

Optimization of antibody binding to FcγRIIIa enhances macrophage phagocytosis of tumor cells

John O. Richards, Sher Karki, Greg A. Lazar, Hsing Chen, Wei Dang, and John R. Desjarlais

Xencor, Inc., Monrovia, California

Abstract

The contribution of Fc-mediated effector functions to the therapeutic efficacy of some monoclonal antibodies has motivated efforts to enhance interactions with Fcγ receptors (FcγR). Although an early goal has been enhanced FcγRIIIa binding and natural killer (NK) cell antibody-dependent cell-mediated cytotoxicity (ADCC), other relevant cell types such as macrophages are dependent on additional activating receptors such as FcγRIIa. Here, we describe a set of engineered Fc variants with diverse FcγR affinities, including a novel substitution G236A that provides selectively enhanced binding to FcγRIIIa relative to FcγRIIb. Variants containing this substitution have up to 70-fold greater FcγRIIIa affinity and 15-fold improvement in FcγRIIIa/FcγRIIb ratio and mediate enhanced phagocytosis of antibody-coated target cells by macrophages. Specific double and triple combination variants with this substitution are simultaneously capable of exhibiting high NK-mediated ADCC and high macrophage phagocytosis. In addition, we have used this unique set of variants to quantitatively probe the relative contributions of individual FcγR to effector functions mediated by NK cells and macrophages. These experiments show that FcγRIIIa plays the most influential role for macrophages and, surprisingly, that the inhibitory receptor FcγRIIb has little effect on effector function. The enhancements in phagocytosis described here provide the potential to improve the performance of therapeutic antibodies targeting cancers. [Mol Cancer Ther 2008;7(8):2517–27]

Introduction

It is well documented that the activating Fcγ receptor (FcγR) FcγRIIIa plays an important role in the therapeutic activity of some monoclonal antibodies. Its clinical relevance is supported by the correlations observed between human FcγRIIIa polymorphism and response to therapy

with the anti-CD20 antibody rituximab (Rituxan; refs. 1–4). These results are consistent with demonstrations in mouse models that FcγR are critical to antibody anticancer activity (5–7). Motivated by this work, several studies have used amino acid engineering (8–10) and glycoengineering (11, 12) to enhance the interactions between the antibody Fc region and FcγRIIIa. Amino acid variants or glycoform modifications have been generated that provide up to 100-fold greater affinity for FcγRIIIa compared with native IgG1, resulting in up to 100-fold improvements in ADCC. The amino acid variants tend to have broader affinity enhancements for multiple FcγR, whereas glycoform perturbations that generate afucosylated antibodies lead to FcγRIIIa-specific affinity improvement. Engineered antibodies using both technologies are currently under development (13).

The role of FcγRIIIa in the efficacies of IgG1-derived antibodies, although more speculative, is important to explore for several reasons. First, R131 FcγRIIIa is associated with greater susceptibility to infectious disease, a relationship that is hypothesized to be due to the critical role of IgG2 in fighting pathogens and the capacity of this isotype to mediate monocyte and neutrophil effector function only with the H131 form (14–16). Second, neuroblastoma patients homozygous for R131 FcγRIIIa had significantly improved progression-free survival when treated with an anti-GD2 murine IgG3 antibody, which has a strong affinity preference for the R131 form of FcγRIIIa. Finally, murine FcγRIV, whose expression on murine effector cells parallels that of human FcγRIIIa, plays a dominant role in the *in vivo* efficacy of anti-CD20 antibodies in mouse models (6, 7).

In contrast to the activating receptors, FcγRIIb elicits negative intracellular signals that down-regulate immune cell function. The therapeutic relevance of FcγRIIb is supported by the improved antibody antitumor activity (5) and greater B-cell depletion (7) observed in FcγRIIb^{-/-} mice, and the correlation observed between the anticancer activity of mouse IgG subclasses and their activating to inhibitory (A/I) ratios (17). These results have led to the hypothesis that A/I ratios are an important variable in determining antibody-mediated effector function (17). Overall, the current view is that the antibody effector functions of monocytes, macrophages, and dendritic cells are governed by the interplay among FcγRIIIa, FcγRIIIa, and FcγRIIb (17–20).

Efforts to improve antibodies by engineering selective binding to FcγRIIIa and FcγRIIIa relative to FcγRIIb have met with marginal success. The difficulty is no doubt due to the high homology of the receptors, particularly between FcγRIIIa and FcγRIIb, which share 93% sequence identity in their extracellular domains and are very similar at the Fc binding interface. We now describe a panel of human antibody Fc variants with a variety of unique FcγR

Received 2/28/08; revised 4/11/08; accepted 4/24/08.

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

Requests for reprints: John R. Desjarlais, Xencor, 111 West Lemon Avenue, Monrovia, CA 91016. Phone: 626-737-8077; Fax: 626-737-8098. E-mail: jrd@xencor.com

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0201

affinities and specificities, including selective engagement of Fc γ RIIIa and Fc γ RIIa relative to Fc γ RIIb. The enhancements in macrophage phagocytosis mediated by these novel variants show that Fc engineering could be used to increase the activity of effector cell types expressing both activating and inhibitory receptors. These variants provide the potential to improve the performance of monoclonal antibodies targeting cancers and infectious diseases and have enabled us to probe the roles of the different Fc γ R in the effector functions of different immune cell types.

Materials and Methods

Construction and Production of Fc Variant Antibodies

The variable region for the humanized anti-epithelial cell adhesion molecule (EpCAM) antibody is an engineered version of sequences that were generated previously (21). Variable region genes were ligated into the vector pcDNA3.1Zeo (Invitrogen) containing the human light chain κ and heavy chain constant regions. Fc mutations were introduced into the heavy chain using QuickChange mutagenesis techniques (Stratagene). All DNA was confirmed by sequencing. Light and heavy chain plasmids were cotransfected into 293T cells and antibodies were purified using protein A chromatography (Pierce). Antibody and receptor concentrations were determined by bicinchoninic acid assay (Pierce).

Receptors, Antibodies, and Cell Lines

Human Fc receptors with His tags were obtained from R&D Systems (Fc γ RI and Fc γ RIIb) or constructed internally (R131 and H131 Fc γ RIIIa and V158 and F158 Fc γ RIIIa). Biacore experiments measuring antibody binding to internally expressed R131 Fc γ RIIIa and the same receptor purchased commercially gave identical affinities (data not shown), indicating consistency between the two sources. Blocking antibodies for ADCC and phagocytosis studies, including anti-Fc γ RI (clone 10.1), anti-Fc γ RII (clone AT10), and anti-Fc γ RIII (clone 3G8), were purchased from GeneTex, AbD Serotec, and BD Biosciences, respectively. Anti-Fc γ RIIb-specific antibody 2B6 (22) was constructed by gene synthesis, subcloned into pcDNA3.1Zeo as a His-tagged chimeric F(ab), and a full-length chimeric antibody. 2B6 antibodies were expressed in 293T cells and purified using nickel affinity or protein A chromatography. Full-length 2B6 was labeled with Percp (Prozyme) according to the manufacturer's instructions. Allophycocyanin (APC)-labeled anti-CD11b, APC-labeled anti-CD14, PE-labeled anti-CD66, and PE-Cyan7-labeled anti-Fc γ RIII (clone 3G8) were purchased from BD Biosciences. FITC-labeled anti-Fc γ RII (clone IV.3) and PE-labeled anti-Fc γ RI (clone 10.1) were purchased from Stem Cell Technologies and eBioscience, respectively. Control IgG used in cell-based assays was a human IgG1 targeting respiratory syncytial virus, the variable region of which was made by gene synthesis. The LS180 cell line was obtained from the American Type Culture Collection.

Determination of Fc Receptor Binding Affinities

Surface plasmon resonance measurements were done using a Biacore 3000 instrument. Antibodies were captured

onto an immobilized protein A/G (Pierce) CM5 biosensor chip (Biacore) generated using standard primary amine coupling. All measurements were done in HBS-EP (Biacore), and glycine buffer (Biacore) was used for surface regeneration. Antibodies (50 nmol/L in HBS-EP) were immobilized on the protein A/G surface for 5 min at 1 μ L/min. Fc receptors in 2-fold serial dilutions (starting at 1 or 2 μ mol/L, six concentrations total) were injected over antibody bound surface for 2 min at 20 μ L/min followed by a 2 or 3 min dissociation phase. After each cycle, the surface was regenerated with glycine buffer. Data were processed by zeroing time and response before the injection of receptor and by subtracting from a reference channel to account for changes due to injections. To correct for baseline drift due to any dissociation of IgG, all experiments were preceded with injection of buffer alone after IgG binding, which was then subtracted from all traces as background as a part of data-processing step termed "double referencing" (23). Kinetic data were fit to a 1:1 binding model (Langmuir) using the BIAevaluation software. Binding curves of the six Fc γ R concentration series were fitted individually. Kinetic variables were used to calculate the equilibrium dissociation constant (K_d) and SD.

