

# Cytokine-induced immune complex binding to the high-affinity IgG receptor, Fc $\gamma$ RI, in the presence of monomeric IgG

Cees E. van der Poel,<sup>1</sup> Roos A. Karssemeijer,<sup>1</sup> Peter Boross,<sup>1</sup> Joke A. van der Linden,<sup>1</sup> Miranda Blokland,<sup>1</sup> Jan G. J. van de Winkel,<sup>2</sup> and Jeanette H. W. Leusen<sup>1</sup>

<sup>1</sup>Immunotherapy Laboratory, Department of Immunology, University Medical Center, Utrecht, The Netherlands; and <sup>2</sup>Genmab, Utrecht, The Netherlands

**Fc $\gamma$ RI is the sole high-affinity immunoglobulin G (IgG) receptor on leukocytes. Its role in immunity and the clearance of opsonized particles has been challenged, as the receptor function may well be hindered by serum IgG. Here, we document immune complex binding by Fc $\gamma$ RI to be readily enhanced by cytokine stimulation, whereas binding of monomeric IgG only modestly increased.**

**Enhanced immune complex binding was independent of Fc $\gamma$ RI surface expression levels. Fc $\gamma$ RI, saturated with prebound IgG, was found capable of effective immune complex binding upon cytokine stimulation. Cytokine-enhanced binding was observed across a variety of immune complexes, including hulgG3- or mlgG2a-opsonized red blood cells, rituximab- or ofatumumab-**

**opsonized B-cell lymphoma, and cetuximab-opsonized glioblastoma cells. This study contributes to our understanding of how Fc $\gamma$ RI can participate in the clearance of opsonized particles despite saturation by monomeric IgG. (*Blood*. 2010;116(24): 5327-5333)**

## Introduction

Fc receptors are receptors for immunoglobulins that play a central role in immunity. On human leukocytes, a myriad of Fc $\gamma$  receptors are expressed; among these receptors, Fc $\gamma$ RI is the only known high-affinity receptor for immunoglobulin G (IgG).<sup>1</sup> Fc $\gamma$ RI is constitutively expressed on monocytes, macrophages, and myeloid dendritic cells. Interferon $\gamma$  (IFN $\gamma$ ) can enhance surface expression of Fc $\gamma$ RI on these cells, and Fc $\gamma$ RI expression can be induced on granulocytes by IFN $\gamma$  or granulocyte colony-stimulating factor (G-CSF) stimulation. In contrast, cytokines such as interleukin-4 (IL-4), IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ ) down-regulate activating Fc receptors, including Fc $\gamma$ RI, and enhance expression of the inhibitory receptor Fc $\gamma$ RIIb.<sup>2,3</sup> In vitro, IFN $\gamma$  treatment leads to increased Fc $\gamma$  receptor-induced cytokine production.<sup>4</sup>

In vivo, the role of Fc $\gamma$ RI in immunity remains unclear, as due to its high affinity Fc $\gamma$ RI is believed to be saturated with monomeric IgG. This has led to the concept that prebound monomeric IgG prevents participation of Fc $\gamma$ RI in clearing immune complexes by extravasated effector cells.<sup>5</sup> Nevertheless, several in vivo studies documented a role for Fc $\gamma$ RI, varying from a contribution during inflammation and autoimmune reactions,<sup>6-8</sup> or, during monoclonal antibody (mAb)-based immunotherapy in melanoma and B-cell lymphoma models,<sup>9,10</sup> to a malaria model in which transgenic expression of human Fc $\gamma$ RI was central for effective mAb treatment.<sup>11</sup> Furthermore, Fc $\gamma$ RI can induce potent proinflammatory signaling compared with Fc $\gamma$ RIIa,<sup>12</sup> and can efficiently mediate both major histocompatibility complex class II (MHC-II) antigen presentation and cross-presentation.<sup>13-16</sup>

It remains unclear how Fc $\gamma$ RI contributes to immune complex clearance in the presence of high IgG levels. For Fc $\alpha$ RI<sup>17,18</sup> and Fc $\gamma$ RIIa<sup>19,20</sup> it has been shown that cytokine stimulation can increase ligand binding of these receptors. This appears analogous to inside-out regulation described for integrins. Many integrins are

expressed in a low-affinity binding state, which can be transformed to a high-affinity form upon cellular activation.<sup>21</sup> We hypothesized that inside-out regulation could contribute to immune complex binding to Fc $\gamma$ R occupied by IgG. Indeed, several studies support intracellular proteins interacting with Fc $\gamma$ RI and possibly affecting ligand binding.<sup>22,23</sup>

In this study, we investigated the effect of cytokine stimulation on Fc $\gamma$ RI ligand binding. Stimulation of Fc $\gamma$ RI-expressing Ba/F3 cells and primary monocytes resulted in increased binding of immune complexes, whereas binding of monomeric IgG was only moderately enhanced. Upon cellular activation, Fc $\gamma$ RI could readily bind immune complexes despite pre-engaged monomeric IgG. Taken together, these data might explain how Fc $\gamma$ RI supports leukocyte interaction with immune complexes.

## Methods

### Antibodies and reagents

Anti-Fc $\gamma$ RI-A647 was from BioLegend (clone 10.1). Unlabeled 10.1 mAb and mouse IgG1/IgG2a isotype were from eBioscience and BD Pharmingen, respectively. Anti-human Glycophorin A mouse IgG2a hybridoma supernatant was described previously;<sup>24</sup> anti-RhD human IgG3 and IgG1 (clones BRAD3 and BIRMA D6, respectively) were from the International Blood Group Reference Laboratory. Human IgG1 anti-RhD clones LHM76/58 and ESD-1 were from Alba Bioscience. Unlabeled polyclonal human IgG3 and IgG1 were purchased from Sigma-Aldrich. Okadaic acid, LY294002, and U0126 were purchased from ALEXIS Biochemicals. Monoclonal antibody IV.3 was isolated from hybridoma supernatant, and IV.3 Fab fragments were made by Fusion Antibodies.

### Cell lines and monocyte isolation

Ba/F3 cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% fetal calf serum, penicillin/streptomycin, and mouse IL-3, as

Submitted April 16, 2010; accepted August 17, 2010. Prepublished online as *Blood* First Edition paper, August 30, 2010; DOI 10.1182/blood-2010-04-280214.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2010 by The American Society of Hematology

described.<sup>18</sup> A1207 and Daudi were kept in RPMI1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

The retroviral vector pMX Fc $\gamma$ RI internal ribosome entry site (IRES) green fluorescent protein (GFP) was described previously.<sup>25</sup> Amphotropic viral particles produced in HEK293T cells were used to transduce Ba/F3 cells. After transduction, Ba/F3-Fc $\gamma$ RI cells were sorted on a FACSAria (BD Biosciences) on green fluorescent protein expression and further subcloned by limited dilution. For all experiments where subclones are shown, results were repeated with a polyclonal line and/or with at least 2 subclones.

