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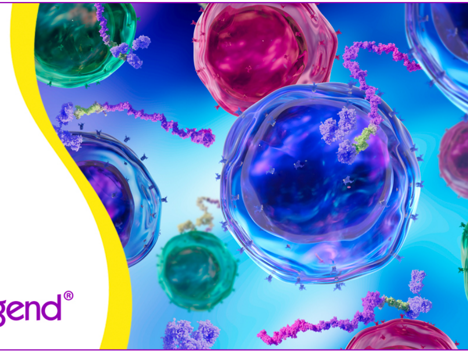

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Cross-Linking of Fcγ Receptor IIa and Fcγ Receptor IIIb Induces Different Proadhesive Phenotypes on Human Neutrophils[1]

Markus Kocher,* Michelle E. Siegel,* Jeffrey C. Edberg,† and Robert P. Kimberly[2]†

Activation of polymorphonuclear leukocytes (PMN) plays an important role in vascular injury associated with systemic vasculitis and in models of autoantibody- and immune complex-mediated disease. The potential role of intravascular activation of PMN, however, is confounded by the observation that some stimuli injected i.v. (e.g., IL-8 and C5a) lead to L-selectin shedding by PMN, which inhibits attachment to endothelium and may be functionally anti-inflammatory. To explore the impact of Fcγ receptor (FcγR)-mediated activation on the PMN adhesive phenotype, FcγRIIa (CD32) and FcγRIIIb (CD16) were targeted with receptor-specific reagents, and the expression of adhesion molecules-mediating rolling (L-selectin) and firm adhesion (CD11b/CD18) was measured. Engagement of either FcγRIIa or FcγRIIIb leads to activation, demonstrated by degranulation (up-regulation of CD66b), and to increased expression of total CD11b/CD18 and functional CD11b/CD18 (I-domain). In contrast, L-selectin shedding induced by PMN FcγR was divergent. Despite the 5- to 10-fold greater expression and engagement at saturation, activation via FcγRIIIb led to little or no change in L-selectin expression. Stimulation of PMN with intact murine anti-receptor IgG1 showed a contribution of FcγRIIa receptor polymorphisms, underscoring the direct influences of FcγR allotypes on receptor function. These observations suggest that FcγRIIIb-mediated activation of circulating PMN may lead to a proadhesive phenotype likely to promote systemic vascular damage. This FcγR-mediated adhesive phenotype will vary with the receptors engaged and their allotypes, which, in turn, reflect properties of the immune complex and the genetics of the host. ***The Journal of Immunology*, 1997, 159: 3940–3948.**

Polymorphonuclear leukocytes (PMN)[3] are important effectors of tissue injury in many inflammatory diseases. They can easily be activated for phagocytosis, the generation of an oxidative burst, and degranulation by a variety of stimuli, including chemotactic agents, cytokines, and immune complexes. There are two structurally and functionally distinct receptors for the Fc region of IgG (FcγR) through which immune complexes can activate PMN (1, 2). FcγRIIa is a transmembrane protein expressing an immunoreceptor tyrosine activation motif in the cytoplasmic domain (1, 2), while FcγRIIIb is a glycosyl phosphatidylinositol-linked protein that has been shown to activate tyrosine kinase activity and raise the intracellular Ca^{2+} concentration (2–4).

Normally, circulating PMN do not adhere to the vascular endothelium, but in inflammatory lesions the sequential engagement of a series of adhesion receptors leads to a multistep program to allow the activated cells to roll along the endothelium, firmly adhere, and then transmigrate across the endothelial wall (5, 6). The initial contact between PMN and endothelial cells is mediated by L-selectin on PMN and leads to tethering and rolling of the PMN (5, 7, 8). The importance of L-selectin-mediated contact in the initiation of PMN rolling under shear flow conditions has been shown in numerous in vitro and in vivo models (9–12). Firm adhesion is then dependent on β_2 integrins (CD11a/CD18 (LFA-1) and CD11b/CD18 (CR3, Mac-1)), which bind to ICAM-1 (CD54) on activated endothelial cells (5). Up-regulation of CD11b/CD18 on PMN, however, is not sufficient to promote firm adhesion (5, 13–15). Expression of an activated form of CD11b/CD18 containing the ligand binding site I domain of the integrin α subunit is required for firm adhesion (5, 13, 16). Typically only a fraction of CD11b (~10%) expresses this exposed binding site (17). Finally, CD31 (PECAM1), expressed on endothelial cells and PMN, is involved in the transmigration of leukocytes across the endothelial cell layer (18). Other PMN surface proteins can also influence the adhesion process. For example, the negatively charged sialophorin (CD43) may act as a repulsion molecule (19–21), while the glycosyl phosphatidylinositol-linked CD66b interacts with E-selectin on endothelial cells and may activate CD11b/CD18 (22).

Numerous physiologic stimuli (including chemotactic factors and many cytokines) can alter the adhesive properties of circulating PMN. Activation of PMN by the chemotactic factor IL-8 induces the up-regulation of CD11b/CD18 and shedding of L-selectin (23). Many other PMN activators, including FMLP, C5a, and leukotriene B_4, induce similar changes in L-selectin and CD11b/CD18 expression. However, some studies have shown that the amount of L-selectin shedding from circulating PMN (or PMN in flow conditions in vitro) negatively correlates with the ability of PMN to initiate contact and rolling on endothelial cell layers (24).

*Research Division, The Hospital for Special Surgery, Cornell University Medical College, New York, NY 10021; and †Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama, Birmingham, AL 35294

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[2] Address correspondence and reprint requests to Dr. Robert P. Kimberly, Division of Clinical Immunology and Rheumatology, University of Alabama, 1900 University Blvd., Birmingham, AL 35294-0006. E-mail address: Robert.Kimberly@ccc.uab.edu

[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; FcγR, Fc region of immunoglobulin G; ICAM-1, intercellular adhesion molecule-1; WG, Wegener's granulomatosis; ANCA, anti-neutrophil cytoplasmic antibody; PR3, proteinase 3; NECA, *N*-ethylcarboxaminoadenosine; MFI, mean fluorescence intensity.

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Under these conditions, L-selectin shedding in the circulation may actually be anti-inflammatory (9).

Several lines of evidence suggest that in autoantibody- and immune complex-mediated inflammatory diseases, there are changes in expression of these critical adhesion molecules that may promote PMN contact, rolling, and firm adhesion to endothelial cells (6). Circulating PMN from patients with systemic lupus erythematosus, a disease characterized by circulating immune complexes, show evidence of activation (increased CD11b/CD18 expression) but little change in surface L-selectin expression (25). Further evidence in Wegener's granulomatosis (WG), a disease characterized by circulating anti-neutrophil cytoplasmic Abs (ANCA) that can engage FcγR (26–28), suggests that despite circulating PMN activation (increased expression of proteinase 3, CD66, and CD11b/CD18), surface L-selectin expression is preserved (29). These observations suggest the possibility that PMN activation through certain FcγR might have some unique characteristics and that, depending on the FcγR repertoire engaged, L-selectin expression might be preserved.