ADCC Assays

ADCC was measured by lactate dehydrogenase release using the Cytotox-ONE Homogeneous Membrane Integrity Assay (Promega). Human peripheral blood mononuclear cells (PBMC) were purified from leukopacks using a Ficoll gradient. DNA genotyping for Fc γ RIIa (position 131) and Fc γ RIIIa (position 158) was carried out using methods by and as a commercial service at Gentris Clinical Genetics. EpCAM⁺ LS180 target cells were seeded into 96-well plates at 20,000 per well and opsonized using antibodies at the indicated concentrations. Triton X-100 and PBMC alone were run as controls. Effector cells were added at 25:1 PBMC/target cells, and plates were incubated at 37°C, 5% CO₂ for 4 h. Cells were then incubated with lactate dehydrogenase reaction mixture for 10 min, and fluorescence was measured using a Wallac Victor2 fluorometer (Perkin-Elmer). All ADCC reactions were done in triplicate. Fluorescence due to spontaneous PBMC and target cell lysis (without antibodies) was subtracted from experimental values (with antibodies), normalized to maximal (Triton) and minimal (no Triton) lysis, and fit to a sigmoidal dose-response. For Fc γ R blocking studies, 10 μ g/mL murine IgG control, anti-Fc γ RII, or anti-Fc γ RIII and a single concentration of opsonizing antibodies (0.316 μ g/mL) were incubated with the target cells for 45 min before the addition of effector cells.

Macrophage Cultures, Phenotype, and Fc γ R Quantitation

CD14⁺ cells were purified from PBMC by EasySep Human Monocyte Enrichment Kit without CD16 depletion (Stem Cell Technologies). Purified CD14⁺ monocytes were cultured in macrophage colony-stimulating factor (Peprotech) at 50 ng/mL for 5 days in a humidified incubator. Differentiated macrophages were identified by combination of anti-CD11b-APC and anti-CD14-APC and

phenotyped for the expression of FcγRI, FcγRIIa, FcγRIIb, and FcγRIII by multiplexed flow cytometry. Briefly, a 1:50 dilution of antibodies that identified the above receptors was incubated on ice for 30 min and washed twice with PBS. Receptor quantification was done using either the Quantum Simply Cellular Mouse Antibody-Binding Standards or the Human Antibody-Binding Standards (Bangs Laboratories) according to the manufacturer's instructions. Briefly, macrophages were dual stained with the combination of anti-CD11b-APC and anti-CD14-APC with either anti-FcγRI-PE, anti-FcγRIIa-FITC, anti-FcγRIIb-Percp, or anti-FcγRIII-PE-Cyan7. The same concentration of antibody that was used to stain FcγR on the macrophages was incubated with the beads. The bead fluorescence intensity was then used as a standard curve to determine the number of receptors on the cells (24).

Phagocytosis Assays

Macrophage antibody-dependent cell-mediated phagocytosis (ADCP) was determined by flow cytometry. Target LS180 cells were labeled with PKH67 (Sigma) and seeded at 25,000 per well into 96-well plates in the presence of 10% human AB serum. Antibodies were diluted serially to 8 half-log concentrations and added to the target cells. Monocyte-derived macrophages were then added at 4:1 effector:target. Cells were spun down briefly and incubated at 37°C for 4 h. Cells were detached from the plate surface with HyQtase, stained with anti-CD11b-APC, anti-CD14-APC, and anti-CD66-PE, washed with PBS, and fixed with 1% paraformaldehyde. Phagocytosis was evaluated on a FACSCanto II flow cytometer (BD Biosciences), and percent phagocytosis was calculated as the number of double-positive cells divided by the total number of tumor cells. The intensity of CD66 staining was used to determine the degree to which tumor cells were internalized. For FcγR blocking experiments, 10 μg/mL anti-FcγRI, anti-FcγRII, anti-FcγRIIb, anti-FcγRIII, control murine IgG, or control human F(ab) were added to the macrophages before the addition of target cells and opsonizing antibodies (0.316 μg/mL).

Results

Engineered Fc Variants Have Diverse and Selective FcγR Affinity Profiles

We have described previously the engineering of antibody Fc variants for improved FcγRIIIa affinity (9). Characterization of two point variants, S239D and I332E, and the corresponding double variant S239D/I332E showed that they provide greater FcγRIIIa affinity and enhanced effector function *in vitro* and *in vivo* relative to native IgG1. Subsequently, we screened a larger set of >900 variants for binding to all FcγR, including FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa.¹ Among the most intriguing receptor affinity profiles from this screen were variants with greater affinity for FcγRIIIa relative to the

inhibitory receptor FcγRIIb. A single mutation, G236A, was identified that fit this target profile. This mutation was combined with I332E and S239D/I332E to generate additional variants I332E/G236A and S239D/I332E/G236A. Variants were constructed in the context of full-length antibodies containing the variable region of a humanized anti-EpCAM antibody (21), expressed in 293T cells, and purified.

Binding affinities of the variant antibodies to all human FcγR relevant to monocytic cells were determined using Biacore. Figure 1A shows example sensorgrams for binding of native IgG1 and G236A variant antibodies to the R131 form of FcγRIIIa. The G236A variant exhibits a marked enhancement in response unit intensity and a slower off-rate relative to native IgG1. Fits of the data for all the variants provided equilibrium K_d values and fold affinities relative to native IgG1 (Table 1). Figure 1B shows a plot of the affinities on a logarithmic scale for binding of each antibody to each receptor. The values obtained for native IgG1 agree well with published data (see footnotes for Table 1). The 4-fold ratio of affinities for binding of native IgG1 to the two FcγRIIIa alleles (V/F158) is also consistent with the literature as is the equivalent affinity of IgG1 for the H131 and R131 alleles of FcγRIIIa (contrasting with the well-known H131 preference of IgG2; ref. 25).

The Biacore data indicate that the G236A mutation provides a 6- to 7-fold enhancement in binding to both isoforms of FcγRIIIa but does not alter affinity for the inhibitory receptor FcγRIIb or FcγRIIIa (Fig. 1B; Table 1). However, it reduces FcγRI affinity ~7-fold. Addition of this mutation to I332E and S239D/I332E imparts its unique profile to these variants—binding of I332E/G236A and S239D/I332E/G236A to FcγRIIIa, and the ratios of FcγRIIIa to FcγRIIb affinities are enhanced relative to the single and double variants, respectively. Addition of G236A again reduces binding to the other activating receptors, although affinity of I332E/G236A and S239D/I332E/G236A for FcγRI remains comparable with or greater than native IgG1, and FcγRIIIa binding is still dramatically improved (6- and 31-fold for binding to the more prevalent F158 isoform by the double and triple variants, respectively). Overall, the variants provide a spectrum of diverse FcγR affinity profiles.

With respect to utility for improving effector function, the reduced affinity to FcγRI potentially makes the single substitution variant G236A suboptimal in spite of its enhanced affinity for FcγRIIIa. The I332E variant provides nearly selective enhancement to FcγRIIIa, with a possible slight increase in affinity for the other receptors. I332E/G236A has an almost ideal Fc receptor profile for enhancing effector function—up to a log greater affinity for all isoforms of FcγRIIIa and FcγRIIIa, improved FcγRIIIa/FcγRIIb and FcγRIIIa/FcγRIIb ratios, and unaltered affinity to FcγRI. The S239D/I332E double variant has greater improvements to FcγRIIIa relative to I332E and alone has enhanced binding to both FcγRIIIa isoforms. A theoretical drawback is that it also binds more tightly to

¹ Unpublished results.

