

Letter to the Editor

Parameters determining the efficacy of CD32 to inhibit activation of Fc ϵ RI in human basophils

To the Editor:

The mechanisms that underlie the success of allergen immunotherapy are varied, and the precise details remain unclear. Immune deviation and blocking antibodies are 2 important candidate explanations, and under the category of blocking antibodies are 2 often discussed possibilities: (1) simple competition for allergen between IgG antibodies developed during immunotherapy and IgE antibodies on the mast cell and basophil cell surface (what we are calling stoichiometric blockade) or (2) interaction between IgG antibodies and CD32 (the low-affinity IgG receptor Fc γ RII) on mast cells^{1,2} or basophils,³⁻⁶ leading to inhibition of the IgE-mediated response.

There is conflicting information about the role of CD32 in this reaction in human subjects. One possible issue is whether human mast cells even express CD32b, the inhibitory IgG receptor. Other issues relate to the relative ability of different IgG subclasses to interact with CD32b or CD32a⁷ and whether CD32a, which is normally considered an activating IgG receptor, acts in an inhibitory capacity in the context of CD32b or cell type.^{4,5}

Human basophils express both CD32a and CD32b,³⁻⁶ and it has been clearly demonstrated that CD32 can mediate inhibition of the IgE-dependent reaction. However, there are a variety of studies that have demonstrated that not all IgG subclasses bind to CD32a or CD32b.⁷ Also, there are polymorphisms in CD32 that influence binding and/or function to certain subclasses.^{7,8} Furthermore, immunotherapy generates different increases in IgG subclasses, and for a variety of reasons, studies have focused on IgG₁ and IgG₄ and very infrequently examine IgG₂ or IgG₃. However, binding studies have shown that IgG₄ does not interact with CD32a or CD32b.⁷ What remains unclear is the relative ability of IgG₁, IgG₂, and IgG₃ to interact with CD32 and the potential for polymorphisms to further differentiate binding.

Using partially enriched human basophils (see the [Methods](#) section in this article's Online Repository at www.jacionline.org) and a series of transfectoma antibodies all using the same complementarity determining region specific for nitrophenyl (NP) but varying the heavy chain subclass (IgE, IgG₁, IgG₂, IgG₃, and IgG₄), the ability of the various IgG subclasses to inhibit IgE-mediated release from basophils sensitized with NP-specific IgE was examined. Three reaction designs were examined, holding IgG constant and varying antigen (which is presumably that natural situation), holding allergen constant and varying IgG, and a third approach presented in the [Results](#) section in this article's Online Repository (see also [Fig E1](#) in this article's Online Repository at www.jacionline.org for a schematic of the experimental design). [Fig 1](#) shows results using the first 2 methods.

By using the first method, the amount of inhibition by IgG was titrated to approximately 50% to detect alteration of the response in either the positive or negative direction when including blockade of CD32 and to not bias the reaction to complete stoichiometric blocking (see the [Results](#) section in this article's

Online Repository). To block CD32 and therefore test the involvement of CD32-mediated inhibition rather than simple stoichiometric blockade, an engineered high-affinity anti-CD32b antibody and a commercial anti-CD32a antibody were used. The densities of CD32a and CD32b were also monitored by using flow cytometry. The results, focusing on the highest concentrations of antigen and antibody ([Fig 1, A-F](#)), indicate that it was difficult to detect functional interaction with CD32b when IgG₁ was used, but IgG₂ and IgG₃ effectively engaged CD32b (the degree of CD32b involvement was measured by the extent of reversal of inhibition when including the CD32b-blocking antibody Ab10523). At lower concentrations of antigen, only stoichiometric inhibition is observed. [Fig E2](#) in this article's Online Repository at www.jacionline.org shows the importance of absolute antigen concentration and the importance of IgG/allergen ratios.

In the second design shown in [Fig 1, G](#) (holding antigen constant and varying IgG), it can be again observed that IgG₁ did not engage CD32b, whereas IgG₂ and IgG₃ did. As shown in [Fig E3](#) in this article's Online Repository at www.jacionline.org, IgG₄ did not cause inhibition. These results also demonstrated that IgG₃ was 10-fold more efficacious in interacting with CD32b than IgG₂, such that only 1 IgG₃ per 20 antigen molecules was necessary to mediate inhibition, whereas approximately 0.5:1 ratios were needed for IgG₂. [Fig 1, B, D, and F](#), also examined the ability to further reverse inhibition through inclusion of CD32a blockade with antibody AbIV.3 (see the [Results](#) section in this article's Online Repository). There was some further reversal by addition of AbIV.3, although the best reversal occurred with CD32b blockade. As discussed in the [Results](#) section in this article's Online Repository, heterogeneity in the relative participation of CD32 was correlated with CD32 expression (see [Table E1](#) in this article's Online Repository at www.jacionline.org), and reversal of IgG₂ and IgG₃ was correlated. Furthermore, as shown in [Fig E4](#), polymorphisms in CD32a and CD32b did not influence the relative participation of CD32. As a low-affinity IgG receptor, CD32 is not thought to engage monomeric IgG effectively, but concentrations of IgG in plasma are very high, and therefore the ability of nonspecific IgG (nsIgG) to inhibit participation of CD32 (by using nsIgG as a substitute for Ab10523) was examined. [Fig E5](#) in this article's Online Repository at www.jacionline.org shows that reversal of CD32's effects occur at an inhibitory concentration of 50% of 150 μ g/mL nsIgG.

These and our previous studies⁶ suggest that there are several important parameters, possibly 5 dimensions, which determine whether it is possible to observe the inhibitory function of CD32 on basophils (see also the [Discussion](#) section in this article's Online Repository at www.jacionline.org). These results place some constraints on how to explore the role of CD32 in the basophil reaction during immunotherapy, and the variability in the published literature on this issue might be accounted for by consideration of the constraints. It would appear that attempts to correlate CD32 function with clinical outcome would require a multiple regression analysis that included parameters that could be measured, although some with some difficulty. These include (1) density of allergen-specific IgE (it is possible to overcome CD32-mediated inhibition with high densities of allergen-

