



# Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions

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**Human monocyte-derived DCs (moDCs) and circulating conventional DCs coexpress activating (CD32a) and inhibitory (CD32b) isoforms of IgG Fc $\gamma$  receptor (Fc $\gamma$ R) II (CD32). The balance between these divergent receptors establishes a threshold of DC activation and enables immune complexes to mediate opposing effects on DC maturation and function. IFN- $\gamma$  most potently favors CD32a expression on immature DCs, whereas soluble antiinflammatory concentrations of monomeric IgG have the opposite effect. Ligation of CD32a leads to DC maturation, increased stimulation of allogeneic T cells, and enhanced secretion of inflammatory cytokines, with the exception of IL-12p70. Coligation of CD32b limits activation through CD32a and hence reduces the immunogenicity of moDCs even for a strong stimulus like alloantigen. Targeting CD32b alone does not mature or activate DCs but rather maintains an immature state. Coexpression of activating and inhibitory Fc $\gamma$ Rb by DCs reveals a homeostatic checkpoint for inducing tolerance or immunity by immune complexes. These findings have important implications for understanding the pathophysiology of immune complex diseases and for optimizing the efficacy of therapeutic mAbs. The data also suggest novel strategies for targeting antigens to the activating or inhibitory Fc $\gamma$ Rb on human DCs to generate either antigen-specific immunity or tolerance.**

## Introduction

mAbs are among the most rapidly growing therapies for the treatment of cancer (1) and autoimmunity (2). Antibodies either fix complement or engage cells of the innate immune system to mediate target cell lysis. The latter process, known as antibody-dependent cellular cytotoxicity (ADCC), requires that the Fc portion of a mAb ligate activating IgG Fc $\gamma$  receptors (Fc $\gamma$ Rb), e.g., Fc $\gamma$ RI (CD64), Fc $\gamma$ RIIa (CD32a), Fc $\gamma$ RIIc (CD32c), or Fc $\gamma$ RIII (CD16), on monocytes, NK cells, neutrophils, or DCs (3). Recent evidence suggests a more indirect effector mechanism, in which Fc $\gamma$ Rb on DCs mediate phagocytosis and enhance cross-presentation of antibody-coated antigens, leading to effective stimulation of both CD4<sup>+</sup> Th1 and CD8<sup>+</sup> CTL effector responses (4–7). Studies in mice show that coligation of the unique inhibitory Fc $\gamma$ RIIb (CD32b) abrogates all of these effects (7, 8).

The activating and inhibitory Fc $\gamma$ Rb on DCs offer rational targets for immunotherapy based on the unique capacity of DCs to play critical roles in both immunity and tolerance (9). Studies in mice have been very promising (7), though translation into the human system has been lacking. Investigators have not been able to distinguish surface CD32a and CD32b when coexpressed on human cells, given their highly homologous extracellular domains (3). In addition, a common genetic polymorphism of CD32a

caused by an arginine (R) to histidine (H) amino acid substitution at position 131 yields divergent avidities for mouse and human IgG ligands (10), which further confounds studies of Fc $\gamma$ R function in the human system.

We have used a recently developed mAb that, unlike any other available reagent, can specifically bind the inhibitory CD32b isoform, as well as block its interaction with IgG, on intact human cells (M.C. Veri et al., unpublished observations). We have evaluated the relative expression of the activating CD16, CD32a, and CD64, in addition to the inhibitory CD32b, on circulating DCs and their precursors as well as on cytokine-induced monocyte-derived DCs (moDCs). We have demonstrated the phenotypic and functional sequelae of ligating either or both the activating CD32a and inhibitory CD32b on immature moDCs. We have also identified factors that modulate the balanced expression of these receptors, which in turn affect the IgG-mediated changes in maturation and function of the DCs themselves. Our findings have important implications for understanding the pathophysiology of diseases mediated by immune complexes and for developing and optimizing antibody- and DC-based therapies for antigen-specific immunity or tolerance. The data also suggest the need for further studies to define the cell biology of enhanced processing and presentation conferred by antigen opsonization.

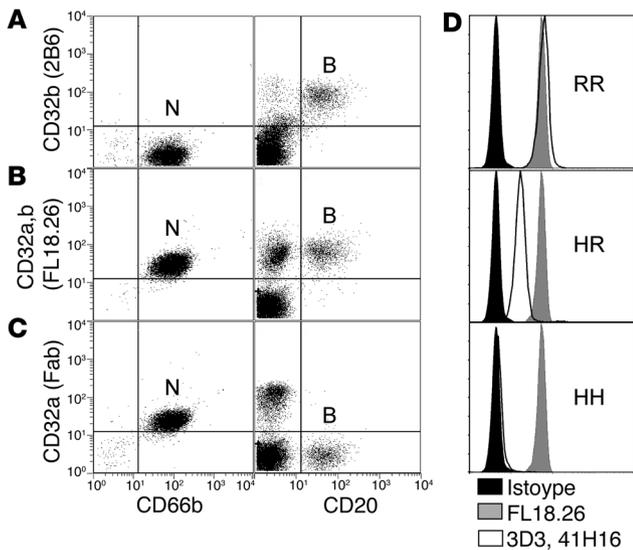
## Results

*Specific mAbs identify CD32 isoforms and CD32a allelic variants by flow cytometry.* We first validated the specificity of mAbs for this study using neutrophils and B cells that express only CD32a or CD32b, respectively, on the cell surface. The novel clone 2B6, which binds extracellular CD32b exclusive of CD32a (M.C. Veri et al., unpublished observations), stained B cells but not neutrophils (Fig-

**Nonstandard abbreviations used:** ADCC, antibody-dependent cellular cytotoxicity; alloMLR, allogeneic mixed leukocyte reaction; Fc $\gamma$ R, Fc $\gamma$  receptor; [<sup>3</sup>H]TdR, methyl-<sup>3</sup>H]thymidine; IVIG, intravenous Ig; MFI, mean fluorescence intensity; moDC, monocyte-derived DC; NHS, normal human serum.

**Conflict of interest:** J.V. Ravetch has a financial interest in MacroGenics Inc., which is managed by Rockefeller University's conflict of interest committee.

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**Figure 1**

2B6 is a novel mAb that specifically detects an extracellular domain of CD32b. Neutrophils and PBMCs were isolated from peripheral blood samples. Cells were stained with various anti-CD32 mAbs and counterstained with anti-CD66b to define neutrophils (N) or anti-CD20 to define B cells (B). (A) mAb 2B6 detected CD32b on B cells but not CD32a on neutrophils. (B) mAb FL18.26 detected CD32a or CD32b, and it stained neutrophils as well as B cells. (C) In contrast, mAb IV.3 (Fab) detected CD32a on neutrophils but not CD32b on B cells. (D) Some mAbs were able to distinguish between the common polymorphic variants of CD32a. mAbs FL18.26 (B) and IV.3 (C) bind both the R<sub>131</sub> and H<sub>131</sub> subtypes of CD32a and stain neutrophils from HH homozygotes, RR homozygotes, and HR heterozygotes equally. mAbs 3D3 and 41H16 recognize only the R<sub>131</sub> subtype (16), shown staining neutrophils from CD32a<sub>131</sub>RR individuals but not from CD32a<sub>131</sub>HH individuals, with intermediate staining of neutrophils from heterozygous (CD32a<sub>131</sub>HR [HR]) individuals.

ure 1A). Clone FL18.26 is not isoform specific (11) and stained neutrophils and B cells (Figure 1B). In contrast, Fab fragments of IV.3 are CD32a specific (12, 13) and detected neutrophils but not B cells (Figure 1C). These data confirm the specificity of 2B6 for CD32b and Fabs of IV.3 for CD32a, thus enabling a clear distinction between activating and inhibitory isoforms of CD32 expressed on the cell surface.