Therefore, we hypothesized that activation by different FcγRs (FcγRIIa and FcγRIIIb) on freshly explanted, unseparated PMN have disparate effects on L-selectin expression. We demonstrate that activation via either FcγRIIa or FcγRIIIb leads to comparable levels of PMN activation (degranulation and increased expression and activation of CD11b/CD18), but L-selectin shedding induced by FcγRIIa and that induced by FcγRIIIb were not comparable. Despite the 5- to 10-fold greater expression and engagement at saturation, activation via FcγRIIIb led to little or no change in L-selectin expression. This observation suggests that FcγRIIIb-mediated activation of circulating PMN may lead to a proadhesive phenotype likely to promote systemic vascular damage. Such properties might allow circulating immune complexes (30, 31) or autoantibodies (such as ANCA (26, 27, 32)) to preserve PMN adhesive capacities while potentiating their proinflammatory cell programs. The role of host FcγR genetics (the FcγRIIa-H131/R131 and FcγRIIIb-NA1/NA2 polymorphisms) will probably influence the spectrum of adhesive phenotypes induced by engagement of FcγR.

Materials and Methods

Blood donors

All donors were healthy volunteers genotyped for the FcγRIIa allelic polymorphisms (FcγRIIa-H131/R131) using an allele-specific dot-blotting assay as described previously (33, 34). The accuracy of the dot-blotting assay was confirmed in selected donors by phenotyping FcγRIIa on PMN and monocytes using the FcγRIIa-R131-specific mAb 41H16 (generously provided by Dr. T. F. Zipf, University of Texas, Houston, TX) as described previously (35, 36). The protocol for phlebotomy was approved by the institutional committee on human rights in research.

Antibodies

Anti-FcγRIII mAb 3G8 IgG and $F(ab')_2$, anti-FcγRII mAb IV.3 Fab, and FITC-labeled IV.3, 3G8, and 22.2 (anti-FcγRI) were obtained from Medarex (Annandale, NJ). The Fab and $F(ab')_2$ were cross-linked with Fab-specific goat anti-mouse $F(ab')_2$ from Jackson ImmunoResearch Laboratories (West Grove, PA). Other mAb included FITC-labeled anti-CD11b (Bear1, mIgG1), anti-CD66b (CD67, mIgG1), and anti-L-selectin (Dreg56, mIgG1) from AMAC (Westbrook, ME). Isotype-matched control Ab was mIgG1-FITC (MOPC21 (Sigma Chemical Co., St. Louis, MO) or ZX3 (AMAC)). The mAb CBRM1/5 (mIgG1, provided by Dr. T. A. Springer, Harvard University) (17), which is specific for a subpopulation of PMN CD11b, was used in indirect assays, and binding was detected with phycoerythrin-conjugated, Fc region-specific, goat anti-mouse $F(ab')_2$ (Jackson ImmunoResearch). Anti-CD43 (DF-T1, mIgG1) was obtained from Dako (Glostrup, Denmark). Anti-CD11a mAb 3A3 (mIgG1) and anti-CD18 mAb31H8 (mIgG1) were purchased from Endogen (Boston, MA). Mouse $F(ab')_2$ were obtained from Jackson ImmunoResearch. Anti-proteinase 3 (anti-PR3) mAb (ANCA, mIgG1, clone CLB-702) was purchased from Research Diagnostics (Flanders, NJ).

Washed whole blood assay

Three or four milliliters of blood was collected into heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ); immediately chilled to 4°C; washed twice in 125 mM sodium chloride, 10 mM phosphate, 5 mM potassium chloride, 5 mM glucose, 1.09 mM $CaCl_2$, and 1.62 mM $MgCl_2$, pH 7.35 (buffer 1), to remove soluble FcγR (which could interfere with subsequent anti-FcγR activation); and resuspended in the original volume. Fifty-microliter aliquots of this washed whole blood were used per tube (Becton Dickinson Labware, Lincoln Park, NJ). The anti-FcγRII- and anti-FcγRIII-specific mAb fragments (5 μg/ml IV.3 Fab and 10 μg/ml 3G8 $F(ab')_2$) were preincubated with aliquots of washed whole blood for 15 min at 4°C. After two washes with buffer 1 at 4°C, the mAb were cross-linked with goat anti-mouse IgG $F(ab')_2$ (35 μg/ml) at 37°C for various periods, followed by two washes with buffer 1 at 4°C. Remaining goat anti-mouse binding sites were then blocked for 15 min with murine $F(ab')_2$ (88 μg/ml), followed by incubation with the detection mAb. Alternatively, stimulation with 3G8 IgG or mIgG1 ANCA at various concentrations was performed at 37°C for the indicated times. Negative control samples were incubated without primary mAbs, and positive controls included cells stimulated with 100 ng/ml PMA (Sigma Chemical Co.) or 1 μM FMLP (Sigma Chemical Co.). All samples were then treated with 1 ml of FACS Lysing Solution (Becton Dickinson Immunocytometry, San Jose, CA) for 10 min at room temperature, washed once with 2 ml of PBS, and analyzed by flow cytometry.

Adhesion assays

Homotypic aggregation of isolated neutrophils (prepared on a discontinuous Ficoll-Hypaque gradient (27)) was measured as previously described (37), except that light microscopy was used to assess clumping. Briefly, 1×10^6 cells were incubated for 10 min at 37°C with stirring with 1.09 mM $CaCl_2$/1.62 mM $MgCl_2$. The stimulus was added, and the cells were incubated for an additional 5 min at 37°C with stirring. The number of cell clumps (≥3 cells) and the remaining unclumped cells were determined using a hemocytometer and by counting the number of clumps vs the number of unclumped cells in a grid. Adherence of stimulated cells to fibrinogen was performed exactly as previously described (17). Briefly, purified cells (4×10^6 cells) were added to fibrinogen-coated culture dishes with or without stimulus for 4 min at 37°C. After 12 washes, the number of bound cells in five random fields at ×400 magnification was determined by light microscopy.

Inhibitors

Genistein was purchased from Life Technologies (Grand Island, NY). 2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), 8-*p*-sulfo-phenyltheophiline, 3,7-dimethyl-1-propargylxanthine, and N^6-cyclopentyl-adenosine were purchased from Research Biochemicals, Inc. (Natick, MA). 5′-*N*-ethyl-carboxyaminoadenosine (NECA) was purchased from Sigma Chemical Co., and misoprostol was provided by Dr. B. Struthers (G. D. Searle, Skokie, IL).

Flow cytometry

The data were collected using a dual laser Cytofluorograph IIS or a FACScan (Becton Dickinson Immunocytometry, San Jose, CA). The instruments were routinely calibrated using fluorescent beads (Sphero Rainbow, Spherotech, Libertyville, IL). The PMN in whole blood were identified by light-scatter properties, and this identification was confirmed by characteristic expression of the human FcγR (FcγRI, FcγRII, and FcγRIII(1)) using the FITC-conjugated IV.3, 3G8, and 22.2.

