anti-CD11b mAb (clone 2LPM19C; Santa Cruz Biotechnology).

Confocal microscopy

The cellular localization of CD11a and CD11b molecules after stimulation with anti-CD66a or CD66b mAb were examined by a capping experiment. Eosinophils (1 $\times$ 10⁶ cells) were activated by incubation with 10 μ g/ml anti-CD66a mAb (GM8G5) or anti-CD66b mAb (80H30), and F(ab')₂ of goat anti-mouse IgG (50 μ g/ml) for 30 min at 37°C. Eosinophils were fixed with 3.7% formaldehyde for 20 min at room temperature, washed with 1% PAB and spread by using cytospin. After staining with FITC-conjugated anti-CD11b mAb (1/200 dilution) or FITC-conjugated anti-CD11a (1/100 dilution) in PAB for 40 min at room temperature and washing (3 $\times$) with 1% PAB, anti-fade reagent was added and images were taken with a LSM 510 confocal laser scanning microscope.

Statistical analysis

All the error bars represent SEM. Two-sided differences between two samples were analyzed with the Student *t* test or Mann-Whitney *U* test. Values of *p* < 0.05 were considered significant.

Results

Cross-linking of CD66b by mAb activates eosinophils

CD66b is a GPI-anchored glycoprotein that is exclusively expressed on human granulocytes (14). Both flow cytometry analyses (Fig. 1A) and Western blot (Fig. 1B) show that CD66b is expressed abundantly on human eosinophils isolated from healthy individuals. CD66a, another member of the human CEA family, is also expressed abundantly on eosinophils (Fig. 1A). Eosinophil CD66b is highly glycosylated, as demonstrated by its migration around a 100 kDa molecular mass range (Fig. 1B), when compared with the 31 kDa predicted polypeptide molecular mass (31). To investigate functions of CD66b in eosinophils, we first examined the effects of CD66b cross-linking by specific mAb on superoxide generation. Eosinophils were incubated with anti-CD66b mAb immobilized onto tissue culture plates through goat anti-mouse IgG Fc-specific Ab. Eosinophils incubated with immobilized anti-CD66b mAb actively generated superoxide in a time-dependent manner; in contrast, eosinophils incubated with immobilized anti-CD66a mAb or medium alone did not generate superoxide (Fig. 2A). The concentration-response study shows that eosinophils were activated by as little as 0.05 μ g/ml anti-CD66b mAb, but they did not respond to 1 μ g/ml anti-CD66a mAb or control mouse IgG1 Ab (Fig. 2B). Other clones of anti-CD66a mAb, including 29H2 (mouse IgG1) and 283340 (mouse IgG2b), did not generate superoxide (data not shown). In contrast, two clones of anti-CD66b mAb, namely 80H30 and GM-2H6, robustly induced superoxide generation (Fig.

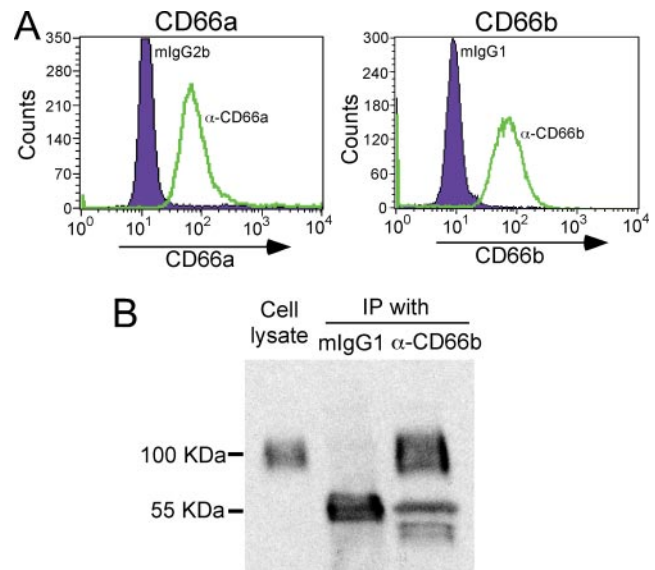


FIGURE 1. Human peripheral blood eosinophils express CD66a and CD66b. *A*, Freshly isolated eosinophils were incubated with mouse anti-CD66a mAb (283340, IgG2b), mouse anti-CD66b mAb (80H30, IgG1), mouse IgG2b Ab, or mouse IgG1 Ab. Primary Abs were detected with PE-conjugated goat anti-mouse Fc-specific F(ab')₂ Ab, and analyzed by flow cytometry. The histograms of one representative experiment from three independent experiments are shown. *B*, CD66b expression was detected by using immunoprecipitation (IP) with anti-CD66b-specific mAb (80H30) or control isotype-matched mouse IgG1 Ab and subsequent immunoblotting with mouse anti-CD66b-specific mAb. The Western blot of one representative experiment from three independent experiments is shown.

2C). Furthermore, F(ab')₂ of anti-CD66b Ab, which had been immobilized directly onto the plates, potentially activated eosinophils (Fig. 2D). Thus, ligation of eosinophil surface CD66b, but not CD66a, by Ab strongly activates eosinophils and induces superoxide generation in an Fc receptor-independent manner.

We examined whether other effector functions of eosinophils are induced by CD66b cross-linking, and we examined functions induced by an authentic stimulus for eosinophils, IL-5, and by a pharmacologic stimulus, PMA. Eosinophil superoxide production induced by cross-linking CD66b was comparable to that induced by IL-5 (10 ng/ml) and ~58% of that induced by PMA (1 ng/ml) (Fig. 3A). As measured by release of a granule protein EDN into supernatants, CD66b cross-linking also induced eosinophil degranulation, (Fig. 3B); the magnitude of eosinophil degranulation induced by CD66b cross-linking was comparable to that induced by IL-5 and ~65% of that induced by PMA. Morphologically, eosinophils adhered and spread onto tissue culture plates coated with anti-CD66b mAb (Fig. 3C). A photomicrograph of polystyrene beads coated with anti-CD66b mAb clearly depicts eosinophils spreading over the spherical bead surface (Fig. 3C). No degranulation or cellular adhesion was observed with eosinophils incubated with immobilized mIgG1 Ab or anti-CD66a mAb. Thus, cross-linking of CD66b triggers various effector functions in eosinophils, including adhesion, superoxide production and degranulation.