Primary monocytes were from healthy donors. Magnetic-activated cell sorting (MACS)-isolated monocytes were obtained from peripheral blood mononuclear cells (PBMCs) using CD14-beads (Miltenyi Biotec).

Red blood cells (RBCs) were from ficoll-separated blood. RBCs were kept in Alsever buffer (Sigma-Aldrich) for a maximum of 1 week at 4°C.

### Monomeric IgG binding

Ba/F3-Fc $\gamma$ RI cells were stimulated as described in "FACS-based EA rosette assay." Next, cells were washed and incubated with various amounts of mouse IgG2a or huIgG3. After a 1-hour incubation on ice, cells were washed and stained with goat F(ab')<sub>2</sub> anti-mouse IgG (or anti-human IgG) phycoerythrin (PE; SouthernBiotech). After washing, cells were fixed with 1% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and analyzed on a FACSCanto II.

### FACS-based EA rosette assay

Fluorescence-activated cell sorting (FACS)-based erythrocytes coated with antibody (EA) rosette assay was adapted from Beekman et al.<sup>26</sup> Briefly, Ba/F3-Fc $\gamma$ RI cells were starved overnight in RPMI1640 containing 1% fetal calf serum (FCS; RPMI 1% FCS). The next day, cells were stimulated for various time points with 30 ng/mL recombinant mouse IL-3 (Pepro-Tech) in RPMI 1% FCS or in RPMI 1% FCS alone (medium control). RBCs were fluorescently labeled with PKH26 (Sigma-Aldrich) according to manufacturer's protocol, opsonized on ice with mIgG2a anti-glycophorin or huIgG3 anti-Rhesus D, and subsequently washed and resuspended in RPMI EDTA (ethylenediaminetetraacetic acid; RPMI1640, 1% FCS, 10mM EDTA). In a 96-well plate,  $1 \times 10^5$  Ba/F3 cells/well were combined with  $1 \times 10^6$  RBCs/well in RPMI EDTA and incubated for 1 hour at 4°C on a shaker. Cells were washed once with cold PBS and resuspended in cold PBS containing 1% PFA. Rosetting was analyzed on a FACSCanto II (BD Biosciences). It was shown previously that bovine IgG does not bind to human Fc $\gamma$ RI.<sup>27</sup>

Blocking mAb 10.1 or isotype control was added at 20  $\mu$ g/mL to Ba/F3-Fc $\gamma$ RI cells before rosette assay and were kept present during assay at 10  $\mu$ g/mL.

Rosette assays using IgG coupled to tosyl-activated Dynabeads was adapted from Bracke et al.<sup>28</sup> Briefly, human IgG1 was coupled to tosyl-activated Dynabeads-M450 (Invitrogen) as described by the manufacturer. IgG-coupled dynabeads were then added to Ba/F3-Fc $\gamma$ RI cells in RPMI EDTA in a ratio of 3.5 beads/cell. Dynabeads were found sufficiently autofluorescent in the PE channel to discriminate between Ba/F3 cells, beads, and Ba/F3 cell-bound beads. Rosettes were visualized on a LSM710 confocal microscope (Zeiss) equipped with a transmission photomultiplier tube for differential interference contrast (DIC) imaging. Pinhole opening was set to maximum.

For competition assays with monomeric ligand, Ba/F3-Fc $\gamma$ RI cells were incubated with various concentrations of polyclonal huIgG3 on ice (Sigma-Aldrich). Unbound IgG was removed by washing Ba/F3-Fc $\gamma$ RI cells before EA rosette assay. For inhibition experiments, Ba/F3-Fc $\gamma$ RI cells were treated for 30 minutes with dimethyl sulfoxide (DMSO), 20  $\mu$ M LY294002, 20  $\mu$ M U-0126, or 1  $\mu$ M okadaic acid (OA) and stimulated with IL-3 in the presence of the inhibitor. Subsequently, cells were washed and subjected to EA rosette assay.

For measuring binding to tumor cells, tumor cell lines were fluorescently labeled with Lavacell (Activemotif), an amino reactive dye, and subsequently opsonized with 0.25  $\mu$ g/mL rituximab (Roche) or ofatumumab (Genmab/GlaxoSmithKline) for Daudi or cetuximab (Merck) for A1207. After washing, the opsonized cells were incubated with Ba/F3-

Fc $\gamma$ RI for 1 hour at 4°C on a shaker. Cells were washed once with cold PBS and fixed in PBS containing 1% PFA. Binding was measured on a FACSCanto II and visualized on a LSM710 confocal microscope (Zeiss) equipped with a transmission photomultiplier tube for differential interference contrast imaging. Pinhole opening was set to maximum.

### Binding assay with primary monocytes

For inhibition of inside-out signaling, monocytes were isolated as described in the previous "Cell lines and monocyte isolation" paragraph. After overnight incubation with 400 U/mL IFN $\gamma$ , monocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) or CellTracker Violet (Invitrogen) and treated with OA for 30 minutes at 37°C. Monocytes were plated out at  $1 \times 10^5$  cells per well in RPMI EDTA containing 5  $\mu$ g/mL IV.3 antigen-binding fragments (Fabs). After 10 minutes incubation on ice,  $1 \times 10^6$  opsonized RBCs were added. Cells were allowed to bind for 1 hour at 4°C on a shaker. After fixation in 1% PFA, binding was analyzed on a FACSCanto II.

For short-term stimulation with interleukins, PBMCs were isolated using ficoll. Cells were allowed to rest for 1 hour in 1% FCS RPMI at 37°C. Next, PBMCs were stimulated with either IL-4 (500 U/mL), IL-6 (200 U/mL), or tumor necrosis factor (TNF) $\alpha$  and IFN $\gamma$  (500 and 400 U/mL, respectively) for 1 hour in 1% FCS RPMI at 37°C. Subsequently, PBMCs were allowed to bind to PKH26-labeled opsonized erythrocytes for 1 hour at 4°C in a 96-well V-bottom plate. After incubation, the plate was spun down and CD14-fluorescein isothiocyanate (FITC) in PBS with 5% normal mouse serum (NMS) was added to each well. The amount of monocyte binding to RBCs was scored as the percentage of CD14, PKH26 double positive events.

### Statistical analysis

Data analysis was performed using Microsoft Excel 2007 and GraphPad Prism 4 software. Sigmoidal dose-response curves were used to fit rosette-binding curves data. Fitted data were tested using the F-test (comparing curves between treatments). Blocking experiments were tested using unpaired Student *t* test. Error bars depict SDs.

