Dimeric FcγR Ectodomains as Probes of the Fc Receptor Function of Anti-Influenza Virus IgG

Bruce D. Wines,*†‡ Hillary A. Vanderven,* Sandra E. Esparon,* Anne B. Kristensen,* Stephen J. Kent,*§‖ and P. Mark Hogarth*†‡

Ab-dependent cellular cytotoxicity, phagocytosis, and Ag presentation are key mechanisms of action of Abs arising in vaccine or naturally acquired immunity, as well as therapeutic mAbs. Cells expressing the low-affinity FcγRs (FcγRII or CD32 and FcγRIII or CD16) are activated for these functions when receptors are aggregated following the binding of IgG-opsonized targets. Despite the diversity of the Fc receptor proteins, IgG ligands, and potential responding cell types, the induction of all FcγR-mediated responses by opsonized targets requires the presentation of multiple Fc regions in close proximity to each other. We demonstrated that such “near-neighbor” Fc regions can be detected using defined recombinant soluble (rs) dimeric low-affinity ectodomains (rsFcγRs) that have an absolute binding requirement for the simultaneous engagement of two IgG Fc regions. Like cell surface–expressed FcγRs, the binding of dimeric rsFcγR ectodomains to Ab immune complexes was affected by Ab subclass, presentation, opsonization density, Fc fucosylation, or mutation. The activation of an NK cell line and primary NK cells by human IgG-opsonized influenza A hemagglutinin correlated with dimeric rsFcγRIIa binding activity but not with Ab titer. Furthermore, the dimeric rsFcγR binding assay sensitively detected greater Fc receptor activity to pandemic H1N1 hemagglutinin after the swine influenza pandemic of 2009 in pooled human polyclonal IgG. Thus these dimeric rsFcγR ectodomains are validated, defined probes that should prove valuable in measuring the immune-activating capacity of IgG Abs elicited by infection or vaccination or experimentally derived IgG and its variants. The Journal of Immunology, 2016, 197: 1507–1516.

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The full protective activity of Abs to many viruses (1–7), including HIV (8–10), simian HIV (11), HSV type 2 (4), and influenza (6). Moreover, a broadly neutralizing Ab (6F12) directed against the influenza A hemagglutinin (HA) stalk region required FcγR engagement for full protective activity (12), making the stalk region a promising vaccine target for neutralization (13) and Fc receptor (FcR)-mediated protection (14, 15). Thus, although HA inhibition by neutralizing Ab is the standard measure of a protective response to vaccination against influenza (16), Ab-dependent cellular phagocytosis (17), Ab-dependent cellular cytotoxicity (7, 18, 19), and other FcγR-dependent functions also contribute to immunity and form part of the optimal vaccine response (14, 20). The capacity of IgG to facilitate FcγR-mediated Ag presentation (21) and induce dendritic cell activation (22) provides another mechanism for Fc-dependent effects in immunity to some infectious agents (23) and immunity more generally (24). Taken together, effector functions triggered by the engagement of FcRs are key contributors to the efficacy of Ab-mediated protection in natural immunity, vaccine responses, and treatments with therapeutic Abs (25, 26).

Despite being fundamental to Ab-mediated immunity, FcR-mediated effector functions are difficult to define and measure, in part because of the different receptors and the plethora of cell types and responses that may be generated. Despite this diversity, FcR activation of cells occurs when Abs aggregated by complexing with Ag present a cluster of Fc regions that cross-link FcRs and trigger subsequent downstream signaling.

Structural studies of the interaction of the human IgG1 Ab Fc region with its various cellular receptors [FcγRI (27, 28), FcγRIIa (29, 30), FcγRIIb (31), and FcγRIIIa (32, 33)], in combination with biosensor studies, defined the atomic basis of the 1:1 interactions of single FcR ectodomains with an IgG1-Fc (34, 35). However, the physiological interaction of IgG immune complexes (ICs) and FcγRs requires avid binding of the complex through the display of multiple Fc regions of “near-neighbor” IgGs to engage and cluster multiple FcγRs on the cell surface (36). Hence, differences in the opsonization of targets by different IgGs influence interactions with FcγRs, chiefly by the density, size (37, 38), and topology of presentation of the Fc regions. Although not easily predicted, these effects may profoundly affect cellular functions (39). Thus, how Ag-bound Abs are presented for sensing by FcγRs underpins effector functions, such as Ab-dependent cellular cytotoxicity and Ab-dependent cellular phagocytosis, but is not simple to quantitate experimentally. Cell-based assays for such key cellular responses (40–45) are difficult to standardize, so there is a need for an assay that simply evaluates the capacity of an immune complex (IC) to present Fcs for avid binding by FcγRs.

In an attempt to recapitulate the avid sensing of ICs by cell surface FcγRs, our approach used a single, biotin-tagged polypeptide containing two FcγR ectodomains where the two Fc-binding modules

*Centre for Biomedical Research, Burnet Institute, Melbourne, Victoria 3004, Australia; †Department of Immunology, Monash University Central Clinical School, Melbourne, Victoria 3004, Australia; ‡Department of Pathology, The University of Melbourne, Melbourne, Victoria 3052, Australia; §Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria 3052, Australia; †Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, The University of Melbourne, Parkville, Victoria 3052, Australia; and ‡Melbourne Sexual Health Centre, Infectious Diseases Department, Alfred Health, Monash University Central Clinical School, Melbourne, Victoria 3004, Australia

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Address correspondence and reprint requests to Prof. P. Mark Hogarth, Centre for Biomedical Research, Burnet Institute, 85 Commercial Road, Melbourne, VIC 3004, Australia. E-mail address: mark.hogarth@burnet.edu.au

Abbreviations used in this article: A450nm, absorbance at 450 nm; C.I., confidence interval; FcR, Fc receptor; HA, hemagglutinin; HSA, human serum albumin; IC, immune complex; rs, recombinant soluble; TNP, trimethylphenyl; WT, wild-type.