Galectin-3 activates eosinophils

We next examined whether galectin-3, a physiologic ligand for both CD66b and CD66a (32), recapitulates the anti-CD66b mAb results. Galectin-3 is a member of a family of β -galactoside-binding animal lectins; it is produced by macrophages and mast cells

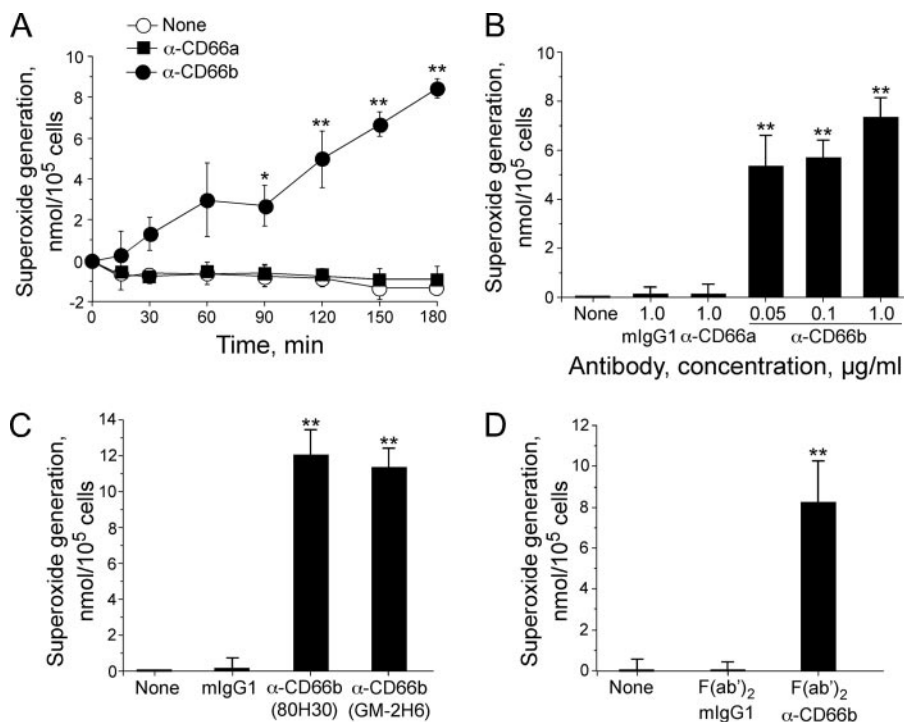


FIGURE 2. Cross-linking of CD66b with Ab activates eosinophils in time- and concentration-dependent manners. *A*, Purified eosinophils were stimulated with anti-CD66a mAb (GM8G5, 1 μ g/ml) or anti-CD66b mAb (80H30, 1 μ g/ml), which had been immobilized onto tissue culture plates through F(ab')₂ of goat anti-mouse IgG. Superoxide generation was measured at times shown. Results show means $\pm$ SEM, $n = 3$. * and **, $p < 0.05$ and $p < 0.01$ compared with no mAb (None). *B*, Purified eosinophils were incubated with mouse IgG1 Ab (1 μ g/ml), anti-CD66a mAb (GM8G5, IgG1, 1 μ g/ml) or anti-CD66b mAb (80H30, IgG1, 0.05–1.0 μ g/ml), which had been immobilized onto plates through F(ab')₂ of goat anti-mouse IgG. After 3 h of incubation, superoxide production was measured by reduction of cytochrome *c*. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with mouse IgG1. *C*, Purified eosinophils were stimulated with mouse IgG1 Ab (1 μ g/ml) or anti-CD66b mAb (clones 80H30 and GM-2H6, both IgG1, 1 μ g/ml), which had been immobilized onto plates through F(ab')₂ of goat anti-mouse IgG. After 3 h of incubation, superoxide production was measured by reduction of cytochrome *c*. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with mouse IgG1. *D*, Purified eosinophils were stimulated with immobilized F(ab')₂ of anti-CD66b mAb (80H30, 50 μ g/ml) or F(ab')₂ of isotype-matched control mouse IgG1 Ab (50 μ g/ml). These Ab had been immobilized directly onto the tissue culture plates. After 3 h of incubation, superoxide production was measured by reduction of cytochrome *c*. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with F(ab')₂ mouse IgG1.

during inflammation (33, 34) and is implicated in eosinophilic airway inflammation in mice (35). At 10 μ g/ml galectin-3 significantly induced superoxide production and degranulation of eosinophils ($p < 0.05$, $n = 3$) (Fig. 4, *A* and *B*). These eosinophil responses to galectin-3 were roughly similar to those induced by cross-linking CD66b with mAb (1 μ g/ml). Cross-linking of CD66a by anti-CD66a mAb (29H2, 1 μ g/ml) did not induce superoxide production or degranulation (Fig. 4, *A* and *B*).

To rule out the potential nonspecific effects of galectin-3, we examined the effects of PI-PLC, an enzyme that specifically removes GPI-anchored proteins from the cell surface. Incubation with PI-PLC decreased the number of CD66b on the eosinophils' surface (Fig. 5*A*). Furthermore, pretreatment with PI-PLC decreased eosinophil superoxide production induced by cross-linking CD66b mAb or by soluble galectin-3 (Fig. 5*B*, $p < 0.01$, $n = 3$). In contrast, superoxide production induced by PMA was not affected by PI-PLC treatment. Thus, not only anti-CD66b cross-linking mAb, but also a natural ligand for CD66b induces eosinophil activation.

Lipid rafts are essential for eosinophil activation induced by CD66b cross-linking

To understand better the molecular mechanisms for eosinophil activation induced by CD66b, we hypothesized that lipid rafts may be involved. Because there were no reports for lipid rafts in eosinophils, we first investigated whether CD66b is localized to lipid

rafts. Unstimulated eosinophils (i.e., incubated with control mouse IgG) and activated cells (i.e., incubated with anti-CD66b mAb) were lysed and a sucrose gradient with ultracentrifugation was used to separate the membrane fractions (30, 36, 37). In unstimulated eosinophils, CD66b was detectable in both lipid rafts and in nonraft fractions; CD66b cofractionated with an authentic lipid raft marker GM1 (30) and with Lyn, which has been mainly detected in the lipid raft fractions (Fig. 6*A*). In stimulated eosinophils, the numbers of CD66b molecules in the lipid raft fractions dramatically increased (Fig. 6*A*), suggesting active recruitment of CD66b from nonraft compartments to raft compartments during activation.