## Results

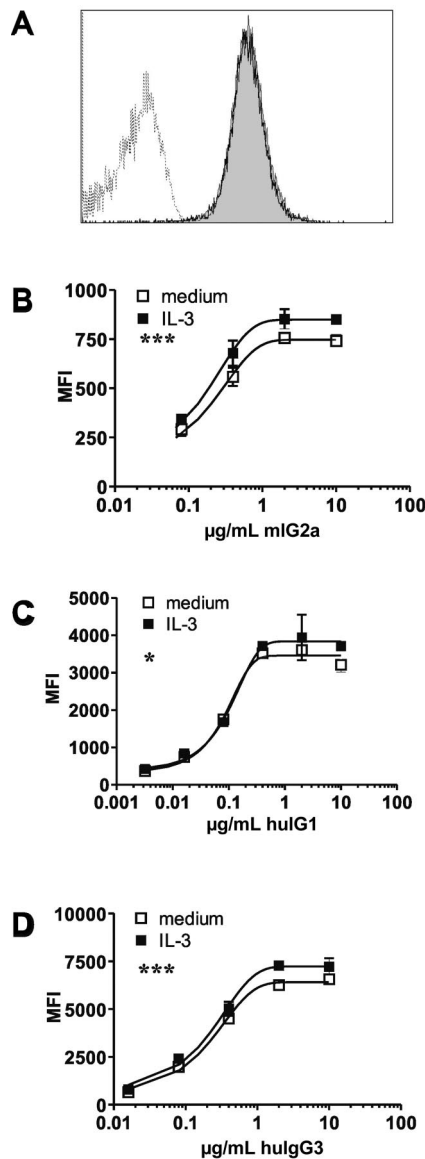
### IL-3 stimulation does not affect surface expression of Fc $\gamma$ RI on Ba/F3-Fc $\gamma$ RI cells

To determine whether Fc $\gamma$ RI is regulated inside out, we used the IL-3-dependent Ba/F3 model. Parental Ba/F3 cells do not express any endogenous Fc $\gamma$ R receptors but do express the Fc $\gamma$  chain (Ho et al<sup>29</sup> and supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). One-hour IL-3-stimulation of an Fc $\gamma$ RI-transduced Ba/F3 subclone (Ba/F3-Fc $\gamma$ RI) did not affect Fc $\gamma$ RI surface expression levels (Figure 1A). Similar results were obtained in a polyclonal line (data not shown). These findings allowed us to perform ligand-binding studies independent of changes in receptor surface expression.

Monomeric IgG binding is a unique characteristic of Fc $\gamma$ RI. IL-3-treated Ba/F3-Fc $\gamma$ RI cells showed a modest, albeit significant, increased binding to mouse IgG2a (mIgG2a), human IgG1, and human IgG3 (huIgG3; Figures 1B-D, respectively). These data demonstrate that short-term stimulation with IL-3 mildly affects monomeric IgG binding in the absence of effects on receptor surface expression levels.

### Inside-out regulation of Fc $\gamma$ RI enhances immune complex binding

To investigate whether treatment of Ba/F3-Fc $\gamma$ RI cells with IL-3-altered immune complex (IC) binding, we used a FACS-based EA rosette assay. This method was previously shown to



**Figure 1.** IL-3 stimulation has no effect on Fc $\gamma$ RI surface expression and slightly increases monomeric IgG binding. Ba/F3-expressing cells were starved overnight and subsequently restimulated with IL-3 for 1 hour. (A) Fc $\gamma$ RI surface expression on a starved (open histogram) and restimulated (closed histogram) subclone of Fc $\gamma$ RI-transduced Ba/F3 cells. Dotted line represents isotype control. (B-D) Binding of monomeric mouse IgG2a (B), human IgG1 (C), or human IgG3 (D) to Fc $\gamma$ RI expressing Ba/F3 cells. Open squares represent starved cells, filled squares represent IL-3 restimulated Ba/F3 cells. Similar results were seen in 3 independent experiments. Datapoints represent means of at least 4 replicates. \* $P < .05$ , \*\*\* $P < .001$ .

correlate with EA-rosette counting by light microscopy.<sup>26</sup> An example of a FACS-based EA rosette assay and gating strategy is shown in supplemental Figure 2.

Upon stimulation with IL-3, Ba/F3-Fc $\gamma$ RI cells exhibited increased binding to mIgG2a-IC compared with control cells, as detected by higher percentages of Ba/F3-Fc $\gamma$ RI:RBC clusters (Figure 2A). Enhanced IC binding was observed as early as 15 minutes after stimulation (Figure 2C), reaching maximum levels by 1 hour. Blocking with Fc $\gamma$ RI-specific mAb 10.1 demonstrated basal binding and IL-3-enhanced binding to be Fc $\gamma$ RI dependent (Figure 2B).

Similar to mIgG2a-IC, Ba/F3-Fc $\gamma$ RI cells showed higher levels of binding of huIgG3-IC upon IL-3 stimulation (Figure 2D).

Furthermore, IL-3 stimulation led to a marked increase in binding of 2 or more opsonized RBCs to Ba/F3-Fc $\gamma$ RI (Figure 2E). Binding to huIgG3-IC was Fc $\gamma$ RI-dependent as determined by receptor blocking with Fc $\gamma$ RI mAb 10.1 (data not shown).

We tested several IgG1 RhD clones (BIRMA D6, ESD-1, and LHM76/58) in the rosette assay. Despite considerable opsonization, IgG1 opsonized RBCs failed to bind to Ba/F3-Fc $\gamma$ RI or primary monocytes (data not shown). This is in concordance with previous reports.<sup>30</sup> It was suggested that in contrast to IgG1, the longer hinge region of IgG3 is capable of overcoming the forces between the negatively charged RBC and the effector cell. Therefore, we used IgG1-coupled beads (huIgG1-IC) to evaluate cytokine-enhanced binding of huIgG1-IC to Ba/F3-Fc $\gamma$ RI. IgG1 coupled beads bound to Ba/F3-Fc $\gamma$ RI cells, whereas bovine serum albumin (BSA)-coupled beads did not. IL-3 stimulation enhanced only binding of IgG1-coated beads, and binding was Fc $\gamma$ RI-dependent, as blocking mAb 10.1 decreased binding to near background levels (Figure 2F). The formation of rosettes with IgG1-coupled beads and Ba/F3-Fc $\gamma$ RI cells could clearly be observed with fluorescence microscopy (Figure 2G).