Statistical analysis

Analysis of flow cytometry listmode data was performed using Cell Quest (Becton Dickinson Immunocytometry). Data are presented as the mean ± SEM. The mean channel fluorescence of histogram data was compared using Student's *t* tests and analysis of variance. Alternatively, nonparametric analysis was performed using Wilcoxon's matched pairs, signed rank test; the Mann-Whitney test; or Friedman's test. A confidence level of $p < 0.05$ was used in all tests except where stated otherwise.

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Results

Cross-linking of either FcγRIIa or FcγRIIIb in whole washed blood induces activation of PMN

To minimize or eliminate potential purification-induced PMN priming (38), we developed an activation procedure using washed whole blood. To establish that cross-linking of FcγRs can induce PMN activation in this system we quantitated degranulation after FcγR cross-linking. Degranulation of PMN is known to increase the surface expression of CD66b from intracellular stores (secondary granules) (39, 40). Using the anti-FcγRIII mAb 3G8 IgG, which cross-links FcγRIII and FcγRII (via the Fc region of 3G8 IgG) (41), rapid up-regulation of surface CD66b was observed (Fig. 1*A*). Maximal CD66b expression was induced with 5 μg/ml mAb 3G8 IgG within 10 min after stimulation (Fig. 1, *B* and *C*). Targeted homotypic cross-linking of FcγRIIa and FcγRIIIb with saturating concentrations of receptor-specific mAbs (mAb IV.3 Fab or mAb 3G8 $F(ab')_2$) and $F(ab')_2$ goat anti-mouse IgG also increased CD66b surface expression (Fig. 1*A*). No significant difference in CD66b expression was found between homotypic or heterotypic cross-linking of PMN FcγR after 10 min of stimulation ($p > 0.05$). These data demonstrate FcγR-induced PMN activation in our washed whole blood assay system.

Both FcγRIIa and FcγRIIIb induce up-regulation CD11b/CD18 on PMN

CD11b/CD18 is expressed on the surface of circulating PMN (Fig. 2*A*) and upon appropriate stimulation can be up-regulated on the surface from intracellular stores (primarily from secretory vesicles, but also specific granules) (42). Homotypic cross-linking of either FcγRIIa or FcγRIIIb on the neutrophil surface led to a significant and uniform increase in CD11b surface expression (from an MFI of 72 ± 4 in the incubated control to an MFI of 179 ± 14 and 173 ± 19 for FcγRIIa- and FcγRIIIb-stimulated cells, respectively; $p < 0.05$ for stimulated vs control; Fig. 2*B*). The expression of CD11b on control cells increased only marginally during the time course of receptor cross-linking at 37°C (Fig. 2*B*). There was no significant difference in CD11b up-regulation induced by either FcγRIIa or FcγRIIIb ($p > 0.05$). Heterotypic cross-linking of FcγRIIa and FcγRIIIb (using mAbs IV.3 Fab+3G8 $F(ab')_2$ and $F(ab')_2$ goat anti-mouse IgG) increased the total surface expression of CD11b further to an MFI of 238 ± 11. The kinetics of CD11b up-regulation were similar after homotypic or heterotypic FcγR cross-linking (Fig. 2*B*). Using the anti-CD18 mAb 31H8, a parallel increase in surface expression of CD18 and CD11b (using mAb Bear1) was observed (results not shown). The increase in CD11b/CD18 surface expression was dependent on protein tyrosine kinase activity, as the protein tyrosine kinase inhibitor, genistein (100 μg/ml), completely blocked up-regulation induced via FcγR cross-linking (not shown). Positive controls in each experiment included stimulation with 100 ng/ml PMA, which increased CD11b expression to an MFI of 398 ± 13 (Fig. 2*B*). In some experiments 1 μM FMLP was used, which increased CD11b expression to comparable levels (not shown). In agreement with earlier studies (43, 44), stimulation of PMN did not result in an increase in surface expression of CD11a (detected with mAb 3A3; results not shown). These results demonstrate that homotypic cross-linking of either FcγRIIa or FcγRIIIb induces rapid and specific up-regulation of the β_2 integrin CD11b/CD18.

Both FcγRIIa and FcγRIIIb induce expression of a CD11b I domain epitope on PMN

Increased CD11b/CD18 surface expression is not necessarily associated with increased adhesiveness. This functional property of

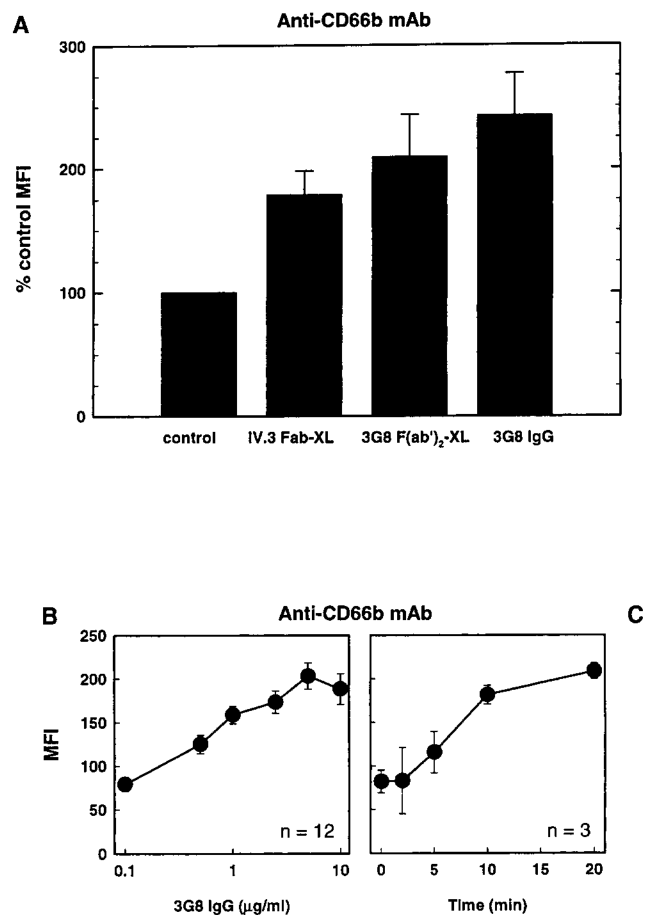

FIGURE 1. Activation of PMN in whole washed blood measured by up-regulation of CD66b after targeted cross-linking of FcγRIIa and FcγRIIIb. *A*, Expression of CD66b on PMN was measured after specific cross-linking of FcγRIIa with IV.3 Fab (IV.3 Fab-XL) and of FcγRIIIb with 3G8 $F(ab')_2$ (3G8 $F(ab')_2$-XL) by goat anti-mouse $F(ab')_2$ for 15 min in whole washed blood. Anti-FcγRIII mAb 3G8 IgG was incubated at a concentration of 5 μg/ml without further cross-linking. The data are shown as the percent increase above control (=100%) MFI ± SEM ($n = 16$). Statistical analysis of the data by Friedman's test indicated that the CD66b expression with all homotypic or heterotypic FcγR-targeted stimulations is significantly increased compared with the control expression, but these are not different from each other ($p < 0.05$). *B*, Dose response of CD66b surface expression measured in MFI on PMN induced by heterotypic cross-linking of FcγRIIIb and FcγRIIa with increasing concentrations of 3G8 IgG for 10 min at 37°C. *C*, CD66b up-regulation after PMN FcγR cross-linking with 5 μg/ml 3G8 IgG at 37°C for increasing lengths of time. Statistical analysis indicated that CD66b was significantly up-regulated in both the time course (*C*) and the dose response (*B*; by analysis of variance, $p < 0.001$). SEM are represented by the error bars, and the number of experiments are indicated.