The R and H variants of CD32a have differing avidities for mouse and human IgG subtypes. Compared with the CD32a<sub>131</sub>R allotype, CD32a<sub>131</sub>H has a higher avidity for complexed human IgG2 and IgG3 (14) and, to a lesser extent, human IgG1 (15). Unlike CD32a<sub>131</sub>H, however, CD32a<sub>131</sub>R binds complexed mouse IgG1 (10). We therefore determined the CD32a phenotypes of all samples used in functional studies, based on staining of neutrophils that express abundant CD32a only. mAbs 3D3 and 41H16 recognize only the R<sub>131</sub> variant of CD32a (16), whereas mAbs FL18.26 and IV.3 recognize both the R<sub>131</sub> and H<sub>131</sub> variants (11, 14). As shown in Figure 1D, FL18.26 stained neutrophils from all CD32a phenotypes with equal intensity. In contrast, 3D3 stained cells homozygous for CD32a<sub>131</sub>R, did not detect cells homozygous for CD32a<sub>131</sub>H, and displayed intermediate staining of heterozygous samples. We confirmed that this method accurately typed U937, THP-1, and K562 cell lines expressing known CD32a subtypes (17, 18) (data not shown). These data

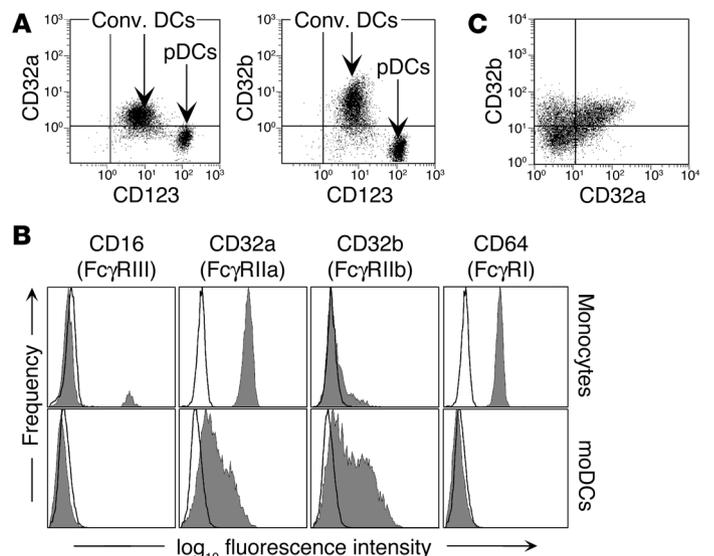
validate this quick method for distinguishing the polymorphic phenotypes of CD32a by flow cytometry (14).

*Subtypes of freshly isolated DCs and DC precursors have distinct FcγR expression profiles.* We studied the differential expression of the activating and inhibitory FcγRs on freshly isolated populations of DCs and their precursors in peripheral blood. We identified “myeloid” or conventional DCs from freshly isolated PBMCs as being lineage (CD3, CD14, CD20, and CD56)<sup>negative</sup>, HLA-DR<sup>bright</sup>, CD11c<sup>+</sup>, and CD123<sup>low</sup>, whereas plasmacytoid DC precursors were lineage<sup>negative</sup>, HLA-DR<sup>bright</sup>, CD11c<sup>negative</sup>, and CD123<sup>bright</sup> (19).

Consistent with previously published results, we found that CD11c<sup>+</sup>CD123<sup>low</sup> conventional DCs in peripheral blood expressed the activating FcγRs CD32a and CD64 but did not express CD16 (4). Almost all circulating conventional DCs also expressed the inhibitory CD32b (*n* = 10; mean ± SD, 92% ± 3.3%) (Figure 2A). We confirmed that freshly isolated plasmacytoid DCs, however, did not express detectable surface levels of CD16, CD32b, or CD64 and expressed minimal to no CD32a (Figure 2A) (20). All CD14<sup>+</sup> monocytes, which are circulating precursors of moDCs, expressed

**Figure 2**

Monocytes, circulating conventional DCs, and cytokine-induced moDCs all express a range of FcγRs, whereas freshly isolated plasmacytoid DCs lack detectable surface expression of all FcγRs. Freshly isolated PBMCs were labeled with fluorochrome-conjugated mAbs. (A) After gating on HLA-DR<sup>bright</sup> PBMCs that were lineage marker negative, CD32a (left) and CD32b (right) were detected on CD123<sup>low</sup> conventional DCs (conv. DCs) but not on CD123<sup>bright</sup> plasmacytoid DCs (pDCs). (B) Monocytes were identified as CD14<sup>+</sup> PBMCs. moDCs were studied as immature cells, gated according to characteristic phenotype (48) but lacking the surface CD83 expression of mature moDCs. Open histograms correspond to isotype controls, and filled histograms represent staining of the indicated FcγR. Most often, CD32a and CD32b were coexpressed on the same subpopulation of moDCs, as shown by a representative sample in C.





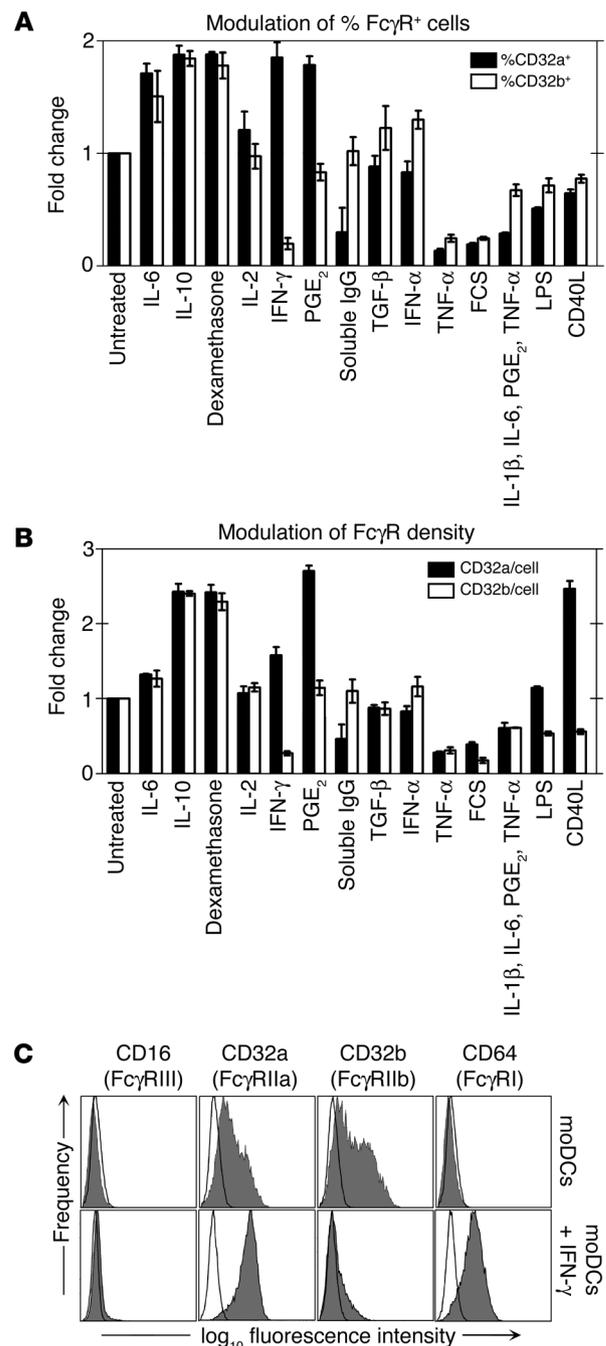
CD32a and CD64 (Figure 2B). Monocyte coexpression of the inhibitory CD32b was highly variable among 30 healthy volunteers, however, ranging from 1% to 48% (mean, 18.1%, SD = 8.3). A separate, but partially overlapping, small subpopulation of CD14<sup>low</sup> monocytes expressed CD16 (Figure 2B) (21, 22). Immature moDCs expressed a balance of CD32a and CD32b similar to that of conventional DCs circulating in fresh blood. On average, 56% ± 10.7% of immature moDCs expressed CD32a (n = 17) and 64% ± 9.4% expressed CD32b (n = 17) (Figure 2B). Most often, these divergent receptors were coexpressed on the same population of cells (Figure 2C). Unlike their monocyte precursors, moDCs lost expression of CD16 and CD64 by day 1-2 of differentiation. Immature moDCs thus provide an excellent model for studying the modulation and function of CD32a and CD32b on the same cells.