We also investigated the roles of these lipid rafts in eosinophil activation induced by CD66b cross-linking. Because cholesterol depletion specifically disrupts lipid rafts without affecting other membrane structures, we used a cholesterol depletion reagent, M β CD, which has been used for other cell types (30, 38–40). By trypan blue exclusion, the M β CD pretreatment did not affect eosinophil viability (data not shown). When cholesterol was depleted, eosinophil adhesion to the anti-CD66b mAb-coated beads was markedly inhibited (Fig. 6*B*, *inset*). Furthermore, M β CD pretreatment nearly abolished eosinophil superoxide production induced by cross-linking CD66b, but showed no effects on superoxide production induced by PMA (Fig. 6*B*). Thus, CD66b is localized to lipid rafts, and their integrity is critical for the functions of CD66b.

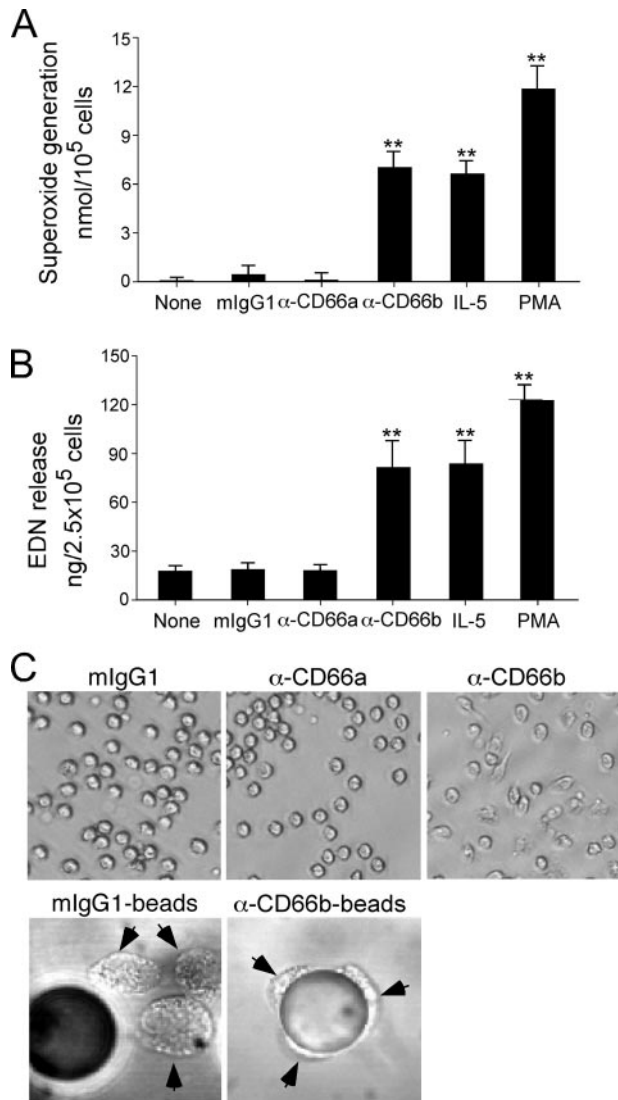


FIGURE 3. CD66b cross-linking induces significant superoxide generation, degranulation, and adhesion. *A*, Eosinophils were incubated with medium only (None), control mouse IgG1 Ab, anti-CD66a mAb (GM8G5), or anti-CD66b Ab (80H30) (Ab and mAb immobilized with F(ab')₂ of goat anti-mouse IgG), IL-5 (10 ng/ml), or PMA (1 ng/ml). After 3 h of incubation, superoxide production was measured as described in Fig. 2*B*. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with mouse IgG1 Ab (for anti-CD66b) or none (for IL-5 and PMA). *B*, After 3 h of incubation with stimuli described in *A*, supernatants were harvested and analyzed for released EDN by RIA. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with mouse IgG1 Ab (for anti-CD66b) or none (for IL-5 and PMA). *C*, *Top panels*: After 3 h of incubation with stimuli described in *A*, cell morphology was recorded under an inverted microscope (original magnification $\times 100$). *C*, *Bottom panels*: Beads were first coated with F(ab')₂ of Fc-specific goat anti-mouse IgG, then mouse IgG1 Ab (*left*) or anti-CD66b mAb (80H30) (*right*). Freshly isolated eosinophils were incubated with beads for 30 min at 37°C. After fixation, photomicrographs were taken ($\times 400$); arrowheads indicate eosinophils.

The Src kinase, Hck, plays a key role in eosinophil activation via CD66b cross-linking

We investigated downstream events of cellular activation induced by CD66b cross-linking. In lymphocytic cells, some Src kinase family proteins localize in lipid rafts where they play a key role in cell activation mediated by GPI-anchored surface molecules (26, 41–44). Eosinophils express four types of Src kinases, including

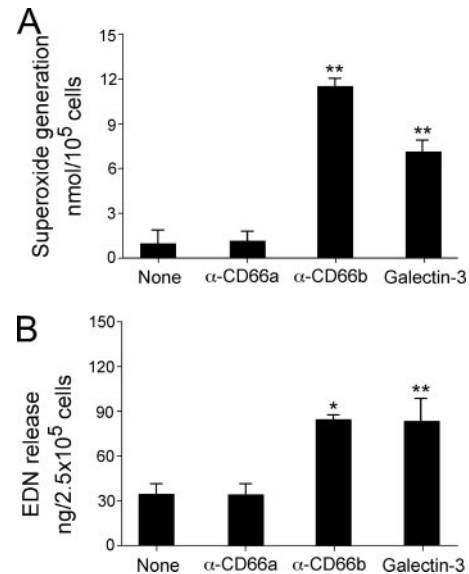


FIGURE 4. Human galectin-3 activates eosinophils. Eosinophils were incubated with medium only (None), immobilized anti-CD66a mAb (29H2) or anti-CD66b mAb (80H30) or soluble galectin-3 (10 μ g/ml) and superoxide generation (*A*) and EDN release (*B*) were measured (see *Materials and Methods* for details). Results show means $\pm$ SEM, $n = 3$. * and **, $p < 0.05$ and $p < 0.01$ compared with anti-CD66a mAb (for anti-CD66b mAb) or none (for galectin-3).

