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Research Article

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Fcγ Receptor IIIb Enhances Fcγ Receptor IIa Function in an Oxidant-dependent and Allele-sensitive Manner

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Abstract

Two classes of receptors for IgG, FcγRIIa and FcγRIIIb, both of which exist in two allelic forms, are expressed on human neutrophils. Neutrophils from normal donors, homozygous for the different allelic phenotypes of FcγRIIIb, have significantly different levels of Fcγ receptor–mediated phagocytosis of IgG-opsonized erythrocytes (EA). However, the observation that FcγRIIIb mediates phagocytosis of specific mAb-targeted erythrocytes poorly suggests that this receptor may influence EA internalization by FcγRIIa in an allele-sensitive fashion. Donors homozygous for the NA1 allele of FcγRIIIb showed greater activation of FcγRIIa after FcγRIIIb cross-linking than donors homozygous for the NA2 allele of FcγRIIIb. This increase in receptor-specific internalization reflects both an increase in ligand binding by FcγRIIa and an increase in internalization efficiency of targets bound. Activation of FcγRIIa by FcγRIIIb is transferable by supernatants from activated cells and is blocked by inhibitors of reactive oxygen species and the H_2O_2–myeloperoxidase–chloride system and by serine protease inhibitors. Thus, cross-linking of FcγRIIIb, which leads to neutrophil degranulation and the generation of reactive oxygen intermediates, in turn alters FcγRIIa avidity and efficiency. These oxidant-mediated changes in FcγRIIa function provide a novel mechanism for receptors to collaborate in both an autocrine and paracrine fashion. The allele sensitivity of these effects suggests that Fcγ receptor polymorphisms may be inherited disease susceptibility factors in host defense against infection and in the development of autoimmunity. (*J. Clin. Invest.* 1995. 95:2877–2885.) Key words: phagocytosis • proteases • Fcγ receptors • oxidants • immunoglobulin G

Introduction

Receptors for the Fc region of IgG, FcγR,[1] provide the crucial link between humoral immunity and the IgG-triggered effector functions by inflammatory cells. Three families of human FcγR (FcγRI, FcγRII, and FcγRIII) have been identified. Within these FcγR families, multiple distinct genes and alternative splicing variants lead to a variety of receptor isoforms that have differences in structure and distinct functional capacities (for reviews see references 1–4). In addition to this diversity, two receptor isoforms found on phagocytic cells have codominantly expressed, allelic polymorphisms—the NA1 and NA2 alleles of FcγRIIIb and the HR–LR alleles of FcγRIIa—which influence FcγR function.

Neutrophils from normal donors, homozygous for the two different allelic phenotypes of FcγRIIIb, have significantly different levels of Fcγ receptor–mediated phagocytosis independent of the allelic phenotype of the second type of Fcγ receptor on neutrophils, FcγRIIa (5). Because experiments using antireceptor mAb-targeted erythrocytes indicate that FcγRIIIb mediates phagocytosis poorly (6), the importance of FcγRIIIb NA phenotype in quantitative EA phagocytosis implies that a model of "one receptor–one function" is inadequate and that FcγRIIIb may collaborate interactively with other receptors. Among the different paradigms of receptor cooperation, there may be physical association of receptors that enhances binding and signaling and interaction independent of physical association. For example, the IL-2 receptor can assemble multiple distinct receptor chains into a complex, and different combinations of α, β, and γ chains have distinct binding and signaling capacities (7, 8). Similarly, TGFβ signals through a heteromeric complex composed of receptor I and receptor II, but a separate nonsignaling TGFβ-binding membrane protein (TGFβ receptor type III) captures the ligand and delivers it to the signaling complex (9). In contrast to these systems, signaling through the T cell receptor upregulates the avidity of CD2 for CD58 (LFA-1) through inside-out signaling requiring specific domains of the CD2 cytoplasmic tail (10, 11). Similarly, activation of β_2 and β_3 integrins as a consequence of receptor phosphorylation is induced by cross-linking other classes of receptors (12–14). Even quantitative surface receptor expression of one receptor may be modified by cross-linking other receptors (15, 16).

Given these precedents for receptor interactions and the suggestion that cross-linking of FcγRIIIb with antireceptor mAb $F(ab')_2$ might augment the phagocytic activity of FcγRIIa that has been engaged independently (17), we sought evidence that FcγRIIIb can augment FcγRIIa-specific function and determined the basis for this effect. Taking advantage of the NA1–NA2 polymorphism as a model system to demonstrate that quantitative augmentation varies with differences in the primary structure of FcγRIIIb, we have shown that cross-linking of

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[1] *Abbreviations used in this paper:* AI, attachment index; E, erythrocytes; EA, IgG-sensitized bovine erythrocytes; E_B biotinylated erythrocytes; E_{AB}, streptavidin coated E_B; E-hIgG2, E coated with human IgG2; E-IV.3, E coated with IV.3 Fab; FcγR, receptors for Fc portion of IgG in human cells; FcγRIIa, 40-kD receptor on human neutrophils and monocytes for Fc portion of IgG; FcγRIIIb, 50–78-kD receptor on human neutrophils for Fc portion of IgG; PABA, *p*-aminobenzamidine; PE, phycoerythrin; PI, phagocytic index; TLCK, N^{α}-tosyl-L-lysyl-chloromethyl ketone.

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FcγRIIIb directly leads to activation of FcγRIIa, a property that can be transferred in supernatants to other neutrophils and that can be blocked by inhibitors of reactive oxygen species and serine proteases. Through coordinate degranulation and generation of reactive oxygen species, FcγRIIIb elicits a cell program that can rapidly activate receptors on both the same and adjacent cells. This novel, oxidant-dependent interaction provides an efficient mechanism for amplification of Fcγ receptor function in neutrophils.

Methods

Subjects. Peripheral blood was collected from 32 disease-free volunteers who ranged in age from 20 to 56 yr (34±8 yr, mean±SD). Protocols for these studies were approved by the Institutional Committee on Human Rights in Research.

Determination of FcγRIIIb and FcγRIIa alleles. Determination of FcγRIIIb alleles, NA1 and NA2, was performed by leukoagglutination as described previously (5). The assignment of NA type was confirmed by immunoprecipitation and flow cytometry with mAbs CLB-FcR gran 1, CLB-gran 11, and GRM1 (18, 19). Phenotyping of donors for the LR–HR alleles of FcγRIIa was performed by quantitative flow cytometry using mAbs 41.H16 and IV.3 as described previously (20, 21). Phenotypic assignment was corroborated by anti-CD3 mitogenesis assays.