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were linked by the FcγRIIA membrane proximal stalk region. In this approach, the selective binding of these dimeric FcγR ectodomains to ICs is dependent on the presentation of pairs of “near-neighbor” Fc regions by the IC to bind both ectodomain modules of the receptor dimer. These dimeric recombinant soluble (rs)FcγR probes recapitulated many properties of the cell surface receptors, including selectivity for binding IgG subclasses and the binding affinities and specificities of the allotypic forms of FcγRIIA and FcγRIIB. Furthermore, the afucosyl form of IgG1 was more active in dimeric rsFcγRIIA binding than the normally glycosylated mAb. The binding of the dimeric rsFcγRIIA probe correlated with NK activation by ICs. This assay is useful for evaluating the functional activity of IgGs binding different Ags and epitopes in and different forms, including mutants, subclasses, and glycoforms.

Materials and Methods

Reagents

Abs and proteins. Human, bovine Fraction V, and human IgG, IgG2, IgG3, and IgG4 myeloma proteins were from Sigma-Aldrich. Human IgG1 mAb b12, its LALA mutant, LL234–235(4A), and afucosyl variant were a gift from Prof. Dennis Burton (The Scripps Institute, La Jolla, CA). High Sensitivity Streptavidin-HRP conjugate was from Pierce (Thermo Scientific, Melbourne, Australia). IgG capture reagent was AffiniPure F(ab’)2 goat anti-human IgG-F(ab’)2 specific (Jackson ImmunoResearch, West Grove, PA). Human serum albumin (HSA), IgG: INTRAGAM P (i.v. Ig normal IgG, 6%), and IgG:Sorbiological (Shanghai, China), polyclonal rabbit anti-human IgG-HRP (Dako, Sydney, Australia), and 3’,5’,5’-tetramethylbenzidine ELISA substrate (Life Technologies/ThermoFisher, Melbourne, Australia).

Generation of BirA ligase–expressing cell lines. A pRESHygro expression vector containing BirA ligase with a C-terminal endoplasmonic retention signal was amplified and a gift from Dr. Amanda Gavin (The University of Melbourne, Institute, La Jolla, CA), with the insert from pDisplay-BirA-ER, a gift from Prof. Dennis Burton (The Scripps Institute, La Jolla, CA). High Sensitivity Streptavidin-HRP conjugate was from Pierce (Thermo Scientific, Melbourne, Australia). Human influenza A virus HA protein was from Sinobiological (Shanghai, China), polyclonal rabbit anti-human IgG-HRP (Dako, Sydney, Australia), and 3’,5’,5’-tetramethylbenzidine ELISA substrate (Life Technologies/ThermoFisher, Melbourne, Australia).

Expression constructs. The production of a chimeric IgG1 comprising a mouse leader and VH sequence (from TB142 anti-trinitrophenyl) [TNP]; American Type Culture Collection) joined to a human IgG1 C region sequence was described previously (49). Chimeric anti-TNP x k chain consisting of TB142VL and human constant k was produced from a codon-optimized construct synthesized by Bioneer Pacific, (Kew, Australia). An IgG4 chimeric FcγR IIa (clone EXPI293F) used in the construction of this construct was codon optimized, synthesized, and transferred into pcR3. Likewise, the TNP-specific mouse VH sequence was joined to a human IgG2 C region sequence (accession no. J00230) using standard molecular biology techniques, and the chimeric Ab sequence was subcloned into pcR3.

FcyR-binding assays using dimeric rsFcγR-biotin. IgG capture reagent, F(ab’)2 goat anti-human IgG, F(ab’)2 F(ab’)2 (10 µg/ml), TNP-BSA (20 µg/ml), or rsFcγR-biotin (1 µg/ml) was prepared in PBS and adsorbed onto wells (plates) to plates (Maxisorp, Nunc). For the analysis of patient samples or IgVs, wells (typically three) were directly coated with 5 µg/ml IgV (INTRAGAM P), and signals from these wells were used to normalize the FcR activity of the test samples. Coated wells were subsequently blocked with PBS containing 1 mM EDTA and 1% (w/v) BSA (Fraction V; Sigma-Aldrich). A 0.5% HSA solution in PBS was used to block wells (plates) (Maxisorp, Nunc). After the capture antibodies were bound and washed, 100 µl of test sample was added to the plates and incubated for 2 h at 37°C. Plates were washed five times with PBS containing 0.05% Tween-20. The Ab-bound plates were incubated with 0.2 µg/ml purified dimeric rsFcγR-biotin or 0.1 µg/ml purified dimeric rsFcγR-biotin, in PBS diluted with 1 mM EDTA, 0.05% Tween-20 (100 µl/well) for 2 h at 37°C. After the wells were washed several times with a mixture of PBS and 0.05% Tween-20, 100 µl of anti-IgG-HRP (1000-fold dilution) in PBS was added to each well and incubated for 1 h at 37°C, followed by 8–10 cycles of washing with wash buffer. High Sensitivity Streptavidin-HRP (Thermo Fisher), 1/10,000 in diluent buffer, was added for 1 h at 37°C, followed by 8–10 cycles of washing with wash buffer and development with TMB Single Solution (Thermo Fisher). The reaction was stopped by addition of an equal volume of 1 M HCl, and absorbance at 450 nm (A450nm) was determined immediately. Delayed determination of absorbance can provide an apparent prozone effect artifact because precipitation of the colorimetric product occurs at high concentration. Bound IgG was measured using polyclonal rabbit anti-human IgG-HRP (Agilent Technologies-Dako), 1/10,000 dilution; Sigma-Aldrich, 1/20,000), and plates were blocked using HSA depleted of IgG by Protein G chromatography (GE (Life Technologies) or 1% FCS in PBS.