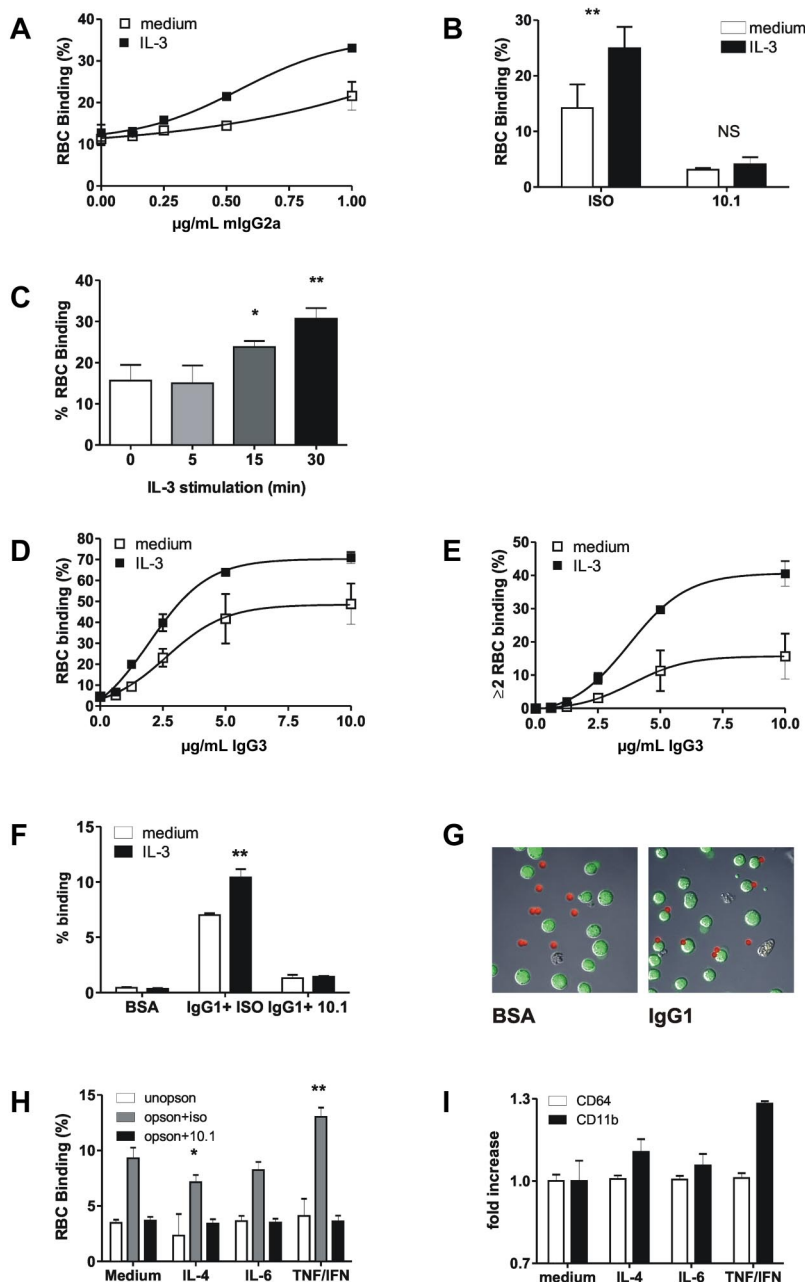
We next assessed whether Fc $\gamma$ RI was similarly regulated in primary monocytes and tested the effect of several proinflammatory cytokines on IC binding. Stimulation with TNF $\alpha$  and IFN $\gamma$ , a known macrophage stimulus,<sup>14</sup> enhanced IC binding by primary monocytes. IC binding was inhibited by Ab 10.1, but not isotype control, supporting the involvement of Fc $\gamma$ RI (Figure 2H and supplemental Figure 3A). Under these conditions, monocytes were positive for surface IgG. Upon stimulation, only slightly increased surface IgG levels were detected, suggesting that TNF $\alpha$  and IFN $\gamma$ -enhanced IC binding to monocytes was not due to a decrease of prebound IgG to Fc $\gamma$ RI after stimulation (supplemental Figure 3B). Furthermore, stimulation did not alter Fc $\gamma$ RI surface expression levels but often (2 of 4 donors) coincided with increased CD11b expression levels (Figure 2I and supplemental Figure 3B). The cytokines IL-6 and IL-4 did not enhance the binding of IC to Fc $\gamma$ RI. Taken together, these data suggested cytokine stimulation increases binding of multivalent ligands (IC) to Fc $\gamma$ RI.

#### Cytokine stimulation enhances Fc $\gamma$ RI binding to opsonized tumor cells

To date, rituximab (RTX) is one of the most successful monoclonal antibodies used in the clinic.<sup>31</sup> Using RTX in combination with a B-cell lymphoma line (Daudi), we studied binding of Ba/F3-Fc $\gamma$ RI to multivalent ligands. IL-3 stimulation led to increased binding of RTX-opsonized Daudi cells to Ba/F3-Fc $\gamma$ RI (Figure 3A). IL-3 induced similar increased binding of Ba/F3-Fc $\gamma$ RI to Daudi cells opsonized with ofatumumab, a newly developed human CD20 mAb (Figure 3A). Binding was Fc $\gamma$ RI-dependent, as evidenced by blocking with mAb 10.1 (supplemental Figure 4). To investigate whether this effect was limited to B-cell lymphoma and CD20 mAb, we tested the human glioblastoma cell line A1207. IL-3 stimulation increased cetuximab-opsonized A1207-binding to Ba/F3-Fc $\gamma$ RI (Figure 3B). Binding of Ba/F3-Fc $\gamma$ RI to opsonized Daudi or A1207 was confirmed using fluorescence microscopy (Figure 3C-D, respectively).

#### Fc $\gamma$ RI inside-out regulation is inhibited by the phosphatase inhibitor, okadaic acid

IL-3 stimulation of Ba/F3 cells leads to activation of the phosphatidylinositol-3-kinase-protein kinase B (PI-3K-PKB) and Raf/