*Various stimuli modulate the balanced expression of CD32a and CD32b on immature moDCs.* We tested the effects of several immune modulators on the balance of activating and inhibitory FcγRs on immature moDCs. Figure 3 shows mean fold changes in the percentage of cells expressing the respective FcγRs (Figure 3A) and the relative changes in FcγR density on the cell surface (Figure 3B) compared with untreated moDCs from the same donors (n = 6 independent experiments). FcγR density was measured as the number of anti-FcγR detection mAbs bound per cell. Mean fluorescence intensities (MFIs) and shifts in MFIs were identical for F(ab')<sub>2</sub> and whole IgG 2B6 (anti-CD32b), which indicates that FcγR staining was mediated by Fab-specific binding and not by interactions with the Fc portions of the detection mAbs. Upregulation of FcγRs by the agents tested did not increase non-specific Fc-mediated binding (Figure 3C).

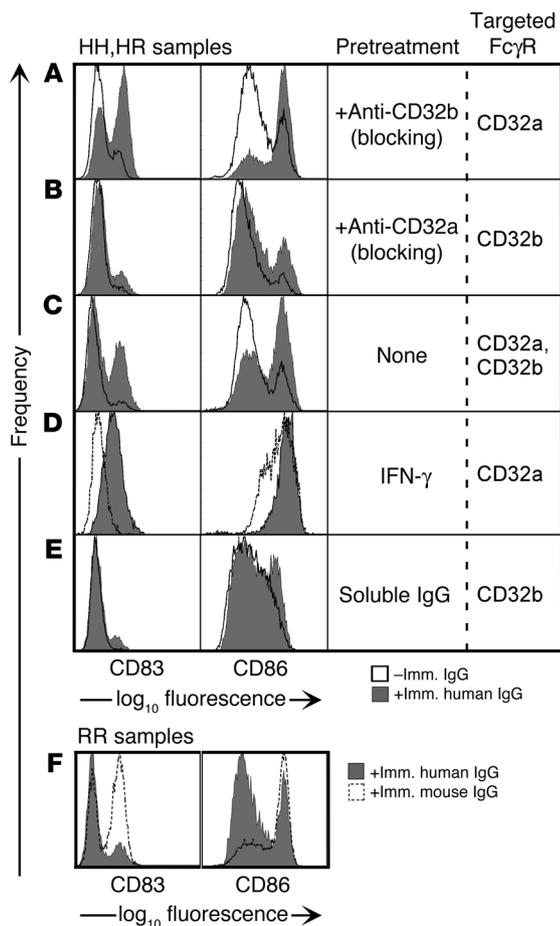
All factors affected only CD32a and/or CD32b expression, or neither, except IL-10 and IFN-γ, which also induced expression of CD16 and CD64, respectively (data not shown). IL-10, IL-6, and dexamethasone all led to proportional increases in expression of CD32a and CD32b without a clear shift favoring either isoform. IFN-γ most potently shifted the balance in favor of activating FcγRs by inducing expression of CD64, increasing the frequency and density of CD32a expression, and exerting opposing effects on CD32b. Conversely, antiinflammatory concentrations of soluble monomeric IgG (0.15 mM), approximating the levels achieved in vivo after administration of intravenous Ig (IVIg), decreased CD32a expression and yielded little to no increase in CD32b expression. Among all factors tested, this led to the greatest relative shift in favor of the

inhibitory FcγRs. CD32a and CD32b did not bind soluble monomeric IgG, which was not detected on the surface of moDCs using anti-human IgG antibodies (data not shown). Hence, receptor occupancy could not account for any change in detection of CD32 isoforms. Culturing cells in the presence of 10% FCS or TNF-α potentially reduced the frequency and density of FcγR expression. All tested maturation stimuli decreased the frequency of CD32a- and CD32b-expressing cells. However, LPS and CD40L, but not the combination of IL-1β, IL-6, TNF-α, and PGE<sub>2</sub> (23), shifted the balance of remaining FcγR-expressing cells in favor of CD32a.

We tested the effects of factors on circulating conventional and plasmacytoid DCs enriched from whole blood after negative selection and cultured in Teflon beakers in 10% normal human



**Figure 3** Various stimuli modulate the balanced expression of CD32a and CD32b on immature moDCs. The indicated reagents were added to cultures of immature moDCs from day 3 to day 6. Expression of FcγRs was measured by flow cytometry (CD16 and CD64 not shown). Analyzed cells were immature or specifically gated for the absence of CD83 in cultures where there was a small amount of maturation (PGE<sub>2</sub> and TNF-α). The mean fold changes (± SD) in the frequency of cells expressing a given FcγR induced by each reagent, compared with untreated cells, are shown in **A**. Density was calculated on the FcγR<sup>+</sup> cells as the number of anti-FcγR antibodies bound per cell using a commercially available kit. The mean fold changes (±SD) in FcγR density induced by the reagents in 5 independent experiments, compared with the averaged FcγR densities on untreated/control moDCs, are shown in **B**. Sample histograms for untreated immature moDCs and IFN-γ-treated immature moDCs are shown in **C**. Open histograms correspond to isotype controls, and filled histograms show staining by the indicated anti-FcγR mAbs.