NK cell–activation assay. NK cell activation was measured by HA:anti-HA IC-dependent induction of intracellular IFN-γ and cell surface CD107a, as previously described (6, 52–54). Briefly, HLA(–) (Nunc, Rochester, NY) were coated with 600 ng of purified HA protein overnight at 4°C in PBS. The wells were washed five times with PBS and incubated with heat-inactivated (56°C for 1 h) sera/plasma or IgV for 2 h at
37°C. Plates were washed seven times with PBS, and 105 PBMCs were added to each well. Healthy donor PBMCs were obtained from buffy packs provided by the Australian Red Cross. PBMCs were isolated by FicolHPaque PLUS (GE Healthcare, Madison, WI), washed with RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine (Life Technologies, Grand Island, NY), frozen in FCS containing 10% DMSO, and stored in liquid nitrogen. thawed PBMCs were washed twice with RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine before addition to each well. Anti-human CD107a allophycocyanin-H7 Ab (clone H4B4; BD Biosciences, SanJose, CA), 5 μg/ml brefeldin A (Sigma-Aldrich), and 5 μg/ml monensin (Golgi Stop; BD Biosciences) were added to the cells and incubated for 5 h at 37°C with 5% CO2. PBMCs were then incubated with 1 mM EDTA to minimize cell adherence to the plate, anti-human CD3 PerCP (clone SP34-2), and anti-human CD56 allophycocyanin (clone B159; both from BD Biosciences) for 30 min at room temperature in the dark. Cells were fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min and permeabilized with FACS Permeabilizing Solution 2 (BD Biosciences) for 10 min. PBMCs were then incubated at room temperature for 1 h with IFN-γ Alexa Fluor 700 (clone B27; BD Biosciences) in the dark. Finally, cells were fixed with 1% formaldehyde and acquired on an LSR II flow cytometer (BD Biosciences).

The NK cell line NK-92 (55 expressing human FcγRIIIa Val158 (GFP-CD16 [176V] NK-92) was used to perform some of the NK cell–activation assays and was kindly provided by Dr. Kerry Campbell (Institute for Cancer Research, Philadelphia, PA). ELISA plate coating was performed as described above. However, following PBS washing, an additional blocking step was performed with PBS containing 5% BSA (Sigma-Aldrich) and 0.1% Tween-20 (U-CyTech) for 2 h at 37°C. Once blocked, plates were washed with PBS and incubated with heat-inactivated sera/plasma or IvIg for 2 h at 37°C. Plates were washed with PBS, and 2 × 105 GFP-CD16 (176V) NK-92 cells were added to each well and incubated at 37°C for 5 h. Anti-human CD107a allophycocyanin (clone A4H3; BD Biosciences) and 1 mM EDTA were added to the cells for 30 min at room temperature in the dark. The GFP-CD16 (176V) NK-92 cells were washed twice with PBS, fixed with 1% formaldehyde, and acquired on an LSR II flow cytometer. Analysis was performed using FlowJo X software version 10.0.7r2 (TreeStar, Ashland, OR).

**Data and statistical analysis.** Statistical analysis was performed with GraphPad Prism version 6.05 (GraphPad, San Diego, CA). Binding data (Figs. 1–4) were fitted using Prism software, to log(agonist) versus response (variable slope, constraining bottom value = 0, the top value was allowed to vary freely). The 95% confidence interval (C.I.) for the individual fitted values for EC50 are indicated graphically for the representative curves in Fig. 1E and in the insets in Fig. 2B–E. Horizontal bars in Fig. 1B and Figs. 2B–E representing EC20 – EC80 were calculated similarly. Cumulative data shown as EC50 with error bars are presented as mean ± 95% C.I. in Figs. 2F, 3K, and 3L. Curve fitting for some low-affinity interactions, using the A450nm value for 250 μg/ml IgG1 and TNP-BSA Ag gave an EC50–3-fold higher than for ICs formed by capture of IgG1 with plate-bound F(ab’2) anti-human F(ab’2) (Fig. 1B, 1E). Likewise, dimeric rsFcγRIIA Phc158 had higher binding activity to the TNBP-BSA: IgG ICs than to the anti-human F(ab’2):IgG complexes (EC50, 250 pM and 1.4 nM, respectively, Fig. 1D, 1E), whereas this trend was not apparent with the higher-affinity Val158 allelic form of dimeric rsFcγRIIA (EC50, 46 and 48 pM, respectively) (Fig. 1C, 1E).

**Results**

**Characterization of dimeric rsFcγRs using model human IgG1 ICs**

The universal requirement for “near-neighbor” clustering of low-affinity FcRs by appropriately arrayed Fc portions in ICs underpins proinflammatory Ab-dependent effector functions. Because cell surface FcγRII and FcγRIII are low-affinity receptors for IgG that avidly bind ICs, we engineered genetic homodimers of their ectodomains, using a flexible linking sequence from the membrane proximal stalk, to generate defined probes that avidly bind to “near-neighbor” Ab pairs in ICs.

The biotin-labeled genetically fused dimeric ectodomains of FcγRIIA His131, FcγRIIIa Val158, and FcγRIIIa Phe158 were produced in cells expressing BirA ligase in the endoplasmic reticulum (46). The activities of these purified human dimeric rsFcγRs were initially characterized by testing their binding activities to different forms of model ICs formed with human IgG1 and TNP-BSA Ag or by capture with anti-F(ab’2). Subnanomolar concentrations of the dimeric rsFcγRIIA and dimeric rsFcγRIIIa had detectable binding to both forms of IgG ICs, whereas the monomeric forms of the proteins had >1000-fold lower binding activity (Fig. 1). The weak binding of the receptor monomers (Fig. 1A) indicates that the avid binding of the receptor dimers requires pairs of AbFc regions that are presented with a proximity to each other that allows the simultaneous binding of the two receptor modules making up the dimeric rsFcγR. It is such “near-neighbor” IgGs that are required for activation of cells via engaging and clustering FcRs.