Reagents. HBSS, RPMI-1640, and IgG-free FCS were from GIBCO Laboratories (Grand Island, NY). FCS was heat inactivated at 56°C for 60 min. Phorbol dibutyrate (PDBu), catalase, superoxide dismutase (SOD), aminotriazole, *p*-aminobenzamidine (PABA), sodium azide, and N^{α}-tosyl-L-lysyl-chloromethyl ketone (TLCK) were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfo-NHS-biotin, NHS-LC-biotin, and streptavidin were obtained from Pierce Chemical Co. (Rockford, IL).

Anti-FcγR mAbs IV.3 (anti-FcγRII, CD32 [22]), and 3G8 (anti-FcγRIII, CD16 [23]) IgG, Fab fragments, or F(ab′)$_2$ were purchased from Medarex, Inc. (Annandale, NJ). Anti-CD16 mAb Leu11b (IgM) and anti-CD35 mAb E11 F(ab′)$_2$ were obtained form Becton Dickinson & Co. (Mountain View, CA) and Research Diagnostics Inc. (Flanders, NJ), respectively. Anti-CD16 mAb 286.5 (24) was generously provided by Dr. Howard B. Fleit (Stony Brook, NY), and F(ab′)$_2$ fragments were prepared by digestion with ficin according to the manufacturer's specifications (Pierce Chemical Co.). Silver stain analysis of SDS-PAGE gels of all Fab fragments and F(ab′)$_2$ preparations indicated that there was no intact IgG. Purified human IgG2 myeloma proteins (hIgG2) were obtained from The Binding Site, Inc. (San Diego, CA). Anti-CD11b/CD18 mAb IB4 (IgG2a, anti-CD18) and IB4 F(ab′)$_2$ were generously provided by Irene Graham (Washington University, St. Louis, MO) (25), and mAb MN41 (IgG1, anti-CD11b) was a gift from Jill Buyon (Hospital for Joint Diseases, New York) (26). Murine IgG1, isotype control for the flow cytometry studies, was obtained from Sigma Chemical Co. Phycoerythrin (PE)-conjugated goat anti-mouse IgG F(ab′)$_2$ and FITC-conjugated rabbit anti-human IgG F(ab′)$_2$ was purchased from Tago Immunochemicals (Burlingame, CA).

Preparation of cells. Fresh anticoagulated human peripheral blood was separated by centrifugation through a discontinuous two step Ficoll-Hypaque gradient (5). Neutrophils (PMNs) were isolated from the lower interface and washed with HBSS. Contaminating erythrocytes were lysed with hypotonic saline (0.02% NaCl) for 20 s, followed by 0.16% NaCl and a final wash with HBSS. After final washes, PMN were resuspended to 5×10^6 cells/ml.

Preparation of erythrocytes. Erythrocytes were coupled to IV.3 Fab (anti-FcγRII mAb) or human IgG2 myeloma protein by a biotin-avidin technique, as described previously (21, 27). To prepare biotinylated E (E_B), 0.5 ml of E (1×10^9 cells/ml) were incubated with sulfo-NHS-biotin (500 μg/ml) for 20 min at 4°C, followed by three washes. E_B at 1×10^9/ml were incubated with an equal volume of streptavidin (250 μg/ml) for 30 min at 4°C. The streptavidin coated E_B (E_{BA}) were then washed and resuspended to 1×10^9 E/ml for immediate use. IV.3 Fab and human IgG2 were biotinylated with NHS-LC-biotin (0.01 mg biotin/mg protein) for 60 min at room temperature. To bind the biotinylated mAb to the E_{BA}, E_{BA} (12.5 μl at 1×10^9/ml) were combined with 5 μl of biotinylated protein (0.01–5 μg) for 45 min (27). After three washes, the anti-FcγRII coated E_{BA} (E-IV.3) and the hIgG2 coated E_{BA} (E-hIgG2) were then resuspended in 125 μl (1×10^8 E/ml) and used immediately. The density of opsonization of E-IV.3 and E-hIgG2 was standardized with flow cytometry for all experiments as described previously (21, 27). To assure maximal levels of internalization by neutrophils, densely opsonized E-IV.3 and E-hIgG2 (as determined by immunofluorescent flow cytometry) were used in the assays of phagocytosis.

Assay of phagocytosis. Quantitation of phagocytosis by PMN was performed as described previously (5, 21). Briefly, cells were resuspended in RPMI at 5×10^6 cells/ml. For FcγRIIIb-induced activation, the PMN were preincubated for 5 min with 3G8 F(ab′)$_2$ (10 μg/ml), which remained present throughout the assay of phagocytosis. Saturating concentrations of all anti-CD16, -CD35, or -CD18 F(ab′)$_2$ fragments (as determined by flow cytometry) were used to cross-link PMN surface receptors in all experiments and were present throughout the assay. In the experiments with PDBu-treated PMN, PDBu (15 ng/ml) was added simultaneously with the phagocytic particle. For inhibition experiments the following reagents were added before the stimulus: SOD (150 μg/ml), catalase (31,000 U/ml), azide (0.1%), PABA (10 mM), or TLCK (0.5 mM). Duplicate samples without inhibitors were the controls for each experimental condition.

To assess internalization of E target particles, PMN (100 μl) were combined with E-IV.3 or E-hIgG2 (125 μl). Because PMN from donors homozygous for the HR allele of FcγRIIa have minimal binding and internalization of E-hIgG2 (21), they were excluded from studies using this probe. The leukocyte–erythrocyte mixtures (ratios of 1:25) were centrifuged at 44 *g* for 3 min and then incubated at 37°C for 15 min to allow for maximum internalization. After hypotonic lysis of noninternalized E, phagocytosis was quantitated by light microscopy. At least 400 cells per slide were counted in duplicate. The data are expressed as phagocytic index (PI, number of ingested erythrocytes per 100 PMN).

To study the capacity of supernatants from stimulated PMN to activate resting cells, PMN were treated with 3G8 F(ab′)$_2$ or PDBu for 5 min, washed twice, and cultured for 15 min at 37°C in RPMI. Supernatants were collected and combined with fresh PMN and target particles. After incubation for 15 min with or without catalase (31,000 U/ml) phagocytosis was quantitated by light microscopy.

Assay of attachment. To quantitate adherence of E target particles to PMN, cells were prepared and combined as described in the assay of phagocytosis above. After centrifugation at 44 *g* for 3 min, the PMN–erythrocyte mixtures were maintained at room temperature for 10 min and then gently resuspended. Adherence of E to PMN was quantitated by light microscopy. Data are expressed as attachment index (AI, number of adherent or internalized erythrocytes per 100 PMN). Under these conditions, < 1% of phagocytes had internalized E.