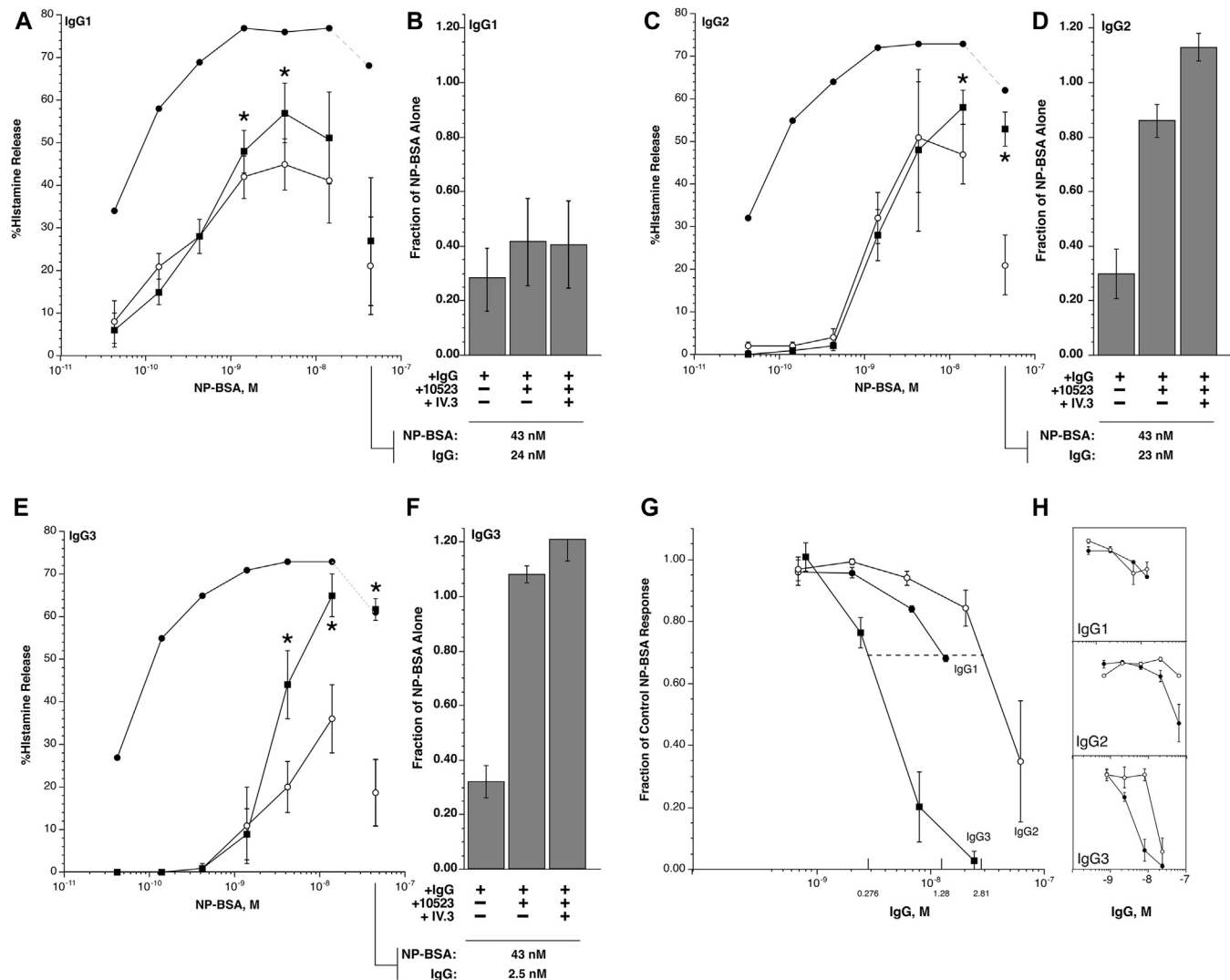


FIG 1. Inhibition of antigen-induced release by human IgG subclass antibodies. Each panel examines one of the 3 human IgG subclass anti-NP antibodies using the protocol described in the text and in the [Methods](#) section in this article's Online Repository. **A, C, and E,** Plots are a composite of 2 reaction conditions with different replication numbers. At 43 nmol/L (3 μ g/mL) NP-BSA, 3 times as much antibody was used as the next highest concentration of NP-BSA, 14 nmol/L (1 μ g/mL; to maintain the same antibody/antigen ratio). Otherwise, the concentration of subclass antibody was held constant for all NP-BSA concentrations of 14 nmol/L or less. Antibody concentrations were 24 (n = 2) and 8 (n = 5) nmol/L for IgG₁ (Fig 1, **A**), 23 (n = 13) and 7.7 (n = 7) nmol/L for IgG₂ (**B**), and 2.5 (n = 13) and 0.83 (n = 8) nmol/L for IgG₃ (Fig 1, **C**). ●, Response to NP-BSA without subclass antibody; ○, response with subclass antibody; ■, response with subclass antibody and Ab10523 at 50 μ g/mL. Asterisks indicate a statistically significant difference ($P < .05$) between subclass antibody with or without Ab10523. **B, D, and F,** Reversal of human IgG subclass inhibition by the combination of blocking antibodies to CD32b and CD32a. **G,** Cells were stimulated with 14 nmol/L NP-BSA (1 μ g/mL), and the concentration of subclass IgG varied (n = 3). ●, IgG₁; ○, IgG₂; ■, IgG₃. **H,** Reversal of inhibition by the inclusion of Ab10523 at 50 μ g/mL for each of the curves shown in Fig 1, **G**.

specific IgE), (2) the subclass of IgG (with IgG₃ >10-fold more potent than IgG₂ > IgG₁ and no IgG₄-mediated inhibition), (3) expression level of CD32, (4) absolute concentration of allergen, and (5) the IgG/allergen ratio. IgG₃ aggregates appear extremely efficacious and potent. However, the effect is sensitive to the precise antibody/antigen ratio, and the range at which any of the subclasses effectively dominate inhibition by engagement with CD32 is narrow. Outside this window, stoichiometric blocking dominates any observed inhibition. Although these are important parameters, our results showing that nsIgG at high enough concentrations can blunt the interaction of antigen-

specific IgG aggregates to interact with CD32 raises important questions on how to study blocking antibodies in the context of CD32. The reagents used to block CD32 are an important part of how to explore a role for CD32, but the inclusion of serum or plasma to present the reaction with blocking antibodies would appear to be problematic for assessing CD32 function. If nsIgG operates in this manner *in vivo*, it would suggest that either there are other conditions that allow IgG antibodies to interact with basophils in circulation or that this reaction is not relevant in circulation but perhaps relevant in tissues at which the concentrations of nsIgG might be more conducive to aggregates

regulating this reaction. In addition, for therapeutics that are designed to work with CD32, use of the IgG₃ subclass might be a potential improvement. Despite the apparent complexity of the requirements for readily observing a CD32 effect, it is possible to find conditions in which this effect is nearly pure (ie, inhibition solely caused by CD32-mediated inhibition with no stoichiometric blocking).

These studies used a single transfectoma series of cloned antibodies, and the bases for the differences in subclass behavior with respect to CD32, although observed in other studies of FcγR subclass affinity,⁷ are unclear. Further study of natural antibodies is needed, but the results suggest that measurement of IgG₂ and IgG₃ antibodies is warranted in immunotherapy studies because it is these 2 subclasses that appear to be the most efficacious in recruiting CD32 into reactions with antigen and antibodies on the human basophil.

Donald MacGlashan, Jr, MD, PhD
Robert G. Hamilton, PhD

From the Department of Medicine, Johns Hopkins Asthma and Allergy Center, Baltimore, Md. E-mail: dmacglas@jhmi.edu.

Disclosure of potential conflict of interest: D. MacGlashan and R. G. Hamilton have received research support from the National Institutes of Health, AI100952.