The dimeric rsFcγRIIA binding to ICs formed with anti-TNP IgG1 and TNP-BSA Ag gave an EC50–3-fold higher than for ICs formed by capture of IgG1 with plate-bound F(ab’2) anti-human F(ab’2) (Fig. 1B, 1E). Likewise, dimeric rsFcγRIIIa Phc158 had higher binding activity to the TNBP-BSA: IgG ICs than to the anti-human F(ab’2):IgG complexes (EC50, 250 pM and 1.4 nM, respectively, Fig. 1D, 1E), whereas this trend was not apparent with the higher-affinity Val158 allelic form of dimeric rsFcγRIIIa (EC50, 46 and 48 pM, respectively) (Fig. 1C, 1E).

**Dimeric rsFcγR binding detects the modified FcγR-binding activity of Fc variants**

The dimeric rsFcγR assays were further evaluated by analyzing the interactions of activating and inactivating variants of IgG1. Mutation of the lower hinge of IgG1, LL(234–235)AA (LALA mutant), greatly diminishes FcγR binding activity and FcR mediated–function (56, 57). The loss of binding activity was recapitulated in the dimeric rsFcγR assays, with dimeric rsFcγRIIA and dimeric rsFcγRIIIa binding only weakly to IgG1-LALA ICs (Fig. 2B–E). In contrast, enhanced binding of dimeric rsFcγRIIA to afucosyl IgG1 (58) was demonstrated with the receptor dimer assay (Fig. 2D, 2E). Both the higher-affinity (Val158, p = 0.008) and the lower-affinity (Phc158, p = 0.016) allelic forms of the receptor had significantly increased affinity to the nonfucosyl IgG (Fig. 2D–F). For dimeric rsFcγRIIIa Val158, the EC50 decreased from 118 ng/ml (86–150, 95% C.I.) with WT IgG to 43 ng/ml (21–65, 95% C.I.) with nonfucosyl IgG; likewise for the Phc158 allele, the EC50 decreased from 275 ng/ml (89–460, 95% C.I.) to 97 ng/ml (55–139, 95% C.I.). Thus, the dimeric rsFcγR assays are useful for discriminating variants of IgG with enhanced or diminished FcR activity.

IC formation measured by determining the bound IgG with anti-IgG, EC20–EC80 for WT IgG1 = 8–151 ng/ml). In contrast, a distinctive feature of the dimeric rsFcγR binding activity was a narrow response profile, with the 20–80% response occurring over an 18-fold increase in IgG concentration (i.e., Fig. 2A, open bar; anti-IgG, EC20–EC80 for WT IgG1 = 8–151 ng/ml). In contrast, a distinctive feature of the dimeric rsFcγR binding activity was a narrow response profile, with the 20–80% response occurring over a 2–3-fold increase in IgG concentration (i.e., Fig. 2B, open bar; FcγRIIa H131, EC20–EC80 WT IgG1 = 150–360 ng/ml). The steeper character of these dimeric rsFcγR response curves can also be described by the numerical equivalent of the Hill constant (Table I). Although anti-IgG binding is typically described by a coefficient near or <1, the binding curves in Fig. 2 are typical of dimeric rsFcγR profiles for binding to IgG1 ICs with coefficients, in this case, ranging from 2.3 to 4.1 (Table 1). The steeper receptor-binding curves reflect the requirement for closely placed “near-neighbor” IgG-Fcs for dimeric rsFcγR binding, which produces a response over a narrow range of IgG concentration that would not be
obvious from measuring Ab binding or titer (e.g., compare Fig. 2A with Fig. 2B–E).

#### Dimeric rsFcγR binding is determined by IgG subclass and presentation of the Fc

The effect of IgG subclass on dimeric rsFcγR binding was investigated using two established methods of forming model ICs (37, 38). First, the capture of IgG by the F(ab')2 anti-human F(ab')2 to form ICs (Fig. 3A) results in the presentation of Fc regions of the IgGs in varied orientations for dimeric rsFcγR binding (Fig. 3C, 3E, 3G, 3I). Second, TNP hapten-specific rIgG and TNP-BSA form ICs (Fig. 3B) in which all IgGs are oriented by the same variable domain:hapten interaction and thus, display a more uniform presentation of Fcs for dimeric rsFcγR binding (Fig. 3D, 3F, 3H, 3J).

Dimeric rsFcγRIIa His131 binding to human IgG1, IgG2, IgG3, or IgG4 captured using F(ab')2 anti-human F(ab')2 to form ICs

### FIGURE 1.

Biotin-labeled dimeric rsFcγRs selectively bind to IgG1 ICs. Human IgG1 (1 μg/ml) was formed into ICs by binding to TNP-BSA (A–D) or by capture with plate-bound F(ab')2 fragments of anti-human F(ab')2 (B–D). These ICs were then reacted with monomeric rsFcγRIIa His131 or monomeric rsFcγRIIa Phe158 (A), dimeric rsFcγRIIa His131 (B), dimeric rsFcγRIIa Val158 (C), or dimeric rsFcγRIIa Phe158 with subsequent detection with streptavidin-HRP (D). (E) Data were fitted using Prism software to log(agonist) versus response (variable slope, constraining bottom value = 0). EC50 values from fitted curves are shown with 95% C.I.