**Figure 4**

Ligation of CD32a or CD32b on immature moDCs has opposing effects on maturation phenotype. (A–E) MoDCs were cultured on plates with immobilized (Imm.) human IgG to ligate FcγRs (filled histograms). CD32a (A) or CD32b (B) was specifically ligated by first incubating moDCs with blocking antibodies against either CD32b or CD32a, respectively. CD32a and CD32b were ligated simultaneously (C) by preincubating moDCs without blocking antibodies. DCs with or without blocking antibodies were also cultured on untreated plates as negative controls (open histograms). Cells were harvested at 48 hours, and DC phenotype was assessed by flow cytometry. Histograms from 1 representative experiment of 8 that used CD32a<sub>131</sub>HH or -HR samples are shown in A–C. Immature IFN-γ-treated moDCs (D) and soluble IgG-treated moDCs (E) were washed to remove these factors and recultured with (filled histograms) or without (open histograms) immobilized human IgG. Cells were harvested at 24–48 hours and phenotype was assessed by flow cytometry. Representative histograms from 1 of 5 separate experiments are shown. (F) In contrast to results obtained from CD32a<sub>131</sub>HH or -HR samples (C, filled histograms), CD32a<sub>131</sub>RR samples were not matured to the same extent after coculture with immobilized human IgG (F, filled histograms; n = 4 experiments). Immobilized mouse IgG1 (F, open histograms), which ligates CD32a but not CD32b in CD32a<sub>131</sub>RR individuals (10), led to maturation that was similar to conditions specifically targeting CD32a on CD32a<sub>131</sub>HH or -HR samples (A, filled histograms). Averaged changes in CD83 and CD86 expression are summarized in Table 1.

serum-RPMI (NHS-RPMI). We added IL-3 to support the viability of plasmacytoid DCs in culture (24). IFN-α, IFN-γ, TGF-β, IL-10, and soluble IgG modulated CD32a and/or CD32b on circulating conventional DCs, which was similar to their effects on cytokine-induced moDCs. Unlike freshly isolated cells, plasmacytoid DCs in culture expressed CD32a, which was modulated by these factors in the same manner as on moDCs (data not shown).

*CD32a and CD32b have opposing effects on DC maturation.* We studied the effects of ligating human IgG to CD32a, CD32b, or both

on immature moDCs, using immobilized IgG to mimic complexed IgG (25). Selective ligation of CD32a matured a subpopulation of moDCs, as evidenced by upregulation of the DC maturation marker CD83 (26) and the costimulatory molecule CD86 (average 58% CD83<sup>+</sup> and 79% CD86<sup>+</sup>; n = 8 experiments; Figure 4A, filled histograms). The frequency of maturation was proportional to the percentage of cells that expressed CD32a. Blocking CD32b in the absence of a ligand for CD32a (recultured in complete medium with 1% NHS, GM-CSF, and IL-4 but without immobilized IgG) did not promote DC maturation (9% CD83<sup>+</sup>; Figure 4A, open histograms) compared with untreated controls (8% CD83<sup>+</sup>; Figure 4C, open histograms) in a total of 8 independent experiments. Ligation of CD32b was associated with a minimal increase in maturation (average 10% increase in CD83; Figure 4B). When the 2 FcγRs were targeted simultaneously, CD32b limited

**Table 1**

Average changes in total moDCs expressing CD83 and CD86

CD32a allotype	Pretreatment	FcγR ligand (immobilized IgG)	Targeted FcγR	Average increase in percent of total moDCs expressing	
				CD83	CD86
CD32a <sub>131</sub> HR, CD32a <sub>131</sub> HH samples	Anti-CD32b	Pooled human IgG	CD32a	49	41
	Anti-CD32a	Pooled human IgG	CD32b	10	12
	None (medium)	Pooled human IgG	CD32a, CD32b	27	31
	IFN-γ	Pooled human IgG	CD32a	49	24
CD32a RR samples	sIgG	Pooled human IgG	CD32b	6	7
	None	Pooled human IgG	CD32a, CD32b	7	13
	None	Mouse IgG1	CD32a > CD32b	45	47

FcγRs on moDCs were ligated by immobilized IgG after pretreatment with blocking mAbs (anti-CD32a, anti-CD32b), IFN-γ, soluble IgG (sIgG), or medium control. Data in the 2 far right columns represent the average increases in the percent of total moDCs expressing the indicated epitope over those not exposed to immobilized IgG.



CD32a-induced maturation (Figure 4, C vs. A, filled histograms;  $P = 0.001$ ). Table 1 summarizes the average increases in the percent of total moDCs expressing CD83 and CD86 after coculture with immobilized IgG, compared with that of similarly treated moDCs cultured without immobilized IgG.

*Cytokine- or soluble IgG-induced shifts in the balance between activating and inhibitory FcγRs affect susceptibility to immobilized IgG-mediated maturation.* We pretreated moDCs with factors that modulated the balance in favor of either activating or inhibitory FcγRs, washed the cells to remove these factors, then recultured the moDCs in complete medium with 1% NHS, GM-CSF, and IL-4, in the presence or absence of immobilized IgG. Culturing IFN-γ-treated DCs in the presence of immobilized IgG led to increased DC maturation compared with cultures lacking immobilized IgG (62% CD83<sup>+</sup> vs. 13% CD83<sup>+</sup> in 4 experiments; Figure 4D). The relative increase in maturation was similar to conditions in which CD32b was blocked before coculture with immobilized IgG (Figure 4A). Conversely, culturing soluble IgG-treated moDCs with immobilized IgG led to only a 6% increase in CD83 expression (Figure 4E). These data indicated that immobilized IgG ligated predominantly activating FcγRs on IFN-γ-treated moDCs or CD32b on IgG-treated moDCs. Pharmacologic modulations of the balance between CD32a and CD32b could therefore affect IgG-mediated maturation.

*Differences in affinity for IgG ligand cause a functional shift in the balance between CD32a and CD32b.* Coculturing untreated moDCs from CD32a<sub>131</sub>HH or CD32a<sub>131</sub>HR individuals with immobilized IgG (Figure 4C, filled histogram) yielded a greater increase in maturation compared with moDCs from RR individuals (average absolute increase in CD83<sup>+</sup> cells: 27% vs. 7%; filled histograms in Figure 4, C and F, respectively;  $P < 0.001$ ). Immobilized mouse IgG1 in the same RR samples, however, led to substantial maturation (Figure 4F, open histograms) and a loss of the discrepancy between the different CD32a<sub>131</sub> phenotypes. In the context of a fixed quantity of FcγRs, differences in ligand avidity can thus cause a shift in the functional balance between CD32a and CD32b.