the receptor can be detected by the mAb CBRM1/5, which has been shown to specifically inhibit receptor-ligand interactions and binds to a neoepitope within the I domain of the α_M subunit of CD11b/CD18 (17). The expression of this neoepitope was therefore measured in parallel to that of total CD11b expression. The CBRM1/5 activation epitope was not found on control unincubated cells (Fig. 2*A*). Minimal expression was found on cells incubated at 37°C, but the level did not change during the time course (Fig. 2*C*). Homotypic cross-linking of either FcγRIIa or FcγRIIIb induced an increase in the I domain expression (to MFIs of 60 ± 9 and 65 ± 12, respectively). Heterotypic cross-linking of

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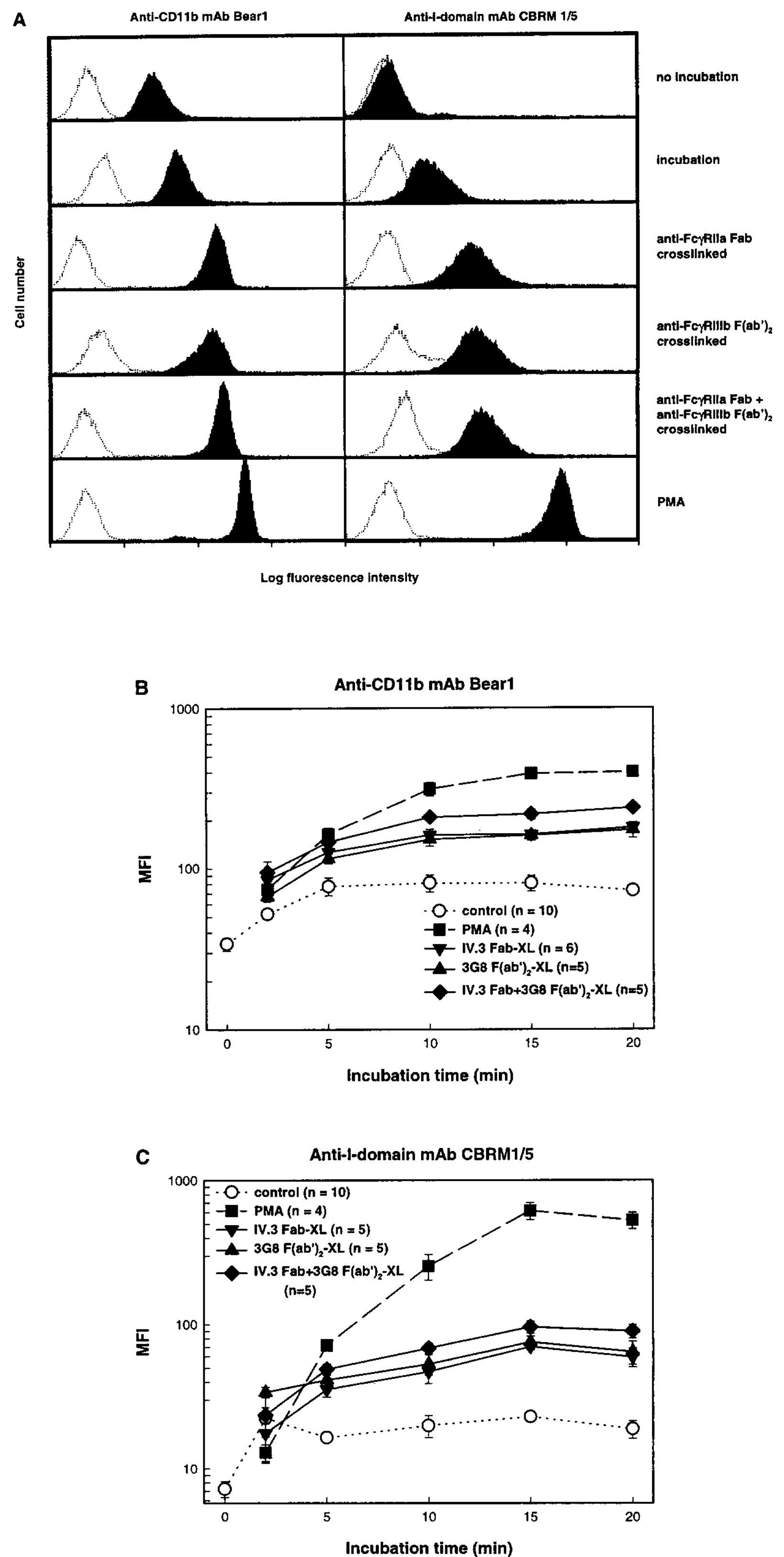

FIGURE 2. Targeted cross-linking of FcγRIIa- and FcγRIIIb-induced functional CD11b/CD18 expression on PMN. *A*, Histograms of a typical experiment showing increased binding of FITC-labeled CD11b mAb Bear1 (solid histograms, *left panels*) and of mAb CBRM1/5 specific for the I-domain ligand binding site detected indirectly with phycoerythrin-labeled goat anti-mouse Fc region-specific $F(ab')_2$ (solid histograms, *right panel*) in washed whole blood after specific cross-linking of FcγRs or incubation with 100 ng/ml PMA for 15 min at 37°C. FITC-labeled mAb MOPC21 was used as isotype-matched control mAb (dotted histograms, *left panels*). Binding of unlabeled mAb MOPC21 was used instead of CBRM1/5 at the same concentration as the isotype control mAb (dotted histograms, *right panels*). A representative experiment from a total of 14 is shown. *B*, Time course of the expression of CD11b/CD18 PMN in washed whole blood after specific cross-linking of FcγRs or incubation with 100 ng/ml PMA for the times indicated, measured with FITC-labeled Bear1. *C*, Time course of the binding of CBRM1/5 after FcγR cross-linking. FcγRIIa was cross-linked with mAb IV.3 Fab (IV.3 Fab-XL) at 5 μg/ml, and FcγRIIIb was cross-linked with mAb 3G8 $F(ab')_2$ (3G8 $F(ab')_2$-XL) at 10 μg/ml, or both were cross-linked together (Both-XL) using $F(ab')_2$-specific goat anti-mouse IgG $F(ab')_2$ for the times indicated in *B* and *C*. Data points and error bars in both panels are the MFI ± SEM from a total of 14 experiments. Statistical significance of the results was assessed by mixed effects analysis of variance and Wilcoxon's matched pair, signed rank test. The time curves of all stimulations are significantly different from the control values or from those induced by PMA ($p < 0.001$). In both *B* and *C*, the stimulations with IV.3 Fab and 3G8 $F(ab')_2$ are not significantly different, but stimulation with both mAb led to a significantly greater increase than that with either alone ($p < 0.05$).