### FIGURE 2.

Characterization of normal and variant IgG1 with dimeric rsFcγR assay. Normal IgG1, nonfucosyl IgG1, and LALA mutant IgG1 were captured using F(ab')2 fragment of anti-human F(ab')2 to form ICs and reacted with HRP-conjugated anti-human IgG (A) or the following human dimeric rsFcγR: FcγRIIa His131 (B), FcγRIIa Arg131 (C), FcγRIIa Val158 (D), or FcγRIIa Phe158 (E). Data were fitted using Prism software to log(agonist) versus response (variable slope, constraining bottom value = 0). Individual fitted values for EC50 ± 95% C.I. are shown as insets in (B)–(E). (F) EC50 values ± 95% C.I. were evaluated by the Mann–Whitney t test. The EC20–EC80 ranges for binding to WT IgG1 were also derived from the curve fitting and are shown as horizontal open bars annotated with the fold ratio of EC80/EC20 in (A)–(E). *p ≤ 0.05, **p ≤ 0.01. f, number of curve fits from independent experiments.
The nature of the IC also influenced dimeric rsFcR binding. The binding differed most markedly for the weakest FeR interactions observed. For example, dimeric rsFcRlla His131 binding activity was just detectable with ICs formed with anti-human F(ab')2 at 5 μg/ml IgG4 (Fig. 3C), whereas when binding to IC of IgG4 formed with the Ag TNP-BSA, the signal was ~6-fold higher at 5 μg/ml IgG4 (Fig. 3D, 3L, EC50 = ~2 μg/ml). For this lowest-affinity FeR interaction, Ab presentation in these different forms of ICs profoundly affects receptor binding. For the higher-affinity interactions, differences between the two methods of IC formation for FeR binding were less apparent. For example, the dimeric rsFcRlla His131 bound similarly to the anti-human F(ab')2:IgG1 and IgG2 ICs (EC50 = 400 and 300 ng/ml respectively, Fig. 3C, 3K) and to the TNP-BSA-IgG1 and IgG2 ICs (EC50 = 280 and 190 ng/ml, respectively, Fig. 3D, 3L).

The dimeric rsFcRlla Arg131 bound anti-human F(ab')2 ICs with the ranking IgG3 > IgG1 > IgG2 > IgG4 (Fig. 3E); a similar hierarchy (IgG1 > IgG2 > IgG4) was apparent with anti-TNP ICs (Fig. 3F, Table II). Thus, the allelic forms of dimeric rsFcRlla recapitulate the IgG subclass binding behavior of their cellular counterparts, and their weak binding to IgG4 is influenced by FeR presentation in different forms of ICs.

Analysis of the allelic variants of dimeric rsFcRlla proteins showed, as expected, that the dimeric rsFcRlla Val158 had greater binding activity to IgG1 IC than the lower-affinity dimeric rsFcRlla Phe158 (EC50 = 260 versus 540 ng/ml Fig. 3K; EC50 = 170 versus 570 ng/ml Fig. 3L). The hierarchies of binding to anti-F(ab')2 ICs (IgG3 > IgG1 > IgG2 > IgG4) and TNP-BSA ICs (IgG1 > IgG2 > IgG4 = nil) (Fig. 3G–L, Table II) were largely comparable to the binding of ICs of the different IgG subclasses to cell surface–expressed FcRlla (34, 37, 38).

It is notable that, in this study (Fig. 3) and in other studies (37, 38), differences occur in FeR binding to ICs made with anti-F(ab')2 or TNP-BSA Ag (Table II). Using the assay to evaluate FcRlla interactions with anti-TNP ICs revealed, similarly to FcRlla, that the weakest interactions between dimeric rsFcRlla and ligand were influenced the most by the manner of formation of the IC. The interactions of ICs of IgG2 and IgG4 with the higher-affinity (Fig. 3G, 3H) and lower-affinity (Fig. 3I, 3J) counterparts, and their weak binding to IgG4 is influenced by FeR presentation in different forms of ICs.

In summary, the dimeric rsFcRls demonstrate binding equivalent to cell surface FcRls with regard to the hierarchy of binding to IgG subclasses, e.g., binding to IgG3 > other subclasses, (see figure 2A in Ref. 37); the subclass specificity of the polymorphic forms of FcR (e.g., binding of IgG2 by FcRlla His131 > FcRlla Arg131); the expected differences in binding strength of the polymorphic forms of FcR (e.g., FcRlla Val158 > FcRlla Phe158); and the appropriately altered binding of LALA hinge mutant and nonfucosyl-variant IgG, b12. In addition, how the Fc is presented influences dimeric rsFcR binding, especially for low-affinity interactions, in particular IgG2 and IgG4 [e.g., FcRlla His131 with IgG4 ICs (Fig. 3C, 3D) and FcRlla with IgG2 (Fig. 3G–J)].
The use of dimeric rsFcγRs in the analysis of anti-influenza A immunity: dimeric rsFcγR binding to serum IgG-opsonized influenza HA