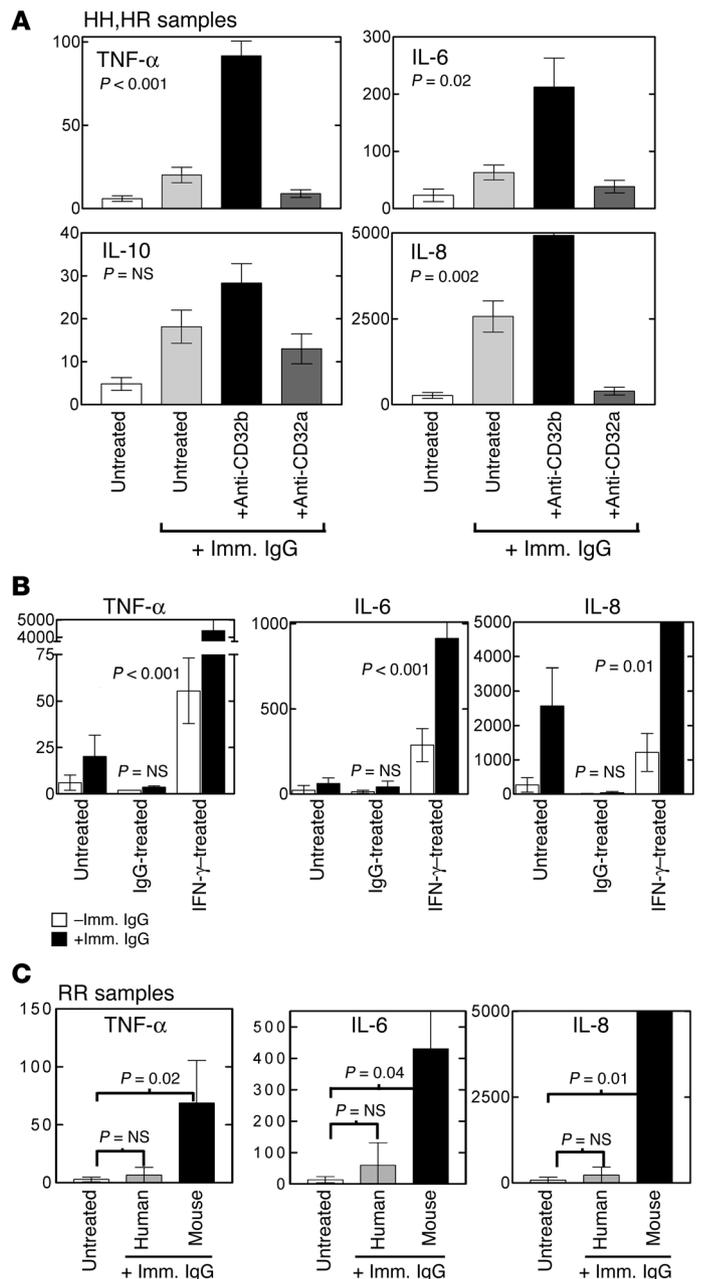
*Coligation of CD32b limits CD32a-mediated cytokine release.* After ligating IgG to CD32a, CD32b, or both, as outlined above, we collected cell-free supernatants at 24–48 hours and measured a panel of cytokines using a multiplexed bead assay. Samples

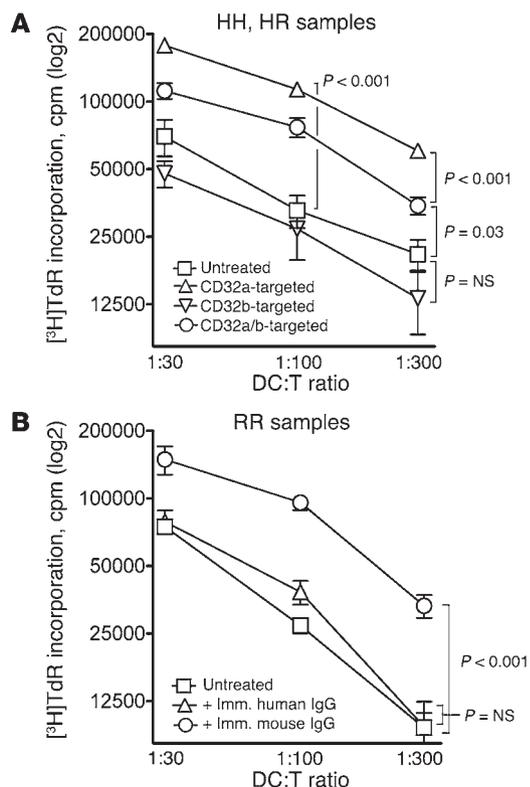
from CD32a<sub>131</sub>HH and -HR individuals are shown in Figure 5A. Simultaneous ligation of CD32b and CD32a (no pretreatment with blocking mAbs) led to suppressed levels of inflammatory cytokines compared with targeting CD32a alone. Differences were statistically significant for TNF-α ( $P < 0.001$ ), IL-6 ( $P = 0.02$ ), and IL-8 ( $P = 0.002$ ) secretion ( $n = 6$  experiments). Significant levels of IL-1β and IL-12p70 were not detected under any condition, and therefore results are not shown. These data offer further functional evidence of the inhibitory role of CD32b.

We also tested cytokine release after ligation of immobilized human IgG to FcγRs on IFN-γ- and soluble IgG-treated immature moDCs (CD32a<sub>131</sub>HH and -HR samples), which favor the respective expression of either CD32a or CD32b. Soluble IgG-treated moDCs cocultured with immobilized IgG did not increase secretion of TNF-α, IL-6, or IL-8, compared with soluble IgG-treated

**Figure 5**

Coligation of CD32b limits CD32a-mediated cytokine release. Immature moDCs were cocultured with immobilized IgG to target FcγRs. After 2 days, supernatants were collected and cytokines measured using a flow cytometry-based multiplexed bead assay. Mean cytokine levels (picograms per milliliter) are plotted on the y axis. (A) For samples derived from CD32a<sub>131</sub>HH or -HR donors, CD32a, CD32b, or both were ligated on immature moDCs after first blocking or not with the mAb to the other isoform ( $n = 6$  independent experiments).  $P$  values for each cytokine reflect differences between CD32a-targeted DCs and DCs on which CD32a and CD32b were targeted simultaneously. (B) IFN-γ- and soluble IgG-mediated shifts in the expression of CD32a and CD32b were also assessed ( $n = 4$  independent experiments).  $P$  values for IFN-γ-treated DCs (top) and IgG-treated DCs (bottom) reflect differences between DCs from respective conditions cocultured with or without immobilized IgG. (C) Immobilized mouse IgG was used as the ligand for FcγRs from CD32a<sub>131</sub>RR samples ( $n = 3$  independent experiments).



**Figure 6**

Targeting CD32a or CD32b affects DC allostimulatory capacity in the MLR. Two days after ligating CD32a, CD32b, both, or neither on immature moDCs, the moDCs were harvested and washed. These moDCs were then recultured at varying doses with  $10^5$  allogeneic T cells in triplicate round-bottomed 96-microwell plates without additional cytokines. DC doses ranged from 3,000 to 300 cells per well, yielding DC:T cell ratios from 1:30 to 1:300.  $^3\text{H}$ TdR uptake by proliferating allogeneic T cells over the last 12 hours of a 4–5 day culture was measured as an index of DC immunogenicity. **(A)** The averaged triplicate values (mean  $\pm$  SEM) for  $^3\text{H}$ TdR incorporation by T cells stimulated in 4 independent experiments using samples derived from CD32a<sub>131</sub>HH or -HR donors are depicted logarithmically ( $\log_2$ ) on the y-axis. **(B)** Experiments using samples from CD32a<sub>131</sub>RR donors were performed in parallel, with immobilized mouse or human IgG ligating Fc $\gamma$ Rs on the immature moDCs. The averaged triplicate values (mean  $\pm$  SEM) from 3 independent experiments are depicted logarithmically ( $\log_2$ ) on the y axis. Differences between conditions in **A** and **B** were tested using a stratified (by DC:T cell ratios) permutation *t* test.

moDC controls (Figure 5B). In contrast, ligating Fc $\gamma$ Rs on IFN- $\gamma$ -treated DCs led to enhanced secretion of TNF- $\alpha$  ( $P < 0.001$ ), IL-6 ( $P < 0.001$ ), and IL-8 ( $P = 0.01$ ) compared with IFN- $\gamma$ -treated DCs cultured without immobilized IgG (Figure 5B). The results were similar to those for DCs on which CD32a alone was ligated (Figure 5A).

