SUPPLEMENTAL METHODS

B. anthracis Strains and Toxins

B. anthracis Sterne strain 34F2 ($pXO1^+$, $pXO2^-$) was obtained from A. Hoffmaster (Centers for Disease Control and Prevention). Bacterial cultures were grown from frozen stocks in brain heart infusion medium (Gibco) at 37^{o}C for 20 h with shaking. Prior to administration to mice, bacteria were washed thrice with PBS to remove toxin. Recombinant protective antigen (PA) and lethal factor (LF) were obtained from Wadsworth Center (New York State Department of Health).

Cell Culture

19D9 hybridoma cells were maintained as previously described (1). Bone marrow-derived macrophages were generated by bone marrow cells derived from mouse FcγRα^{null} mice or mouse FcγRα^{null} expressing human FcγRs. Cells were differentiated into macrophages by incubation for 7 d in RPMI1640 supplemented with 2 mM L-glutamine, 10% FBS, MEM non-essential amino acids (Invitrogen, Life technologies), 5 ng ml^{-1} recombinant murine IL-3 (Peprotech) and 5 ng ml^{-1} recombinant murine M-CSF (Peprotech). All cells were maintained at 37^{o}C, 5% CO_2.

Generation of mouse-human chimeric mAbs and Fc variants

For the generation of mouse-human chimeric 19D9 mAb, the variable regions of the heavy and light chains were cloned from 19D9 hybridoma (mouse IgG1,κ) by 5' RACE as previously described (2). Briefly, total RNA was isolated from 19D9 hybridoma cells and cDNA was generated using primers specific for mouse IgG1 and mouse κ constant regions. PCR-amplified heavy and light chain variable region fragments were cloned to hIgG1 and human κ constant region vectors. For the generation of Fc domain variants, site-directed mutagenesis using specific primers was performed based on the QuikChange site-directed

mutagenesis Kit II (Agilent Technologies), as previously described (3). Mutated plasmid sequences were validated by direct sequencing (Genewiz).

Recombinant Protein Expression and Purification

Antibodies and soluble human FcγR ectodomains were generated by transient transfection of 293T cells, as described previously (3). Antibodies were purified by Protein G Sepharose 4 Fast flow (GE Healthcare) and His-tagged recombinant proteins using His-Tag isolation and pull-down dynabeads (Invitrogen). Purity was assessed by SDS-PAGE and Coomassie blue staining and was estimated to be >90%. For antibody preparations, endotoxin (LPS) contamination was quantified by the Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod) and levels were <0.005 EU mg^{-1}.

PA-specific ELISA

Recombinant PA (1 μg ml^{-1}; 50 μl/well) was immobilized onto high-binding 96-well microtiter plates (Nunc) and following overnight incubation at 4^{o}C, plates were blocked with PBS + 2% (w/v) BSA + 0.05% (v/v) Tween 20 for 2 h. After blocking, plates were incubated for 1 h with serially-diluted IgG antibodies (starting at 5 μg ml^{-1} with serial twofold dilutions in PBS), followed by incubation with HRP-conjugated goat anti-human IgG (1 h; 1:5000; Sigma). Plates were developed using the TMB two-component peroxidase substrate kit (KPL) and reactions stopped with the addition of 1 M phosphoric acid. Absorbance at 450nm was immediately recorded using a SpectraMax Plus spectrophotometer (Molecular Devices).

Surface Plasmon Resonance (SPR)

All experiments were performed with a Biacore T100 SPR system (Biacore, GE Healthcare) at 25^{o}C in HBS-EP+ buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20). For the measurement of anti-PA affinity of 19D9 IgG variants, IgG antibodies (diluted at 20 μg ml^{-1} in 10 mM sodium acetate, pH 4.5) were immobilized on Series S CM5 chips by amine coupling at a density of 1000 RU. Recombinant PA were

injected though flow cells at a flow rate of 30 µl min^{-1} with the concentration ranging from 3.9 – 1000 nM (serial twofold dilutions). Association time was 120 s followed by 600 s dissociation. At the end of each cycle, sensor surface was regenerated with 50 mM NaOH (30 µl min^{-1}; 30 s). Background binding to blank immobilized flow cells was subtracted and affinity constants were calculated using BIAcore T100 Evaluation software using the 1:1 Langmuir binding model. For the determination of the affinity of human IgG1 Fc domain variants for human FcγRs, soluble ectodomains of human $FcγRIIa^{R131}$, FcγRIIb, and $FcγRIIIa^{F158}$ diluted at 20 µg ml^{-1} in 10 mM sodium acetate, pH 4.5 were immobilized on Series S CM5 chips by amine coupling resulting in a density of 2000 RU. Recombinant IgG samples were injected through flow cells at seven different concentrations (ranging from 2000-31.25 nM; serial twofold dilutions) at a flow rate of 30 µl min^{-1} for 120 s, followed by a 300-s dissociation step. After each assay cycle, the sensor surface was regenerated with a 30-s injection of 25 mM NaOH at a flow rate of 30 µl min^{-1}.