There is increasing evidence that Fc-mediated functions are important in clearance of influenza infections (6, 12), but typical assays to measure such responses through activation of cells or killing of target cells remain cumbersome. The dimeric rsFcγR-binding assay was evaluated in the context of Abs to this common viral pathogen. Dimeric rsFcγR-binding activity to ICs formed between influenza HA and human IgG Abs was assessed using IgVg and HA from the 2009 H1N1 pandemic virus and from an H3N2 virus. Because IgVg is prepared from the sera of thousands of individuals, it is composed of the IgG Ab repertoire at a population level and opsonizes all epitopes of HA for which specific IgG molecules exist in the population. Like the model ICs (Figs. 2, 3), H3N2 HA opsonized with IgVg (Fig. 4) showed a steeper dimeric rsFcγR-binding profile (Fig. 4B, 4C) compared with the broader profile for the detection of opsonizing IgG (anti-IgG, $EC_{20}$–$EC_{80}$ = 20–275 μg/ml, Fig. 4A). The estimated $EC_{50}$ values for dimeric rsFcγR binding (rsFcγRIIa His131 $EC_{50}$ $∼$ 370 μg/ml; rsFcγRIIa Val158 $EC_{50}$ $∼$ 250 μg/ml) were several fold above the $EC_{50}$ for IgG binding (anti-IgG $EC_{50}$ $= 74 \mu g/ml$), a feature also consistent with the model ICs. Enumeration of the $EC_{50}$ values for dimeric rsFcγR binding could only be approximated by curve fitting because the binding did not reach saturation ($r^2 = 0.88–0.92$, Fig. 4B, 4C). The same trends were apparent in dimeric rsFcγRIIa and dimeric rsFcγRIIIa binding to ICs formed by IgVg opsonization of A (H1N1)pdm09 HA, although the levels of opsonization and dimeric rsFcγR binding were less than for the HA from H3N2 A/Perth/16/2009 (Fig. 4B, 4C). For input concentrations of IgVg < 100 μg/ml, opsonization of HA by IgG, although well detected by anti-IgG, is sparse and therefore, bound IgGs cannot simultaneously engage the two FcγR-binding modules of the dimeric rsFcγRIIa or dimeric rsFcγRIIIa. The relatively steep reaction profiles of the dimeric rsFcγR-binding curves dictate that, rather than determining end point titer, a meaningful measure of the FcR activity for comparing different sera is the dimeric rsFcγR-binding signal at a fixed Ab concentration (or plasma/serum dilution) within the $EC_{20}$–$EC_{80}$ range of the assay.

FcR activity of IgVg-opsonized H1N1 pandemic HA mirrors HA-inhibition activity

Fc-mediated functional responses to the HA of novel influenza A strains entering the population, such as the 2009 swine influenza, A(H1N1)pdm09 (A/California/04/2009) exist, pre-exposure via cross-reactive Abs. Upon exposure via infection or vaccination, strain-specific HA inhibition titers are increased, and Fc-mediated
population of PBMCs by these ICs was measured by intracellular stain for IFN- 
point titer (NK-92 cells expressing Fc 
with ICs formed with plasmas diluted at 1:40 for the capacity to activate 
tested (Fig. 5B). Consistent with the dimeric rsFc 
relevance for the dimeric rsFc 
(Fig. 5C). The correlation between these differing, but contempora-
2010 was mirrored by a contemporaneous peak in HA-inhibition titer 
measure of a correlate of immunity, the peak in Fc 
G0 (IvIg), after the 2009 H1N1 pandemic. Thus, IvIg was 
pooled IgG (IvIg), after the 2009 H1N1 pandemic. Therefore, we assessed whether the di-
meric rsFc 
activity toward virally infected cells also increases. Furthermore, NK cell–activating capacity of HA Abs trended to-
ard an increase in pooled IgG preparations after the 2009 H1N1 influenza pandemic (54). Therefore, we assessed whether the di-
meric rsFc 
assay correlates with HA activity of HA Abs at the population level, as represented in 
pooled IgG (Ivlg), after the 2009 H1N1 pandemic. Thus, Ivlg was 
used to opsonize H1N1 pandemic HA, and dimeric rsFc 
blocking was measured. Ivlg preparations during 2010 showed higher dimeric rsFc 
blocking-binding activity, suggesting that the FcR-activating capacity of Abs specific for the HA of influenza virus A(H1N1)pdm09 was increased subsequent to the emergence 
of the pandemic in 2009 (Fig. 5A). Interestingly, this increased dimeric rsFc 
activity appeared to be transient, peaking and declining in 2010. When the dimeric rsFc 
activity was compared pre-2009 with 2010, the postpandemic increase in receptor 
activity was significant (p = 0.0003) over the three Ab concentrations 
tested (Fig. 5B). Consistent with the dimeric rsFcR assay being a measure of a correlate of immunity, the peak in FcR activity in 2010 was mirrored by a contemporaneous peak in HA-inhibition titer 
(Fig. 5C). The correlation between these differing, but contemporane-
ous, Ab functions (Fig. 5D, p < 0.0001) suggests a biological 
relevance for the dimeric rsFcγR assay and lends support to the 
idea of coordination of Ab functions in immunity. 

**Dimeric rsFcγR binding is a predictor of NK cell activation** 

Next, the relationship between dimeric rsFcγR binding and cellular 
FcγR effector function was investigated. The ability of dimeric 
rsFcγR activity to correlate with cellular activation by HA-specific 
Abs was tested by comparison with the activation of NK-92 cells 
expressing FcγRIIa Val158, a well-established system for mea-
suring Ab-mediated NK activation. Plasma from individuals was 
used to separately opsonize HA of Perth (H3N2) and to activate 
NK-92-FcγRIIa Val158 cells and for binding dimeric rsFcγRIIa 
Val158. The cell and dimeric rsFcγRIIa activities correlated strongly 
(p < 0.0001), validating use of the dimeric rsFcγRIIa receptor 
to predict cellular responses (Fig. 6). Thus, the FcR dimer assay 
marks individuals on the basis of the NK cell–activating potential 
of their anti-HA IgG Abs. Next, the ability of the dimeric rsFcγR 
assay to predict the capacity of anti-HA ICs to activate fresh blood 
primary NK cells was assessed. The plasma from 30 individuals 
was used to opsonize A(H1N1)pdm09 HA, and these ICs were 
used to activate NK cells and bind dimeric FcγR (Fig. 7). NK 
cell activation (intracellular IFN-γ and/or CD107a surface ex-
pression) correlated more strongly with binding of the dimeric 
rsFcγRIIa Val158 (p = 0.005, Fig. 7C) than the anti-HA IgG Ab 
titer (p = 0.027, Fig. 7A) or Ab EC50 (p = 0.79, Fig. 7B). Thus, dimeric rsFcγRIIa binding activity to IgG-opsonized HA corre-
lates with IC capacity for NK activation. 