**Figure 2. Stimulation of primary monocytes and Ba/F3-Fc $\gamma$ RI cells results in enhanced IC binding.** Percentage of Ba/F3-Fc $\gamma$ RI cells binding RBCs opsonized with IgG2a (A-C) or human IgG3 (D-E). Open squares represent starved cells, filled squares represent starved Ba/F3-Fc $\gamma$ RI cells stimulated with IL-3 for 1 hour. (B) EA rosette assay in the presence of Fc $\gamma$ RI-specific mAb 10.1 (10.1) or isotype control (iso). \*\* $P < .01$ ; NS: not significant,  $t$  test. (C) Effect of duration of IL-3 stimulus on IC binding. \* $P < .05$ ; \*\* $P < .01$ ,  $t$  test. (D) Binding of 1 or more RBCs to Ba/F3 cells. (E) Binding of 2 or more RBCs to Ba/F3 cells. Datapoints represent the means of at least 3 replicates. Similar results were obtained in 3 independent experiments. \*\*\* $P < .001$ . (F) Binding of Ba/F3-Fc $\gamma$ RI cells to BSA or IgG1-coupled beads in the presence of 10.1 mAb (10.1) or isotype control (ISO). Similar results were obtained in 3 independent experiments. \*\* $P < .01$ ,  $t$  test. (G) Rosetting of BSA and IgG1-coupled beads with Ba/F3-Fc $\gamma$ RI cells visualized with fluorescence microscopy. Beads are detected in the red channel, Ba/F3-Fc $\gamma$ RI in the green channel. (H) Binding of unopsonized (unopson) and opsonized (opson) erythrocytes to primary monocytes in the presence of 10.1 mAb (10.1) or isotype control (iso). Data represent means of triplicates measured from 1 donor. Similar results were seen in 3 of 4 donors. \* $P < .05$ ; \*\* $P < .01$ . (I) Fc $\gamma$ RI (CD64) and CD11b expression on CD14 $^{+}$  gated monocytes. Shown is the fold increase in surface expression relative to unstimulated (medium) cells. Data represent mean from triplicates.

mitogen-activated protein kinase/extracellular-signal-regulated kinase (Raf/MEK/ERK) pathways. It has been shown that Fc $\alpha$ RI inside-out regulation in Ba/F3 cells is dependent on PI-3K and protein phosphatase 2 (PP2a).<sup>17,18</sup> We investigated whether similar signaling pathways underlie Fc $\gamma$ RI inside-out regulation. In our model, both a PI3-Kinase inhibitor (LY294002)<sup>32</sup> and a MEK1/2 inhibitor (U0126)<sup>33</sup> did not affect IL-3-enhanced binding to mIgG2a-IC (Figure 4A). OA, a PP2a inhibitor,<sup>34</sup> completely inhibited the IL-3-enhanced binding.

To investigate whether Fc $\gamma$ RI was similarly regulated on primary monocytes, we stimulated monocytes overnight with IFN $\gamma$  before OA treatment. OA efficiently inhibited binding of opsonized RBCs to monocytes in a dose-dependent manner (Figure 4B). Importantly, treatment of OA did not affect Fc $\gamma$ RI-expression levels (supplemental Figure 5). In these assays, binding of IC to Fc $\gamma$ RII was prevented by the addition of Fc $\gamma$ RII-specific IV.3 Fab before adding IC. Fc $\gamma$ RI-dependent binding of opsonized RBC was

confirmed with mAb 10.1 (Figure 4C). These data suggest that protein phosphatases play a role during Fc $\gamma$ RI inside-out regulation.

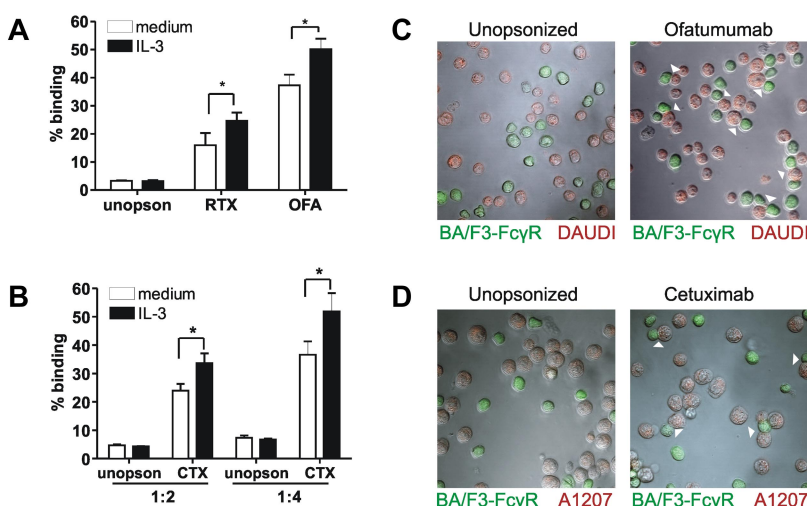
#### Cytokine stimulation enhances binding of immune complexes to saturated Fc $\gamma$ RI

Inside-out regulation of Fc $\gamma$ RI in the Ba/F3 model mainly affected binding to multivalent ligands (immune complexes) rather than monomeric IgG. We hypothesized that this shift could contribute to immune complex binding of Fc $\gamma$ RI already saturated by monomeric ligands.

By saturating Ba/F3-Fc $\gamma$ RI with monomeric IgG3 after stimulation, we studied whether opsonized RBCs were able to bind to IgG-occupied Fc $\gamma$ RI. In unstimulated cells, preincubation with as little as 5  $\mu\text{g/mL}$  monomeric IgG3 blocked RBC binding to Ba/F3-Fc $\gamma$ RI cells to near background levels. Stimulation with

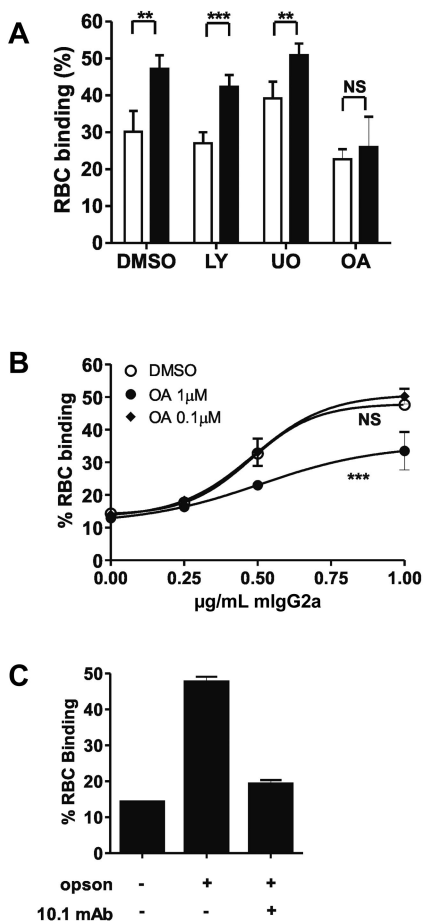


**Figure 3. Cellular activation leads to enhanced FcγRI-mediated binding of opsonized tumor celllines.** (A) Percentage of Ba/F3- FcγRI cells binding to Daudi cells opsonized with Rituximab (RTX), Ofatumumab (OFA) or unopsonized (unopson) Daudi cells. E/T ratio was 1/2. (B) Percentage of Ba/F3- FcγRI cells binding to Cetuximab (CTX) or unopsonized (unopson) A1207. E/T ratio was either 1/2 or 1/4. Data represent the means of at least 3 replicates. FcγRI-blocking mAb 10.1 was used to investigate FcγRI-dependent binding (not shown) \**P* < .05. Experiment was repeated 3 times yielding similar results. (C-D) Binding of IL-3 stimulated Ba/F3-FcγRI to Ofatumumab-opsonized Daudi cells (C) or Cetuximab-opsonized A1207 cells (D) determined with fluorescence microscopy. Ba/F3-FcγRI cells are shown in green, Daudi and A1207 cells in red. Arrowheads denote contacts between Ba/F3-FcγRI and tumor cells.