Immunolabelling and Flow Cytometry

Adherent bone marrow-derived macrophages were detached following incubation (15 min) with PBS, 5 mM EDTA, 1% FCS at 4°C. Cells were washed and incubated with FITC-conjugated anti-human FcγR antibodies (anti-CD64, clone 10.1, BD Biosciences; anti-CD32a, clone IV.3, StemCell technologies; anti-CD32b, clone 2B6; anti-CD16a/b, clone 3G8 BD Biosciences; and anti-CD16b, clone 1D3, AbD Serotec). Following immunolabelling, cells were washed and analyzed by flow cytometry using a BD FACS Calibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo analysis software (Treestar).

In Vitro Cytotoxicity Assay

Bone marrow-derived macrophages ($6x10^4$ cells/well) were plated into 96-well plates and incubated for 24 h at 37°C (5% CO_2). Cells were washed gently with PBS and treated with lethal toxin (300 ng ml^{-1} protective antigen (PA), 300 ng ml^{-1} lethal factor (LF)) in the presence of variable amounts of 19D9 hIgG1 Fc variants in HBSS supplemented with 0.1%

FCS. Following a 4-h incubation at 37^{o}C, cell supernatants were collected and lactate dehydrogenase (LDH) was quantified using the Cytotoxicity Detection Kit (Roche) following manufacturer's recommendations. Absorbance at 450nm was recorded using a SpectraMax Plus spectrophotometer (Molecular Devices). Background absorbance at 620nm was subtracted and LDH was expressed as percent cytotoxicity in relation to maximal LDH levels obtained from cells incubated with 0.6% Triton X-100.

Mice and In Vivo Protection Experiments

Mice deficient for all classes of murine FcγRs (FcγRα^{null}) and FcγR humanized mice have been previously generated and extensively characterized (4). FcγR humanized mice expressed the following human FcγRs: FcγRI, FcγRIIa^{R131}, FcγRIIb, FcγRIIIa^{F158}, and FcγRIIIb. For in vivo protection experiments, mice were 8-14 weeks of age. Unless otherwise stated, 19D9 or isotype control (clone 4-4-20; anti-fluorescein isothiocyanate (FITC)) antibody was administered i.p. 3 h prior to i.v. challenge with *B. anthracis* Sterne (10^4). Mouse survival was monitored daily.

Statistical Analysis

Quantitative data from multiple experiments are presented as mean ± SEM. Two-way analysis of variance (ANOVA) was used to test for differences in in vitro protection assays and where statistically significant effects were observed, post-hoc analysis using Bonferroni *t*-test was performed. IC_{50} values (amount of antibody (μg ml^{-1}) required to protect BMDMφ from LeTx-induced cytotoxicity by 50%) were calculated by non-linear regression fit analysis (variable slope) of *log* transformed (*log* mAb concentration) data. For in vivo protection experiments, survival rates were analyzed with the *log*-rank test. *P* values of <0.05 were considered to be statistically significant. Data were analyzed with Graphpad Prism software.

Study Approval

All in vivo experiments were performed in compliance with federal laws and institutional guidelines and have been approved by the Rockefeller University Institutional Animal Care and Use Committee.

SUPPLEMENTAL TABLES

Table S1: Affinity of 19D9 Fc variants for human FcγRs

19D9 hIgG1 Variant	FcγRIIa*		FcγRIIb		FcγRIIIa#	
	KD ($x10^{-6}$)	Fold	KD ($x10^{-6}$)	Fold	KD ($x10^{-6}$)	Fold
Wild Type	1.33	1.0	2.7	1.0	2.22	1.0
N297A	n.d.b.		n.d.b.		n.d.b.	
G236A/S239D/A330L/I332E	0.104	12.8	1.67	1.6	0.144	15.4
S267E/L328F	0.03	44.3	0.024	112.5	n.d.b.	

Values represent KD (μM); * R131 variant; #F158 variant. Binding constants were obtained by SPR analysis with immobilized FcγRs and soluble mAbs. n.d.b., no detectable binding.

Table S2: Affinity of 19D9 Fc variants for PA

19D9 hIgG1 Variant	KD (M) Mean	SD
Wild Type	$1.73x10^{-8}$	$1.1x10^{-10}$
N297A	$1.78x10^{-8}$	$2.5x10^{-10}$
G236A/S239D/A330L/I332E	$1.79x10^{-8}$	$1.5x10^{-10}$
S267E/L328F	$1.77x10^{-8}$	$3.5x10^{-10}$

Affinities were determined using recombinant PA and calculated by fitting the SPR sensograms to the 1:1 Langmuir binding model.

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