To distinguish the effect of activation stimuli on FcγRIIa-mediated internalization from any effect on FcγRIIa-mediated adherence, incubation mixtures with PMN-erythrocyte ratios of 1:1 were used. In preliminary studies, it was determined that under these conditions all erythrocytes were bound to PMN, thus eliminating the potential to increase phagocytosis by increasing adherence. Triplicate tubes were set up, with the first tube used to determine AI and document the absence of free (nonadherent) E by light microscopy. Under these conditions, with fixed and limited adherence, the AI for E-IV.3 and E-hIgG2 were 106±25 attached E/100 PMN and 102±30, respectively. The remaining replicates were incubated with or without activating agents [3G8F(ab′)$_2$ (10 μg/ml)] and/or inhibitors [SOD (150 μg/ml)] at 37°C for 15 min and after lysis of noninternalized E, internalization was quantitated.

Immunofluorescent flow cytometry. Fresh leukocytes (5×10^5 in PBS with 0.1% BSA) were incubated with saturating amounts of specific mAb or isotype controls for 30 min at 4°C. After two washes with cold PBS containing 0.1% BSA, cells were incubated with saturating concentrations of PE-conjugated goat anti-mouse IgG F(ab′)$_2$ for 30 min at 4°C, followed by washing twice with cold PBS/0.1% BSA. E coated with human IgG were stained with FITC-conjugated rabbit anti–human IgG F(ab')$_2$ and E coated with IV.3 Fab were stained with PE-conjugated goat anti–mouse IgG F(ab')$_2$, followed by washes with cold PBS/0.1% BSA.

After staining, cell-associated immunofluorescence was quantitated on a Cytofluorograf IIS (Becton Dickinson and Co., San Jose, CA) with a 2151 computer as described previously (5). For each experiment, the instrument was calibrated with quantitative FITC and PE microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC) to allow for assessment of both absolute and relative levels of immunofluorescence.

Data analysis. For assessment of the relative phagocytic capacity of individuals with different FcγRIIIb alleles, all experiments were performed in a matched-pairs experimental design. Accordingly, each subject homozygous for a given FcγRIIIb allele was studied in comparison to a second subject, homozygous for the other allele and matched for the same phenotype of FcγRIIa (e.g., NA1-LR vs. NA2-LR).

The effects of activating stimuli on FcγR-mediated phagocytosis and attachment are presented as % control [100 × experimental PI (or AI)/control PI(or AI)]. Data are displayed as mean±SEM. The effects of stimuli or inhibitors were compared using a paired *t* test (two-tailed). A probability of 0.05 was used to reject the null hypothesis that there is no difference between the groups or conditions.

Results

Allele-sensitive differences in FcγRIIIb-induced activation of FcγRIIa. PMN from donors homozygous for the NA1 and NA2 alleles of FcγRIIIb in PMN differ in their capacities to mediate internalization of IgG-sensitized erythrocytes. To determine the relative roles of FcγRIIIb and FcγRIIa in contributing to this difference, we compared FcγRIIa-mediated internalization in NA homozygous donors and the capacity of the two alleles of FcγRIIIb to enhance FcγRIIa function. PMN from normal donors homozygous for NA1 and NA2 alleles and matched for identical FcγRIIa alleles were studied simultaneously in a matched-pairs design. In NA1 and NA2 homozygotes both baseline expression of FcγRIIa and FcγRIIIb and specific FcγRIIa-mediated phagocytosis are equivalent (21). Cross-linking of FcγRIIIb with 3G8 F(ab')$_2$ in donors homozygous for the NA1 allele markedly increased internalization of erythrocytes coupled to anti-FcγRII mAb IV.3 Fab (E-IV.3), an FcγRII-specific probe (21, 27) (Fig. 1 *A*; $P < 0.006$). In contrast, in donors homozygous for the NA2 allele, cross-linking of FcγRIIIb did not activate FcγRIIa-mediated internalization of E-IV.3 (NA1 vs. NA2, $P < 0.005$; $n = 11$). Cell surface expression of FcγRIIa did not change after FcγRIIIb cross-linking in either group (NA1 103±2% control; NA2 101±1% control; $n = 4$). Furthermore, the capacity to activate FcγRIIa function was not intrinsically different in individuals homozygous for either NA1 or NA2; PDBu-induced enhancement of E-IV.3 internalization (which is independent of FcγRIIIb) was identical for both groups (NA1 vs. NA2: 400±306% control vs. 434±212%, $n = 6$ pairs). These findings indicate that the capacity of FcγRIIIb to augment FcγRIIa function varies with FcγRIIIb structure and is a specific property of FcγRIIIb.

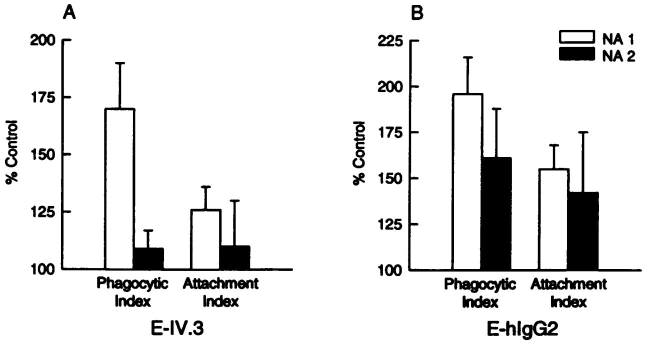

Figure 1. Cross-linking of FcγRIIIb activates FcγRIIa-mediated phagocytosis: allele-sensitivity. PMN from normal donors homozygous for NA1 and NA2 alleles and matched for identical FcγRIIa alleles were studied simultaneously in a matched-pairs design. PMN from each donor were preincubated with 3G8 F(ab')$_2$ (10 μg/ml) or control medium for 5 min and then combined with (*A*) E-IV.3 or (*B*) E-hIgG2. The effects of 3G8 F(ab')$_2$-induced activation on E-IV.3 phagocytosis and attachment is presented as % control [100 × $\mathrm{PI}_{3\mathrm{G8}\ \mathrm{F(ab')2}}$ (or AI)/$\mathrm{PI}_{\mathrm{control}}$ (or AI)]. Control PI and AI for E-IV.3 were 114±16 E-IV.3 internalized/100 PMN and 203±32 E-IV.3 attached/100 PMN, respectively, and for E-hIgG2 105±12 E-hIgG2 internalized/100 PMN and 152±26 E-hIgG2 attached/100 PMN, respectively. Baseline AI and PI for NA1 and NA2 homozygotes were indistinguishable. (*A*) Cross-linking FcγRIIIb with 3G8 F(ab')$_2$ in donors homozygous for the NA1 allele increased internalization of E-IV.3 (NA1 vs. control $P < 0.006$), whereas in donors homozygous for the NA2 allele there was no increase in FcγRIIa-mediated internalization (NA1 vs. NA2 $P < 0.005$, $n = 11$ pairs). In NA1 homozygotes, there was a trend toward increased adherence of E-IV.3 in activated cells (NA1 vs. control $0.1 > P > 0.05$; NA1 vs. NA2 P = NS; $n = 6$ pairs), but no increase in surface expression of FcγRII ($n = 4$). (*B*) In donors homozygous for the NA1 allele, cross-linking FcγRIIIb with 3G8F(ab')$_2$ amplified internalization of E-hIgG2 more than in NA2 donors (NA1 vs. NA2 $P < 0.02$; NA1 vs. control $P < 0.001$; NA2 vs. control $P < 0.05$, $n = 12$ pairs). Cross-linking of both the NA1 and NA2 alleles of FcγRIIIb resulted in comparable increments in attachment of E-hIgG2 ($n = 5$).