**Discussion** 

FcγRs and IgG Abs are increasingly understood to play key roles in 
immunity to pathogens, vaccine responses, and autoimmunity. Although many effector functions can result from FcγR activation, 
all are triggered when multiple Fc regions of IgGs in ICs bind 
and cluster receptors to initiate signaling (59, 60). To identify IgG 
molecules that bind to Ag and are sufficiently closely spaced to 
bind and cluster FcγRs, we produced defined dimeric soluble 
forms of FcγRIIa and FcγRIIa as mimics of neighboring, sig-
naling FcγRs on the cell surface. The usefulness of these probes 
was tested using IgG ICs formed with model Ags and with human 
Abs to influenza HA. 

A general feature of the assay was a higher threshold and steeper 
reaction profile for dimeric rsFcγR binding to IC than the bind-
ing of anti-IgG. This follows from the requirement for divalent 
binding of the dimeric rsFcγR by bridging the closely spaced Fcs 
of neighboring Abs. At IgG concentrations above the EC50 
for opsonization, relatively small differences in the levels of opso-
nizing IgG presumably achieve a critical density of presented Fc 
regions that result in large increases in dimeric rsFcγR-binding activity. 

The use of the dimeric rsFcγR assays demonstrated expected 
hierarchies of IgG subclass binding IgG3 > IgG1 ~ IgG2 >> IgG4 
for FcγRIIa (H131) interactions and IgG3 > IgG1 >> IgG2 ~ IgG4 
for FcγRIIa (Arg131) interactions (Fig. 3, Table II), which were 
comparable to the reported activities of the cell surface FcγRs (34, 
37, 38); the FcγRIIa Phe158 allele has weaker binding activity 
than the Val158 allelic form, and both FcγRIIa Val158 and FcγRIIa 

![FIGURE 6. The dimeric rsFcγRIIa Val158 assay correlates with NK-92- FcγRIIa Val158 activation by influenza A HA, A/Perth/16/2009 (H3N2). ICs were formed by reacting plasma from 30 individuals with plate-bound HA A/Perth/16/2009 (H3N2). ICs formed with plasmas diluted at 1:80 were assessed for the binding of dimeric rsFcγRIIa Val158 and compared with ICs formed with plasmas diluted at 1:40 for the capacity to activate NK-92 cells expressing FcγRIIa Val158. Dimeric rsFcγRIIa Val158 binding was normalized using binding in wells directly coated with Ivlg (5 μg/ml). The two assays were correlated using nonparametric Spearman analysis and fitted by linear regression.](image)

![FIGURE 7. Dimeric rsFcγR binding has superior correlation with donor NK activation by influenza A HA, A(H1N1)pdm09 than with anti-IgG end point titer or EC50. ICs were formed by reacting plasma from 30 individuals with plate-bound HA from A(H1N1) pdm09 virus. Activation of the CD56+ NK cell population of PBMCs by these ICs was measured by intracellular stain for IFN-γ and/or surface expression of CD107a and was correlated with IgG end point titer (A), IgG 1/EC50 (B), and dimeric FcγRIIa Val158 binding, normalized using binding in wells directly coated with Ivlg (5 μg/ml) (C). Each pair of assays was correlated using nonparametric Spearman analysis and fitted by linear regression.](image)
Phε¹⁵ showed the expected higher binding activity to afucosylated IgG1; FcγRIIa and FcγRIIa binding to the weaker interacting subclasses, IgG2 and IgG4, was affected by the nature of the IC; dimeric rsFcγR-binding activity to IgG-opsonized Ag showed a higher threshold and steeper reaction profile than the binding of anti-IgG; as expected, receptors failed to bind to IgG1 containing the established FcR binding–inactivation LALA lower hinge mutant; and dimeric rsFcγR binding activity of ICs correlated with NK activity measured using FcγRIIa-expressing NK-92 cells or primary NK cells and detected a transient increase in the activity of anti-H1 pdm HA Abs in IgG prepared during 2010 postpandemic H1N1 influenza. This last finding demonstrated that the dimeric rsFcγR assay was at least as capable as cell-based assays (54) in revealing changes in Ab activity.

The strength of engagement of FcγRs by IgG depends on Fc-intrinsic properties, such as amino acid residues at the interaction interfaces and glycosylation, but it also depends on how an Ab reacts with Ag to form an IC or opsonized target. Interestingly, the subclasses IgG2 and IgG4, which have weaker interactions with FcγRs, exhibited different FcγRIIa- and FcγRIIIa-binding activities when oriented by capture with anti-F(ab')₂ compared with when they were bound by TNP-BSA Ag. Both of these methods of forming ICs are described in studies that defined cellular FcγR interactions and the effect of IC size on these interactions (37, 38). FcγRII and FcγRIII binding to Fc are low-affinity interactions for which the 1:1 intrinsic FcR:Fc reactions have been well described. However, the biological interactions of FcγRII and FcγRIII with ICs are multivalent and even with the divalent interactions described in this article, the net outcome of weak but avid binding reactions was profoundly affected by differences in ligand presentation in the IC. This phenomenon is important in evaluating the activities of therapeutic Abs, particularly agonist mAbs (61), developed in low FcR-binding formats, such as IgG2 or IgG4 backbones. Thus, although the Fc-intrinsic effects can be engineered by altering amino acid residues and glycosylation, the consequences of opsonization-related effects are difficult to predict because Abs are highly diverse, with ~10³¹ possible Ab:Ag interactions (62).