Hck, Fgr, Lyn, and Lck (45, 46). Therefore, one or more Src kinases may be involved in eosinophil activation through CD66b. Eosinophils were activated by cross-linking CD66b, and changes

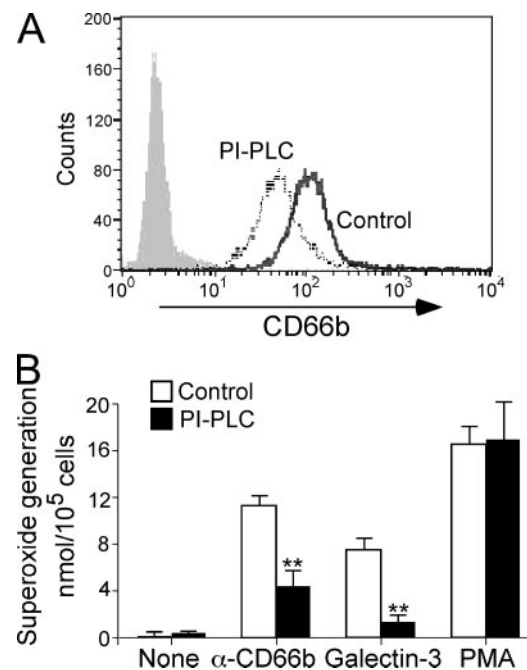


FIGURE 5. Human galectin-3 uses GPI-anchored CD66b to activate eosinophils. *A*, Eosinophils were preincubated with PI-PLC (1U) (broken line) or medium (control, solid line) for 1 h at 37°C, before flow cytometric analysis to evaluate GPI-anchored CD66b expression. The histogram of one representative experiment from three independent experiments is shown. *B*, After preincubation with PI-PLC or medium (control), cells were incubated with medium alone (None), immobilized anti-CD66b (80H30, 1 μ g/ml), soluble galectin-3 (10 μ g/ml), or PMA (1 ng/ml). Superoxide generation was measured (see Fig. 2*B*). Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with cells stimulated with anti-CD66b Abs or galectin-3 without PI-PLC pretreatment.

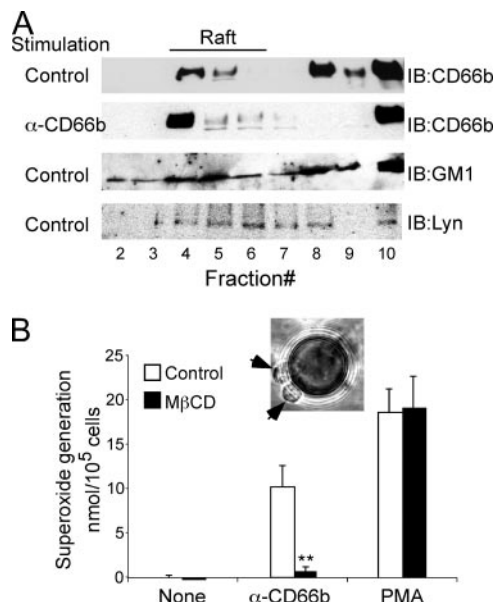


FIGURE 6. CD66b is constitutively localized in lipid rafts and its localization is essential for eosinophil activation through CD66b. *A*, Eosinophils (2×10^7 cells) were first incubated with anti-CD66b or control mouse IgG1, and then CD66b was cross-linked with Fc-specific goat anti-mouse F(ab')₂. Activation was stopped with cold PBS, and cells were lysed. Lysates were applied to a sucrose gradient followed by ultracentrifugation. Each fraction (numbered 2–10 from top to bottom fractions) was analyzed by using Western blots with anti-CD66b, anti-GM1, or anti-Lyn; the anti-GM1 and anti-Lyn were used as lipid raft fraction markers. The Western blots of one representative experiment from three independent experiments are shown. *B*, After pretreatment with MβCD, eosinophils were stimulated as described in Fig. 2. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with cells stimulated with immobilized anti-CD66b and no pretreatment with MβCD. *Inset* photomicrograph shows cholesterol-depleted eosinophils after incubation with anti-CD66b coated beads (details in *Materials and Methods*). Arrowheads show eosinophils ($\times 400$).

in the phosphorylation levels of Hck, Fgr, Lyn, and Lck were analyzed by immunoprecipitation and by immunoblot with anti-phosphotyrosine mAb (47). Increased phosphorylation of Hck was observed within 1 min after CD66b cross-linking; it reached a peak at 5 min and decreased gradually thereafter (Fig. 7*A*). These results were reproducible among three different eosinophils donors and showed a $193 \pm 30\%$ increase in Hck phosphorylation at 30 min (mean $\pm$ SEM, $p < 0.05$, $n = 3$). An *in vitro* kinase assay using an exogenous substrate, MBP, showed that the enzymatic activity of Hck also increased within 10 min of CD66b cross-linking (Fig. 7*B*). Similar experiments were repeated with Fgr, Lyn, and Lck, but we did not detect increased tyrosine phosphorylation or increased kinase activity of these molecules after CD66b cross-linking (data not shown). To examine the role of Hck activation in eosinophil effector functions, eosinophils were pretreated with the Src kinase specific inhibitor, PP1. Both superoxide generation and EDN release induced by CD66b cross-linking were abolished by PP1 treatment, but superoxide generation and EDN release induced by PMA were not affected by PP1 (Fig. 7, *C* and *D*). Thus, Hck likely plays a key role in eosinophil activation mediated by CD66b cross-linking.

CD66b is physically associated with a β_2 integrin, CD11b, and induces clustering of this molecule

Integrin adhesion molecules, such as $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) and $\alpha_4\beta_1$ (VLA-4, CD49d/CD29) are likely important for eosinophil recruitment into inflammation sites in allergic diseases. These integrins, particularly β_2 integrin, can regulate eosinophil degranulation in response to stimulation with various physiologic secretagogues (48, 49). Furthermore, ligation of CD11b by mAb, without other exogenous stimuli, triggers eosinophil activation (50). Because CD66b cross-linking induced tight adhesion of eosinophils and their effector functions (Fig. 3), we investigated the potential interaction between CD66b and CD11b. Eosinophils incubated with anti-CD66b mAb and F(ab')₂ of goat anti-mouse IgG showed distinct clustering and capping of CD11b (Fig. 8*A*). In