The suggestion of an FcγRIIIb-induced increase in the attachment index of E-IV.3 in NA1 individuals (Fig. 1 *A*) raised the possibility that the FcγRIIIb cross-linking might alter ligand binding capacity of FcγRIIa in addition to amplification of phagocytosis. Therefore, to examine FcγRIIIb-driven activation of FcγRIIa in the context of an FcγRIIa-specific natural ligand that might be more sensitive to changes in receptor avidity, we explored the attachment and phagocytosis of erythrocytes coupled to human IgG2 (E-hIgG2). We and others have previously shown that E-hIgG2 do not bind FcγRIIIb but do bind efficiently to the LR (131-H) allelic form of FcγRIIa (21, 28). In PMN from homozygous NA1 individuals 3G8 F(ab')$_2$ preincubation significantly increased phagocytosis of E-hIgG2 ($P < 0.001$; Fig. 1 *B*). In contrast to the experiments with E-IV.3, however, NA2 donors also were capable of increasing FcγRIIa-mediated phagocytosis of E-hIgG2 ($P < 0.05$). When matched for FcγRIIa alleles, stimulated PMN from NA1 donors showed significantly greater increases in FcγRIIa function than PMN from NA2 ($P < 0.02$). The increase in E-hIgG2 internalization was greater than that in E-IV.3 for each group, which may reflect the clear increase in the attachment index for E-hIgG2 in both NA1 and NA2 donors (Fig. 1 *B*). Assessment of the percentage of cells supporting attachment demonstrated that the increase in attachment index reflects both a change in the percent attachment and the number of E-hIgG2 attached per cell.

To distinguish the effect of FcγRIIIb-mediated activation on attachment to FcγRIIa per se from its effect on internalization and to establish that both processes were indeed affected, internalization was assessed at limiting PMN–erythrocyte ratios. With a ratio of 1:1, all erythrocytes are bound to PMN,

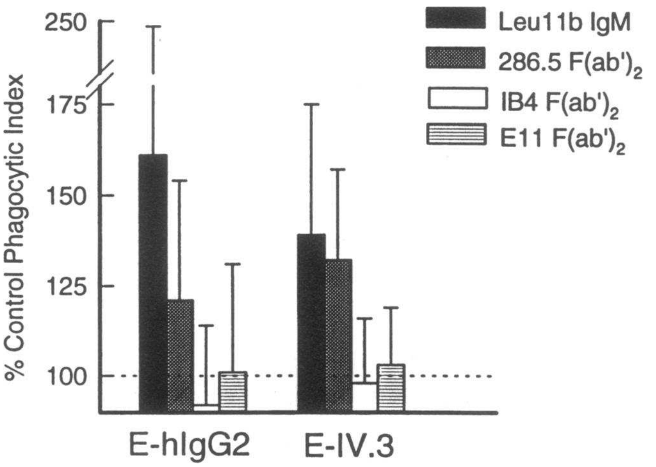

Figure 2. Cross-linking CD16, but not CD35 or CD18, activates FcγRIIa. PMN were treated with saturating concentrations of the anti-CD16 mAbs Leu11b (IgM) or 286.5 F(ab')$_2$, anti-CD18 (IB4 F(ab')$_2$), anti-CD35 (E11 F(ab')$_2$), or control medium and incubated with either E-IV.3 or E-hIgG2. Phagocytosis was determined by light microscopy. Data are expressed as % control ($100 \times PI_{stimulated}/PI_{control}$). Internalization of both FcγRIIa-specific probes is enhanced by two distinct methods of cross-linking FcγRIIIb. Leu11b: E-hIgG2 $P < 0.02$, $n = 16$; E-IV.3 $P < 0.004$, $n = 12$; 286.5 F(ab')$_2$: E-hIgG2 $P < 0.02$, $n = 13$; E-IV.3 $P < 0.001$, $n = 16$; and E11 F(ab')$_2$ (n = 8-9) and IB4 F(ab')$_2$ (n = 6-11) P = NS.

which eliminates the potential to increase phagocytosis by increasing adherence. Using this system of controlled adherence (E-IV.3 AI: 106±25 attached E/100 PMN and E-hIgG2 AI: 102±30), treatment with 3G8 F(ab')$_2$ still resulted in a consistent increase in FcγRIIa-mediated phagocytosis (E-IV.3, 191±22% control PI, $n = 5$, $P < 0.003$; E-hIgG2, 167±15% control PI, $n = 6$, $P < 0.007$). Thus, cross-linking of FcγRIIIb resulted in both increased attachment and enhanced efficiency of internalization of E's attached to FcγRIIa (Fig. 1).

To exclude the possibility that activation of FcγRIIa might reflect a unique property of mAb 3G8, several different anti-CD16 mAbs of differing isotypes were studied. PMN from NA1 homozygotes or NA1–NA2 heterozygotes were treated with saturating concentrations of mAb 286.5 F(ab')$_2$ (a mIgG1 recognizing a ligand-binding site epitope) (24), Leu11b (a mIgM), or control medium. Internalization of both E-IV.3 and E-hIgG2 was significantly augmented by both of these anti-CD16 mAbs (Fig. 2). The lack of a requirement for an intact IgG heavy chain in the cross-linking antibody is underscored by experiments with the IgM anti-CD16 mAb Leu11b. Furthermore, the activation of FcγRIIa by intact Leu11b argues against the possibility that contaminating residual proteases from the digestion of IgG are the activators of FcγRIIa. In contrast to results with 3G8 F(ab')$_2$, 286.5 F(ab')$_2$, and Leu11b, incubation of PMN with F(ab')$_2$ fragments of anti-CD35 (E11) or anti-CD18 (IB4) mAbs did not alter FcγRIIa function (Fig. 2). These results emphasize the importance of CD16 cross-linking as a specific trigger for the activation of FcγRII.