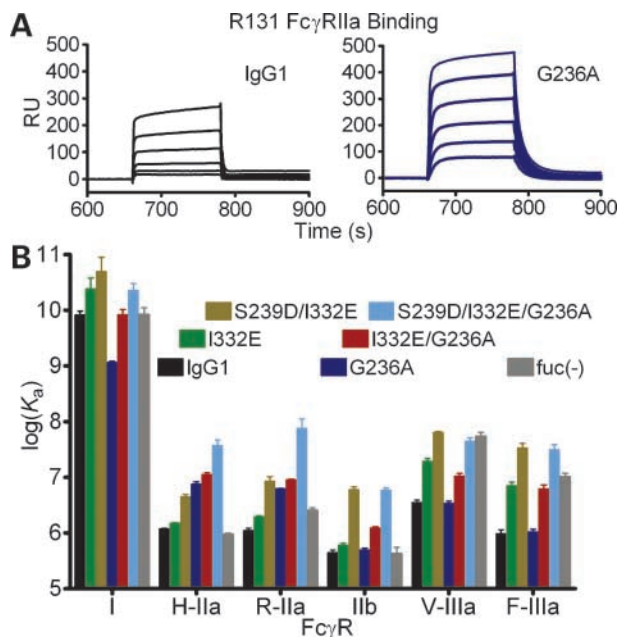


Figure 1. Fc variant antibodies exhibit a diverse range of FcγR affinity profiles. Binding affinities to all the relevant human FcγR were determined using Biacore. **A**, example Biacore sensorgrams measuring binding to R131 FcγRIIIa show that the G236A variant (*right*) has an enhanced response unit (RU) intensity and slower off-rate relative to native IgG1 (*left*). **B**, equilibrium K_d values were obtained from Langmuir fits of the Biacore data, and affinities for each of the FcγR were plotted as the log of the K_a . Mean \pm SD. The Fc variants display diverse Fc receptor affinities: I332E and fuc(-) show nearly selective enhanced binding to FcγRIIIa, and G236A shows selectively enhanced binding to FcγRIIIa relative to FcγRIIb (IIa/IIb ratio = 13-15). Addition of G236A similarly results in improved FcγRIIIa/FcγRIIb ratios for the double and triple variants I332E/G236A and S239D/I332E/G236A. Affinities, fold affinities relative to native IgG1, and IIa/IIb ratios are given in Table 1.

FcγRIIb. Addition of G236A, although not affecting the absolute FcγRIIb affinity, dramatically improves its FcγRIIIa/FcγRIIb ratio while still leaving it with a very high FcγRIIIa affinity relative to native IgG1.

As an additional comparator, we generated an afucosylated [fuc(-)] version of the IgG1 anti-EpCAM antibody. Removal of fucose from the complex carbohydrate attached at N297 improves binding only to FcγRIIIa (26, 27). This profile is consistent with a model for enhancement involving alleviation of a steric interaction between Fc fucose and carbohydrate on the receptor (26), a mechanism that should only enhance affinity to human FcγRIIIa/b due to lack of an asparagine at the analogous position in the other receptors. We produced fuc(-) antibody by expressing the native IgG1 anti-EpCAM in the Lec13 CHO line (28). Antibodies expressed in this cell line are consistently ~90% fuc(-) in contrast to normal CHO lines that produce ~2% fuc(-) antibody (29). Biacore data showed that fuc(-) enhanced affinity only for FcγRIIIa (Fig. 1B), consistent with results from other studies (26, 27) and the receptor glycosylation model (26). FcγRIIIa affinity enhancements for fuc(-) antibody were 16- and 10-fold for the V158 and F158 alleles, respectively (Table 1).

Fc Variants with Increased Affinity for FcγRIIIa Enhance ADCC Mediated by Natural Killer Cells

Variant antibodies were evaluated for their capacity to mediate ADCC activity using PBMC as effector cells. Target cells were EpCAM⁺ LS180, a colon adenocarcinoma cell line. PBMC were alltyped for V/F158 FcγRIIIa and H/R131 FcγRIIIa polymorphism, and lysis was measured using release of lactate dehydrogenase. Improvements in both EC₅₀ and maximal lysis relative to native IgG1 were observed for all the variants except G236A, which showed a reduction in ADCC activity (Fig. 2A). Similarly, the addition of G236A to I332E reduced maximal activity relative to the single I332E variant, although it maintained improved potency relative to native IgG1. In contrast, the S239D/I332E/G236A variant showed only a modest decline relative to the double variant potentially due to their much higher FcγRIIIa affinities (Table 1). Similar results were observed when the ADCC assay was carried out with PBMC from donors having the low responder R/R131 FcγRIIIa and F/F158 FcγRIIIa genotype (data not shown).

A general trend was observed for a dependence of ADCC activity on FcγRIIIa affinity. To explore this more quantitatively, the negative log of the EC₅₀ for each variant was plotted versus the log of its corresponding association constant (K_a) for each of the receptors. A strong correlation was observed between ADCC activity and affinity for FcγRIIIa ($r^2 = 0.93$ and 0.97 for V158 and F158, respectively; Fig. 2B), despite the fact that binding assays measured monomeric IgG, whereas ADCC assays assessed activity of opsonized cells with enhanced avidity for low affinity FcγR. The relationship with FcγRIIIa was distinctly stronger than correlations with the other activating receptors ($r^2 = 0.34-0.57$; data not shown). To corroborate these results, we used blocking antibodies to selectively inhibit FcγRII or FcγRIII. Whereas an antibody that blocks both FcγRIIIa and FcγRIIb had no effect on ADCC activity, blocking FcγRIIIa dramatically inhibited ADCC by the variants and native IgG1 (Fig. 2C). As an additional confirmation of the importance of FcγRIIIa, we determined ADCC for fuc(-) antibody and compared the results with those obtained for the I332E/G236A and S239D/I332E double variants. Enhancements in activity were observed by both the variants and fuc(-) antibody (Fig. 2D), with the rank order of improvement consistent with their FcγRIIIa affinity.

The strong dependence on FcγRIIIa suggests that ADCC activity by PBMC is dominated by natural killer (NK) cells, which express only this receptor among the FcγR [with few exceptions (30)]. To directly determine the role of NK cells in mediating ADCC, we tested our antibodies using PBMC depleted of NK cells. Consistent with previous studies (31, 32), ADCC activity was abolished for native IgG1 as well as the variants tested (data not shown). Taken together, the data confirm that under the conditions of this assay ADCC activity by PBMC is mediated primarily by FcγRIIIa on NK cells.

Fc Variants with Increased FcγRIIIa Affinity Enhance ADCC by Macrophages

To evaluate the FcγR dependence for a cell type with a more complex receptor expression profile, we investigated the capacity of the Fc variant antibodies to promote phagocytosis by macrophages. Monocytes were purified from PBMC and differentiated into macrophages using macrophage colony-stimulating factor, confirmed by the expression of CD14 and CD11b. Multivariant flow cytometry was used to determine FcγR expression levels. As seen in Fig. 3A, the macrophage phenotype consisted of FcγRIIIa, FcγRIIb, FcγRIII, and low levels of FcγRI. Bead-based quantification was used to obtain an approximate receptor number (24). Consistent with the distribution in the cytometry profiles, FcγRIIIa had the highest level of expression followed by FcγRIIb, FcγRIII, and FcγRI (Fig. 3B).

To evaluate phagocytosis, PKH67-labeled LS180 target cells were opsonized with the various anti-EpCAM antibodies and incubated with macrophages. Macrophages were stained with anti-CD14-APC and anti-CD11b-APC, and LS180 tumor cells were stained with anti-CD66-PE. With this triple staining method, macrophages that phagocytose tumors should be double positive for PKH67 and CD11b/CD14. Target cells that are internalized should also be less intensely stained for CD66 compared with residual tumor cells. As shown in Fig. 3C, native IgG1 significantly increased phagocytosis compared with controls, and activity was enhanced even further by the S239D/I332E variant.

To compare the potencies of the various Fc-modified antibodies, phagocytosis was measured as a function of antibody concentration for each of the variants using H/H131 FcγRIIIa and V/F158 FcγRIIIa macrophages (Fig. 4A). Substantial improvements in both half-maximal effective concentration (EC₅₀) and maximal phagocytosis relative to native IgG1 were observed for all the variants. The I332E variant showed modest improvement followed by S239D/I332E. The FcγRIIIa-selective G236A variant provided substantial improvements to activity by itself. Its combination with the other two variants led to further enhancements, with the triple mutant showing the highest activity. Improvements by fuc(-) antibody were comparable with those of I332E, consistent with their similar FcγR profiles. Repeat experiments using different donors consistently resulted in enhancements, although the degree of improvements varied from donor to donor. Greater enhancements were observed using macrophages possessing the R/R131 FcγRIIIa and F/F158 FcγRIIIa genotype (Fig. 4B) due in part to the poor activity of native IgG1 with this donor. Further experiments are required to evaluate the significance of allelic differences on macrophage activity.