REFERENCES

1. Daeron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J Clin Invest* 1995;95:577-85.
2. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 1996;379:346-9.
3. Kepley CL, Cambier JC, Morel PA, Lujan D, Ortega E, Wilson BS, et al. Negative regulation of FcεpsilonRI signaling by FcγammaRII costimulation in human blood basophils. *J Allergy Clin Immunol* 2000;106:337-48.
4. Cady CT, Powell MS, Harbeck RJ, Giclas PC, Murphy JR, Katial RK, et al. IgG antibodies produced during subcutaneous allergen immunotherapy mediate inhibition of basophil activation via a mechanism involving both FcγammaRIIA and FcγammaRIIB. *Immunol Lett* 2010;130:57-65.
5. Cassard L, Jonsson F, Arnaud S, Daeron M. Fcγamma receptors inhibit mouse and human basophil activation. *J Immunol* 2012;189:2995-3006.
6. Macglashan D Jr, Moore G, Muchhal U. Regulation of IgE-mediated signalling in human basophils by CD32b and its role in Syk down-regulation: basic mechanisms in allergic disease. *Clin Exp Allergy* 2014;44:713-23.
7. Warmerdam PA, van de Winkel JG, Vlug A, Westerdaal NA, Capel PJ. A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. *J Immunol* 1991;147:1338-43.
8. Li X, Wu J, Carter RH, Edberg JC, Su K, Cooper GS, et al. A novel polymorphism in the Fcγgamma receptor IIB (CD32B) transmembrane region alters receptor signaling. *Arthritis Rheum* 2003;48:3242-52.

<http://dx.doi.org/10.1016/j.jaci.2015.10.043>

METHODS

Materials, buffers, and antibodies

The following were purchased: piperazine-N,N'-bis(2-ethanesulfonic acid [PIPES]), BSA, ethyleneglycol-bis-(β -aminoethylether)-N,N',N'-tetraacetic acid, EDTA, and D-glucose; RPMI 1640 containing 25 mmol/L HEPES and L-glutamine (BioWhittaker, Walkersville, Ma); Percoll (Pharmacia, Piscataway, NJ); tris(hydroxymethyl)-aminomethane and Tween-20 (Bio-Rad Laboratories, Hercules, Calif); mouse anti-human IgE antibody (6061P; Hybridoma Reagent Laboratory, Baltimore, Md); goat polyclonal anti-hIgE antibody generated and enriched, as described previously^{E1}; the anti-Fc ϵ RI antibody CRA-1 (eBioscience, San Diego, Calif); mouse IgG₁ anti-penicillin antibody (Fitzgerald, Acton, Mass); anti-CD32a antibody and clone IV.3 (STEMCELL Technologies, Seattle, Wash); control mouse IgG₁ (Zymed, San Francisco, Calif); control mouse IgG_{2b} (eBioscience); anti-CD63 antibody (clone H5C6; BD Biosciences, San Jose, Calif); anti-IL-3 receptor-phycoerythrin mIgG_{2a} (BD Pharmingen, San Jose, Calif); anti-mouse IgG_{2b}-Alexa Fluor 647 (Molecular Probes, Invitrogen, Carlsbad, Calif); and human IgG (Cappel Laboratories, Malvern, Pa). BPO(32)-HSA (pencilloyl-human serum albumin) was synthesized, as described previously.^{E2} NP(30)-BSA was synthesized by using previously published methods.^{E3} PIPES-albumin-glucose (PAG) buffer consisted of 25 mmol/L PIPES, 110 mmol/L NaCl, 5 mmol/L KCl, 0.1% glucose, and 0.003% HSA. PAGCM was defined as PAG supplemented with 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Lactic acid IgE elution buffer was 0.01 mol/L lactic acid, 0.14 mol/L NaCl, and 0.005 mol/L KCl at pH 3.9.^{E4,E5}

There were several antibodies used for studying CD32b that were developed at Xencor (Monrovia, Calif). The engineered CD32b-specific antibody Ab10523 was developed at Xencor, and its selectivity and affinity are presented in a previous report.^{E6} This antibody selectively binds to CD32b with an affinity that is 400-fold greater than that of human IgG. The commercial version of anti-CD32a (AbIV.3) retains an Fc domain that could potentially bind to CD32b, and therefore in all experiments AbIV.3 could only be used in conjunction with Ab10523 so that both CD32b and CD32a were blocked. The concentration used to block binding of IgG to CD32b (50 μ g/mL) was chosen to be high to mitigate against possible differences in affinity of the IgG subclasses for CD32; 5 μ g/mL has been shown to block IgG binding.^{E6}

The transfectomas that were used to generate the NP-specific IgG₁, IgG₂, IgG_{3b}, IgG₄, and IgE antibodies were obtained from Hybridoma Reagent Laboratory and were previously obtained from the designers.^{E7} There was very limited availability of NP-specific IgG₄ (concentrations were limited to the 20 ng/mL range), and stock concentrations of purified NP-specific IgG₁ were limited to 13 μ g/mL. All IgG concentrations were measured by using an in-house ELISA with standardized anti-IgG antibodies: HP6045P for capture and HP6043B (biotinylated) for detection.^{E8} It was assumed but not validated that these reagents detected each subclass equally in the ELISA format. The prepared IgG solutions, even at dilutions close to or at the stock concentration, did not alter histamine release induced by anti-IgE antibody or induce release on their own.

Basophil purification and reaction conditions

Basophils were obtained by means of venipuncture (this study was approved by the Johns Hopkins Institutional Review Board, #NA-00080662) and enriched on a 2-step Percoll gradient.^{E9} Purity ranged from 4% to 35%. Culturing of purified basophils was typically in medium consisting of RPMI-1640 supplemented with 8 μ g/mL gentamicin and 0.03% RNase-free BSA and either 1 or 10 ng/mL IL-3. The 10 ng/mL concentration of IL-3 was used for aggregate-induced secretion, whereas 1 ng/mL was used for most other experiments. Culture periods were 18 to 24 hours at cell densities of 2 million total cells/mL medium. For many experiments, a portion of the endogenous IgE was removed by treating the cells with lactic acid buffer (on ice for 10 seconds with buffer before adding 1.5 mL of EB to neutralize the pH).^{E5} Sensitization by anti-NP-specific IgE was titrated for an optimal CD32 effect, and for cells cultured with IL-3, 0.25 μ g/mL anti-NP-specific IgE was typical. In other experiments sensitization with up to 5 μ g/mL anti-NP-specific IgE was done. After washing, the

cells were stimulated in PAGCM buffer under various conditions. If aggregates of IgG and antigen were used, a mixture of antigen and antibody was prepared for at least 15 minutes before adding the aggregate to the cells. The histamine release reaction time was typically 30 minutes at 37°C. Histamine release was measured from supernatants of stimulated cells by using automated fluorimetry.^{E10}

Flow cytometry

Flow cytometry was performed on cells fixed in 2% paraformaldehyde for 20 minutes at room temperature before blocking with 4% BSA-PBS overnight. Gating for basophils was based on forward scatter/side scatter gates and labeling with anti-IL-3 receptor antibody.^{E11} If CD32a receptors were the target for labeling, the protocol included 20 μ g/mL Ab10523 because clone IV.3 (anti-CD32a) could bind to CD32b through its Fc region. If CD32b receptors were the target, the "control" labeling included 20 μ g/mL Ab10523, as well as 1 μ g/mL Ab10523-Alexa Fluor 647, whereas detection of CD32b included only 1 μ g/mL Ab10523-Alexa Fluor 647. Because Ab10523 does not bind to CD32 through its Fc region, no blocking of CD32a was necessary.^{E6} In general, mean net fluorescence is used. In some experiments the readout of basophil activation was the change in expression of CD63. Gating of basophils in these experiments included forward scatter/side scatter, IL-3 receptor (phycoerythrin) and Fc ϵ RI (with CRA-1 antibody), and detection anti-CD63 antibody. Secondary labeling was with anti-mIgG_{2b}-Alexa Fluor 488 (to detect CRA-1) and anti-mIgG₁-Alexa Fluor 647 (to detect CD63). Calibration beads were used to ensure the flow cytometer was producing similar results between daily and weekly measurements.