Activation of FcγRIIa-mediated phagocytosis is oxidant dependent. Gresham et al. (29) have demonstrated that phorbol esters can stimulate neutrophil internalization of IgG-sensitized E by both FcγRIIa and FcγRIIIb through an oxidant-dependent mechanism. Given the ability of PDBu to augment FcγRIIa-specific function, we evaluated the possibility that activation of FcγRIIa by PDBu and FcγRIIIb might use a similar mechanism. Internalization by FcγRIIa was unaffected by incubation with 0.5 mM H_2O_2, but in the presence of aminotriazole (20 mM), an inhibitor of catalase that can be released from endogenous neutrophil stores and metabolize H_2O_2, internalization was significantly increased by 0.5 mM H_2O_2 (E-IV.3 = 147±19% control PI, $n = 11$, $P < 0.03$; E-hIgG2 = 133±10, $n = 10$; $P < 0.01$). Given these observations providing direct evidence that oxidants can affect receptor-specific function, we examined the effects of SOD and catalase, inhibitors of reactive oxygen metabolites. Neither SOD nor catalase altered baseline receptor-specific phagocytosis (Fig. 3). PDBu significantly augmented FcγRIIa-specific phagocytosis, an effect blocked by SOD and catalase (Fig. 3) in a manner similar to their effects on EA phagocytosis (29). In the presence of aminotriazole, PDBu resulted in an even greater enhancement of FcγRIIa-mediated internalization compared with PDBu alone (50% increase). FcγRIIIb-mediated phagocytosis was negligible at baseline and unaffected by phorbols (30). Most importantly, the increase in FcγRIIa phagocytosis induced by cross-linking FcγRIIIb was also inhibited in the presence of either SOD or catalase (Fig. 3). In contrast, heat-denatured and inactivated SOD did not block either PDBu- or FcγRIIIb-induced stimulation (PDBu with denatured SOD = 372±69% control PI E-hIgG2; 3G8 F(ab')$_2$ with denatured SOD = 214±38% control PI). These findings suggest that activation of FcγRIIa is mediated by generation of reactive oxygen intermediates and provide strong evidence that FcγRIIIb triggers their generation.

Reactive oxygen intermediates are found in cell supernatants, and therefore we considered the possibility that the erythrocyte probe might be modified by exposure to active supernatants and lead to the spurious conclusion that intrinsic FcγRIIa properties are being altered. Supernatants from PDBu-treated PMN were active in enhancing FcγRIIa-mediated binding and phagocytosis of unstimulated PMN (Fig. 4). As predicted, the activation induced by supernatants was oxidant dependent and blocked by catalase (Fig. 4). Similar results from experiments with supernatants from 3G8 F(ab')$_2$-stimulated PMN (144±11% control, $P < 0.04$) indicated that this effect was not simply a carry over of the stimulus per se from stimulated to unstimulated cells, but due to soluble factors in supernatants from activated cells. Having established the activity of stimulated supernatants, we tested their capacity to directly modify phagocytic probes. Treatment of both E-IV.3 and E-hIgG2 with supernatants from stimulated PMN did not alter their internalization (107±8% control and 97±2% control, respectively), indicating not only that the oxidants are acting on the phagocytes rather than on the E's but also that oxidants may act in a paracrine fashion.

Because 3G8 F(ab')$_2$ pretreatment can induce an increase in both ligand binding and phagocytic efficiency, we examined the question of whether oxidants contributed to both effects. Inclusion of SOD in incubation mixtures of stimulated PMN abrogated the increase in attachment of E-hIgG2 (Fig. 5 *A*). Similarly, with limiting PMN/erythrocyte ratios, SOD blocked the augmentation of FcγRIIa-mediated phagocytosis of E-IV.3 or E-hIgG2 by FcγRIIIb cross-linking (Fig. 5 *B*). Thus both components of FcγRIIIb-stimulated phagocytosis by FcγRIIa are oxidant dependent.

"Auto-opsonization" does not mediate FcγRIIIb-induced augmentation of phagocytosis. We considered the possibility

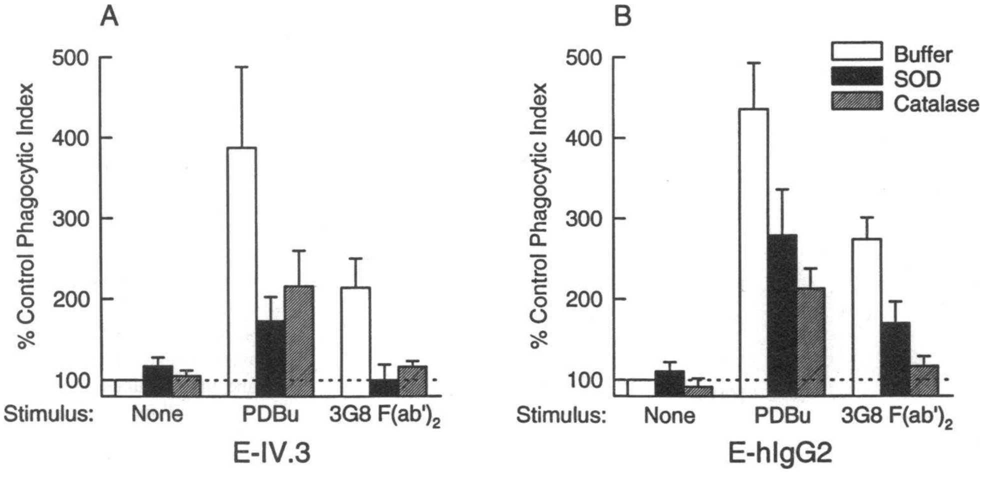

Figure 3. Activation of FcγRIIa-mediated phagocytosis is oxidant dependent. PMN treated with PDBu (15 ng/ml), 3G8 F(ab′)$_2$ (10 μg/ml), or control medium and incubated with either E-IV.3 (*A*) or E-hIgG2 (*B*) in the presence of SOD (150 μg/ml), catalase (31,000 U/ml), or buffer. Data are expressed as % control (100 × $PI_{stimulated}/PI_{control}$). Activation of FcγRIIa by either PDBu and cross-linking FcγRIII is markedly decreased in the presence of inhibitors of reactive oxygen metabolites. PDBu-induced activation: E-IV.3: SOD vs. no SOD $P < 0.04$, $n = 5$; E-hIgG2: SOD vs. no SOD $P < 0.05$; catalase vs. no catalase $P < 0.02$, $n = 5$. 3G8 F(ab′)$_2$-induced activation: E-IV.3: SOD vs. no SOD $P < 0.02$; $n = 5$; catalase vs. no catalase $P < 0.04$, $n = 5$; E-hIgG2 internalization : SOD vs. no SOD $P < 0.02$; $n = 6$; catalase vs. no catalase $P < 0.01$, $n = 6$. PMN from NA1 homozygotes or NA1–NA2 heterozygotes were used in experiments with 3G8F(ab′)$_2$.