To quantify the relative importance of the individual FcγR in macrophage ADCC, we plotted the log of the affinities (K_a) against the negative log of the EC₅₀ values for each of the receptors (Fig. 4C). In contrast to the ADCC analysis, the strongest correlation was seen with FcγRIIIa ($r^2 = 0.79$ and 0.80), and poor correlations were seen with FcγRI ($r^2 = 0.00$) and FcγRIIIa ($r^2 = 0.09$ and

Table 1. Binding affinities of Fc variant antibodies for human Fc receptors

	FcγRI		H131 FcγRIIIa		R131 FcγRIIIa		FcγRIIb		IIa/IIb ratio*		V158 FcγRIIIa		F158 FcγRIIIa	
	K_d (nmol/L) [†]	Fold [‡]	K_d (μmol/L) [†]	Fold [‡]	K_d (μmol/L) [†]	Fold [‡]	K_d (μmol/L) [†]	Fold [‡]	H131	R131	K_d (μmol/L) [†]	Fold [‡]	K_d (μmol/L) [†]	Fold [‡]
Native IgG1	0.12 ± 0.02 [§]	1.0	0.85 ± 0.03	1.0	0.91 ± 0.10	1.0	2.3 ± 0.3	1.0	2.7	2.5	0.28 ± 0.03 [¶]	1.0	1.0 ± 0.2 ^{**}	1.0
G236A	0.86 ± 0.05	0.14	0.13 ± 0.01	6.5	0.161 ± 0.003	5.7	2.0 ± 0.2	1.2	15	12	0.30 ± 0.03	0.93	0.97 ± 0.13	1.0
I332E	0.041 ± 0.023	2.9	0.67 ± 0.02	1.3	0.52 ± 0.03	1.8	1.7 ± 0.1	1.4	2.5	3.3	0.052 ± 0.007	5.4	0.14 ± 0.02	7.1
I332E/ G236A	0.12 ± 0.03	1.0	0.089 ± 0.007	9.6	0.110 ± 0.003	8.3	0.81 ± 0.04	2.8	9.1	7.4	0.095 ± 0.012	3.0	0.16 ± 0.03	6.3
S239D/ I332E	0.020 ± 0.016	6.0	0.23 ± 0.02	3.7	0.12 ± 0.02	7.6	0.17 ± 0.02	14	0.74	1.4	0.016 ± 0.001	18	0.030 ± 0.006	33
S239D/ I332E/ G236A	0.044 ± 0.015	2.7	0.027 ± 0.007	31	0.013 ± 0.006	70	0.17 ± 0.02	14	6.3	13	0.023 ± 0.004	12	0.032 ± 0.008	31
fuc(-)	0.12 ± 0.04	1.0	1.1 ± 0.1	0.77	0.40 ± 0.05	2.3	2.4 ± 0.8	0.96	2.2	6.0	0.018 ± 0.003	16	0.098 ± 0.016	10

*IIa/IIb ratio = K_d (FcγRIIIa) / K_d (FcγRIIb) for the H131 (left) or R131 (right) FcγRIIIa allotype.

[†] K_d s were obtained from global Langmuir fits of Biacore data (mean ± SD). $K_a = 1 / K_d$.

[‡] Fold = K_d (IgG1) / K_d (variant).

[§] Literature value for the binding of native IgG1 to FcγRI by Biacore is 0.110 nmol/L (46).

^{||} Literature values for the binding of native IgG1 to FcγRIIb by Biacore and calorimetry vary from 0.95 to 3.8 μmol/L (26, 47, 48).

[¶] Literature values for the binding of native IgG1 to V158 FcγRIIIa by Biacore and calorimetry vary from 0.21 to 0.75 μmol/L (9, 26, 49).

^{**} Literature values for the binding of native IgG1 to F158 FcγRIIIa by Biacore and calorimetry vary from 2.7 to 5.0 μmol/L (26, 49). The ratio of affinities for binding of native IgG1 to the two FcγRIIIa alleles (K_d [F158] / K_d [V158]) is 4 in the current study. Literature values for this ratio by Biacore and calorimetry vary from 5 to 7 (26, 49).

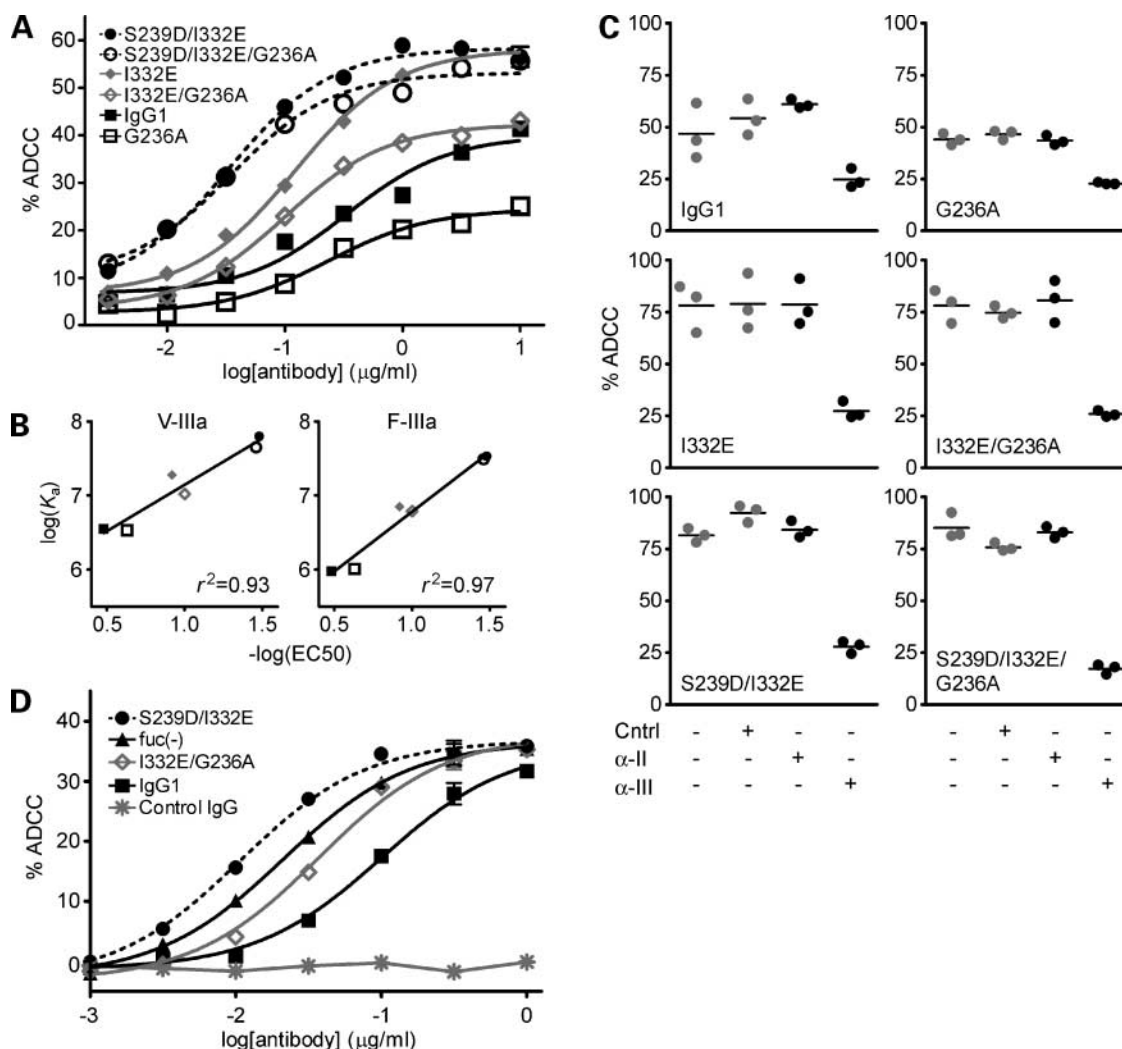


Figure 2. ADCC activity is Fc γ R1IIa and NK cell dependent. **A**, ADCC was determined by measuring lysis by PBMC of EpCAM⁺ LS180 target cells opsonized with varying concentrations of anti-EpCAM antibodies. Fc γ R1IIa genotype was V/F158. All the variants, except G236A, increased ADCC relative to native IgG1, eliciting improvements in both EC₅₀ and maximal lysis. Mean \pm SE of triplicate wells. **B**, plots of Fc γ R1IIa affinities ($\log[K_a]$; Table 1) versus ADCC activity ($-\log(\text{EC}_{50})$) show strong correlations. Correlations with the other activation receptors were weaker ($r^2 = 0.53$, $r^2_{\alpha\text{-IIa}} = 0.34$, and $r^2_{\alpha\text{-IIIa}} = 0.57$; data not shown). Symbols are as shown in **A**. **C**, use of selective blocking antibodies confirms the importance of Fc γ R1IIa. ADCC mediated by native IgG1 or Fc variant antibodies was determined as described in **A**, except that 10 $\mu\text{g/ml}$ blocking antibodies selective for Fc γ R1II (α -II) or Fc γ R1III (α -III) were added to the LS180 tumor cells along with 0.316 $\mu\text{g/ml}$ opsonizing antibodies before the addition of PBMC. Gray, control samples containing no antibody (all -) or nonspecific murine IgG (Cntrl +); black, blocking samples (α -II +, α -III +). The results show that blocking Fc γ R1III dramatically inhibited ADCC, whereas blocking Fc γ R1II had no effect. **D**, consistent with the binding results, fuc(-) antibody mediates an ADCC activity level intermediate between variants with lower (I332E/G236A) and higher (S239D/I332E) Fc γ R1IIa affinity. ADCC was determined as described in **A**, and Fc γ R1IIa genotype for the experiment was V/F158. Mean \pm SE of triplicate wells. Control IgG was anti-respiratory syncytial virus IgG1.