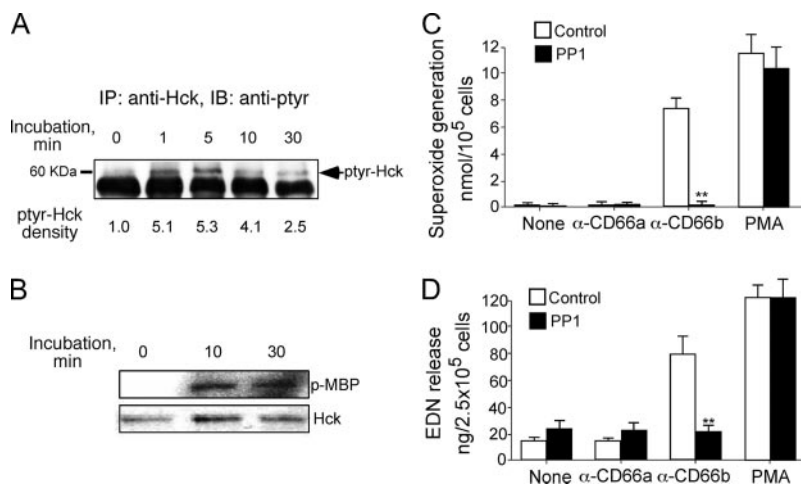


FIGURE 7. Hck plays a key role in eosinophil activation through CD66b. *A*, Eosinophils ((5×10^6) /sample) were incubated with anti-CD66b Ab (80H30, 10 μ g/ml) and cross-linked with F(ab')₂ of Fc-specific goat anti-mouse IgG for the times shown. After immunoprecipitation with anti-Hck Ab, the Hck phosphorylation level was detected by Western blotting with anti-phosphotyrosine 4G10 Ab (anti-ptyr). The Western blot of one representative experiment from three independent experiments is shown. The densitometric analyses of Hck phosphorylation normalized to time 0 as 1.0 are listed below the bands. *B*, Eosinophils were stimulated by immobilized anti-CD66b on the coated plates for 10 or 30 min at 37°C. Cell lysates were immunoprecipitated with anti-Hck; the immunoprecipitated complexes were used in an *in vitro* kinase assay; the eluates were separated by 10% SDS-PAGE; and the dried gel was exposed to film. MBP was used as an exogenous substrate for the *in vitro* kinase assay. The blot of one representative experiment from three independent experiments is shown. *C* and *D*, Eosinophils were pretreated with a Src kinase-specific inhibitor, PP1 (10 μ M) or with medium only (control) for 30 min at 37°C. Eosinophils were then incubated with medium alone (None), immobilized anti-CD66a (GM8G5), immobilized anti-CD66b (80H30), or PMA. Results show means $\pm$ SEM, $n = 3$. **, $p < 0.01$ compared with cells stimulated by cells stimulated by immobilized anti-CD66b Ab without PP1 pretreatment.

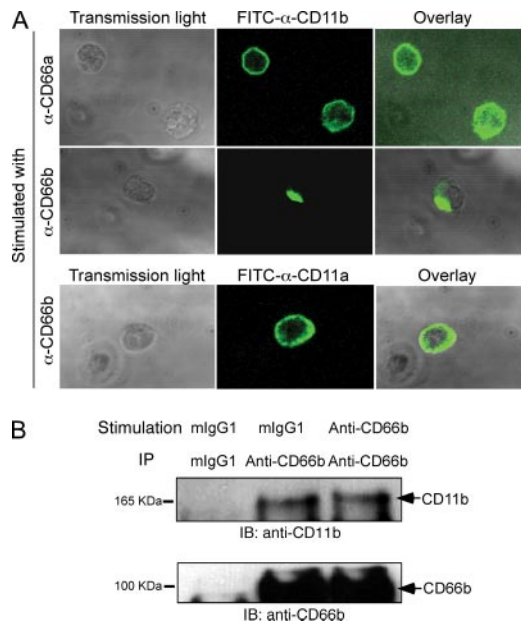


FIGURE 8. CD66b is physically associated with and induces clustering of a β_2 integrin, CD11b. *A*, CD66a or CD66b were cross-linked with mouse anti-CD66a mAb (GM8G5, IgG1) or anti-CD66b mAb (80H30, IgG1) (10 μ g/ml) and F(ab')₂ of goat anti-mouse IgG (50 μ g/ml) for 30 min at 37°C. Cellular localization of CD11a and CD11b was detected by staining with FITC-anti-CD11a or FITC-anti-CD11b mAb. Transmission light and fluorescence images were taken with a Zeiss LSM 510 confocal laser scanning microscope ($\times 400$). The results of one representative experiment from three independent experiments are shown. *B*, Freshly isolated eosinophils (5×10^6 cells) were incubated with immobilized anti-CD66b mAb (80H30, IgG1) or isotype-matched control mouse IgG1 Ab. Lysate from each group was immunoprecipitated with anti-CD66b mAb or control mouse IgG1 Ab. Subsequently, CD11b and CD66b were detected with anti-CD11b Ab and anti-CD66b Ab by using Western blot, respectively. The Western blots of one representative experiment from three independent experiments are shown.

contrast, eosinophils incubated with anti-CD66a mAb and F(ab')₂ of goat anti-mouse IgG showed that CD11b was distributed evenly over the cells' surfaces. Anti-CD66b mAb did not induce clustering of another β_2 integrin molecule, CD11a, suggesting a specific interaction between CD66b and CD11b.

To investigate their interactions at a molecular level, both unstimulated (i.e., incubated with control mouse IgG) and activated (i.e., incubated with anti-CD66b mAb) eosinophils were lysed and immunoprecipitated with anti-CD66b mAb; the molecules associated with CD66b were depicted by Western blot analysis. In unstimulated eosinophils, both CD11b and CD66b were detectable in the immunoprecipitants with anti-CD66b mAb (Fig. 8*B*). In stimulated eosinophils, the amount of CD11b molecules in the CD66b immunoprecipitants did not change, suggesting that CD11b is constitutively associated with CD66b without cellular activation.

Discussion

Herein, we characterized the functions of CD66b in human eosinophils. The CD66b glycoprotein is highly expressed on the eosinophils' surface, and cross-linking the CD66b with specific mAb or a ligand, galectin-3, induces tight cellular adhesion, superoxide generation, and degranulation. The magnitudes of the eosinophil effector functions induced by cross-linking the CD66b molecule were comparable to those induced by an authentic agonist, IL-5. Furthermore, independent of the eosinophil activation induced by CD66b cross-linking, CD66b constitutively localizes in lipid rafts.