that stimulated PMN might release the complement component C3, which becomes activated and bound to the erythrocyte probe ("auto-opsonization") (31, 32). Pretreatment of the probe with active supernatants did not confer enhanced phagocytosis. Nonetheless, we blocked the ligand-binding site for C3bi on complement receptor 3 with mAb MN41 IgG (26), but this did not prevent enhancement of FcγRIIa function (E-hIgG2 internalization by PDBu-stimulated PMN: 443% unstimulated PI, $n = 4$, $P < 0.03$). Recognizing that CD11b/CD18 may be activated by H_2O_2 (33) and that PDBu amplification of EA phagocytosis can be blocked by pretreatment of PMN with IB4 F(ab′)$_2$ (34), an mAb against the β-chain of CR3 (25), we tested the effects of IB4 on FcγRIIIb-induced effects. As shown in Fig. 6, internalization of E-IV.3 and E-hIgG2 was enhanced to a similar extent with or without IB4 F(ab′)$_2$ (25 μg/ml). Taken together these experiments do not support a role for CD11b/CD18 in the activation of FcγRIIa. However, a role for leukocyte response integrin or integrin-associated protein, which have the potential to amplify FcγR function in other systems (35–37), cannot be excluded.

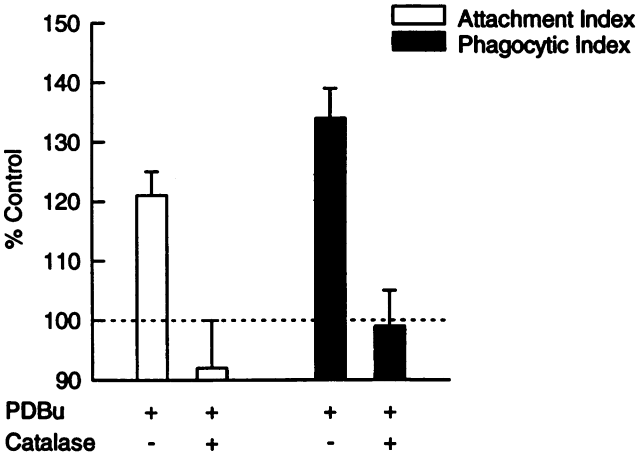

Figure 4. Supernatants from stimulated PMN activate FcγRIIa. PMN were treated with PDBu (15 ng/ml) for 5 min, washed twice, and cultured for 15 min at 37°C in RPMI. Supernatants were collected and combined with fresh PMN and E-IV.3. After incubation for 15 min with or without catalase (31,000 U/ml) attachment and phagocytosis were determined ($n = 5$–8). Data are expressed as % control [100 × $PI_{stimulated}$ (or AI)/$PI_{control}$ (or AI)]. For AI: control vs. PDBu $P < 0.02$, PDBu vs. catalase $P < 0.04$. For PI: control vs. PDBu $P < 0.0001$, PDBu vs. catalase $P < 0.01$.

Chlorinated oxidants and serine proteases participate in FcγRIIa activation. Proteolysis by serine proteases has been proposed as a mechanism for enhanced ligand binding by FcγRIIa in human monocytes (38, 39). Because PMN can use the H_2O_2–myeloperoxidase–chloride system to generate chlorinated oxidants capable of activating such protease zymogens and inactivating protease inhibitors (40–43), we considered the possibility that the generation of hypochlorous acid might be the mechanism underlying oxidant-dependent enhancement of FcγRIIa-mediated ligand binding. H_2O_2 is a substrate for the generation of HOCl, and O_2^- plays a role in regulating myeloperoxidase activity and may increase H_2O_2 availability by oxidizing other substrates (44, 45). While inhibition of activation by both catalase and superoxide dismutase is consistent with a role for HOCl, the more potent inhibition of FcγRIIa activation by catalase supports this model.

To examine the contribution of HOCl to oxidant-dependent FcγRIIIb-driven activation of FcγRIIa, we assessed the ability of methionine, a scavenger that rapidly reacts with HOCl to yield methionine sulfide (46), and sodium azide, an inhibitor of myeloperoxidase, to abrogate activation. Constitutive internalization of E-hIgG2 was not significantly altered by coincubation with 20 mM methionine (131±13% control), whereas 3G8 F(ab′)$_2$-induced amplification of E-hIgG2 phagocytosis was significantly decreased ($P < 0.008$; Fig. 7). Methionine also inhibited the 3G8 F(ab′)$_2$-induced increase of E-IV.3 phagocytosis ($P < 0.04$; Fig. 7) and decreased PDBu-induced enhancement (no methionine vs. methionine: 271±55 vs. 141±13% of unstimulated PI, $n = 6$, $P < 0.04$). Coincubation with sodium azide (0.1%) significantly decreased the FcγRIIIb-induced enhancement of FcγRIIa phagocytic function (E-hIgG2 $P < 0.01$; E-IV.3 $P < 0.001$, Fig. 7). The PDBu-induced activation also