0.24). Interestingly, however, when variants were grouped into subsets that either included the Fc γ R1IIa-selective G236A mutation (*open symbols*) or did not (*closed symbols*), correlations with Fc γ R1 and Fc γ R1IIa emerged, particularly for Fc γ R1IIa ($r^2 = 0.87$ -1.0), with some *P* values indicating significance between the subsets and all of the data. Finally, contrary to expectations for the inhibitory receptor, Fc γ R1Ib affinity did not negatively correlate with phagocytosis. This result suggests that, for macrophages, greater interaction with Fc γ R1Ib, despite significant expression, does not inhibit antibody-dependent phagocytic effector function.

Blocking Antibodies Confirm Fc-Engineered Targeting of Fc γ R1IIa and Show Its Importance in Macrophage Phagocytosis

To confirm the importance of Fc γ R1IIa in macrophage-mediated ADCC, specific Fc γ R blocking antibodies were included in the assay before addition of tumor cells and antibodies. For native IgG1 and all variants, simultaneous blocking of Fc γ R1IIa and Fc γ R1Ib had the greatest inhibitory effect on phagocytosis (Fig. 5A) with H/H131 V/F158 macrophages. Inhibition of Fc γ R1II was most dramatic with G236A. Modest inhibition was observed when Fc γ R1 or Fc γ R1IIa were blocked, indicating that these activating

receptors also contribute. Their role is most apparent by the dramatic reduction in phagocytosis observed when all three of the activating Fc γ R are simultaneously blocked. Investigation of the role of Fc γ RIIb was enabled by an antibody (2B6) selective for this receptor, the specificity of which has been shown in the literature (22) and confirmed in our own work (data not shown). Blocking Fc γ RIIb alone had little or no effect (Fig. 5A) on phagocytosis mediated by any of the variants. These blocking experiments indicate a dominant role for Fc γ RIIa in mediating macrophage phagocytosis, relatively lower contributions from Fc γ RI and Fc γ RIII, and minimal effect from Fc γ RIIb. Isolation of phagocytosis to single receptors, carried out by combining blocking antibodies, confirmed these results, particularly the importance of Fc γ RIIa for variants containing the G236A mutation (Supplementary Fig. S1).²

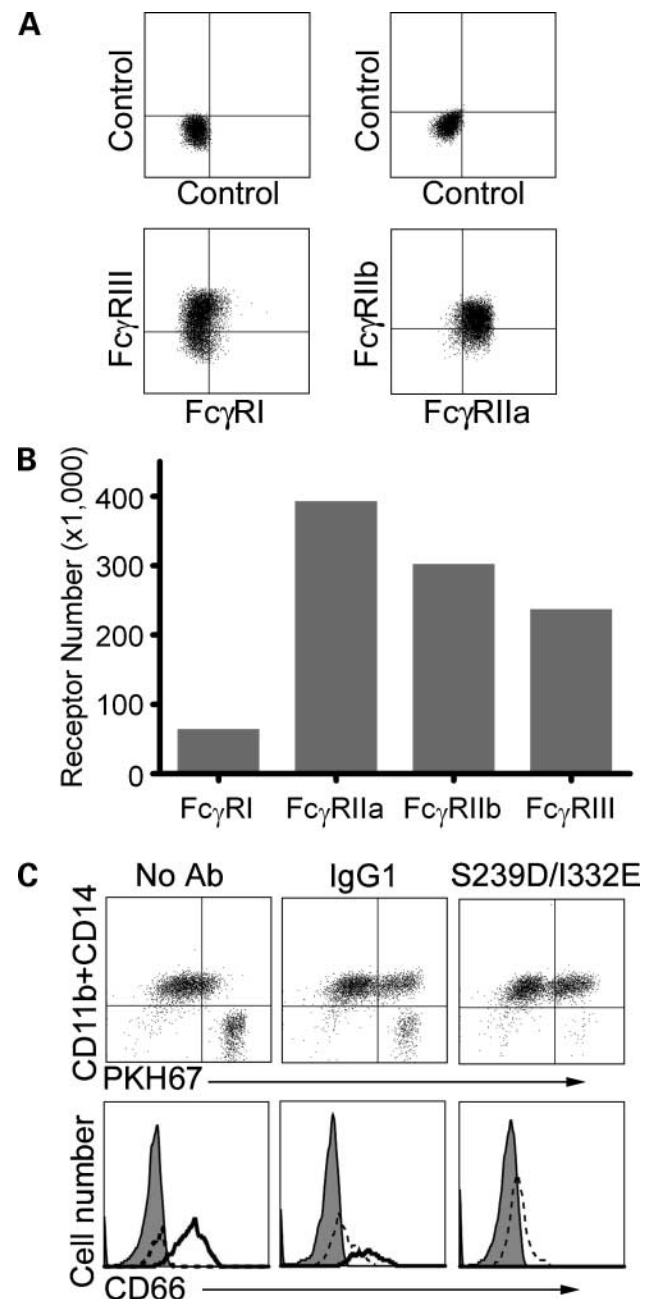
Fc γ RIIb Does Not Suppress Macrophage Phagocytosis

Fc γ RIIb has been reported to inhibit macrophage phagocytosis by modulating the threshold of activation (19). However, as discussed above, our variant series showed no systematic relationship between affinity for Fc γ RIIb and phagocytic potency, nor did we observe an effect on phagocytosis when Fc γ RIIb was selectively blocked. However, in these studies the controls for all but one of the variants already exhibited nearly maximal phagocytosis, making it difficult to observe any further enhancement with inhibitory receptor blockade. We therefore repeated the Fc γ RIIb blocking experiments with macrophages from the same H/H131 V/F158 donor using a lower concentration of the antibodies. Consistent with the

prior study, Fc γ RIIb blockade again had no effect (Fig. 5B). Similar results were observed with macrophages from donors having the low responder R/R131 F/F158 genotype (Supplementary Fig. S1).²

Discussion

Modification of antibodies to optimize Fc γ R affinities, such as described here and in previous work (8–12), has become a promising strategy for improving their therapeutic activity. Despite significant progress, however, the ideal profile(s) of Fc γ R affinities and selectivities remain undefined. Accordingly, we continue to engineer additional



² Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Figure 3. Fc γ R expression on monocyte-derived macrophages and Fc γ R-mediated phagocytosis were determined using multivariate flow cytometry. Purified CD14⁺ monocytes were cultured with macrophage colony-stimulating factor for 5 days, and macrophages were identified by the combination of anti-CD11b-APC and anti-CD14-APC. **A**, cytometry profiles of CD11b⁺CD14⁺ cells gated for individual Fc γ R indicate high levels of Fc γ RIIa, Fc γ RIIb, and Fc γ RIII and low levels of Fc γ RI. **B**, approximate receptor number on purified macrophages was determined using a bead-based antibody binding kit. Consistent with the flow cytometry data, the highest expression was seen for Fc γ RIIa followed by Fc γ RIIb and Fc γ RIII; Fc γ RI expression was much lower. **C**, cytometry profiles illustrating the three-color phagocytosis assay. LS180 adenocarcinoma target cells were labeled with PKH67, opsonized with varying concentrations of antibodies, and cocultured with macrophages for 4 h; cells were then stained and evaluated by flow cytometry. Y axis, macrophages (stained with anti-CD14-APC and anti-CD11b-APC); X axis, tumor cells (stained with PKH67). Macrophages that phagocytose tumors are double positive for PKH67 and CD11b/CD14 (top right quadrants), and target cells that are internalized show less intense CD66 staining compared with residual tumor cells. Bottom, histograms for macrophages (filled), phagocytosed tumor cells (dotted line), and residual tumor cells (solid line). Native IgG1 significantly increased phagocytosis compared to control (No Ab), and activity was enhanced further by the S239D/I332E variant.

