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Brief Report **Immunology**

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Human IgG Fc domain engineering enhances antitoxin neutralizing antibody activity

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The effector activity of antibodies is dependent on engagement with Fcγ receptors (FcγRs) and activation of the associated intracellular signaling pathways. Preclinical evaluation of therapeutic humanized or chimeric mAbs to study the interactions of their Fc regions with FcγRs is hampered by substantial structural and functional FcγR diversity among species. In this report, we used mice expressing only human FcγRs to evaluate the contribution of FcγR-mediated pathways to the neutralizing activity of an anti-anthrax toxin chimeric mAb. We observed that the protective activity of this mAb was highly dependent upon FcγR engagement, with minimal protection against anthrax toxin observed in FcγR-deficient mice following mAb administration. We generated anti-anthrax toxin mAbs with specific Fc domain variants with selectively enhanced affinity for particular human FcγRs and assessed their activity in FcγR-humanized mice. We determined that Fc domain variants that were capable of selectively engaging activating FcγRs substantially enhanced the in vitro and in vivo activity of anthrax toxin-neutralizing antibodies. These findings indicate that the application of Fc domain engineering is a feasible strategy to enhance toxin-neutralizing activity and suggest that engineered antitoxin antibodies will have improved therapeutic efficacy.

Introduction

The development of hybridoma technology has revolutionized medicinal therapeutics, making possible the generation of highly specific mAbs with efficacy against a wide range of diseases. While Fab-antigen interactions play a crucial role in the protective activity of an antibody, it is now apparent that coupling the Fab-mediated recognition with Fc effector activity is crucial for optimal in vivo activity for protection against microbial pathogens and their toxins (1–5). Fcγ receptors (FcγRs) are capable of either cellular activation through immunoreceptor tyrosine-based activation motif–dependent activation of intracellular tyrosine kinases or the inhibition of activation through recruitment of phosphatases to the immunoreceptor tyrosine-based inhibition motif domain and are thereby categorized into 2 broad classes: activating and inhibitory (6). The cellular outcome of IgG interaction with FcγRs is governed by the affinity of the Fc domain for the specific receptor and the expression pattern of those receptors on the effector cells. Since most effector cells coexpress activating and inhibitory FcγRs, it is the ratio of the binding affinities of a specific IgG Fc to these receptors that will determine the outcome of the IgG-FcγR interaction (7). Indeed, differences in the capacity of an IgG molecule to engage activating or inhibitory FcγRs are a determining factor for the in vivo activity of a particular IgG subclass or variant (3, 7).

Antibody-mediated neutralization of bacterial toxins was classically considered to be a direct process that relied solely on the ability of the variable region of antibodies to bind toxins. However, recent findings suggest that effective in vivo protection against microbial pathogens and their toxins requires both Fab recognition and Fc binding to FcγRs for optimal activity (1, 8–10). This in turn suggests that it may be possible to enhance the toxin-neutralizing activity of antibodies by engineering the Fc domain to selectively engage certain classes of FcγRs. Indeed, engineering of the Fc region of an immunoglobulin can increase its protective efficacy against different pathogens and improve effector functions, including antibody-dependent cell-mediated cytotoxicity and opsonization (11–13).

Over the past decade, significant advances have been made to generate humanized and mouse-human chimeric mAbs to reduce toxicity and enhance various effector functions (14). Murine or nonhuman primate model systems are commonly used for the preclinical evaluation of these mAbs and for the study of the Fc-FcγR interaction for humanized/chimeric antibodies, even though these models poorly reflect the structural diversity and the unique expression pattern of human FcγRs of human leukocytes (15–18). Therefore, we have recently developed a mouse model in which the mouse FcγRs were deleted and all the human FcγRs were expressed as transgenes, recapitulating the human-specific expression pattern (19). Since these mice retain functional FcγR binding and signaling activities, their use facilitates the assessment of the neutralization activity of human mAbs in a context closely related to the human FcγR system. Here, we used the well-characterized anthrax toxin neutralization model (1, 10) to study the role of FcγR-mediated pathways in its neutralization activity using FcγR-humanized mice and mouse-human chimeric forms of a protective mAb. Additionally, we report that specific Fc domain variants of this mAb present significantly augmented in vitro and in vivo neutralization activity through selective engagement of particular classes of human FcγRs.