Statistics

For most of these studies, paired analysis was possible because of the nature of the experimental design. Figures show error bars for SEMs. For comparisons of dose-response curves, the area under the curve was used to decide whether there were gross differences in 2 dose-response curves (eg, inhibition with or without Ab10523). ANOVA with Tukey *post hoc* analysis was used to isolate individual concentration differences.

Genotyping

DNA from whole blood was isolated with the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, Md) and analyzed with 2 probe sets by using the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif). The primers and reporters for both CD32a and CD32b were designed by Life Technologies (Grand Island, NY), and the set for CD32a was part of their pre-existing inventory of validated primer/reporter sets. As such, Life Technologies only provides the context sequence as information about the set; the single nucleotide polymorphism (SNP) was rs1801274 (H131R), and the context sequence was AATGGAAAATCCCA GAAATTCTCCC[A/G]TTTGATCCCACCTTCTCCATCCCA. The SNP for CD32b was rs1050501 (I232T); CCTAGCTCCCAGCTCTTCAC and CCCTACAGCAGCAACAATGG were the forward and reverse primers, respectively, and CGCTACAGCAGTCCCAGT (VIC) and CGCTACAG CAATCCCAGT (FAM) were the 2 reporter sequences.

Optimizing inhibition by IgG

A series of transfectoma antibodies were used that retain the same complementarity determining region (and therefore the same target epitope) and only modify the subclass to explore the relative ability of IgG subclasses to regulate IgE-mediated secretion. This circumvents issues related to different affinities for the epitope. As noted previously, 5 related reagents were used; IgE, IgG₁, IgG₂, IgG₃, and IgG₄ were obtained by using recombinant technology to each bind the *p*-nitrophenyl ligand (which is presented as the multivalent antigen NP[30]-BSA). Our previous studies on CD32 signaling also showed that the level of expression of CD32 determined its functional efficacy, and it is not uncommon to observe very poor CD32-mediated inhibition in freshly isolated peripheral blood basophils. Therefore basophils

were cultured overnight in IL-3, which has been shown to upregulate both CD32a and CD32b expression and improve their function, to provide some consistency and readily measurable functional effects.^{E6,E12} After overnight culture with 1 ng/mL IL-3, basophils were treated for 10 seconds with lactic acid to dissociate about 50% of endogenous IgE to expand the number of unoccupied receptors, and the cells were sensitized with a density of NP-specific IgE conducive to observing inhibition by CD32b (ie, 0.25 μ g/mL NP-specific IgE; see below), as determined in pilot testing. As found previously,^{E6} higher densities of sensitization blunted the inhibition caused by interaction with CD32 (data not shown). For some experiments, CD32a and CD32b expression was assessed by using flow cytometry. There are several ways that testing for inhibition by CD32b could be done, but the method that best mimics conditions *in vivo* holds the concentration of antibody constant while the concentration of antigen is varied. *A priori*, it was not known what antibody/antigen ratio would be optimal for inhibition of the IgE-mediated reaction for each of the subclasses. It was decided that for a given concentration of antigen, in this case 1 μ g/mL NP-BSA (14.3 nmol/L, which is just below a concentration that would be considered supraoptimal), the amount of IgG₁, IgG₂, and IgG₃ would be titrated to generate approximately 50% inhibition. This was chosen so that stoichiometric blocking would not be so effective that a test for reversal of inhibition would not be possible (eg, all epitopes bound or sterically blocked and inaccessible to cell-surface IgE). Because the antigen concentration is decreased and the antibody concentration is held constant, it would be expected that in some concentration regimens, the antibody/antigen ratio would result in complete stoichiometric blocking. The concentration of each subclass (determined by total IgG ELISA) to achieve approximately 50% inhibition was quite different for IgG₁ and IgG₂ compared with IgG₃; the molar antibody/antigen ratios were approximately 0.6:1, 0.5:1, and 0.06:1 for IgG₁, IgG₂, and IgG₃, respectively. The 10-fold difference between IgG₁/IgG₂ and IgG₃ was unexpected, and therefore the antibodies were tested for their immunoreactivity, but in each case an average of 98% $\pm$ 2% of each subclass was found to be immunoadsorbed by an NP-BSA-sepharose immunosorbent bead (and not a BSA-bead immunosorbent). These ratios at the 1 μ g/mL NP-BSA concentration or greater were used for many of the experiments.

To examine whether CD32b was involved in inhibition by IgG₁, IgG₂, and IgG₃, the engineered antibody Ab10523 was included in the cell buffer for 5 minutes before addition of NP-BSA. The Fc of this engineered antibody does not interact with CD32, but the antibody binds with high affinity through its Fab and effectively blocks binding of IgG, even in aggregate form (including the blocking of the Fc of the anti-CD32a antibody AbIV.3, see below, to CD32b).^{E13} Mixtures of antigen and IgG subclass were formed by combining 4-fold higher concentrations of each (1:1 mixing results in 2-fold concentration) at least 15 minutes before mixing with an equal volume of cells with or without CD32 blocking antibodies (resulting in the concentrations noted in Fig 1).

RESULTS

The general scheme for these studies is shown in Fig E1. Three methods were used to explore the conditions necessary to observe a role for CD32 in mediating the IgE response.

Subclass-mediated inhibition of IgE-mediated secretion

There was a strong correlation between inhibition mediated by IgG₂ and IgG₃ ($r = 0.93$, $P = .0002$) at the highest concentration of NP-BSA (3 μ g/mL or 43 nmol/L) and 22 nmol/L IgG₂ or 2.4 nmol/L IgG₃ (highest concentration in Fig 1, C and E). For IgG₁ to IgG₃, there were regions of the antigen dose-response curve in which the IgG subclass effectively inhibited the IgE-mediated reaction (higher antibody/antigen ratios), but this inhibition was insensitive to blocking CD32b with Ab10523. Of the 3 subclasses that reversed inhibition, IgG₃ was the most sensitive to reversal. The hierarchy of efficacy related to CD32

was as follows: IgG₃ > IgG₂ > IgG₁. Unlike IgG₂ or IgG₃, no instance was found of IgG₁-mediated inhibition of the response being completely reversed by inclusion of Ab10523; typically, reversal was no greater than 50% and was highly variable among basophil preparations. It is worth noting that for IgG₃ and IgG₂, it was possible to arrange conditions for an almost pure CD32-mediated inhibition in that reversal was complete. These conditions were used for the genotyping studies below. These conditions were also used to compare the relative efficacy of CD32-mediated inhibition on freshly isolated versus cultured basophils (day 0 vs day 1). On day 0, Ab10523-dependent reversal of the inhibition caused by inclusion of either IgG₂ or IgG₃ was weak. For example, using IgG₃ to block reversal by Ab10523 was quite variable and averaged 27% $\pm$ 11% ($n = 7$, $P = .046$, and IgG₃ inhibited, without Ab10523, on average, 32% $\pm$ 5%) rather than the approximately 100% reversal observed for the same conditions after culture with IL-3 (data not shown).