The HH/HR versus RR phenotypes have functional significance in cytokine release assays. Compared with samples from CD32a<sub>131</sub>HH and -HR individuals (Figure 5A), coculturing moDCs from CD32a<sub>131</sub>RR individuals with immobilized human IgG did not significantly enhance cytokine production (Figure 5C). Because the CD32a<sub>131</sub>R subtype has a high affinity for murine IgG1 (10), however, immobilized mouse IgG (Figure 5C) led to marked increases in TNF- $\alpha$  ( $P = 0.02$ ), IL-6 ( $P = 0.04$ ), and IL-8 ( $P = 0.01$ ) levels that were similar to those in CD32a-ligated DCs from CD32a<sub>131</sub>HH or -HR individuals (Figure 5A).

Taken together, the results indicate that shifts in the expression of CD32a and CD32b have functional consequences for moDC cytokine secretion. CD32a variants also determine moDC susceptibility to human or mouse Ig and hence the efficacy of certain mAb therapies (27).

Targeting CD32a versus CD32b has opposing effects on DC stimulatory capacity in the allogeneic mixed leukocyte reaction. We targeted CD32a, CD32b, both, or neither, on immature moDCs and measured effects on DC immunogenicity using allogeneic mixed leukocyte reactions (alloMLRs). Results from 5 experiments using CD32a<sub>131</sub>HH and -HR samples are shown in Figure 6A. CD32a-targeted DCs were the most potent stimulators of allogeneic T cells ( $P < 0.001$ ; Figure 6A, triangles) because of the maturation effect of ligating CD32a. Coligation of CD32b (circles) limited the absolute increase in stimulatory capacity otherwise medi-

ated by targeting CD32a alone ( $P < 0.001$ ). Specifically ligating CD32b (inverted triangles) did not support moDC maturation, so the stimulatory activity was no different than that in untreated immature DCs (squares). This is further evidence of the opposing functions of CD32a and CD32b on immature moDCs. These results also indicate that CD32b can actively inhibit CD32a-mediated DC activation and immunogenicity, even in the context of a very strong stimulus like alloantigen.

The HH/HR versus RR phenotypes also affect reactivity to alloantigens in the alloMLR. Ligating immobilized human IgG to Fc $\gamma$ Rs on immature moDCs from CD32a<sub>131</sub>RR individuals did not significantly enhance immunogenicity (Figure 6B, triangles) compared with untreated immature moDCs (Figure 6B, squares). Coculturing these samples with immobilized mouse IgG (Figure 6B, circles), however, led to a marked increase in allostimulation ( $P < 0.001$ ). This again confirms the functional significance of the CD32a<sub>131</sub> polymorphism and exemplifies how differences in the avidity of CD32a for IgG can create a functional shift in the overall balance between activating and inhibitory Fc $\gamma$ Rs.

## Discussion

Previous studies of CD32 expression and function on human DCs (4, 25, 28) could not distinguish between activating CD32a and inhibitory CD32b. We believe this is the first distinct characterization of CD32a and CD32b expression, modulation, and function on circulating human DCs, their precursors, and cytokine-induced moDCs. Nearly all circulating conventional DCs and a major population of moDCs express both CD32a and CD32b, which have opposing effects on IgG-mediated maturation and function of DCs. Ligating complexed human IgG to CD32a matures and activates moDCs in proportion to the frequency of CD32a expression, without any apparent bystander effect. This supports potent DC function in the alloMLR, a standard assay of DC immunogenicity, and in the release of proinflammatory cytokines. Ligand of CD32b significantly limits these immunogenic functions.

Cross-linking CD32a by complexed IgG induces phosphorylation of a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) (29). In contrast, CD32b bears a cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM). Coligation of CD32b initiates tyrosine phosphorylation of the ITIM that inhibits ITAM-mediated events (29). Coexpression of these divergent



receptors, which share almost identical ligand-binding domains, therefore establishes a threshold of DC activation.

CD32b plays a major role in regulating immune responses in mice. Genetic deletion of CD32b predisposes to autoimmunity (30) and results in pathologically enhanced immune responses (31). We have studied the expression and modulation of CD32a and CD32b on the surface of live human cells with an antibody that detects an extracellular domain of CD32b. Unlike previously available reagents, this mAb does not cross-react with CD32a and finally permits an assessment of surface-expressed FcγRs. This is more physiologically relevant than prior methods that measured total CD32b protein or mRNA from cell lysates, which include the large intracellular compartment in addition to surface FcγRs.

Monocytes have emerged as the predominant effectors of ADCC against tumor cells *in vivo* (32). In contrast to their constitutive expression of the activating FcγRs, CD32a and CD64, monocyte expression of the inhibitory CD32b is surprisingly diverse, ranging from 1–48% among 30 healthy volunteers. This previously unrecognized variability in the balance between activating and inhibitory FcγRs in the resting state may influence the quality and/or magnitude of antibody-based immune responses.

Perturbations in the balance between activating and inhibitory FcγRs may affect the pathophysiology of autoimmune diseases (33) or the efficacy of antibody-based therapies (8, 34). For example, TNF- $\alpha$ -mediated suppression of CD32a and CD32b may lead to decreased immune complex clearance *in vivo*. This may be one mechanism by which TNF- $\alpha$  contributes to an immune complex-mediated disease like rheumatoid arthritis (35). While dexamethasone increases CD32a expression, it also increases CD32b and could shift the balance in favor of the inhibitory FcγRs, accounting for a treatment effect of steroids on autoimmune diseases. This may also underlie the negative effect of steroids on ADCC against tumors (36). Increased CD32b expression in other settings may actually improve therapeutic responses to IVIG (34). The addition of any adjunctive treatment to mAb therapy must therefore consider the consequences this has on therapeutic efficacy, owing to alterations in the ratio of activating to inhibitory FcγRs.

In a murine model of immune thrombocytopenic purpura, Samuelsson et al. showed that IVIG requires the presence of CD32b to mediate protective antiinflammatory effects and that IVIG increases CD32b expression on splenic macrophages (34). We found that antiinflammatory concentrations of soluble monomeric IgG decrease CD32a expression on immature moDCs without significantly affecting CD32b. Accordingly, soluble IgG-treated DCs do not gain allostimulatory capacity (data not shown) or increase production of inflammatory cytokines after coculture with immobilized IgG. The net effect on the balance between activating and inhibitory FcγRs on murine macrophages and human DCs is similar, however, and yields the same functional sequelae. Our findings are therefore consistent with those from mouse studies showing that the overall balance between activating and inhibitory FcγRs is pertinent to the mode of action of IVIG (34).

Several lines of evidence show that optimum immune rejection of tumors or infectious pathogens requires coordinated cellular and humoral immune responses (37, 38). Though most often studied for their ability to stimulate antigen-specific T cells, DCs can also potentially affect innate (39) and humoral (40) immune responses. We found that ligating CD32a on moDCs leads to secretion of IL-10 and IL-6, which stimulate B cells and plasma cells, and TNF- $\alpha$  and IL-8, which serve as chemoattractants for other innate

effector cells like neutrophils. Despite the apparent Th2 type of response, and the absence of IL-12p70, CD32a-targeted DCs were able to stimulate more potent allogeneic T cell proliferation compared with control DCs. This reveals a unique type of maturation through which DCs can play a key role in recruiting a diversified immune response against antibody-coated pathogens or tumors.