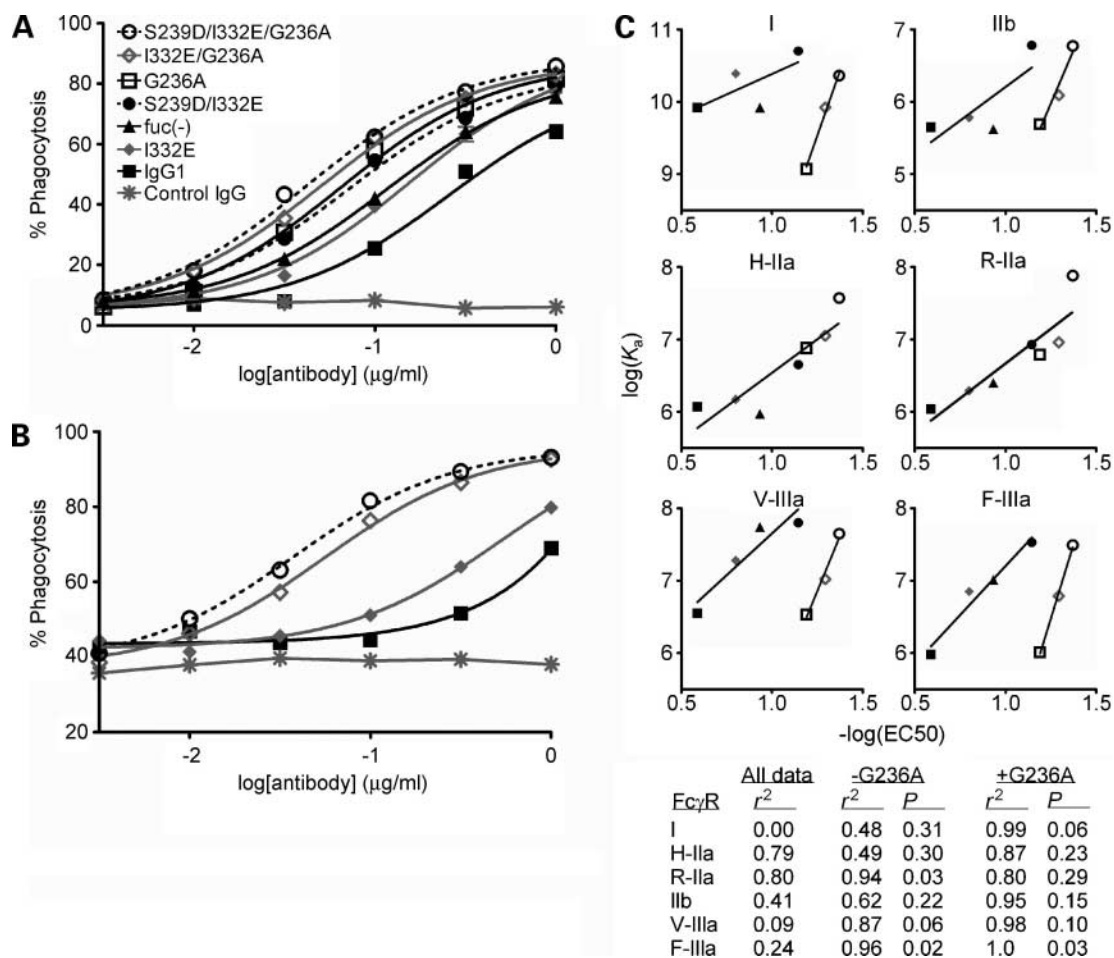


Figure 4. Fc variants with increased affinity for Fc γ RIIa enhance macrophage phagocytosis. The phagocytosis assay was carried out as described in Fig. 3. **A**, macrophage phagocytosis was measured as a function of antibody concentration. Percent phagocytosis was determined as the number of double-positive cells divided by the total number of PKH67-positive cells. Fc γ RIIa and Fc γ RIIIa genotypes were H/H131 and V/F158, respectively. Mean \pm SE for triplicate wells. Representative of two independent experiments. Substantial improvements in EC₅₀ and maximal activity relative to native IgG1 were seen for all variants, with the triple mutant showing the highest activity. Control IgG for A and B was anti-respiratory syncytial virus IgG1. **B**, greater phagocytosis enhancements were observed using macrophages possessing the R/R131 Fc γ RIIa and F/F158 Fc γ RIIIa genotype. Data represents the mean \pm SE for triplicate wells. Symbols are as shown in **A**. **C**, plots of Fc γ R affinities ($\log[K_a]$; Table 1) versus ADCC activity ($-\log[EC_{50}]$) show a strong correlation for Fc γ RIIa, no correlation for Fc γ RI and Fc γ RIIIa, and a distinctly nonnegative correlation for Fc γ RIIb. However, correlations significantly improved, particularly for Fc γ RIIIa, when antibodies were grouped as low (-G236A) and high (+G236A) Fc γ RIIa affinity subsets. Lines in the plots are based on all data for Fc γ RIIa and \pm G236A subsets for Fc γ RI, Fc γ RIIb, and Fc γ RIIIa. EC₅₀ data are from the experiment shown in **A**, and affinities are from Table 1. Symbols are as shown in **A**. The table shows the correlations (r^2) for all data, the -G236A subset, and the +G236A subset as well as the P values for the significance between the subsets and all data.

Fc γ R selectivities through amino acid substitution and use the variants to further define the precise Fc γ R binding profiles for optimal activity of the relevant immune cells. Our specific goals for this study were 2-fold. First, we set out to engineer and evaluate the benefit of variants with selective affinity enhancement for Fc γ RIIIa and Fc γ RIIa relative to Fc γ RIIb. Second, we wished to characterize at a detailed affinity level the contribution of individual receptors to the effector functions of cells with both simple (NK cells) and complex (macrophages) Fc γ R expression.

NK cells are unique among effector cells in that they typically express only the activating receptor Fc γ RIIIa and are not subject to inhibition by Fc γ RIIb. When PBMC were used as effectors, lysis was mediated almost completely by

the NK cell population, shown by the strong correlation with Fc γ RIIIa affinity, the substantial reduction in activity when blocked with anti-Fc γ RIIIa but not anti-Fc γ RII antibodies, and the complete lack of activity upon NK cell depletion. The optimal receptor profile for NK-mediated ADCC is clearly greater affinity for Fc γ RIIIa. Overall, the work here on NK cells, a simple system with respect to Fc receptors, gave no surprises and served more as a control for our approach to studying effector functions using the Fc variants. Moreover, the absolute dependence of these standard ADCC assays on NK cells cautions against extrapolating relative variant activities into clinical settings where multiple effector cells and cytotoxic mechanisms are involved.

Macrophages are a more complex cell type with respect to Fc receptor expression. They express various levels of all the activating and inhibitory Fc γ R subject to regulation by cytokines. Studies on the Fc γ R dependence of human macrophage phagocytosis have shown that qualitatively both Fc γ RIIa and Fc γ RIIIa can mediate phagocytosis of antibody-coated cells but that Fc γ RIIIa expression is more heterogeneous (18, 33, 34). In our studies, macrophages cultured in macrophage colony-stimulating factor expressed high levels of Fc γ RIIa, lower but still significant levels of Fc γ RIIb and Fc γ RIIIa, and low levels of Fc γ RI. The most important finding was that antibody variants with improved Fc γ RIIa affinity, contributed most markedly by the G236A mutation, enhanced macrophage-mediated phagocytosis relative to native IgG1. The importance of Fc γ RIIa was supported by the strong correlation observed between its affinity and phagocytosis and confirmed by the prominent reductions in activity observed when it was blocked using anti-Fc γ RII antibody. The effect of blocking Fc γ RII was most significant for native IgG1 and variants with high affinity for Fc γ RIIa, the most dramatic of which was G236A. However, the effect of anti-Fc γ RII was also

observed for I332E and S239D/I332E, two variants that improve affinity to Fc γ RIIIa more than Fc γ RIIa. Together with the slight but consistent reductions in phagocytosis observed when Fc γ RI and Fc γ RIIIa were blocked, the data suggest that these receptors play a complimentary role to Fc γ RIIa. Indeed, nearly perfect correlations between phagocytosis EC₅₀ and Fc γ RIIIa affinity were observed when variants were separated into high (+G236A) and low (-G236A) Fc γ RIIa affinity subsets. This analysis is not conclusive due to the minimal number of points. Nonetheless, this relationship suggests that Fc γ RIIa is a coarse knob for macrophage phagocytosis, setting the maximal level of activity that is tuned more finely by Fc γ RIIIa.