The proportion of antibody/antigen was held constant across a range of antigen concentrations to test the importance of absolute antigen concentration and IgG/antigen ratio (design 3 indicated in Fig E1). Using the ratio for effective engagement of CD32b for IgG₃ (NP-BSA at 14 nmol/L [1 μ g/mL] and IgG₃ at 0.8 nmol/L [0.14 μ g/mL] in Fig 1, E), Fig E2, A, shows the results for the IgG₃ subclass, maintaining this same antigen/antibody ratio across the dose-response curve. The result suggested little broadening of the Ab10523-sensitive range (compare these results with Fig 1, E), and it can be seen that concentrations of less than 1.4 nmol/L (100 ng/mL) NP-BSA still showed no reversal when CD32b was blocked. IgG₂ and IgG₁ showed similar results (data not shown). The relative importance of the antibody/antigen ratio differs for the 3 subclasses (Fig E2, B). By holding the antibody concentration constant and varying the antigen concentration, the antibody/antigen ratio quickly grows (in the log-domain of concentrations tested) to levels at which the available NP binding sites on the antigen are likely saturated with antibody (or sterically hindered), blocking access of the antigen to cell-surface IgE. The expectation is that all inhibition curves might be relatively steep, and this was found for IgG₂ and IgG₃. Fig E2, B, shows that relative to IgG₂ and IgG₃, IgG₁ showed a shallow inhibition curve. In our previous study of CD32 function,^{E6} a murine IgG₁ antibody was used to make aggregates with penicillin antigens, and it also showed an unexpected shallow inhibition curve.

Enhancement by IgG₄

The results shown in Fig E3 indicate that IgG₄ mediated enhancement of the antigen response. For IgG₄, no inhibition was noted at the highest concentrations of NP-BSA, but as noted in the Methods section, the antibody available for testing was very limited so that only lower concentrations of NP-BSA could be tested at antibody/antigen ratios that approximated the ratios used of IgG₁, IgG₂, and IgG₃. However, at these lower concentrations, there was enhancement when the stimulus strength was weak (this was observed in both antigen dose-response curves and titration of NP-specific IgE density, data not shown for the latter), and there was no enhancement for concentrations of NP-BSA greater than those shown in the figure (data not shown). Fig E3 shows this enhancement at the low end of the NP-BSA dose-response curve. IgG₄ antibody alone

did not cause histamine release (data not shown). IgG₂ and IgG₃ were tested at concentrations similar to IgG₄, and no enhancement was observed; in fact, there was modest inhibition of the NP-BSA response, and the enhanced response seen with the combination of IgG₄ and NP-BSA was inhibited with the additional inclusion of IgG₂ or IgG₃ (data not shown). These results do not preclude the possibility that higher concentrations of IgG₄ could mediate stoichiometric blocking of the IgE-mediated response, and it is possible that this particular transfectoma of IgG₄ does not behave like natural IgG₄. However, the prior reports that IgG₄ does not interact with CD32 are concordant with no inhibition by IgG₄ when IgG₂ and IgG₃ do inhibit. It is not yet clear what process produces the enhancement of release, although there is a report that sometimes IgG antibodies can enhance binding of antigen to IgE.^{E14}

Genotype versus functional inhibition

Previous studies have noted that IgG₂ (and to a lesser extent IgG₃) does not bind to one of the polymorphic variants of CD32a (aa131 = arginine) but does bind well to aa131 = histidine and that CD32b does not show this polymorphism.^{E15-E19} The genotype of the donor was examined for its relationship to relative inhibition by IgG₂ or IgG₃ and to the additional reversibility with CD32a blockade. Genotyping the donors showed an allelic frequency that was not statistically different ($P > .05$, χ^2 test) than expected. The literature suggests that the allelic frequency of the A SNP (leading to H131) is approximately 0.5, leading to a 1:2:1 proportion for the AA:AG:GG genotypes. In our population of 28 donors, the ratio was 0.39:0.50:0.11. For the CD32b SNP that alters its function,^{E18,E19} the ratio of genotypes was 0.86:0.07:0.07 (AA:AG:GG, with the GG genotype being homozygous for the poorer functioning isoleucine substitution; $P > .05$, χ^2 test against expected allelic frequency of 0.9 for the A SNP). Fig E4 separates the results shown in Fig 1, D and F, according to genotype. There was no meaningful difference among the various conditions with respect to genotype. Notably, for IgG₂, being homozygous for the His variant did not lead to greater inhibition or increase the difference between blocking with Ab10523 alone and the combination of Ab10523 plus AbIV.3 (comparing the ratio of [Ab10523 + AbIV.3]/Ab10523 for the H/H and H/R variants, $P = .75$). There was only one case of the R/R CD32a variant tested, and it was not apparently different. In addition, there were 2 cases of a homozygous presence of the I252 CD32b variant that has been reported to function less effectively than the dominant form of CD32b.^{E18,E19} Inhibition of the IgE-mediated response was effective whether using IgG₂ or IgG₃ and was reversed with Ab10523.

Both subjects were heterozygous for H131 CD32a, and there was no additional IgG₂ or IgG₃ aggregate-induced histamine release in nonsensitized cells at antibody/antigen ratios for IgG₂ of 0.5:1 or 2:1 and ratios for IgG₃ of 0.05:1 or 2:1. Parenthetically, aggregates of IgG₂ or IgG₃ and NP-BSA across a broad range of concentrations (42–336 nmol/L allergen in 0.05:1.0 to 1:1 ratios with IgG₂/IgG₃) in the absence of basophil sensitization with NP-specific IgE did not induce direct secretion regardless of the CD32a genotype.

Receptor expression for both CD32a and CD32b was also determined in these studies. The average increase in CD32b and CD32a expression between days 0 and 1 (the day of functional

testing) was 3.9 ± 0.3 – and 3.8 ± 0.5 –fold, respectively. Based on our previous studies,^{E6} the choice of culturing in 1 ng/mL IL-3 was made to bias the upregulation toward CD32b because we were specifically interested in whether IgG₂ bound to CD32b. In some of these experiments ($n = 4$), a comparison was made between expression changes at 1 versus 10 ng/mL IL-3, and as found previously, upregulation did not change for CD32b between 1 and 10 ng/mL (1.05 ± 0.2 -fold for ratio of expression, 10 vs 1 ng/mL) but did further increase for CD32a (1.8 ± 0.36 -fold for ratio of expression, 10 vs 1 ng/mL), and this was statistically different between CD32a and CD32b ($P = .022$). There was an approximately 5-fold and 4-fold range of expression for CD32a and CD32b, respectively, on day 1 among the subjects tested, and there was an inverse relationship between the expression level of CD32a, CD32b, or the sum of CD32a and CD32b and the degree of inhibition by IgG₂ and IgG₃ (Table E1). There was no relationship between receptor expression and genotype and no relationship between expression and the reversal by the addition of Ab10523 in the context of IgG₂ aggregates or the residual reversal by the addition of AbIV.3 (the difference between Ab10523 alone and the combination of Ab10523 plus AbIV.3 for IgG₂ aggregates).