FcγRIIa and FcγRIIIb further induced expression of the CBRM1/5 epitope (to an MFI of 90 ± 10; Fig. 2*C*), an increase that was significantly different from that induced by FcγRIIa or FcγRIIIb alone ($p < 0.05$). Positive controls included stimulation with 100 ng/ml PMA (CBRM1/5 MFI of 528 ± 69 after 20-min stimulation; Fig. 2*C*).

It has been shown that 30% of the total CD11b/CD18 molecules express the I domain after stimulation with PMA (17). Based on the relative expression of the I domain after FcγR stimulation, heterotypic cross-linking of FcγRIIa and FcγRIIIb induced I domain expression on 7% of the total number of CD11b/CD18 molecules on the PMN surface. This compares very well to the reported 10% I

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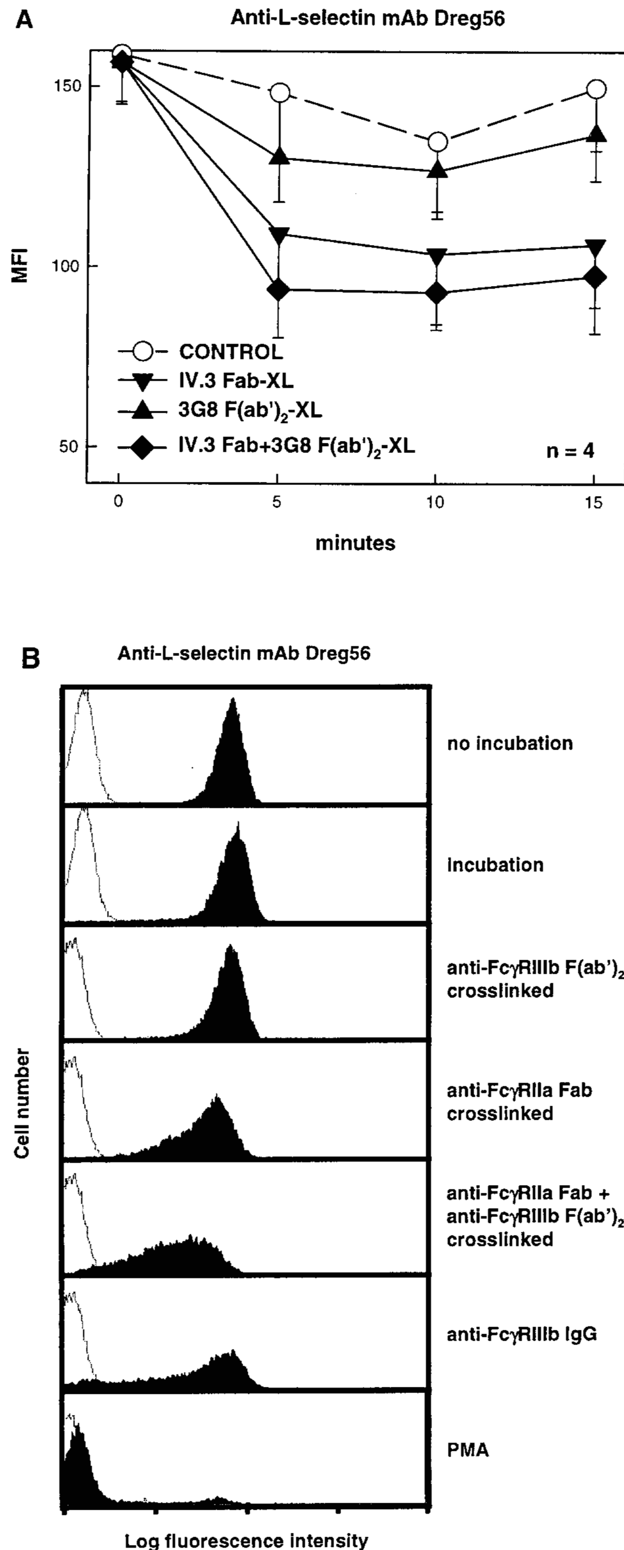

FIGURE 3. Targeted cross-linking of FcγRIIa, but not of FcγRIIIb, induced decreased L-selectin expression on PMN. Expression of L-selectin on PMN in washed whole blood after specific cross-linking of FcγR or incubation with PMA was measured by flow cytometry with FITC-labeled L-selectin mAb Dreg56. *A*, Washed whole blood cells with cross-linked FcγRIIa by mAb IV.3 Fab (IV.3 Fab-XL), with cross-linked FcγRIIIb using mAb 3G8 F(ab')$_2$ (3G8 F(ab')$_2$-XL), or with both FcγR cross-linked together (Both-XL) were incubated for the indicated times. Control cells with no mAb preincubation were also incubated with goat anti-mouse IgG F(ab')$_2$. Cells were stained with FITC-labeled L-selectin mAb, and the MFI of the granulocyte population measured by flow cytometry is shown. The average MFI from four experiments is plotted with the SEM. Statistical analysis indicated that L-selectin expression induced by IV.3 Fab was significantly lower than that induced by 3G8 F(ab')$_2$ ($p < 0.05$), but the effect of both mAb together was not significantly different from that of IV.3 Fab, nor was the effect of 3G8 F(ab')$_2$ different from the control value. *B*, Histograms of a typical experiment showing decreased binding of Dreg56 after cross-linking FcγR for 15 min at 37°C (solid histograms). FITC-labeled mAb MOPC21 (murine IgG1) was used as an isotype-matched control (dotted histograms). Anti-FcγRIIa mAb IV.3 Fab were used at 5 μg/ml, and anti-FcγRIIIb mAb 3G8 F(ab')$_2$ were used at 10 μg/ml and cross-linked as described in *Materials and Methods*. 3G8 IgG was used at 5 μg/ml, and the respective PMA concentration was 100 ng/ml. A representative experiment from a total of seven is shown, and in this case the donor was heterozygous for FcγRIIa$^{H131/R131}$.

domain expression induced by 0.1 μM FMLP, which was sufficient to mediate >90% of the cell adhesion to ICAM-1 and fibrinogen (17). To directly determine the adhesive potential of FcγR-stimulated cells, we assessed both homotypic aggregation (a CD11b/CD18-dependent process) (37) and binding to fibrinogen (dependent on expression of the I domain) (17). Heterotypic cross-linking with mAb 3G8 IgG induced a strong homotypic aggregation response indistinguishable from that induced by FMLP (32 vs 37 clumps in a defined field (see *Materials and Methods*; with 1.9×10^5 and 1.4×10^5 total nonclumped cells remaining; $n = 3$). In addition, stimulation of PMN with 10^{-7} M FMLP or 10 μg/ml 3G8 IgG induced binding to fibrinogen-coated plates (117 and 49 bound cells in a defined field (see *Materials and Methods*), respectively; $n = 3$). Negative controls included isotype control (MOPC21), anti-CR1 mAb HB8592, and anti-CD55 mAb IA10 (≤2 clumps and ≤10 fibrinogen-bound cells in all cases). These data demonstrate that cross-linking of PMN FcγR is sufficient to stimulate increased CD11b/CD18 expression, increased I domain expression, and increased PMN adhesion.