In contrast to the activating receptors, Fc γ RIIb played a minimal role in regulating tumor cell phagocytosis. Even variants with increased binding to Fc γ RIIb showed enhanced ADCC, and a negative correlation was not observed between Fc γ RIIb affinity and ADCC activity. These data suggest that biasing affinity toward activating receptors is sufficient for increased phagocytic activity. This result is in striking contrast to the growing paradigm that Fc γ RIIb modulates the activation threshold for cells

Downloaded from <http://aacrjournals.org/mct/article-pdf/7/8/2517/1881251/2517.pdf> by guest on 18 September 2024

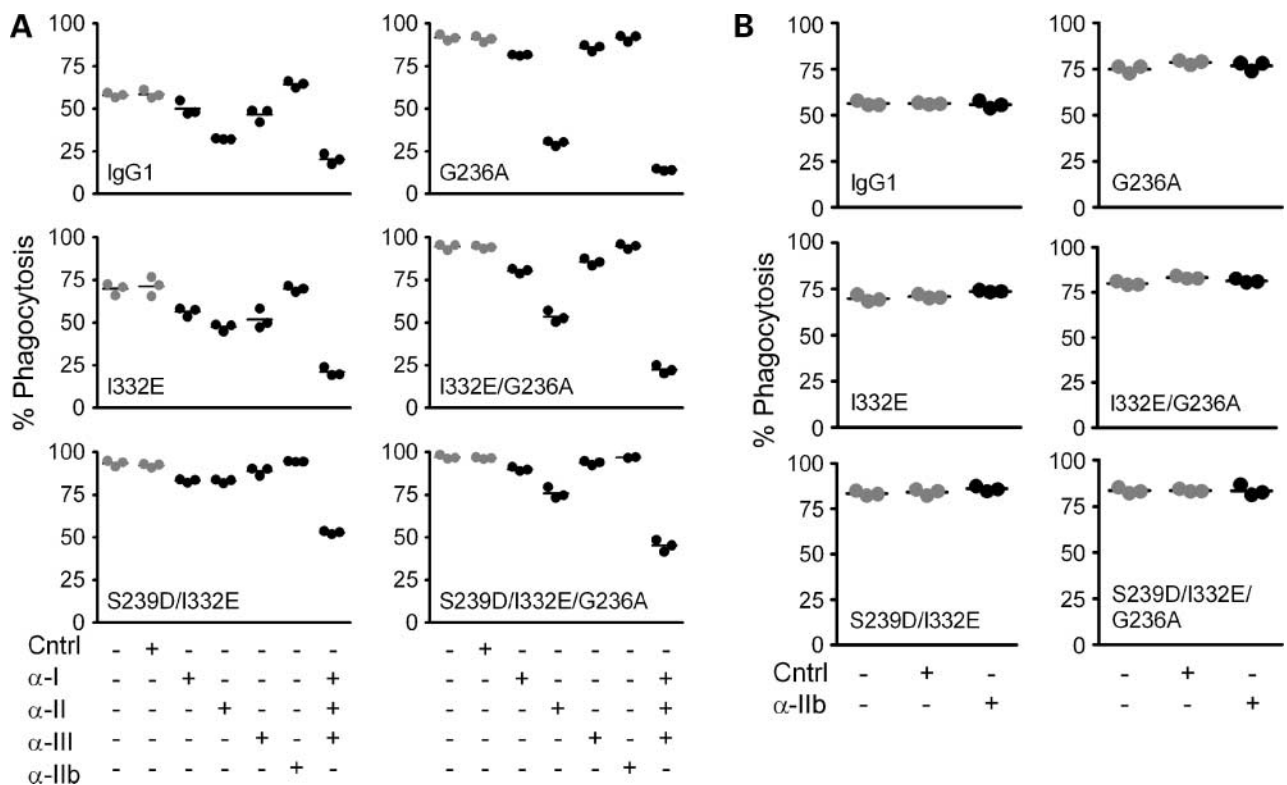


Figure 5. Fc γ RIIa dominates macrophage phagocytosis. **A**, use of selective blocking antibodies confirms the importance of Fc γ RIIa. ADCC was determined as described in Fig. 4, except selective Fc γ R blocking antibodies were added to the macrophages at 10 μ g/mL before the addition of tumor cells and 0.316 μ g/mL opsonizing antibodies. *Gray*, controls containing no antibody (all -) or nonspecific murine IgG (*Ctrl* +); *black*, samples with blocking antibodies. For native IgG1 and all variants, blocking both Fc γ RIIa and Fc γ RIIb (α -II +) had the greatest inhibitory effect, especially for the Fc γ RIIa-selective G236A variant. In contrast, blocking Fc γ RIIb alone (α -IIb +) had no effect. Some inhibition was seen with individual Fc γ RI (α -I +) or Fc γ RIII (α -III +) blockade, and the most dramatic reduction was seen with simultaneous blockade of all three activating Fc γ R, indicating a dominant role for Fc γ RIIa and contributing roles for Fc γ RI and Fc γ RIII. **B**, Fc γ RIIb plays a minimal role. Blocking studies with anti-Fc γ RIIb were repeated using a lower concentration of opsonizing antibodies (0.10 μ g/mL) so that any enhancement due to inhibitory receptor blockade could more readily be observed. Although phagocytosis was below maximal for all controls, Fc γ RIIb blockade again had no effect. *Gray*, controls containing no antibody (all -) or nonspecific human F(ab) (*Ctrl* +). Fc γ RIIa and Fc γ RIIIa genotypes for both experiments (**A** and **B**) were H/H131 and V/V158, respectively. These results are representative of at least two independent experiments.

that express it, and the hypothesis that A/I ratios (17) are a variable in determining antibody-mediated effector function. The regulatory role of FcγRIIb in macrophage phagocytosis has been reported in studies in which cytokines, particularly interleukin-4, have been used to modulate FcγRIIb expression (19, 35). In these studies, however, there was no simultaneous evaluation of the effect on activating receptors, and more recent work has shown that interleukin-4 reduces their expression (36). The *in vivo* role of FcγRIIb is supported by the greater antibody antitumor activity and increased B-cell depletion observed in FcγRIIb^{-/-} mice (5, 7). The reason(s) for the disconnect between these studies and the results for human macrophages observed in our work is not clear, although it is worth emphasizing that the enhanced activities observed in the FcγRIIb^{-/-} mice were generally observed at subtherapeutic doses of antibody. Overall, the results may indicate that the qualitative roles of the different FcγR determined from perturbations such as heterologous expression, blocking, and knockout studies do not directly define the optimal Fc receptor affinity profile(s) for a given cell type.

In the context of antitumor therapy, many questions remain as to the importance of NK cells relative to other FcγR-bearing effector cells despite their prominent reputation for mediating ADCC. Although they are the simplest link between the clinical correlations observed between FcγRIIIa polymorphism and outcome in anti-CD20 therapy, macrophages and dendritic cells also express FcγRIIIa. The relevance of macrophages and dendritic cells to antibody antitumor mechanism has been implicated by the observed capacity of macrophages and dendritic cells to infiltrate tumors (37, 38), the monocyte dependence of antibody-mediated efficacy in mouse studies (6, 7), and the growing evidence that antibody therapy can induce an adaptive immune response (39, 40). For example, mice deficient of tissue macrophages were found to be more resistant to antibody-mediated B-cell depletion (6). Furthermore, specific mouse IgG isotypes and engineered antibodies with increased binding to FcγRIV, present on murine monocytes and neutrophils but not NK cells, have shown enhanced B-cell depletion, and blocking FcγRIV greatly diminishes this activity (10).

In human therapy, the improved survival observed for neuroblastoma patients having the higher-affinity allele of FcγRIIIa (R131 for mouse IgG3) would seem to support a role for myeloid lineage cells in efficacy (41) at least for some indications. Although such correlations have also been observed in non-Hodgkin's lymphoma and breast cancer for rituximab and trastuzumab, respectively (3, 42), a recent study suggests that these correlations are due to linkage disequilibrium between FcγRIIIa and FcγRIIIa (43), consistent with the lack of affinity preference of IgG1 for either FcγRIIIa allele. Additional myeloid cells include neutrophils and dendritic cells, both of which express FcγRIIIa. We are currently evaluating the capacity of the variants described here to improve neutrophil ADCC, which is known to be strongly dependent on FcγRIIIa (44). Dendritic cells, likewise, have been shown to be dependent

on FcγRIIIa for immunocomplex-stimulated maturation (45), an important factor in cross-presentation of tumor-derived antigens and activation of antitumor cytotoxic T lymphocytes (20). A major goal for the field of therapeutic antibodies is to converge Fc engineering capabilities with clear definitions of both the optimal FcγR profile(s) for different effector cells and the relevance of each cell type to the treatment of a particular cancer.