Reversal of CD32-mediated inhibition by nsIgG

The observation that Ab10523 with or without AbIV.3 could reverse the inhibition of aggregates at certain concentrations and antibody/antigen regimens raised the question of whether nonspecific hIgG at high concentrations could effectively compete for CD32 binding. Basophils were stimulated as detailed in the experiments for Fig 1 but in the presence of nonspecific hIgG at concentrations ranging from 5 to 0.05 mg/mL or Ab10523. Reversal was just as effective as inclusion of Ab10523, with a median effective concentration for reversal of approximately 150 μ g/mL (Fig E5). nsIgG also reversed inhibition of NP-specific IgG₂ used at the concentration (rightmost point in Fig 1, C) to obtain the primarily CD32-mediated effect (data not shown).

To augment this observation, similarly prepared basophils were examined in simple buffer versus whole serum. Because whole serum is difficult to work with on the automated fluorometer, the readout of the basophil response was expression of CD63. In a pilot study it was first established that the reversible inhibition measured in experiments like that shown in Fig 1 worked similarly, irrespective of whether measuring histamine release or CD63 expression (data not shown). The comparison between simple buffer and serum showed that the ability of IgG₃ to initiate inhibition was markedly blunted; inhibition by the inclusion of IgG₃ antibody relative to NP-BSA alone was 0.25 ± 0.10 but in the presence of 90% serum was 0.93 ± 0.03 ($n = 3$). This result suggests that the natural presence of nsIgG in serum blunted the ability of IgG₃ aggregates to interact with CD32b.

DISCUSSION

It was necessary to establish a controlled modification of a specific IgG to address questions about the interaction of IgG subtypes with CD32. This was accomplished by using a well-characterized transfectoma series of antibodies that also included an IgE antibody to the same epitope. It is expected that this choice will avoid the potential issue of the various IgG subclasses binding to an epitope with different affinities or binding to

different epitopes with different affinities. However, it does not precisely mimic conditions *in vivo* where not all epitopes will necessarily be shared between IgE and IgG. However, it should be noted that even though IgG and IgE antibodies might use different epitopes, this does not preclude steric interference between nearby but distinct epitopes. Therefore in this context the use of a common epitope for IgE and IgG subclasses binding to a multivalent antigen is not too dissimilar from the natural situation. In these circumstances a mixture of stoichiometric blocking and interaction with CD32 will be a characteristic of many antigen-antibody complexes. It is for this reason that a test for CD32 interaction requires a good CD32 blocking antibody, and despite the potentially complex overlapping of the 2 kinds of blocking, it was possible to find conditions that established a nearly pure CD32 effect in that Ab10523 completely reversed inhibition by IgG₂ and IgG₃. It should be noted that this only occurs at high antigen concentrations and with a restricted range of antibody/antigen ratios. Low but functional antigen concentrations do not generate an aggregate that interacts well with CD32; stoichiometric blocking is the rule in this regime.

As noted previously, published studies suggest that IgG₂ should not interact with the R131 variant of CD32, and because all CD32b is the R131 variant, IgG₂ should not interact with CD32b.^{E15} In contrast, one might have expected to observe some effect of genotype on the participation of CD32a. Based on the excellent correlation between inhibition mediated by IgG₃ and IgG₂ aggregates and the inverse correlation between receptor expression and inhibition by the aggregates, it is apparent that both subclasses interact effectively with both CD32a and CD32b and that expression levels of these receptors dominate any potentially subtle effect of CD32a genotype (His/His, His/Arg, or Arg/Arg). These results suggest that regardless of the subclass of IgG, the reaction would likely be dominated by the effects of CD32b, and the results also suggest, as noted by others,^{E12,E20} that CD32a appears to act in an inhibitory capacity. These results also suggest that there would be no possibility of a pure CD32a-mediated reaction during which an aggregate of IgG₂ and antigen in the absence or presence of cell-surface antigen-specific IgE could selectively bind to CD32a of the H131 form to induce activation. In fact, we found no direct activation by IgG₂ (or IgG₃) aggregates (without IgE sensitization), even with the homozygous H131 form of CD32a.

If the ELISA measurements of the concentrations of each subclass are valid, the results also suggest that IgG₃, with its extended hinge and rigid structure, bound more effectively to CD32a and CD32b and that 10-fold lower concentrations of IgG₃ were needed to functionally engage CD32 on the basophil. Although IgG₁ does appear to engage CD32, the results were variable, with little or no reversal by Ab10523 (eg, see Fig 1, H). This is an unexpected result that indicates that it is not entirely clear how to view the capacities of sera from patients undergoing immunotherapy to engage CD32. In serum, based on World Health Organization standards, IgG₁, IgG₂, and IgG₃ account for 60%, 29%, and 8% of the total IgG in healthy adults. From this perspective, the dominant CD32-dependent functional effect belongs to IgG₃ (13% of IgG₁ concentrations but 10-fold more potent in these assays and also more efficacious). At certain concentrations and antibody/antibody ratios, IgG₂ and IgG₃ are functionally similar with respect to their engagement of CD32 for inhibition of IgE-mediated release. Stoichiometric inhibition (simple blocking of IgE binding by masking binding epitopes)

might be the primary role of IgG₁. Because the study was performed with a single transfectoma cloned antibody series, the results will need to be substantiated in the future with natural antibodies. The increase in the efficacy of IgG₃ is interesting, although the precise role of IgG₃ relative to the other subclasses in the expression of allergic diseases or efficacy of treatments like immunotherapy is not a well-explored area. Most studies of allergen immunotherapy, for example, have only measured IgG₁ and IgG₄, partially because of early difficulties finding good anti-subclass antibodies for detecting IgG₂ or IgG₃ and the complicated methods for measuring these 2 subclasses. However, there is some early evidence that IgG₂ and IgG₃ levels do increase during immunotherapy,^{E21} although the predominant conclusion is that IgG₁ and IgG₄ are the most relevant to monitor (see the review by Djurup^{E22}). The absence of IgG₃ has been associated with problems in managing airway infections, and this has an effect on asthma exacerbations.^{E23} IgG₂ and IgG₃ antibodies are thought to be induced to specific types of antigens, such as IgG₂ to polysaccharide antigens or glycosylated proteins, so that it might be important to differentiate between allergens that can drive the generation of these subclasses and factor this knowledge into the conditions that lead to CD32 involvement.