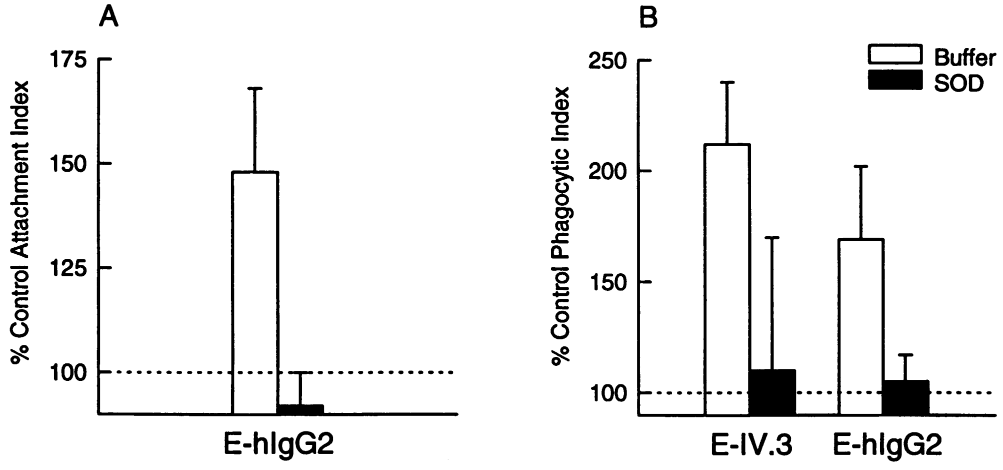

Figure 5. The increased efficiency of both FcγRIIa binding and internalization is oxidant dependent. (*A*) PMN in the presence or absence of SOD (150 μg/ml) were treated with 3G8 F(ab')$_2$ (10 μg/ml) or control medium and incubated with E-hIgG2 and attachment was assessed. Inclusion of SOD abrogated the FcγRIIIb-driven amplification of E-IgG2 binding (SOD vs. no SOD: $P < 0.04$, $n = 5$). (*B*) To specifically assess phagocytosis, we fixed and limited attachment (1:1 ratios of E to PMN) as described in Methods. Rosetted PMN were incubated with 3G8 F(ab')$_2$ or control medium, in the presence or absence of SOD. The 3G8 F(ab')$_2$-induced increase in the efficiency of FcγRIIa-mediated internalization was inhibited by SOD (no SOD vs. SOD: E-IV.3 $P < 0.03$, $n = 6$; E-hIgG2 $0.1 > P > 0.05$, $n = 3$).

decreased 50% ($P < 0.01$). These data support a role for chlorinated oxidants that may interact directly with cellular targets to induce FcγRIIa activation or may facilitate the capacity of proteases to alter the receptor–ligand interactions.

A role for proteases in FcγRIIa function has been suggested by the observations that the serine protease inhibitor, TLCK, decreases FcγRIIa-mediated binding of EA in human monocytes (38, 39). As expected for the E-IV.3 probe, neither basal levels (86±14% control PI, $n = 6$) nor 3G8 F(ab')$_2$-stimulated levels of internalization (no TLCK vs. TLCK: 156±10 vs. 138±12% of unstimulated PI) were significantly affected by TLCK. In contrast, basal internalization of E-hIgG2, a probe sensitive to changes in ligand-binding site, was moderately decreased in the presence of 0.5 mM TLCK (76±7% control, $n = 5$, $P < 0.03$), while 3G8 F(ab')$_2$-stimulated enhancement was dramatically reduced from 217±22% of unstimulated PI to 126±11% ($n = 8$, $P < 0.02$). As further evidence for the role of inhibition of serine proteases, we preformed a series of experiments in the presence of PABA, a specific reversible serine protease inhibitor. Similar to TLCK, PABA significantly reduced 3G8 F(ab')$_2$ activation of internalization of E-hIgG2 (no PABA vs. PABA: 239±104 vs. 130±31% of unstimulated PI, $n = 7$, $P < 0.01$), whereas the effect on E-IV.3 was minimal (no PABA vs. PABA: 211±41 vs. 170±61% of unstimulated PI, $n = 7$, P = NS). Taken together, these observations suggest that activation-induced proteolytic effects contribute to augmentation of FcγRIIa function primarily through changes in binding detected by native ligand. Facilitation of this process by chlorinated oxidants provides a rapid mechanism by which neutrophils can use the FcγRIIIb-activated NADPH oxidase system and granular constituents (47, 48) in a cooperative manner to modulate the binding capacity of FcγRIIa without altering quantitative receptor expression. The ability of methionine and azide to inhibit the activation-induced increase in E-IV.3 phago-

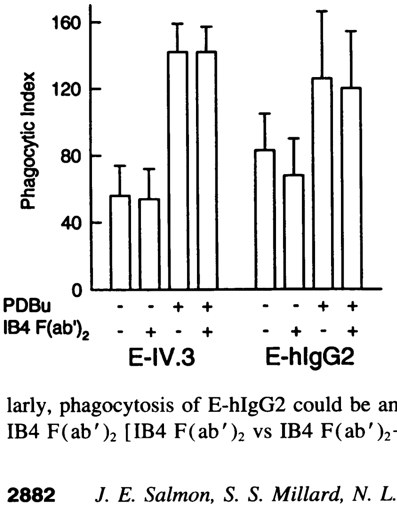

Figure 6. Anti-CD18 mAb IB4 F(ab')$_2$ does not inhibit activation of FcγRIIa. PMN were pretreated with IB4 F(ab')$_2$ (10 μg/ml) before incubation with E-IV.3 or E-hIgG2. Internalization of E-IV.3 was enhanced to a similar extent with or without IB4 F(ab')$_2$ (control vs. PDBu $P < 0.02$; IB4 F(ab')$_2$ vs. IB4 F(ab')$_2$+PDBu, $n = 4$, $P < 0.004$). Similarly, phagocytosis of E-hIgG2 could be amplified in the presence of IB4 F(ab')$_2$ [IB4 F(ab')$_2$ vs IB4 F(ab')$_2$+PDBu, $n = 6$, $P < 0.01$].

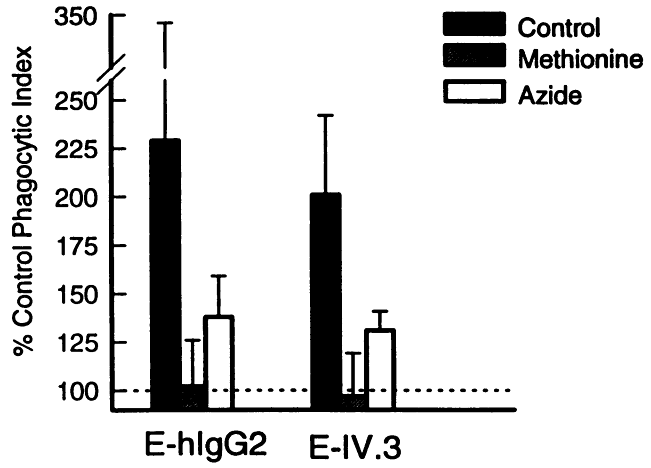

Figure 7. Chlorinated oxidants participate in FcγRIIa activation. PMN were incubated with the HOCl scavenger methionine (20 mM); sodium azide (0.1%), an inhibitor of myeloperoxidase; or buffer for 5 min at 37°C before activation with 3G8 F(ab')$_2$. Internalization of E-hIgG2 and E-IV.3 in stimulated and unstimulated cells was quantitated. Duplicate samples of activated cells without methionine (or azide) and unactivated cells with methionine (or azide) were the controls for each experimental condition. Activated phagocytosis of both FcγRIIa-specific probes was inhibited in the presence of methionine (E-hIgG2: no methionine vs. methionine, $n = 8$, $P < 0.008$; E-IV.3: no methionine vs. methionine, $n = 6$, $P < 0.04$). Similarly, azide inhibited activation of FcγRIIa (E-hIgG2: no azide vs. azide, $n = 7$, $P < 0.01$; E-IV.3: no azide vs. azide, $n = 9$ $P < 0.001$).

cytosis suggests that effects of oxidants, such as HOCl, extend beyond changes in ligand binding.