FcγRIIa and FcγRIIIb induced differential changes in L-selectin expression

Expression of L-selectin is important for initial PMN contact-initiated rolling on activated endothelial cells under shear flow conditions. Activation-induced shedding of this receptor has been reported, which may impinge on the ability of activated PMN to migrate from the circulation to endothelial cells and tissues (9, 24). Homotypic cross-linking of PMN FcγRIIa led to a rapid decrease in surface expression of L-selectin expression (Fig. 3, *A* and *B*). This shedding was dependent on protein tyrosine kinase activity, as shown by >80% inhibition with genistein (results not shown). No significant change in expression occurred on control cells (cells incubated at 37°C; Fig. 3*A*). PMA (100 ng/ml), used as a positive control, led to virtually complete L-selectin shedding indicated by an MFI not different from that of the isotype control Ab (Fig. 3*B*). Complete shedding of L-selectin after stimulation with FMLP (1 μM) was also observed (not shown). Remarkably, homotypic cross-linking of FcγRIIIb led to little or no change in L-selectin expression that did not reach statistical significance ($p > 0.05$; Fig. 3). Heterotypic cross-linking induced with IV.3 Fab+3G8 F(ab')$_2$ and F(ab')$_2$ goat anti-mouse IgG (or induced by mAb 3G8 IgG) decreased L-selectin expression to levels indistinguishable from those produced by FcγRIIa alone ($p > 0.05$; Fig. 3). Consistent with other studies, we observed a decrease in CD43 (sialophorin) surface expression after PMN stimulation with PMA but no change in expression after stimulation with FMLP (21, 45). Similarly, no change in CD43 expression was observed after

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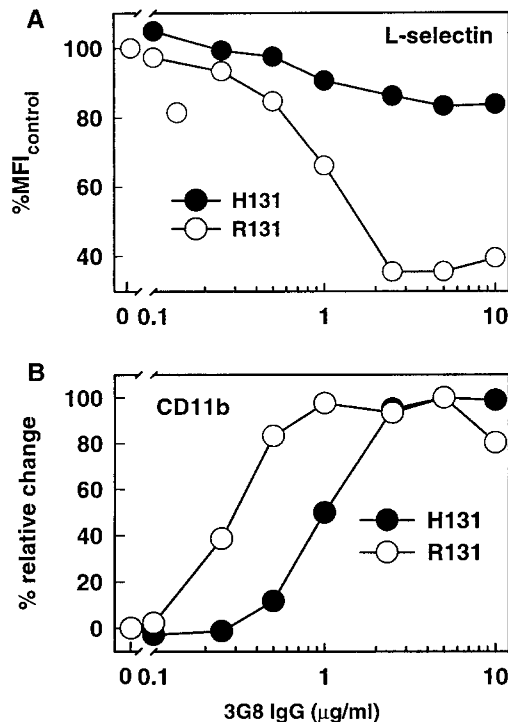

FIGURE 4. Effects of the allelic polymorphism of FcγRIIa on the dose-response curve of 3G8 IgG-mediated heterotypic cross-linking of FcγRIIIb and FcγRIIa on PMN. Washed whole blood of donors homozygous for $FcγRIIa^{R131}$ (R131) or for $FcγRIIa^{H131}$ (H131) was incubated with increasing concentrations of mAb 3G8 for 10 min at 37°C. Cells were stained with FITC-labeled L-selectin (*A*) or CD11b (*B*) mAbs, and the mean fluorescence of the granulocyte population was measured by flow cytometry. For L-selectin, the percentages relative to the basal level of the control samples were calculated, and a representative experiment of a total of three is shown (*A*). For CD11b, the percentages relative to the maximally measured up-regulation were calculated, and a representative experiment of a total of five is shown (*B*). The experiments were performed with matched donors in a paired fashion.

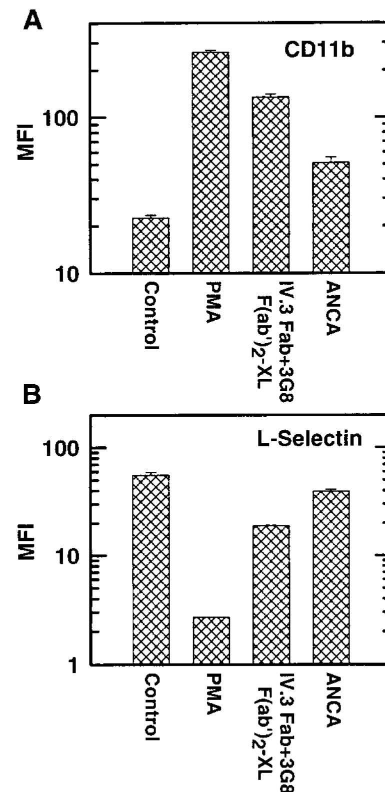

FIGURE 5. ANCA induces up-regulation of CD11b and shedding of L-selectin on PMN. Whole washed blood was stimulated with 20 μg/ml ANCA (anti-PR3 mAb CLB-702), 20 μg/ml murine IgG1 (control), PMA, or heterotypic FcγR cross-linking (using mAbs IV.3 Fab+3G8 $F(ab')_2$ followed by $F(ab')_2$ goat anti-mouse IgG) at 37°C for 20 min as described in *Materials and Methods*. The surface expression of CD11b (*A*) and L-selectin (*B*) on the PMN was measured by flow cytometry. Individual samples were performed in triplicate, and a representative experiment from a total of four is shown. The isotype control was not statistically different from unstimulated control cells (not shown). All stimulated samples were significantly different from the control ($p < 0.02$).

cross-linking of either PMN FcγR (not shown). These data indicate that FcγRIIa-mediated, but not FcγRIIIb-mediated, PMN activation can induce L-selectin shedding.