REFERENCES

- E1. Adkinson NF Jr. Measurement of total serum immunoglobulin E and allergen-specific immunoglobulin E antibody, 2nd ed. Washington (DC): American Society of Microbiology; 1980.
- E2. MacGlashan DW Jr, Lichtenstein LM. Studies of antigen binding on human basophils. I. Antigen binding and functional consequences. *J Immunol* 1983; 130:2330-6.
- E3. Weinberger JZ, Greene MI, Benacerraf B, Dorf ME. Hapten-specific T-cell responses to 4-hydroxy-3-nitrophenyl acetyl. I. Genetic control of delayed-type hypersensitivity by VH and I-A-region genes. *J Exp Med* 1979;149:1336-48.
- E4. Pruzansky JJ, Grammer LC, Patterson R, Roberts M. Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. *J Immunol* 1983;131:1949-53.
- E5. MacGlashan DW Jr. Relationship between Syk and SHIP expression and secretion from human basophils in the general population. *J Allergy Clin Immunol* 2007;119:626-33.
- E6. MacGlashan D Jr, Moore G, Muchhal U. Regulation of IgE-mediated signalling in human basophils by CD32b and its role in Syk down-regulation: basic mechanisms in allergic disease. *Clin Exp Allergy* 2014;44:713-23.
- E7. Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 1987;166:1351-61.
- E8. Hamilton R. Human IgG subclass measurements in the clinical laboratory. *Clin Chem* 1987;33:1707-25.
- E9. Warner JA, Yancey KB, MacGlashan DW Jr. The effect of pertussis toxin on mediator release from human basophils. *J Immunol* 1987;139:161-5.
- E10. Siraganian RP. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 1974;57:383-94.
- E11. MacGlashan DW Jr. Endocytosis, re-cycling and degradation of unoccupied FcεRI in human basophils. *J Leuk Biol* 2007;82:1003-10.
- E12. Cassard L, Jonsson F, Arnaud S, Daeron M. Fcγ receptors inhibit mouse and human basophil activation. *J Immunol* 2012;189:2995-3006.
- E13. Chu SY, Vostiar I, Karki S, Moore GL, Lazar GA, Pong E, et al. Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and FcγRIIb with Fc-engineered antibodies. *Mol Immunol* 2008;45: 3926-33.
- E14. Denepoux S, Eibensteiner PB, Steinberger P, Vrtala S, Visco V, Weyer A, et al. Molecular characterization of human IgG monoclonal antibodies specific for the major birch pollen allergen Bet v 1. Anti-allergen IgG can enhance the anaphylactic reaction. *FEBS Lett* 2000;465:39-46.
- E15. Warner PA, van de Winkel JG, Vluga A, Westerdaal NA, Capel PJ. A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. *J Immunol* 1991;147:1338-43.
- E16. Ramirez J, Fernandez-Sueiro JL, Lopez-Mejias R, Montilla C, Arias M, Moll C, et al. FCGR2A/CD32A and FCGR3A/CD16A variants and EULAR

- response to tumor necrosis factor- α blockers in psoriatic arthritis: a longitudinal study with 6 months of followup. *J Rheumatol* 2012;39:1035-41.
- E17. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fc γ receptors and their polymorphic variants for human IgG subclasses. *Blood* 2009;113:3716-25.
- E18. Li X, Wu J, Carter RH, Edberg JC, Su K, Cooper GS, et al. A novel polymorphism in the Fc γ receptor IIB (CD32B) transmembrane region alters receptor signaling. *Arthritis Rheum* 2003;48:3242-52.
- E19. Kyogoku C, Dijstelbloem HM, Tsuchiya N, Hatta Y, Kato H, Yamaguchi A, et al. Fc γ receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. *Arthritis Rheum* 2002;46:1242-54.
- E20. Cady CT, Powell MS, Harbeck RJ, Giclas PC, Murphy JR, Katial RK, et al. IgG antibodies produced during subcutaneous allergen immunotherapy mediate inhibition of basophil activation via a mechanism involving both Fc γ RIIA and Fc γ RIIB. *Immunol Lett* 2010;130:57-65.
- E21. Devey ME, Wilson DV, Wheeler AW. The IgG subclasses of antibodies to grass pollen allergens produced in hay fever patients during hyposensitization. *Clin Allergy* 1976;6:227-36.
- E22. Djurup R. The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen-specific immunotherapy. *Allergy* 1985;40:469-86.
- E23. Sakai E, Yamamoto T, Yamamoto K, Mizoguchi Y, Kaneno H, Ihashi M, et al. IgG3 deficiency and severity of 2009 pandemic H1N1 influenza. *Pediatr Int* 2012;54:758-61.

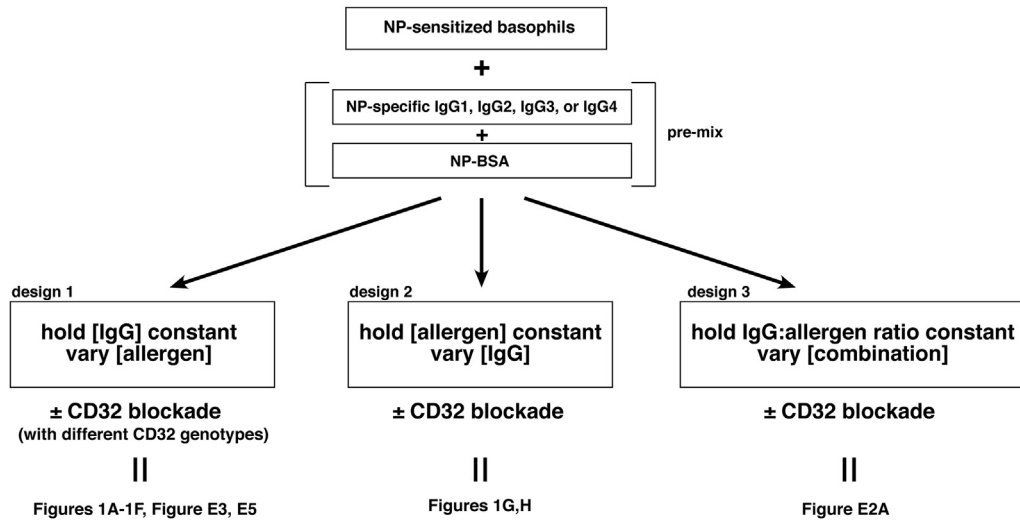


FIG E1. Experimental design diagram using NP-specific IgE for basophil sensitization followed by allergen challenge (NP-BSA) in the presence of different NP-specific IgG subclass antibodies with and without CD32 blockade (with Ab10523). Three basic design types were used, as noted in the diagram, and the manuscript figures associated with each design are indicated. *Brackets* indicate "concentration" of the molecule.

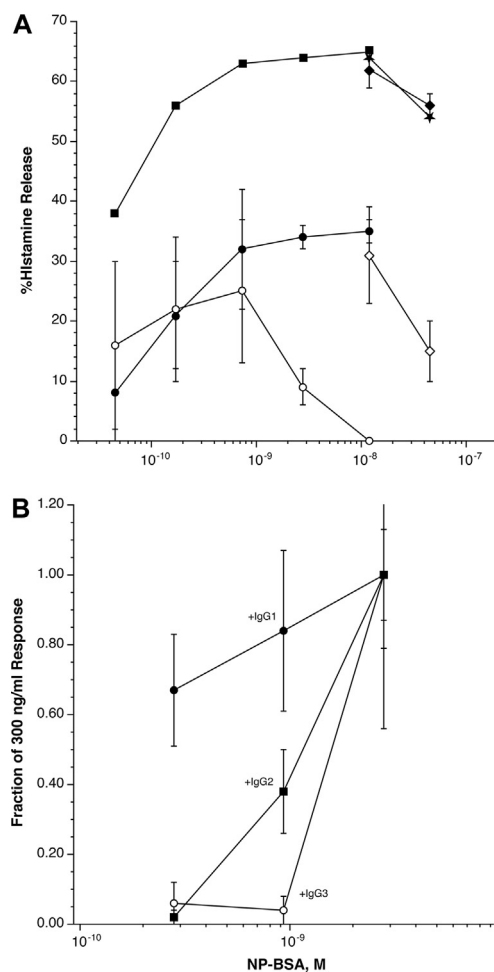


FIG E2. Reversal of CD32-mediated inhibition while maintaining a constant antibody/antigen ratio. **A**, Composite plot of 2 antibody/antigen ratios: ■, control response to NP-BSA; ○, response to the NP-BSA and antibody/antigen ratio of 0.20:1 ($n = 3$); ●, response to the NP-BSA and antibody/antigen ratio of 0.20:1 in the presence of Ab10523 at 50 $\mu\text{g}/\text{mL}$; ★, control response to NP-BSA; ◇, response to the NP-BSA and antibody/antigen ratio of 0.06:1 ($n = 4$); ◆, response to the NP-BSA and antibody/antigen ratio of 0.06:1 in the presence of Ab10523 at 50 $\mu\text{g}/\text{mL}$. **B**, By using results from Fig 1, A-C (only the inhibition curves without Ab10523), the results are plotted as the fraction of 300 ng/mL response.