Similar findings regarding the expression and function of CD32b on human moDCs were recently published (41). We did not find, however, that CD32b blockade increased secretion of IL-12p70. Our results are consistent with previous reports of absent or diminished IL-12p70 production after triggering CD32a on human monocytes (42) and moDCs (25, 43). This discrepancy may derive from differences in methodology, e.g., the specific conditions of FcγR blockade or subsequent removal versus inclusion of excess blocking mAb from DC cultures. We also used immobilized IgG to ligate the unblocked activating FcγRs, instead of relying on circulating immune complexes in serum that were diluted 100-fold in culture (41). This may have led to differences in the extent of FcγR cross-linking and hence the divergent biological responses. We also blocked CD32b and recultured moDCs in the presence of 1% serum without immobilized IgG, and we did not find a statistically significant increase in moDC maturation or activation. We do not know whether blockade of CD32a in the other system would have prevented maturation and IL12p70 production or whether an alternative activation pathway might have been operative.

Blockade of cell activation has unique implications for DCs. While mature DCs are the most potent stimulators of immunity, immature DCs can mediate the opposite task of generating antigen-specific tolerance (9). We have shown that engaging CD32b, by first blocking CD32a or pretreating DCs with soluble IgG, limits IgG-mediated maturation and activation of moDCs, even in the context of a very strong immunogen such as alloantigen. CD32b helps maintain B cell tolerance (3), but we believe this is the first evidence that CD32b may play an active role in limiting DC maturation and hence in inducing T cell tolerance as well. That CD32b can mediate the uptake of immune complexes (44) suggests a rationale for targeting antigens to this receptor to induce antigen-specific tolerance, and such studies are underway. It also implies that tumor cells coated with therapeutic mAbs could inadvertently initiate an inhibitory pathway with undesirable results.

In the setting of fixed numbers of CD32a and CD32b surface epitopes, differences in functional avidity for IgG can shift their functional balance. In our studies using human IgG as ligand, the maturation and functional sequelae are most evident in samples from donors displaying the high-binding CD32a<sub>131</sub> alleles. Samples with low-affinity receptors are less affected by immobilized human IgG but are instead matured and activated by mouse IgG1. This indicates one of the mechanisms by which CD32a polymorphisms may affect immune responses to self or pathogenic/tumor antigens (27, 45). It also highlights how the species of IgG ligand can shape laboratory (6) or clinical studies (46) involving human FcγRs.

These data have shown that subsets of human DCs express a pair of receptors that share a common ligand but mediate opposing functions. These can elicit divergent immune responses. The balance of available activating versus inhibitory FcγRs determines the net response. We have altered this balance by using blocking mAbs, modulating the expression of activating and/or inhibitory FcγRs, or using ligands of differing affinities for the respective FcγRs. These findings have important implications for elucidating the



pathophysiology of autoimmune diseases, optimizing mAb therapies, engineering mAbs to target specific FcγRs (47), and rationally targeting Ags to FcγRs on DCs *in vitro* or *in vivo*.

## Methods

**Media, sera, and Igs.** Complete RPMI 1640 was supplemented with 10 mM HEPES, 1% penicillin/streptomycin (Media Laboratory, Memorial Sloan-Kettering Cancer Center [MSKCC]), 50 μM 2-ME (Invitrogen Corp.), 1% L-glutamine (Invitrogen Corp.), and heat-inactivated NHS (Gemini Bio-Products). All media and reagents were endotoxin free. Stock solution of therapeutic-grade sterile 10% pooled human IgG (Gamunex; gift of Bayer HealthCare, Biological Products Division) was 100% monomeric by fast protein liquid chromatography (data not shown). Pooled human IgG, mouse IgG1 (Sigma-Aldrich), and mouse IgG Fabs (Jackson ImmunoResearch Laboratories Inc.) were diluted immediately before use in sterile PBS.

**Cytokines.** Sterile recombinant, endotoxin-, pyrogen-, carrier-, and mycoplasma-free human cytokines used to support generation of moDCs *in vitro* and/or to modulate FcγR expression included GM-CSF (Berlex); IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, TGF-β1, TNF-α (all from R&D Systems); IFN-α2b (INTRON A; Schering Corp.); IFN-γ-1b (Actimmune; InterMune); and PGE<sub>2</sub> (Calbiochem).

**Cell purification and generation of DCs.** Informed consent was obtained for research specimen collection using protocols approved by the Institutional Review and Privacy Board of Memorial Hospital, MSKCC. PBMCs were obtained from whole blood or leukocyte concentrates (Blood Bank, MSKCC) by density centrifugation over Ficoll-Paque PLUS (endotoxin-free, no. 17-1440-03; Amersham Pharmacia Biotech). Neutrophils were purified from the rbc pellet after hypotonic lysis of erythrocytes.

Circulating blood DCs were enriched from buffy coats by negative immunomagnetic selection according to the manufacturer's instructions (Stem-Sep Human Dendritic Cell Enrichment Cocktail; StemCell Technologies). Enriched populations were cultured in 10% NHS with IL-3 (10 ng/ml) to support CD11c<sup>+</sup>CD123<sup>bright</sup> plasmacytoid DCs (24) or without IL-3 for CD11c<sup>+</sup>CD123<sup>low</sup> "myeloid" or conventional DCs. Immature moDCs were generated over 5–6 days from tissue culture plastic-adherent PBMCs (CD14<sup>+</sup> monocyte precursors) in the presence of GM-CSF (1000 IU/ml) and IL-4 (500 IU/ml) in complete RPMI-1% NHS (48). Fresh medium and cytokines were replenished every 2 days.

**Antibodies and secondary reagents for staining and blocking.** Direct FITC-, PE-, allophycocyanin-, PE-cyanine-5- (PC-5-), and PC-7-conjugated mouse anti-human mAbs included anti-CD3, anti-CD11c, anti-CD14, anti-CD16, anti-CD19, anti-CD20, anti-CD56, anti-CD64, anti-CD83(-FITC), anti-CD86, anti-CD123, anti-HLA-DR (BD Biosciences – Pharmingen); and anti-CD83(-PE) (Immunotech). Anti-human CD32 mAbs included clone 41H16 (kind gift of Jan G.J. van de Winkel, University Medical Center, Utrecht, The Netherlands); clone IV.3 (HB-217; ATCC), with Fab fragments produced by the MSKCC Monoclonal Antibody Core Facility; clone 2B6 [described below; unconjugated and FITC-conjugated mouse IgG1 and FITC-conjugated chimeric F(ab')<sub>2</sub>; MacroGenics]; PE-conjugated clone CIK5 (CALTAG Laboratories); and FITC-conjugated clones FL18.26 and 3D3 (BD Biosciences – Pharmingen). Isotype controls included the appropriate fluorochrome-conjugated or unconjugated mouse IgG1, IgG2a, or IgG2b (DakoCytomation and BD Biosciences – Pharmingen). Unconjugated primary Igs were secondarily stained with FITC- or PE-conjugated F(ab')<sub>2</sub> fragments of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). Flow cytometry studies used a Cytomics FC 500 (Beckman Coulter), gating for collection and analysis of at least 10,000 live cells.