Cholesterol depletion and disruption of lipid rafts blocks eosinophil activation and adhesion mediated by CD66b. Cross-linking CD66b activates a Src kinase, Hck, and the cellular functions are completely abrogated with the Src kinase specific inhibitor, PP1. These observations suggest a model wherein lipid rafts, as a member structure, and Hck, as a signaling molecule, are essential for eosinophil activation mediated by CD66b cross-linking.

The CD66b glycoprotein is GPI-anchored to the cell membrane and is constitutively localized in lipid rafts; it does not have cytoplasmic domains and may be unable to interact directly with intracellular signaling molecules. However, Hck can also be localized in lipid rafts (51). Thus, CD66b could directly interact with Hck through lipid-lipid interactions between the GPI of CD66b and the palmitoylate of Hck. In addition, CD66b cross-linking could induce an accumulation of microdomain lipid rafts, resulting in the formation of macromolecules and inducing an accumulation and activation of signal-related molecules, including Hck. Finally, other transmembrane proteins might interact with CD66b and directly activate the intracellular signaling pathways. For example, integrins can function as adaptors in GPI-anchored protein-mediated cell activation (52). Our observations suggest that CD11b (i.e., α -chain of a β_2 integrin) is constitutively associated with CD66b and that they form a macromolecular complex after CD66b ligation (Fig. 8*A*). Furthermore, cross-linking of CD11b, without any other exogenous stimulus, leads to tyrosine phosphorylation of several intracellular proteins and induces eosinophil degranulation (50). Although another CEA family member, CD66a, is highly expressed on the eosinophils' surface, cross-linking of CD66a did not activate eosinophils. Unlike CD66b, CD66a has conventional transmembrane anchors as well as a 74-aa cytoplasmic domain (10). The unique molecular features of CD66b, including the GPI anchor, localization to lipid rafts, and interactions with CD11b, may explain this molecule's potent ability to trigger eosinophil functions. Thus, several nonmutually exclusive hypotheses are possible for CD66b-mediated mechanisms involved in the activation and effector functions of eosinophils. Future experiments that use molecular biological approaches, such as small interfering RNA, will be necessary to elucidate the precise mechanisms for CD66b-mediated eosinophil activation.

The unique characteristics of CD66b molecules, which are highly glycosylated, GPI-anchored, and localized in lipid rafts, may suggest two biological insights for innate immunity and molecular interactions. First, the highly glycosylated CD66b may be a receptor for lectin-type molecules generated by other inflammatory cells or be an innate receptor for the products of microorganisms. For example, activated macrophages and mast cells produce galectin-3 during inflammation (34, 53), and galectin-3 induces the production and release of proinflammatory mediators by eosinophils, likely through CD66b (Fig. 4). Galectin-3 may also decorate the cell surface carbohydrate molecules on microorganisms; this would allow eosinophils to recognize potential targets (54, 55). Furthermore, CEA family molecules directly recognize the carbohydrate cell surface structure of microorganisms (56). Second, CD66b may physically interact with other membrane molecules and form macromolecular complexes of transmembrane proteins and signaling molecules within the lipid rafts. These macromolecular complexes may then act as a nucleus to generate intracellular activation signals and to induce the effector functions of eosinophils. Indeed, the CEA family molecules, including CD66b, induce homophilic and heterophilic receptor aggregation and are considered to be part of a molecular receptor complex on the cell surface (10). In eosinophils, one of the components of these macromolecular complexes is likely CD11b (Fig. 8). In the future, the isolation and characterization of the molecular complexes containing

CD66b on the surface of human eosinophils will be particularly important. These analyses should include studies of signal transduction pathways used by these receptor complexes and should search for additional natural ligands.

Despite an extensive search, the genes encoding the CD66b protein have not been identified in mice or rats (57). In contrast, CD66b homologues have been identified in two primate species, baboon and African green monkey (58). Thus, CEA family molecules are probably undergoing rapid evolution; there must be strong selection pressures on these molecules, such as exposures to microorganisms and parasites. Mouse eosinophils are relatively resistant to various activation stimuli (59) and eosinophil degranulation is rare in mouse models of eosinophilic inflammation (60). Could this lack of vigorous activation of mouse eosinophils compared with human eosinophils be due to the lack of CD66b? Thus, it will be important to generate mice transgenic for human CD66b, to examine the functions of eosinophils from these mice in vivo and in vitro, and to evaluate the roles of eosinophils more critically in immunity, host immunity and disease pathology.

In conclusion, the cross-linking of CD66b by specific Abs or a ligand, galectin-3, activates a Src family kinase, Hck, and triggers tight cellular adhesion and production and release of proinflammatory mediators in human eosinophils. Furthermore, CD66b is localized in lipid rafts and is physically associated with an adhesion molecule, CD11b; upon cross-linking, CD66b induces a marked clustering of CD11b molecules. CD66b may be a versatile positive regulator of eosinophil effector functions through recognition of their environmental ligands and/or physical interactions with other membrane molecules. Future studies will identify the importance of CD66b during inflammation, host immunity, and allergic diseases and will elucidate potential therapeutic approaches to target this molecule.

Acknowledgments

We thank Diane Squillace for technical assistance, Cheryl R. Adolphson for editorial assistance, and LuRaye S. Eischens for secretarial help.

Disclosures

The authors have no financial conflict of interest.