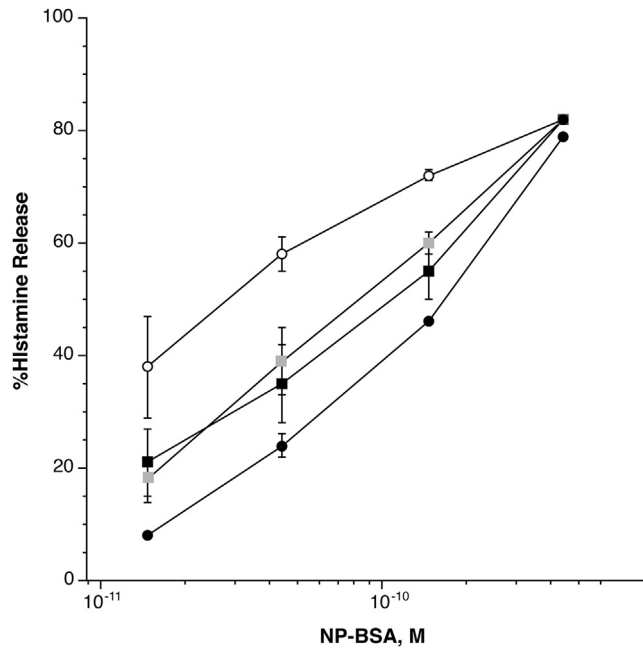


FIG E3. Enhancement of antigen-induced release by human IgG₄ subclass antibody. ●, Response to NP-BSA without subclass antibody; ○, response with subclass antibody; ■, response with subclass antibody and Ab10523 at 50 µg/mL; □, subclass antibody and Ab10523 plus AbIV.3.

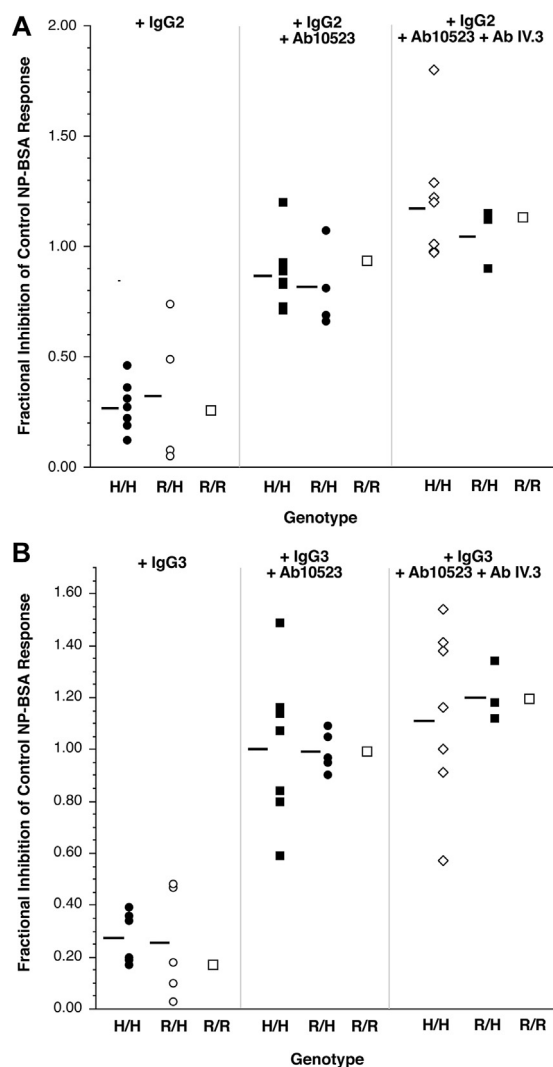


FIG E4. Working with results from experiments like that shown in Fig 1, E-G, responses of the individual genotypes for IgG₂- and IgG₃-mediated inhibition with or without Ab10523 and with or without AbIV.3. Each point is a different experiment. Horizontal bars show the mean of the donor scatterplots.

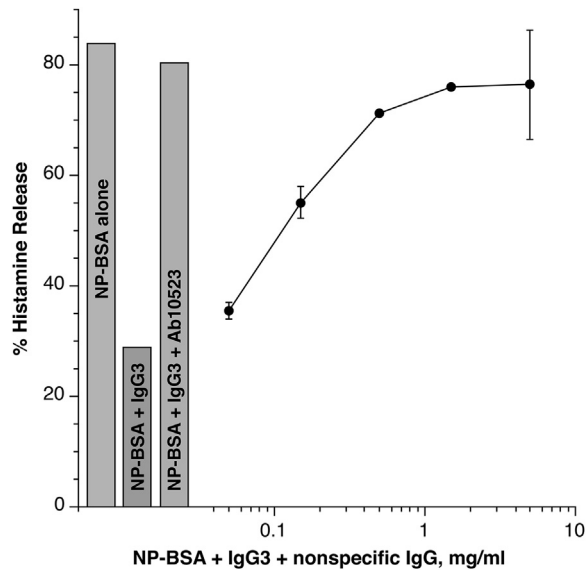


FIG E5. Reversal of IgG₃-mediated inhibition of histamine release by nonspecific hIgG. After overnight culture with 1 ng/mL IL-3, sensitized cells were stimulated with 43 nmol/L NP-BSA plus 9.9 nmol/L NP-specific IgG₃ (premixed 15 minutes before stimulation) in the absence or presence of several concentrations of nsIgG (n = 2) or Ab10523.

TABLE E1. Correlation coefficients for various combinations of CD32a and CD32b expression and the extent of inhibition by NP-specific IgG₂ and IgG₃ (n = 9)

Expression	IgG ₂	IgG ₃
CD32a	−0.73 (<i>P</i> = .02)	−0.65 (<i>P</i> = .05)
CD32b	−0.60 (<i>P</i> = .08)	−0.65 (<i>P</i> = .06)
CD32a + CD32b	−0.70 (<i>P</i> = .03)	−0.70 (<i>P</i> = .03)

Individual data presented in Fig 4, in which CD32a and CD32b receptor levels were measured by using flow cytometry, were correlated with the inhibition caused by either IgG₂ or IgG₃. For these 2 antibodies, all of the inhibition at the concentrations used was reversible with Ab10523 and AbIV.3.