Discussion

Neutrophils from normal donors, homozygous for the two different allelic phenotypes of FcγRIIIb, have significantly different levels of Fcγ receptor–mediated phagocytosis (1). However, experiments using antireceptor mAb-targeted erythrocytes indicate that FcγRIIIb mediates phagocytosis poorly (2, 30) and raise the possibility that FcγRIIIb may collaborate with FcγRIIa on neutrophils for internalization of EA. Such an interactive mechanism would require that FcγRIIIb have the capacity to influence FcγRIIa-mediated phagocytosis and that this capacity demonstrates quantitative differences between donors homozygous for the two different FcγRIIIb alleles. Indeed, initial observations have suggested that cross-linking of FcγRIIIb with antireceptor mAb can augment the phagocytic activity of FcγRIIa on PMN (17). Therefore, we systematically explored both the capacity of FcγRIIIb to influence FcγRIIa in an allele-sensitive fashion and the mechanisms underlying this effect.

Our data indicate that cross-linking of FcγRIIIb does activate FcγRIIa for phagocytosis. Anti-CD16 mAbs of differing isotypes enhanced FcγRIIa function, demonstrating that priming is not a unique property of single mAb and is not dependent on IgG heavy chain. Furthermore, $F(ab')_2$ fragments of anti-CD35 or -CD18 mAbs did not alter FcγRIIa function, underscoring the importance of CD16 cross-linking as a specific trigger for the activation of FcγRII. The increase in FcγRIIa-specific internalization reflects both an increase in ligand-mediated binding of erythrocytes and an increase in internalization efficiency of targets bound. This activation does not involve FcγRIIIb serving as a ligand-binding, "capture" receptor delivering ligand directly to FcγRIIa (49–51), because FcγRIIIb does not bind hIgG2 although we recognize the possibility that this might occur with some human IgG1 and IgG3. The mechanism of activation is dependent on the FcγRIIIb-driven generation of oxidants, and at least part of the increase in avidity of FcγRIIa appears to rely on a proteolytic modification. In addition, an oxidant-dependent increase in FcγRIIa-mediated internalization also occurs. The capacity of supernatants from stimulated PMN to enhance FcγRIIa function in unstimulated PMN precludes the need for a physical association between FcγRIIa and FcγRIIIb. These oxidant-dependent mechanisms provide the opportunity not only for autocrine stimulation of Fcγ receptor function but also for paracrine stimulation of different cells and perhaps even different receptor species.

The mechanism of altered avidity involves, at least in part, proteolysis. Previous work by Van de Winkel et al. (38, 39) suggested that a proteolytic process was involved in FcγRIIa-mediated binding in monocytes. Although those studies did not formally exclude the possibility that a change in surface charge or other properties rather than direct receptor modification was involved, our studies indicating a change in avidity for ligand-driven binding of E-hIgG2 but not for mAb-driven binding of E-IV.3 support the concept of direct modification of FcγRIIa. Of course, modification of an as yet unrecognized accessory molecule participating in binding cannot be excluded. The ability of catalase and SOD to block this effect strongly implicates oxidants in this process, and the inhibition of FcγRIIa activation in the presence of methionine, a scavenger for HOCl, or azide, an inhibitor of myeloperoxidase, suggests that neutrophils can use the H_2O_2–myeloperoxidase system to generate chlorinated oxidants, such as HOCl, which activate proteinase zymogens and inactivate proteinase inhibitors to begin a protease cascade. Because quantitative receptor expression did not change, the modulation of FcγRIIa is unlike that for other leukocyte receptors and counterreceptors, such as CD16, CD43, L-selectin, and TNF receptor, which regulate surface expression through enzymatic cleavage (15, 16, 52). Rather, conformational changes affecting the ligand binding site appear most likely.

In addition to a change in avidity, our data clearly demonstrate an increase in receptor-mediated internalization efficiency. Although the incomplete blockade of activation of TLCK or PABA could reflect either incomplete enzyme inhibition or the presence of several different mechanisms of activation, both the increase in E-IV.3 internalization in the absence of an increase in binding and the increase in phagocytosis in the limiting attachment paradigm unambiguously demonstrate an enhancement of the efficiency of receptor-mediated internalization. As with the change in avidity, this process involves the FcγRIIIb-mediated generation of oxidants and is inhibitable by both catalase and SOD. Given the rapid time frame for these effects, it is unlikely that modulation of transcription factors plays a significant role although oxidants are known to affect NF-κB and others (53–55). In contrast, oxidants can rapidly influence levels of quantitative tyrosine phosphorylation, and tyrosine phosphorylation is essential for Fcγ receptor–initiated functions (56–58). Indeed, synergy between FcγRII and FcγRIIIb for activation of the respiratory burst and phagocytosis requires tyrosine phosphorylation of FcγRII (30, 59). Of course, changes in phosphorylation states could provide mechanisms for both avidity modulation ("inside-out" signaling [12–14]) and for internalization.

Our observations provide the basis for better understanding of the mechanisms whereby FcγRIIIb homozygous donors can differ in quantitative EA phagocytosis. More importantly, however, our data indicate a mechanism for receptor collaboration in an autocrine fashion without the requirement of either ligand-mediated or -independent direct physical interaction between receptor species. Of course, our results do not preclude a physical interaction of some fraction of FcγRIIIb and FcγRIIa, as suggested by resonance energy transfer studies (60, 61), nor do they preclude other molecules such as CR3 from playing a role in FcγRIIa priming (59); however, they do suggest a more general mechanism for FcγRIIIb that has the potential to influence or "collaborate" with a broader range of receptors (30) in both an autocrine and a paracrine fashion. Indeed, the target cells for a paracrine effect need not be restricted to other phagocytes, and thus these paracrine effects may provide a foundation for Fcγ receptor alleles on phagocytes to influence other components of the immune system.

In this context, these observations suggest that FcγR polymorphisms may be considered inherited disease susceptibility factors in host defense against infection and in the development of autoimmunity. For example, the current work showing that NA2 homozygotes are relatively ineffective in amplifying FcγRIIa is consistent with the observation that individuals homozygous for FcγRIIIb-NA2, especially when combined with homozygosity for FcγRIIa-HR and with a terminal complement component deficiency, are more likely to develop serious meningococcal infection (62). Interactions with other components of the immune system through modulation of protein tyrosine kinase activities, transcription factors, and other mechanisms remain to be explored.

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