**Production of 2B6 mAb against CD32b.** Human CD32a-transgenic mice were immunized with a recombinant protein containing the complete extracellular domain of human CD32b. Spleen cells from immunized mice

were fused with a proprietary myeloma cell line (MacroGenics Inc.). Resulting mAbs secreted by the hybridomas were screened by ELISA for differential binding of the inhibitory and activating isoforms of CD32. Specificity and selectivity were further confirmed by surface plasmon resonance analysis and flow cytometry of stained Burkitt lymphoma-derived cell lines and human peripheral blood leukocytes. 2B6 was a CD32b-blocking clone selected among several anti-CD32b-secreting hybridomas for its ability to react with CD32b at high titers with no binding to CD32a. Furthermore, clone 2B6 blocked binding of aggregated human IgG to CD32b (M.-C. Veri et al., unpublished data).

**Modulation of FcγRs.** The following specific factors were added to immature moDC cultures starting on day 3 or 4: IL-1β (2 ng/ml), IL-2 (6,000 IU/ml), IL-3 (10 ng/ml), IL-6 (1,000 IU/ml), IL-10 (25 ng/ml), IFN-γ (1,000 U/ml), IFN-α2b (1,000 U/ml), PGE<sub>2</sub> (5 μM), TGF-β (10 ng/ml), and TNF-α (5 ng/ml). IFN-γ-treated DCs were very adherent and required 5 minutes incubation with 0.4 μM EDTA for harvesting. Pooled human IgG was added to yield a final concentration approaching that achieved after therapeutic administration of IVIG (0.15 mM). Sterile therapeutic grade dexamethasone (American Regent Laboratories) was added to yield a final concentration of 0.1 μM. All conditions used only 1% NHS, except the one condition in which FCS was evaluated. Cells were harvested for phenotypic or functional studies on day 6–7.

To assess the effects of different maturation stimuli on FcγR expression, the following were added to separate cultures of immature moDCs on day 6–7: cocktail of inflammatory cytokines (IL-1β [2 ng/ml], IL-6 [1,000 IU/ml], TNF-α [10 ng/ml]) and PGE<sub>2</sub> (5 μM) (23), LPS (10 ng/ml), or CD40L-transfected murine fibroblasts (5 DCs per 1 CD40L-expressing fibroblast; kind gift of Jacques Banchereau, Baylor Institute for Immunology Research, Dallas, Texas, USA) (49). Cells were harvested 2 days later and evaluated for effects on FcγR expression. Maturation was confirmed by upregulation of cell-surface CD83 on more than 90% of cells. Gating on HLA-DR<sup>bright</sup> cells during cytofluorographic analysis excluded CD40L-transfected fibroblasts.

**Quantitative flow cytometry.** The frequencies of respective FcγR<sup>+</sup> cells per population, and relative changes thereof, were calculated as the number of viable immature (CD14-CD83-HLA-DR<sup>+</sup>) DCs with more intense staining than that of the isotype control, divided by the number of viable moDCs in the analysis gate. To quantify receptor density per cell, we used standardized antibody-binding beads (Quantum Simply Cellular; Bangs Laboratories Inc.), which comprise 4 populations with varying capacities to bind mouse IgG (e.g., detection mAbs) and 1 nonbinding blank population. The beads were stained and evaluated in parallel with moDCs using the same anti-FcγR detection reagents. A standard calibration curve was generated by plotting the known antibody-binding capacity of each bead population against the measured MFI of the bound detection mAb. We then calculated the number of detection mAbs bound per cell from the MFI of each sample using the standard curve. Whole IgG1 formulations of clones CIK5 (anti-CD32a) and 2B6 (anti-CD32b) were used at saturating concentrations to quantify CD32a and CD32b. Staining with Fab fragments of clone IV.3 and F(ab')<sub>2</sub> fragments of 2B6 yielded the same changes in the frequency of CD32a<sup>+</sup> or CD32b<sup>+</sup> DCs and shifts in MFI.

**Ligation of FcγRs with immobilized IgG.** We immobilized IgG on plastic plates to mimic the effect of complexed IgG (25). Pooled human or mouse IgG (both 1.0 mg/ml) was added to each well of a 96-well round-bottom tissue culture-treated plate. Plates were washed 4 times with PBS after overnight incubation to yield immobilized IgG. For selective ligation of CD32a, harvested day 5–6 immature moDCs were washed with PBS; blocked with anti-CD32b (clone 2B6, 5 μg/ml) by incubation at 4°C for 30 minutes; washed with PBS 3 times to remove excess blocking mAb; resuspended in complete medium supplemented with 1% NHS, GM-CSF, and IL-4; and recultured in 96-well plates



with immobilized IgG (10<sup>5</sup> cells/0.1 ml/well). For selective ligation of CD32b, cells were blocked with mAb IV.3 (10 µg/ml) before reculture with or without immobilized IgG. Preincubation without mAbs allowed simultaneous ligation of CD32a and CD32b. For each condition, cells added to plates lacking immobilized IgG served as negative controls. For experiments that used immobilized mouse IgG1, plates with immobilized Fab fragments of mouse IgG served as negative controls to rule out an effect of xenogeneic protein. Where indicated, treated moDCs were washed to remove excess soluble IgG or IFN-γ before reculture with or without immobilized IgG.

**T lymphocytes.** T cells for alloMLRs were obtained from tissue culture plastic-nonadherent PBMCs, then further purified by nonadherence and elution from nylon wool columns (Polysciences Inc.). Purity was greater than 90–95% based on CD3 expression.

**Cytokine measurement by cytometric bead array.** Supernatants of DCs were collected 2 days after FcγR ligation, immediately frozen, and thawed once for assay. Levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were measured simultaneously using a Human Inflammation Cytometric Bead Array (CBA) Kit (BD Biosciences – Pharmingen) according to the manufacturer’s instructions.

**AlloMLRs.** DCs were harvested 48 hours after FcγR ligation, extensively washed, then cocultured with 10<sup>5</sup> purified allogeneic T cells in triplicate round-bottomed microwells (Costar 3799; Corning Inc.) at variable ratios from 1:30 to 1:300 (DC:T cells), in complete RPMI 1640 supplemented with 10% NHS. Methyl-<sup>3</sup>H]thymidine (<sup>3</sup>H]TdR; 1 µCi/well; New England Nuclear, PerkinElmer) was added to each well on day 4–5, and plates were harvested 10–12 hours later using a Harvester 96 Mach III (Tomtec). Proliferation of responder T cells was based on <sup>3</sup>H]TdR incorporation, which was measured using a microplate scintillation counter (TopCount NXT; PerkinElmer).

**Biostatistics.** The 2-sample *t* test was used to compare expression of CD83 and CD86, and concentrations of IL-10, IL-6, and TNF-α between experi-

mental conditions. Due to truncation of values at 5,000, the Mann-Whitney *U* test was used to compare concentrations of IL-8. We analyzed differences in <sup>3</sup>H]TdR uptake between conditions in the alloMLR across a range of DC:T cell ratios. The differences were tested using a stratified (by DC:T cell ratios) permutation *t* test, where the subject was the permutation unit, in order to maintain the correlation structure between T cell doses. *P* values less than or equal to 0.05 were considered statistically significant.

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