Disclosure of Potential Conflicts of Interest

The authors have financial interests in Xencor, including employment and stock options.

Acknowledgments

We thank Igor Vostiar, Patrick Joyce, Araz Eivazi, and Sreenivas Laxmanan for technical contributions, Bassil Dahiyat and Madhav Dhodapkar for helpful discussions, and David Szymkowski and Marie Ary for assistance with the article.

References

- Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood* 2002;99:754–8.
- Kim DH, Jung HD, Kim JG, et al. FCGR3A gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. *Blood* 2006;108:2720–5.
- Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003;21:3940–7.
- Treon SP, Hansen M, Branagan AR, et al. Polymorphisms in FcγRIIIA (CD16) receptor expression are associated with clinical response to rituximab in Waldenstrom's macroglobulinemia. *J Clin Oncol* 2005;23:474–81.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443–6.
- Uchida J, Hamaguchi Y, Oliver JA, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 2004;199:1659–69.
- Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific engagement of Fcγ receptors regulates B lymphocyte depletion during CD20 immunotherapy. *J Exp Med* 2006;203:743–53.
- Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR. *J Biol Chem* 2001;276:6591–604.
- Lazar GA, Dang W, Karki S, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* 2006;103:4005–10.
- Stavenhagen JB, Gorlatov S, Tuailon N, et al. Fc optimization of therapeutic antibodies enhances their ability to kill tumor cells *in vitro* and controls tumor expansion *in vivo* via low-affinity activating Fcγ receptors. *Cancer Res* 2007;67:8882–90.
- Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 1999;17:176–80.
- Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, et al. Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol Bioeng* 2004;87:614–22.
- Desjarlais JR, Lazar GA, Zhukovsky EA, Chu SY. Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective. *Drug Discov Today* 2007;12:898–910.
- Salmon JE, Edberg JC, Brogle NL, Kimberly RP. Allelic polymorphisms of human Fcγ receptor IIA and Fcγ receptor IIIB. Independent mechanisms for differences in human phagocyte function. *J Clin Invest* 1992;89:1274–81.
- Sanders LA, Feldman RG, Voorhorst-Ogink MM, et al. Human

- immunoglobulin G (IgG) Fc receptor IIA (CD32) polymorphism and IgG2-mediated bacterial phagocytosis by neutrophils. *Infect Immun* 1995;63:73–81.
16. van Sorge NM, van der Pol WL, van de Winkel JG. FcγR polymorphisms: implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* 2003;61:189–202.
17. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin G subclass activity through selective Fc receptor binding. *Science* 2005;310:1510–2.
18. Munn DH, McBride M, Cheung NK. Role of low-affinity Fc receptors in antibody-dependent tumor cell phagocytosis by human monocyte-derived macrophages. *Cancer Res* 1991;51:1117–23.
19. Tridandapani S, Siefker K, Teillaud J-L, Carter JE, Wewers MD, Anderson CL. Regulated expression and inhibitory function of FcγRIIb in human monocytic cells. *J Biol Chem* 2002;277:5082–9.
20. Dhodapkar KM, Kaufman JL, Ehlers M, et al. Selective blockade of inhibitory Fcγ receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A* 2005;102:2910–5.
21. Lazar GA, Desjarlais JR, Jacinto J, Karki S, Hammond PW. A molecular immunology approach to antibody humanization and functional optimization. *Mol Immunol* 2007;44:1986–98.
22. Veri MC, Gorlatov S, Li H, et al. Monoclonal antibodies capable of discriminating the human inhibitory Fcγ-receptor IIB (CD32B) from the activating Fcγ-receptor IIA (CD32A): biochemical, biological and functional characterization. *Immunology* 2007;121:392–404.
23. Myszkowski DG. Improving biosensor analysis. *J Mol Recognit* 1999;12:279–84.
24. Lenkei R, Andersson B. Determination of the antibody binding capacity of lymphocyte membrane antigens by flow cytometry in 58 blood donors. *J Immunol Methods* 1995;183:267–77.
25. Parren PW, Warmerdam PA, Boeijs LC, et al. On the interaction of IgG subclasses with the low affinity FcγRIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. *J Clin Invest* 1992;90:1537–46.
26. Ferrara C, Stuart F, Sondermann P, Brunker P, Umansky P. The carbohydrate at FcγRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. *J Biol Chem* 2006;281:5032–6.
27. Masuda K, Kubota T, Kaneko E, et al. Enhanced binding affinity for FcγRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. *Mol Immunol* 2007;44:3122–31.
28. Ripka J, Adamany A, Stanley P. Two Chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose. *Arch Biochem Biophys* 1986;249:533–45.
29. Shields RL, Lai J, Keck R, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002;277:26733–40.
30. Ernst LK, Metes D, Herberman RB, Morel PA. Allelic polymorphisms in the FcγRIIC gene can influence its function on normal human natural killer cells. *J Mol Med* 2002;80:248–57.
31. Ortaldo JR, Woodhouse C, Morgan AC, Herberman RB, Cheresch DA, Reisfeld R. Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. *J Immunol* 1987;138:3566–72.
32. Abdullah N, Greenman J, Pimenidou A, Topping KP, Monson JR. The role of monocytes and natural killer cells in mediating antibody-dependent lysis of colorectal tumour cells. *Cancer Immunol Immunother* 1999;48:517–24.
33. van de Winkel JG, Anderson CL. Biology of human immunoglobulin G Fc receptors. *J Leukoc Biol* 1991;49:511–24.
34. Indik ZK, Park JG, Hunter S, Mantaring M, Schreiber AD. Molecular dissection of Fcγ receptor-mediated phagocytosis. *Immunol Lett* 1995;44:133–8.
35. Joshi T, Ganesan LP, Cao X, Tridandapani S. Molecular analysis of expression and function of hFcγRIIb1 and b2 isoforms in myeloid cells. *Mol Immunol* 2006;43:839–50.
36. Pricop L, Redecha P, Teillaud JL, et al. Differential modulation of stimulatory and inhibitory Fcγ receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol* 2001;166:531–7.
37. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002;23:549–55.
38. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting *in vivo* elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res* 2005;65:3437–46.
39. Dhodapkar KM, Krasovskiy J, Williamson B, Dhodapkar MV. Anti-tumor monoclonal antibodies enhance cross-presentation of cellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. *J Exp Med* 2002;195:125–33.
40. Taylor C, Hershman D, Shah N, et al. Augmented HER-2 specific immunity during treatment with trastuzumab and chemotherapy. *Clin Cancer Res* 2007;13:5133–43.
41. Cheung NK, Sowers R, Vickers AJ, Cheung IY, Kushner BH, Gorlick R. FCGR2A polymorphism is correlated with clinical outcome after immunotherapy of neuroblastoma with anti-GD2 antibody and granulocyte macrophage colony-stimulating factor. *J Clin Oncol* 2006;24:2885–90.
42. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and response to trastuzumab-based treatment in patients with HER-2/neu-positive metastatic breast cancer. *Proc AACR*; 2007 Apr 17; Los Angeles; 2007.
43. Hatjiharissi E, Hansen M, Santos DD, et al. Genetic linkage of FcγRIIa and FcγRIIIa and implications for their use in predicting clinical responses to CD20-directed monoclonal antibody therapy. *Clin Lymphoma Myeloma* 2007;7:286–90.
44. Nagarajan S, Venkiteswaran K, Anderson M, Sayed U, Zhu C, Selvaraj P. Cell-specific, activation-dependent regulation of neutrophil CD32A ligand-binding function. *Blood* 2000;95:1069–77.
45. Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J Clin Invest* 2005;115:2914–23.
46. Paetz A, Sack M, Thepen T, et al. Recombinant soluble human Fcγ receptor I with picomolar affinity for immunoglobulin G. *Biochem Biophys Res Commun* 2005;338:1811–7.
47. Maenaka K, van der Merwe PA, Stuart DI, Jones EY, Sondermann P. The human low affinity Fcγ receptors IIa, IIb, and III bind IgG with fast kinetics and distinct thermodynamic properties. *J Biol Chem* 2001;276:44898–904.
48. Mimura Y, Sondermann P, Ghirlando R, et al. Role of oligosaccharide residues of IgG1-Fc in FcγRIIb binding. *J Biol Chem* 2001;276:45539–47.
49. Okazaki A, Shoji-Hosaka E, Nakamura K, et al. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcγRIIIa. *J Mol Biol* 2004;336:1239–49.