IL-3, however, led Ba/F3-FcγRI to bind opsonized RBCs effectively (Figure 5A, B). Binding was lower compared with unoccu-

pled FcγRI but quickly reached a plateau, which was maintained at saturating levels of monomeric IgG3 (20 μg/mL). At this concentration, FcγRI was saturated by IgG3 as evidenced by surface IgG staining (Figure 5C). These data document that cytokine stimulation can lead to FcγRI IC binding despite saturation of the receptor with monomeric IgG.

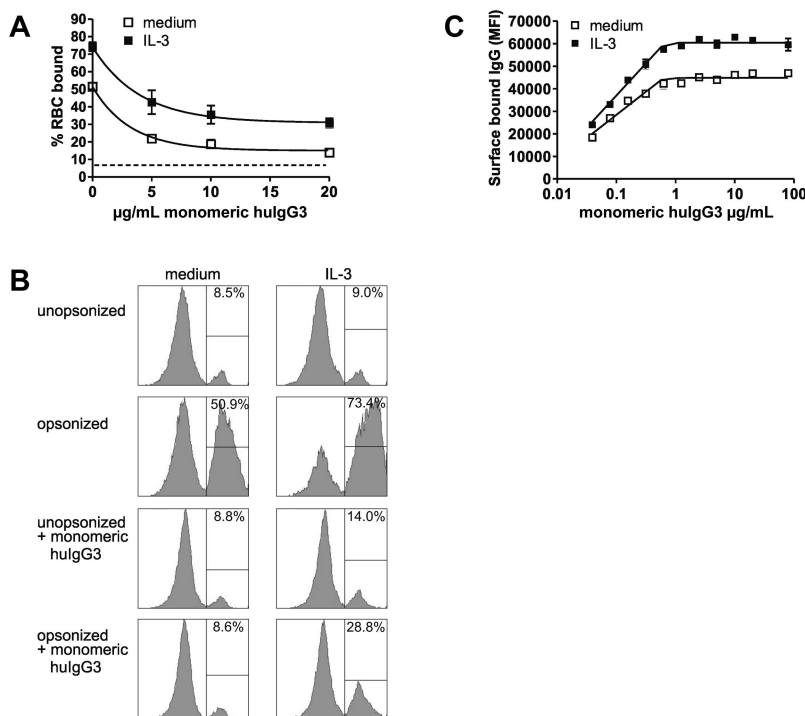


**Figure 4. OA inhibits cytokine-enhanced FcγRI IC binding on primary monocytes and Ba/F3- FcγRI cells.** (A) Ba/F3-FcγRI cells were treated with DMSO, LY294002 (LY), U-0126 (UO) or OA and stimulated with IL-3. IC Binding was tested using mlgG2a-opsonized RBCs. Data represent means of at least 4 replicates. Similar results were obtained in 3 independent experiments. \*\**P* < .01; \*\*\**P* < .001; NS: not significant. (B) IFNγ stimulated monocyte binding to mlgG2a-opsonized RBCs after OA treatment. \*\*\**P* < .001, comparing 1 μM OA treatment with DMSO fitted curve; NS: not significant, comparing 0.1 μM OA treatment with DMSO treated fitted curve. (C) Effect of FcγRI blocking mAb 10.1 binding of mlgG2a-opsonized RBCs to FcγRI. Similar results were seen in 3 independent experiments.

## Discussion

The role of FcγRI in immunity remains unclear, as it is unknown how a high-affinity Fcγ receptor can bind immune complexes. Extravasating monocytes originate from an IgG rich milieu, and it is believed that due to the high affinity of FcγRI, this receptor may well be saturated with monomeric IgG. Only after de novo synthesis has FcγRI been postulated to contribute to the clearance of IgG-opsonized particles. Here, we show that FcγRI ligand-binding can be regulated inside out (Figures 2-4) and that this regulation impacts IC binding to a saturated receptor (Figure 5). Inside-out regulation represents a fast process, as IL-3 enhanced binding could be detected within 15 minutes after stimulation (Figure 2C). Cytokine-enhanced IC binding was observed with different ICs, including tumor cell lines opsonized with IgG1-therapeutic mAbs, as well as mouse IgG2a- and human IgG3-opsonized RBCs and human IgG1-coupled beads (Figures 2-3). Increased binding induced by cytokines was not associated with differences in receptor-surface expression levels. The presence of EDTA in our binding assays makes it unlikely that integrin interactions are involved during cytokine-enhanced IC binding. Furthermore, we did not observe increased binding to unopsonized cells upon stimulation.

Increased binding of ligand upon cellular activation was particularly striking when multivalent ligands (ICs) were used (Figure 3). Monovalent ligands showed a small increase in binding (Figure 1) upon stimulation. This could be due to small aggregates in our IgG preparations, as IgG dimers formed with mouse anti-human κ light-F(ab')<sub>2</sub> showed a small increase in binding upon stimulation (data not shown). Because the increase in binding was most apparent using multivalent ligands, we speculate that the main mechanism involved in regulating IC binding is likely due to alterations in receptor mobility and/or receptor clustering. Photo-bleach experiments with an FcαRI-YFP fusion protein suggested increased mobility of the receptor upon IL-3 stimulation in Ba/F3



**Figure 5. Effect of cellular activation on immune complexes binding to saturated Fc $\gamma$ RI.** (A) Binding of Ba/F3-Fc $\gamma$ RI to hulgG3-opsonized RBCs (y-axis) after incubating Ba/F3-Fc $\gamma$ RI with varying amounts of monomeric hulgG3 (x-axis). Dotted line represents binding of Ba/F3-Fc $\gamma$ RI to unopsonized RBCs. (B) Percentage of Ba/F3-Fc $\gamma$ RI binding to RBCs. One representative sample of triplicates is shown. (C) Amounts of cell-bound Ba/F3-Fc $\gamma$ RI IgG3 after incubation with varying amounts of monomeric human IgG3 as detected by flow cytometry. Experiments were repeated 3 times yielding similar results.

cells (Honing et al, unpublished data). On the other hand, dimerization of Fc $\gamma$ RI has been previously suggested<sup>35</sup> and Fc $\gamma$ RI has been shown to associate with plasma membrane microdomains.<sup>36</sup> Indeed, Fc $\gamma$ RI-binding capacity could be altered upon cholesterol depletion. A model where lipid raft localization and subsequent clustering affects ligand binding has also been suggested for Fc $\gamma$ RIIA.<sup>37</sup> It is important to note that the inside-out regulation described here is probably different from the active “trapping” of IC by Fc $\gamma$ R as suggested by Dale et al,<sup>38</sup> as our binding experiments were performed at 4°C. Cytokine-enhanced ligand binding was capable of inducing IC binding despite the presence of prebound IgG to Fc $\gamma$ RI. It is possible that the affinity of Fc $\gamma$ RI might differ between antigen-bound (in IC) and antigen-unbound IgG (prebound IgG to the receptor). However, it seems likely that such differences would be antigen dependent.