Influence of FcγRIIa alleles on adhesion receptor changes

The amount of L-selectin shedding induced by PMN activation with mAb 3G8 IgG was less than that observed with mAb Fab heterotypic cross-linking (Fig. 3*B*). To investigate the basis for the differential heterotypic activation of L-selectin shedding (Fig. 3*B*), we considered the possibility that the ability of FcγRIIa to bind the Fc region of mAb 3G8 IgG will be influenced by which FcγRIIa alleles are expressed by the donor (41). The R131 allele binds mIgG1 (the subclass of mAb 3G8 IgG) with much higher affinity than the H131 allele (46). While the L-selectin shedding induced by cross-linked mAb IV.3 was consistently strong and comparable between different donors, the shedding induced with 3G8 IgG was variable and in many cases less than the mAb IV.3-stimulated decrease (Fig. 3*B*). Indeed, in donors homozygous for $FcγRIIa^{R131}$, mAb 3G8 IgG induced significant L-selectin shedding (to the same level as cross-linked anti-FcγRIIa), while very little shedding of L-selectin from donors homozygous for $FcγRIIa^{H131}$ was observed (Fig. 4*A*). The impact of this polymorphism in the mAb 3G8 IgG-stimulated expression of CD11b was also observed, but only at subsaturating concentrations of mAb (Fig. 4*B*). This is presumably due to the ability of FcγRIIIb (when stimulated with saturating concentrations of mAb) to induce high levels of CD11b up-regulation independently of FcγRIIa. These results support the direct mAb observations showing that FcγRIIIb does not significantly contribute to the FcγR-induced shedding of L-selectin and reinforce the importance of host FcγR genetics in Ab/autoantibody-mediated inflammatory responses (2).

ANCA induces up-regulation of CD11b and down-regulation of L-selectin on PMN

Incubation of whole washed blood with 20 μg/ml anti-PR3 mAb for 20 min at 37°C induced a significant up-regulation of CD11b surface expression on neutrophils (Fig. 5*A*; $p < 0.001$; $n = 4$). Incubation with 20 μg/ml isotypically matched murine IgG1 did not lead to any increase. The magnitude of CD11b up-regulation varied among donors but was not significantly different from heterotypic cross-linking of FcγR ($n = 4$). Similarly, we observed ANCA induced down-regulation of L-selectin (Fig. 5*B*), but the magnitude of ANCA-induced L-selectin down-regulation was significantly less than that observed after heterotypic cross-linking of FcγR ($p < 0.011$; $n = 4$).

Effects of inhibitors on the induced changes in surface markers

Increases in intracellular cAMP levels down-regulate numerous pathways in PMN (47–50). It has previously been found that

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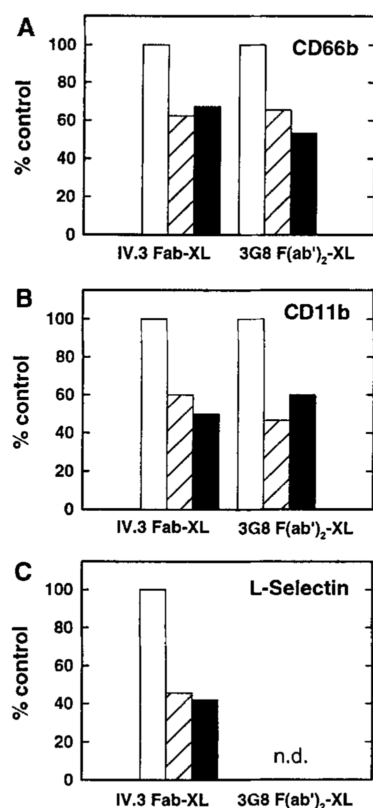

FIGURE 6. Effects of misoprostol and NECA on the changes in surface expression of CD66b, CD11b, and L-selectin on PMN induced by cross-linking of FcγRIIa or FcγRIIIb. Whole washed blood was preincubated with mAb IV.3 Fab (IV.3 Fab-XL) or mAb 3G8 F(ab')$_2$ (3G8 F(ab')$_2$-XL) either with no inhibitor (white bars) or in the presence of 10^{-5} M misoprostol (hatched bars) or 10^{-6} M NECA (black bars) as described in *Materials and Methods*. XL, cross-linked. The surface expression of CD66b (*A*), CD11b (*B*), and L-selectin (*C*) was measured by flow cytometry after staining the cells with the respective specific FITC-labeled mAbs. Data are expressed as the difference in MFI between the control samples and the respective cross-linked samples. A representative experiment of a total of three is shown. n.d., not done.

cAMP modulators can block FMLP-induced up-regulation of CD11b/CD18 on PMN (47). Similarly, the adenosine A2-receptor agonist NECA, which elevates intracellular cAMP levels, has been shown to inhibit FcγR phagocytosis by PMN (51). Pretreatment of whole washed blood with 10^{-6} M NECA resulted in significant (but not complete) inhibition of homotypic FcγRIIa- and homotypic FcγRIIIb-induced PMN degranulation (CD66b expression) and up-regulation of CD11b on PMN (Fig. 6, *A* and *B*). PMN FcγRIIa-induced L-selectin shedding was also partially inhibited by NECA (Fig. 6*C*). The inhibitory effect of NECA on the FcγR-induced up-regulation of CD11b could be reversed by the specific competitors of NECA (52): 10^{-4} M 3,7-dimethyl-1-propargylxanthine and 10^{-4} M 8-*p*-sulfo-phenyltheophiline (not shown). Furthermore, the adenosine A2-receptor-specific agonist, 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine (CGS 21680; 10^{-5} M), partially inhibited FcγR-mediated CD11b up-regulation, while the adenosine A1-receptor agonist, N^6-cyclopentyl-adenosine (10^{-6} M), was inefficient in altering FcγR-mediated changes in CD11b expression (not shown). Misoprostol, a stable PGE$_1$ analogue, also induces a rise in the intracellular cAMP concentration (50, 53). Comparable inhibition of PMN FcγR-induced changes in CD66b, CD11b, and L-selectin expression was achieved by pretreatment of washed whole blood with 10^{-5} M misoprostol (Fig. 6). These results indicate that changes in cAMP levels can partially block FcγR-induced changes in L-selectin and CD11b/CD18 expression, but do not allow us to distinguish the pathways leading to the differential shedding of L-selectin and up-regulation of CD11b by FcγRIIa and FcγRIIIb.

Discussion

We have shown in this study that activation of PMN via FcγR can lead to different adhesive phenotypes depending on which FcγR is engaged. While both FcγRIIa and FcγRIIIb induce degranulation and up-regulation of CD11b/CD18, only FcγRIIa induces L-selectin shedding. Similarly, 3G8 IgG induces strong down-regulation of L-selectin on PMN from donors homozygous for FcγRIIa^{R131}, which binds murine IgG1 (46), but not from donors homozygous for FcγRIIa^{H131}, which has very low affinity for murine IgG1 (46) reinforcing the lack of FcγRIIIb-induced shedding of L-selectin. In parallel with the increase in total CD11b/CD18 expression, the I-domain of CD11b, which contains the ligand binding site for ICAM-1 and fibrinogen (17) (and is recognized by mAb CBRM1/5), was also up-regulated on PMN by homotypic cross-linking of either FcγRIIa or FcγRIIIb. The level of I domain epitope expression induced by FcγR-mediated cell activation compares well with published values that have been shown to mediate most of the firm PMN adhesion to ICAM-1- and fibrinogen-coated substrate in vitro (17). Indeed, FcγR-induced PMN binding to fibrinogen and PMN homotypic aggregation was observed and was indistinguishable in magnitude from FMLP-induced adhesion/aggregation.