References

- Kita, H., C. R. Adolphson, and G. J. Gleich. 2003. Biology of Eosinophils. In *Middleton's Allergy Principles and Practice*, 6th Ed. N. F. Adkinson, J. W. Yunginger, W. W. Busse, B. S. Bochner, S. T. Holgate, and F. E. R. Simons, eds. Mosby-Year Book, Inc., Philadelphia, PA, pp. 305–332.
- Horie, S., G. J. Gleich, and H. Kita. 1996. Cytokines directly induce degranulation and superoxide production from human eosinophils. *J. Allergy Clin. Immunol.* 98: 371–381.
- Abu-Ghazaleh, R. I., T. Fujisawa, J. Mestecky, R. A. Kyle, and G. J. Gleich. 1989. IgA-induced eosinophil degranulation. *J. Immunol.* 142: 2393–2400.
- Kroegel, C., T. Yukawa, G. Dent, P. Venge, K. F. Chung, and P. J. Barnes. 1989. Stimulation of degranulation from human eosinophils by platelet-activating factor. *J. Immunol.* 142: 3518–3526.
- Tedla, N., C. Bandeira-Melo, P. Tassinari, D. E. Sloane, M. Samplaski, D. Cosman, L. Borges, P. F. Weller, and J. P. Arm. 2003. Activation of human eosinophils through leukocyte immunoglobulin-like receptor 7. *Proc. Natl. Acad. Sci. USA* 100: 1174–1179.
- Nutku, E., H. Aizawa, S. A. Hudson, and B. S. Bochner. 2003. Ligand of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 101: 5014–5020.
- Munitz, A., I. Bachelet, R. Eliashar, A. Moretta, L. Moretta, and F. Levi-Schaffer. 2006. The inhibitory receptor IRP60 (CD300a) suppresses the effects of IL-5, GM-CSF, and eotaxin on human peripheral blood eosinophils. *Blood* 107: 1996–2003.
- Elsner, J., R. Hochstetter, K. Spiekermann, and A. Kapp. 1996. Surface and mRNA expression of the CD52 antigen by human eosinophils but not by neutrophils. *Blood* 88: 4684–4693.
- Munitz, A., I. Bachelet, R. Eliashar, M. Khodoun, F. D. Finkelman, M. E. Rothenberg, and F. Levi-Schaffer. 2006. CD48 is an allergen and IL-3-induced activation molecule on eosinophils. *J. Immunol.* 177: 77–83.
- Hammarstrom, S. 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin. Cancer Biol.* 9: 67–81.
- Torsteinsdottir, I., N. G. Arvidson, R. Hallgren, and L. Hakansson. 1999. Enhanced expression of integrins and CD66b on peripheral blood neutrophils and eosinophils in patients with rheumatoid arthritis, and the effect of glucocorticoids. *Scand. J. Immunol.* 50: 433–439.
- Zhao, L., S. Xu, G. Fjaertoft, K. Pauksen, L. Hakansson, and P. Venge. 2004. An enzyme-linked immunosorbent assay for human carcinoembryonic antigen-related cell adhesion molecule 8, a biological marker of granulocyte activities in vivo. *J. Immunol. Methods* 293: 207–214.
- De Haas, M., J. M. Kerst, C. E. van der Schoot, J. Calafat, C. E. Hack, J. H. Nuijens, D. Roos, R. H. van Oers, and A. E. von dem Borne. 1994. Granulocyte colony-stimulating factor administration to healthy volunteers: analysis of the immediate activating effects on circulating neutrophils. *Blood* 84: 3885–3894.
- Eades-Perner, A. M., J. Thompson, H. van der Putten, and W. Zimmermann. 1998. Mice transgenic for the human C6M6 gene express its product, the granulocyte marker CD66b, exclusively in granulocytes. *Blood* 91: 663–672.
- Mawhorter, S. D., D. A. Stephany, E. A. Ottesen, and T. B. Nutman. 1996. Identification of surface molecules associated with physiologic activation of eosinophils: application of whole-blood flow cytometry to eosinophils. *J. Immunol.* 156: 4851–4858.
- Mengeler, H. J., T. Maikoe, J. A. Raaijmakers, J. W. Lammers, and L. Koenderman. 1995. Cognate interaction between human lymphocytes and eosinophils is mediated by β 2-integrins and very late antigen-4. *J. Lab. Clin. Med.* 126: 261–268.
- Cheng, P. C., M. L. Dykstra, R. N. Mitchell, and S. K. Pierce. 1999. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* 190: 1549–1560.
- Stoddart, A., M. L. Dykstra, B. K. Brown, W. Song, S. K. Pierce, and F. M. Brodsky. 2002. Lipid rafts unite signaling cascades with clathrin to regulate BCR internalization. *Immunity* 17: 451–462.
- Gaus, K., E. Chklovskaya, B. Fazekas de St. Groth, W. Jessup, and T. Harder. 2005. Condensation of the plasma membrane at the site of T lymphocyte activation. *J. Cell Biol.* 171: 121–131.
- Harder, T., and K. R. Engelhardt. 2004. Membrane domains in lymphocytes: from lipid rafts to protein scaffolds. *Traffic* 5: 265–275.
- Field, K. A., D. Holowka, and B. Baird. 1997. Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J. Biol. Chem.* 272: 4276–4280.
- Anderson, H. A., E. M. Hiltbold, and P. A. Roche. 2000. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat. Immunol.* 1: 156–162.
- Poloso, N. J., A. Muntasell, and P. A. Roche. 2004. MHC class II molecules traffic into lipid rafts during intracellular transport. *J. Immunol.* 173: 4539–4546.
- Chaturvedi, A., Z. Siddiqui, F. Bayiroglu, and K. V. Rao. 2002. A GPI-linked isoform of the IgD receptor regulates resting B cell activation. *Nat. Immunol.* 3: 951–957.
- Liang, F., R. Z. Qi, and C. F. Chang. 2001. Signalling of GPI-anchored CD157 via focal adhesion kinase in MCA102 fibroblasts. *FEBS Lett.* 506: 207–210.
- Suzuki, T., N. Kiyokawa, T. Taguchi, T. Sekino, Y. U. Katagiri, and J. Fujimoto. 2001. CD24 induces apoptosis in human B cells via the glycolipid-enriched membrane domains/rafts-mediated signaling system. *J. Immunol.* 166: 5567–5577.
- Itzhaky, D., N. Raz, and N. Hollander. 1998. The glycosylphosphatidylinositol-anchored form and the transmembrane form of CD58 associate with protein kinases. *J. Immunol.* 160: 4361–4366.
- Ide, M., D. Weiler, H. Kita, and G. J. Gleich. 1994. Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J. Immunol. Methods* 168: 187–196.
- Zhu X., K. J. Hamann, N. M. Muñoz, N. Rubio, D. Mayer, A. Hernreiter, and A. R. Leff. 1998. Intracellular expression of Fc γ RIII (CD16) and its mobilization by chemoattractants in human eosinophils. *J. Immunol.* 161: 2574–2579.
- Legler, D. F., O. Micheau, M. A. Doucey, J. Tschopp, and C. Bron. 2003. Recruitment of TNF receptor 1 to lipid rafts is essential for TNF α -mediated NF- κ B activation. *Immunity* 18: 655–664.
- Thompson, J. A., F. Grunert, and W. Zimmermann. 1991. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J. Clin. Lab. Anal.* 5: 344–366.
- Feuk-Lagerstedt, E., E. T. Jordan, H. Leffler, C. Dahlgren, and A. Karlsson. 1999. Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils. *J. Immunol.* 163: 5592–5598.
- Saada, A., F. Reichert, and S. Rotshenker. 1996. Granulocyte macrophage colony stimulating factor produced in lesioned peripheral nerves induces the up-regulation of cell surface expression of MAC-2 by macrophages and Schwann cells. *J. Cell Biol.* 133: 159–167.
- Frigeri, L. G., and F. T. Liu. 1992. Surface expression of functional IgE binding protein, an endogenous lectin, on mast cells and macrophages. *J. Immunol.* 148: 861–867.
- Zuberi, R. I., D. K. Hsu, O. Kalayci, H. Y. Chen, H. K. Sheldon, L. Yu, J. R. Appar, T. Kawakami, C. M. Lilly, and F. T. Liu. 2004. Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma. *Am. J. Pathol.* 165: 2045–2053.
- Thorpe, E. B., and T. M. Gallagher. 2004. Requirements for CEACAMs and cholesterol during murine coronavirus cell entry. *J. Virol.* 78: 2682–2692.
- Young, R. M., D. Holowka, and B. Baird. 2003. A lipid raft environment enhances Lyn kinase activity by protecting the active site tyrosine from dephosphorylation. *J. Biol. Chem.* 278: 20746–20752.
- Lacour, S., A. Hammann, S. Graziade, D. Lagadic-Gossman, A. Athias, O. Sergeant, G. Laurent, P. Gambert, E. Solary, and M. T. Dimanche-Boitrel.