Several mouse models have shown involvement of Fc $\gamma$ RI during immunotherapy. We found that ligand binding by mouse Fc $\gamma$ RI is at least differently regulated compared with human Fc $\gamma$ RI. Ligand binding to IC by mouse bone marrow–derived dendritic cells (DCs) and bone marrow–derived macrophages was not affected by cytokine stimulation or OA treatment (supplemental Figure 5). Thus, mouse Fc $\gamma$ RI is not capable of inside-out regulation, or else it is dependent on different stimuli. Mouse and human Fc $\gamma$ RI both bind IgGs with high affinity; however, they show only 19% sequence similarity with the intracellular, c-terminal tail.

Previously, dephosphorylation of Fc $\alpha$ RI was shown to regulate ligand binding and to be dependent on PP2A.<sup>17</sup> Unlike Fc $\alpha$ RI, however, serine phosphorylation of the cytosolic tail of Fc $\gamma$ RI  $\alpha$ -chain is unlikely to play a role during inside-out regulation, because a truncated receptor lacking all serines was found to respond to cytokine stimulation (data not shown). Strikingly, PI-3K inhibition did not affect cytokine-enhanced IC binding to Fc $\gamma$ RI, suggesting distinct mechanisms underlying Fc $\gamma$ RI regulation compared with Fc $\alpha$ RI.

In earlier reports, we published the interaction between the cytosolic tail of Fc $\gamma$ RI and periplakin<sup>22,26</sup> to affect receptor-ligand binding. Ba/F3-Fc $\gamma$ RI cells did not show periplakin-dependent inside-out regulation, as truncation of Fc $\gamma$ RI and overexpression of c-terminal periplakin did not affect cytokine-enhanced ligand binding (data not shown). This could be due to low expression levels of endogenous periplakin in Ba/F3 cells.

Overall, our data suggest that Fc $\gamma$ RI saturated with monomeric IgG is capable of binding multivalent immune complexes. This finding contributes to the Fc $\gamma$  receptor field, as it suggests that Fc $\gamma$ RI can participate in clearance of IgG-opsonized particles despite saturation with monomeric ligand. Furthermore, it opens the field for targeted immunotherapy to this receptor. Fc $\gamma$ RI-targeted immunotherapy can be achieved via bispecific antibodies or the use of aglycosylated IgGs to bind selectively to Fc $\gamma$ RI.<sup>39</sup> Studies using a chimeric receptor consisting of carcinoembryonic antigen–specific scFv fused to the cytosolic tail of Fc $\gamma$ RI have demonstrated the potent cytotoxic effects mediated through this receptor.<sup>40</sup> Direct targeting of Fc $\gamma$ RI by expressing scFv-H22 on tumor cell lines showed similar results.<sup>41</sup>

Our data provide new insights into the role of Fc $\gamma$ RI in biology, although the precise mechanism underlying Fc $\gamma$ RI inside-out regulation needs to be elucidated. Furthermore, our finding that Fc $\gamma$ RI can contribute to immune complex binding warrants further research into the efficacy and role of Fc $\gamma$ RI during immunotherapy.

## Acknowledgments

We thank G. Spierenburg and K. Gaiser for cell sorting, M. Jansen for assisting with mouse models, and P. Coffey for critically reading the manuscript.

C.E.v.d.P. was supported by a Dutch Science Fund (NWO-ALW2PJ/05 088), and P.B. was supported by a grant from the Association of International Cancer Research (AICR 06-368).

## Authorship

Contribution: C.E.v.d.P., R.A.K., and P.B. designed and performed research and analyzed and interpreted data; J.A.v.d.L. and M.B.

performed research; and C.E.v.d.P., J.G.J.v.d.W., and J.H.W.L. designed research and wrote the manuscript.

Conflict-of-interest disclosure: Ofatumumab is a product of Genmab/GlaxoSmithKline and J.G.J.v.d.W. is an employee of Genmab. The remaining authors declare no competing financial interests.

Correspondence: J. H. W. Leusen, University Medical Centre Utrecht, Department of Immunology KC02.085.2, Laboratory for Immunotherapy, Lundlaan 6, 3584 EA Utrecht, The Netherlands; e-mail: jleusen@umcutrecht.nl.