Synergistic interactions between FcγRIIIb and FcγRIIa have been described in the phagocytic response and in the respiratory burst (54, 55). While we have no direct evidence of functional synergism between FcγRIIa and FcγRIIIb in the present study, heterotypic cross-linking of these receptors did lead to further increases (relative to homotypic cross-linking of either receptor) in CD11b/CD18 expression. One potential mechanism of enhanced heterotypic responses is the FcγRIIIb-induced tyrosine phosphorylation of FcγRIIa (54). In an attempt to dissociate the FcγRIIa and FcγRIIIb contributions to these changes in adhesion receptor expression, we used the PGE1 analogue misoprostol and the adenosine A2-receptor analogue NECA. NECA has been shown to differentially effect monocyte FcγRIa- and FcγRIIa-mediated phagocytosis (52). However, both FcγRIIa- and FcγRIIIb-mediated degranulation and up-regulation of CD11b/CD18 expression were partially inhibited to comparable levels by these modulators of cAMP (NECA and misoprostol). This is in contrast to the complete inhibition of FMLP-stimulated up-regulation of CD11b/CD18 and adhesion by modulators of cAMP (47, 48). This suggests that FcγRIIa and FcγRIIIb certainly share some signaling properties, while the dissociation of L-selectin shedding in the absence of pharmacologic inhibitors indicates that FcγRIIa and FcγRIIIb must also engage some distinct signaling elements (3, 56).

The recruitment of phagocytic cells to an inflammatory site is facilitated by activated endothelial cells expressing increased levels of E-selectin, P-selectin, and ICAM-1 (5). While these molecules are able to mediate rolling and adhesion of PMN (10), the accumulation of circulating leukocytes in mice lacking L-selectin on leukocytes is greatly diminished (9, 57). Indeed, activation-induced shedding of PMN L-selectin has been shown to reduce the transmigration of cells across endothelial cells (24), and induction of shedding by anti-inflammatory agents prevents PMN attachment (58). Conversely, inhibition of L-selectin shedding leads to increased accumulation of PMN on activated endothelial cells under shear flow conditions (8). The importance of L-selectin in initial PMN attachment to and rolling on endothelial cells has been

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underscored by the observation that L-selectin mediates faster rolling and stronger tethering than either E- or P-selectin (12, 59), and that it is particularly suited for the initiation of PMN attachment to endothelial cells under flow conditions (60). Furthermore, L-selectin cross-linking itself can signal the up-regulation of CD11b/CD18 (61). We show here that cross-linking of FcγR leads to significant, but not complete, shedding of L-selectin. Since FcγRIIIb, unlike FcγRIIa, induces only marginal shedding of L-selectin, the resulting adhesive phenotype of PMN will clearly be influenced by the nature of the FcγR-dependent stimulus. This may have implications in vivo in situations where there might be preferentially engagement of individual FcγR by IgG autoantibodies or immune complexes (30).

After the initiation of PMN rolling, firm adhesion to endothelial cells is mediated in large measure by the β_2 integrins (especially CD11b/CD18). Stimulation of PMN with FMLP leads to the rapid up-regulation of surface CD11b/CD18 from intracellular stores (secretory vesicles) (43). Other stimuli (such as PMA) may also lead to expression of CD11b/CD18 from specific granules. Our data show that rapid CD11b/CD18 up-regulation from intracellular stores is mediated by homotypic cross-linking of either FcγRIIa or FcγRIIIb (Fig. 2). There was no evidence for up-regulation of CD11a/CD18 upon PMN activation (results not shown) (43). Interestingly, functional blocking of CD11b/CD18-mediated adhesion of PMN to ICAM-1 and fibrinogen has indicated that the 10% subset of CD11b/CD18 molecules identified by CBRM1/5 after 0.1 μM FMLP stimulation mediates >90% of the adhesive activity of CD11b/CD18 (17). This suggests that the 7% of CD11b/CD18 molecules expressing the CBRM1/5 epitope after FcγR cross-linking may be sufficient to mediate cell adhesion, particularly under conditions with very little L-selectin shedding (Fig. 5). Indeed, FcγRIIIb-mediated activation would induce such a phenotype. The role of direct interactions between FcγRIIIb and CD11b/CD18 (54, 62–64) in the modulation of the adhesive potential of PMN remains to be determined.

The severity of the systemic vasculitis that characterizes many autoimmune diseases, including WG, has been correlated to serum levels of autoantibodies (65). ANCA are found in the circulation of patients with many forms of systemic vasculitis. The most common ANCA target in WG is PR3 (66, 67). Small amounts of PR3 may be found associated with the plasma membrane of circulating PMN and monocytes, thereby becoming accessible to circulating autoantibodies and supporting a possible role in the pathogenesis of WG (68). Anti-MPO, anti-lactoferrin, and other ANCA have been found to be strongly associated with other autoimmune diseases (65, 67, 69). ANCA can bind to the surface of PMN and induce cell activation (26, 27, 70), and a role for FcγR in this ANCA-induced PMN activation has been shown (26–28, 65). PMN from patients with WG show signs of activation (increased CD66 and PR3 surface expression) but no change in L-selectin expression (29). Likewise, in the immune complex-mediated disease systemic lupus erythematosus, circulating PMN have increased surface expression of CD11b/CD18 (which can be correlated with the disease severity) but no parallel decrease in L-selectin expression (25). These data suggest that in contrast to other inflammatory mediators (FMLP, IL-8, and C5a), PMN activation by FcγR leads to a distinct pattern of activation, with relative preservation of L-selectin expression (31). The ability of FcγRIIIb, but not FcγRIIa, to reproduce this phenotype suggests that some autoantibody- and immune complex-induced PMN activation may proceed primarily through FcγRIIIb, which has been shown to be the dominant FcγR mediating PMN activation by certain immune complexes (30).

FcγRIIIb-mediated PMN activation with maintained L-selectin expression provides a mechanism by which PMN activated in the circulation may be able to adhere to and initiate damage of endothelial cells. In autoantibody- and immune complex-mediated diseases, therefore, we anticipate that the differential engagement of FcγRIIa or FcγRIIIb will alter the inflammatory potential of preactivated PMN to induce endothelial cell damage, especially in the absence of immune complex deposition per se in the vessel walls. The role of host FcγR genetics, including the FcγRIIa-H131/R131 and FcγRIIIb-NA1/NA2 polymorphisms, will undoubtedly influence the spectrum of inflammatory damage. Indeed, we have preliminary evidence that the FcγRIIIb-NA1 allele shows an association with the development of renal disease in patients with WG (71). The therapeutic targeting of individual FcγR may allow specific regulation of this proadhesive phenotype.

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