2004. Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells. *Cancer Res.* 64: 3593–3598.
39. Marwali, M. R., J. Rey-Ladino, L. Dreolini, D. Shaw, and F. Takei. 2003. Membrane cholesterol regulates LFA-1 function and lipid raft heterogeneity. *Blood* 102: 215–222.
40. Nguyen, D. H., and D. Taub. 2002. Cholesterol is essential for macrophage inflammatory protein 1 β binding and conformational integrity of CC chemokine receptor 5. *Blood* 99: 4298–4306.
41. Davy, A., N. W. Gale, E. W. Murray, R. A. Klinghoffer, P. Soriano, C. Feuerstein, and S. M. Robbins. 1999. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. *Genes Dev.* 13: 3125–3135.
42. Bohuslav, J., V. Horejsi, C. Hansmann, J. Stockl, U. H. Weidle, O. Majdic, I. Bartke, W. Knapp, and H. Stockinger. 1995. Urokinase plasminogen activator receptor, β 2-integrins, and Src-kinases within a single receptor complex of human monocytes. *J. Exp. Med.* 181: 1381–1390.
43. Brown, D. 1993. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. *Curr. Opin. Immunol.* 5: 349–354.
44. Stefanova, I., V. Horejsi, I. J. Ansotegui, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 254: 1016–1019.
45. Stafford, S., C. Lowell, S. Sur, and R. Alam. 2002. Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation. *J. Immunol.* 168: 1978–1983.
46. Lynch, O. T., M. A. Giembycz, I. Daniels, P. J. Barnes, and M. A. Lindsay. 2000. Pleiotropic role of lyn kinase in leukotriene B₄-induced eosinophil activation. *Blood* 95: 3541–3547.
47. Barlic, J., J. D. Andrews, A. A. Kelvin, S. E. Bosinger, M. E. DeVries, L. Xu, T. Dobransky, R. D. Feldman, S. S. Ferguson, and D. J. Kelvin. 2000. Regulation of tyrosine kinase activation and granule release through β -arrestin by CXCR1. *Nat. Immunol.* 1: 227–233.
48. Horie, S., and H. Kita. 1994. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. *J. Immunol.* 152: 5457–5467.
49. Nagata, M., J. B. Sedgwick, M. E. Bates, H. Kita, and W. W. Busse. 1995. Eosinophil adhesion to vascular cell adhesion molecule-1 activates superoxide anion generation. *J. Immunol.* 155: 2194–2202.
50. Kato, M., R. T. Abraham, S. Okada, and H. Kita. 1998. Ligation of the β 2 integrin triggers activation and degranulation of human eosinophils. *Am. J. Respir. Cell Mol. Biol.* 18: 675–686.
51. Robbins, S. M., N. A. Quintrell, and J. M. Bishop. 1995. Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Mol. Cell. Biol.* 15: 3507–3515.
52. Stockinger, H. 1997. Interaction of GPI-anchored cell surface proteins and complement receptor type 3. *Exp. Clin. Immunogenet.* 14: 5–10.
53. Liu, F. T., D. K. Hsu, R. I. Zuberi, I. Kuwabara, E. Y. Chi, and W. R. Henderson, Jr. 1995. Expression and function of galectin-3, a β -galactoside-binding lectin, in human monocytes and macrophages. *Am. J. Pathol.* 147: 1016–1028.
54. Fradin, C., D. Poulain, and T. Jouault. 2000. β -1,2-Linked oligomannosides from *Candida albicans* bind to a 32-kilodalton macrophage membrane protein homologous to the mammalian lectin galectin-3. *Infect. Immun.* 68: 4391–4398.
55. Van den Berg, T. K., H. Honing, N. Franke, A. van Remoortere, W. E. Schiphorst, F. T. Liu, A. M. Deelder, R. D. Cummings, C. H. Hokke, and I. van Die. 2004. LacdiNAc-glycans constitute a parasite pattern for galectin-3-mediated immune recognition. *J. Immunol.* 173: 1902–1907.
56. Sauter, S. L., S. M. Rutherford, C. Wagener, J. E. Shively, and S. A. Hefta. 1993. Identification of the specific oligosaccharide sites recognized by type 1 fimbriae from *Escherichia coli* on nonspecific cross-reacting antigen, a CD66 cluster granulocyte glycoprotein. *J. Biol. Chem.* 268: 15510–15516.
57. Beauchemin, N., P. Draber, G. Dveksler, P. Gold, S. Gray-Owen, F. Grunert, S. Hammarstrom, K. V. Holmes, A. Karlsson, M. Kuroki, et al. 1999. Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp. Cell Res.* 252: 243–249.
58. Zhou, G. Q., Y. Zhang, and S. Hammarstrom. 2001. The carcinoembryonic antigen (CEA) gene family in non-human primates. *Gene* 264: 105–112.
59. Kobayashi, T., K. Iijima, and H. Kita. 2003. Marked airway eosinophilia prevents development of airway hyper-responsiveness during an allergic response in IL-5 transgenic mice. *J. Immunol.* 170: 5756–5763.
60. Malm-Erfjelt, M., C. G. Persson, and J. S. Erfjelt. 2001. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 24: 352–359.