## References

- Bruhns P, Iannascoli B, England P, et al. Specificity and affinity of human Fc $\gamma$  receptors and their polymorphic variants for human IgG subclasses. *Blood*. 2009;113(16):3716-3725.
- Pricop L, Redecha P, Teillaud JL, et al. Differential modulation of stimulatory and inhibitory Fc $\gamma$  receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol*. 2001;166(1):531-537.
- Tridandapani S, Wardrop R, Baran CP, et al. TGF- $\beta$  1 suppresses [correction of suppresses] myeloid Fc $\gamma$  receptor function by regulating the expression and function of the common gamma-subunit. *J Immunol*. 2003;170(9):4572-4577.
- Boruchov AM, Heller G, Veri MC, et al. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J Clin Invest*. 2005;115(10):2914-2923.
- Nimmerjahn F, Ravetch JV. Fc $\gamma$  receptors as regulators of immune responses. *Nat Rev Immunol*. 2008;8(1):34-47.
- Barnes N, Gavin AL, Tan PS, et al. Fc $\gamma$ RI-deficient mice show multiple alterations to inflammatory and immune responses. *Immunity*. 2002;16(3):379-389.
- Baudino L, Nimmerjahn F, Azeredo da Silveira S, et al. Differential contribution of three activating IgG Fc receptors (Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV) to IgG2a- and IgG2b-induced autoimmune hemolytic anemia in mice. *J Immunol*. 2008;180(3):1948-1953.
- Ioan-Facsinay A, de Kimphe SJ, Hellwig SM, et al. Fc $\gamma$ RI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity*. 2002;16(3):391-402.
- Bevaart L, Jansen MJ, van Vugt MJ, et al. The high-affinity IgG receptor, Fc $\gamma$ RI, plays a central role in antibody therapy of experimental melanoma. *Cancer Res*. 2006;66(3):1261-1264.
- Minard-Colin V, Xiu Y, Poe JC, et al. Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV. *Blood*. 2008;112(4):1205-1213.
- McIntosh RS, Shi J, Jennings RM, et al. The importance of human Fc $\gamma$ RI in mediating protection to malaria. *PLoS Pathog*. 2007;3(5):e72.
- Dai X, Jayapal M, Tay HK, et al. Differential signal transduction, membrane trafficking, and immune effector functions mediated by Fc $\gamma$ RI versus Fc $\gamma$ RIIIa. *Blood*. 2009;114(2):318-327.
- Keler T, Guyre PM, Vitale LA, et al. Targeting weak antigens to CD64 elicits potent humoral responses in human CD64 transgenic mice. *J Immunol*. 2000;165(12):6738-6742.
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.
- Liu C, Goldstein J, Graziano RF, et al. Fc $\gamma$ RI-targeted fusion proteins result in efficient presentation by human monocytes of antigenic and antagonist T cell epitopes. *J Clin Invest*. 1996;98(9):2001-2007.
- Heijnen IA, van Vugt MJ, Fanger NA, et al. Antigen targeting to myeloid-specific human Fc $\gamma$ RI/CD64 triggers enhanced antibody responses in transgenic mice. *J Clin Invest*. 1996;97(2):331-338.
- Bakema JE, Bakker A, de Haij S, et al. Inside-out regulation of Fc $\alpha$ RI (CD89) depends on PP2A. *J Immunol*. 2008;181(6):4080-4088.
- Bracke M, Nijhuis E, Lammers JW, Coffey PJ, Koenderman L. A critical role for PI 3-kinase in cytokine-induced Fc $\alpha$ -receptor activation. *Blood*. 2000;95(6):2037-2043.
- Kanters D, ten Hove W, Luijk B, et al. Expression of activated Fc $\gamma$ RI discriminates between multiple granulocyte-priming phenotypes in peripheral blood of allergic asthmatic subjects. *J Allergy Clin Immunol*. 2007;120(5):1073-1081.
- Koenderman L, Hermans SW, Capel PJ, van de Winkel JG. Granulocyte-macrophage colony-stimulating factor induces sequential activation and deactivation of binding via a low-affinity IgG Fc receptor, hFc $\gamma$ RII, on human eosinophils. *Blood*. 1993;81(9):2413-2419.
- Banno A, Ginsberg MH. Integrin activation. *Biochem Soc Trans*. 2008;36(Pt 2):229-234.
- Beekman JM, Bakema JE, van de Winkel JG, Leusen JH. Direct interaction between Fc $\gamma$ RI (CD64) and periplakin controls receptor endocytosis and ligand binding capacity. *Proc Natl Acad Sci U S A*. 2004;101(28):10392-10397.
- Miller KL, Duchemin AM, Anderson CL. A novel role for the Fc receptor gamma subunit: enhancement of Fc gamma R ligand affinity. *J Exp Med*. 1996;183(5):2227-2233.
- Boot JH, Geerts ME, Aarden LA. Functional polymorphisms of Fc receptors in human monocyte-mediated cytotoxicity towards erythrocytes induced by murine isotype switch variants. *J Immunol*. 1989;142(4):1217-1223.
- Beekman JM, van der Poel CE, van der Linden JA, et al. Filamin A stabilizes Fc gamma RI surface expression and prevents its lysosomal routing. *J Immunol*. 2008;180(6):3938-3945.
- Beekman JM, Bakema JE, van der LJ, et al. Modulation of Fc $\gamma$ RI (CD64) ligand binding by blocking peptides of periplakin. *J Biol Chem*. 2004;279(32):33875-33881.
- Symons DB, Clarkson CA. Genomic organization and sequence of the extracellular domain exons of the bovine Fc gamma RI receptor, and evidence for restricted binding of ruminant IgG to U937 cells. *Mol Immunol*. 1992;29(11):1407-1413.
- Bracke M, Lammers JW, Coffey PJ, Koenderman L. Cytokine-induced inside-out activation of Fc $\alpha$ RI (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. *Blood*. 2001;97(11):3478-3483.
- Ho AS, Wei SH, Mui AL, Miyajima A, Moore KW. Functional regions of the mouse interleukin-10 receptor cytoplasmic domain. *Mol Cell Biol*. 1995;15(9):5043-5053.
- Kumpel BM, Hadley AG. Functional interactions of red cells sensitized by IgG1 and IgG3 human monoclonal anti-D with enzyme-modified human monocytes and FcR-bearing cell lines. *Mol Immunol*. 1990;27(3):247-256.
- Lim SH, Beers SA, French RR, et al. Anti-CD20 monoclonal antibodies: historical and future perspectives. *Haematologica*. 2010;95(1):135-143.
- Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*. 1994;269(7):5241-5248.
- Favata MF, Horiuchi KY, Manos EJ, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem*. 1998;273(29):18623-18632.
- Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J*. 1988;256(1):283-290.
- Harrison PT, Allen JM. High affinity IgG binding by Fc $\gamma$ RI (CD64) is modulated by two distinct IgSF domains and the transmembrane domain of the receptor. *Protein Eng*. 1998;11(3):225-232.
- Beekman JM, van der Linden JA, van de Winkel JG, Leusen JH. Fc $\gamma$ RI (CD64) resides constitutively in lipid rafts. *Immunol Lett*. 2008;116(2):149-155.
- Boumazou S, Hart SP, Chamberlain LH, Glennie MJ, Dransfield I. Association of Fc $\gamma$ RIIIa (CD32a) with lipid rafts regulates ligand binding activity. *J Immunol*. 2009;182(12):8026-8036.
- Dale BM, Traum D, Erdjument-Bromage H, Tempst P, Greenberg S. Phagocytosis in macrophages lacking Cbl reveals an unsuspected role for Fc gamma receptor signaling and actin assembly in target binding. *J Immunol*. 2009;182(9):5654-5662.
- Jung ST, Reddy ST, Kang TH, et al. Aglycosylated IgG variants expressed in bacteria that selectively bind Fc $\gamma$ RI potentiate tumor cell killing by monocyte-dendritic cells. *Proc Natl Acad Sci U S A*. 2010;107(2):604-609.
- Biglari A, Southgate TD, Fairbairn LJ, Gilham DE. Human monocytes expressing a CEA-specific chimeric CD64 receptor specifically target CEA-expressing tumour cells in vitro and in vivo. *Gene Ther*. 2006;13(7):602-610.
- Bevaart L, Goldstein J, Vitale L, et al. Direct targeting of genetically modified tumour cells to Fc $\gamma$ RI triggers potent tumour cytotoxicity. *Br J Haematol*. 2006;132(3):317-325.