The dimeric rsFcγR assay described in this article has a number of advantageous features. Dimeric rsFcγRs are genetically defined dimers, have site-specific biotinylation, and, importantly, bind ligand without further complexing (e.g., to streptavidin) to provide multivalency. The intrinsic FcγRIIa and FcγRIIIa interactions with IgG are 1:1 and were thoroughly characterized by surface plasmon resonance assays (e.g., BIAcore, figure 3 in Ref. 37), but biological responses mediated by these receptors result from multivalent interactions. The assay of the dimeric receptor interaction with two Fcs described in this article is a measure of the minimal avid interaction of IgG with the low-affinity FcγRs. The dimeric rsFcγR binds to adjacent Fcs when presented by two Abs in close proximity. The correlation of receptor activity assayed off the cell membrane with activity in a cellular context, and indeed with in vivo biology, is difficult to evaluate. Nonetheless, it can be noted that other cell-free approaches, which clearly produced key insights (20, 63–66), require receptor complexing or immobilization resulting in measuring higher or ill-defined valency interactions, respectively. In contrast, dimeric FcγR binding measures the spatial relationship of pairs of Abs complexed with Ag, with the effects of receptor polymorphism, Ab subclass, and glycosylation contributing to the overall measure of FcγR activity.

Furthermore, the use of FcγR dimers is informative and does not require specialized equipment. It needs only limited amounts of sample and can be used to evaluate the FcγR activities of many types of IgG Abs bound to different Ags. Central to the assay is the biotinylated dimer rsFcγR, which could be easily incorporated into powerful, parallel high-throughput bead-based assays (20, 63, 64, 66–68), adapting these for detecting closely spaced Ab pairs. Evaluating the quality of IgG responses for FcγR activation will be of particular importance for understanding natural (67) and vaccine-induced immunity (6, 18, 20, 24, 69) to viruses and other infectious diseases. The detection of a transient increase in the activity of IgG from 2010 toward H1N1 pandemic HA, which correlated with HA-inhibition titer and NK activation, indicates that the dimeric rsFcγR assay may provide a useful measure of immunity. Moreover, dimeric rsFcγR may be of value for evaluating therapeutic IgG and IgG mixtures. Therapeutic IgG Abs have revolutionized the treatment of some cancers and rheumatoid arthritis; however, the application of therapeutic Abs to infectious disease may be more challenging in situations in which strain differences and a need for sterilizing immunity may necessitate the use of Ab cocktails of increasing complexity and functionality (70, 71). Indeed, such cocktails of Abs are used for treatment of rabies virus (72), toxins (73), and, most recently, Ebola (74). The

| Table I. Shape “H” coefficients from IC-binding curves |
| --- |
| Figure | Probe | H* | 95% C.I. |
| Fig. 2A | Anti-IgG (opsonization) | 1.0 | 0.67–1.2 |
| Fig. 2B | Dimeric rsFcγRIIa His¹³¹ | 3.3 | 1.8–4.7 |
| Fig. 2C | Dimeric rsFcγRIIa Arg¹³¹ | 4.1 | 3.3–4.8 |
| Fig. 2D | Dimeric rsFcγRIIa Val¹⁵⁸ | 2.3 | 1.7–2.9 |
| Fig. 2E | Dimeric rsFcγRIIa Phe¹⁵⁸ | 3.1 | 2.1–4.1 |

*H is a descriptor of steepness of the response curve numerically equivalent to the Hill coefficient.

| Table II. Reactivity of dimeric rsFcγRs with IgG ICs |
| --- |
| Dimeric rsFcγR | IC Format | Binding Hierarchy |
| FcγRIIa His¹³¹ | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | >> | IgG4 |
| Fig. 3C | TNP-BSA | IgG1 | ≥ | IgG2 | >> | IgG4 |
| Fig. 3D | Anti-F(ab')² | IgG2 | > | IgG1 | ~ | IgG2 | ~ | IgG4 |
| FcγRIIa Arg¹³¹ | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | >> | IgG4 |
| Fig. 3E | TNP-BSA | IgG1 | > | IgG2 | ~ | IgG4 |
| FcγRIIa Val¹⁵⁸ | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | >> | IgG4 |
| Fig. 3F | TNP-BSA | IgG1 | > | IgG2 | ~ | IgG4 |
| FcγRIIa Phe¹⁵⁸ | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | >> | IgG4 |
| Fig. 3G | TNP-BSA | IgG1 | > | IgG2 | ~ | IgG4 |
| FcγRIIIa | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | ~ | IgG4 |
| Fig. 3H | TNP-BSA | IgG1 | > | IgG2 | ~ | IgG4 |
| FcγRIIIa | Anti-F(ab')² | IgG3 | > | IgG1 | ~ | IgG2 | ~ | IgG4 |
| Fig. 3I | TNP-BSA | IgG1 | > | IgG2 | ~ | IgG4 |

Binding hierarchy for dimeric rsFcγR derived from Fig. 3.
FcR-activating mixture of such Abs is greatly greater than for single therapeutic Abs, except in situations in which the target Ags are highly abundant (such as CD20 on B cell tumors) or have repeating epitopes. The engineered FcRy ectodomain dimers described in this article measure the content of closely spaced, and thus, receptor-aggregating, pairs of “near-neighbor” IgG Abs on opsonized targets. Clearly, they are important tools for the prediction of FcRy activation by Ag-specific IgG and thereby, the evaluation of Ab responses to vaccines, infections, and therapeutic Abs.

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Disclosures

The authors have no financial conflicts of interest.

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