Results and Discussion

Toxin neutralization was previously thought to be the sole result of blocking toxin activity through Fab-antigen interactions. However, our recent observations indicate that FcγR-mediated path-

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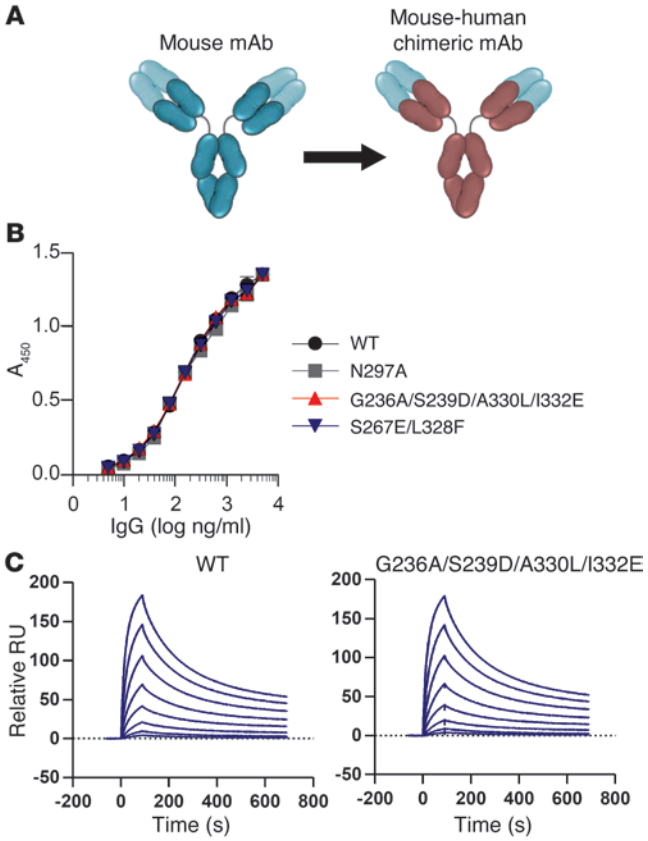

Figure 1
Generation and characterization of mouse-human chimeric anti-PA mAb. (**A**) Representation of the mouse-human chimeric anti-PA (19D9) mAb. The constant regions of the heavy (CH1-3) and light chain (Cκ) of mouse IgG1, κ (parental mAb) were replaced with those of hIgG1 and Cκ, respectively. Recombinant 19D9 hIgG1 wild-type and Fc domain variants were generated. No difference in their affinity and specificity for PA was evident among the various Fc domain variants, as assessed by (**B**) ELISA and (**C**) surface plasmon resonance (affinity values are shown in Supplemental Table 2).

ways, such as Fc-mediated toxin uptake by effector cells like macrophages, contribute substantially to the neutralizing activity of anti-anthrax toxin mAbs (1, 10). Given the previously described requirement for FcγRs for the activity of anti-anthrax mouse mAb 19D9 (1), we have generated a mouse-human chimeric IgG form of this antibody as a necessary precursor to a fully human therapeutic antibody. The constant regions of the heavy and light chains (mouse IgG1,κ) were replaced with those of human IgG1 (hIgG1) and κ constant regions, respectively (Figure 1A).

Similarly, we have shown previously that generation of switch variants of the parental 19D9 mAb and comparison of their neutralization potencies resulted in more effective neutralizing antibodies, while not effecting their binding affinity or specificity, such that their relative efficacy was IgG2a>IgG2b>IgG1 (1). This hierarchy of mouse IgG subclass activity results from the differential binding of these subclasses to specific mouse FcγRs, as previously determined (7). Mouse IgG2a binds with a log higher affinity to the activation receptor FcγRIV, while mouse IgG1 preferentially engages the inhibitory FcγR, FcγRIIb (7). These differences in the capacity of mouse IgG subclasses to engage activating or inhibitory FcγRs was previously shown to be predictive of the in vivo antibody-dependent cell-mediated cytotoxicity activity in several mouse models (7, 19). In contrast to the murine IgG subclasses, no specific human IgG subclass exists that binds selectively to a particular class of human FcγRs conferring enhanced in vivo activity, such as mouse IgG2a, which interacts selectively with activating FcγRs (7).

Since the main determining factor for the Fc-FcγR interactions is the amino acid backbone of the Fc region, we generated specific mutants of hIgG1 19D9 within the Fc domain region that participates in the Fc-FcγR interface. We characterized the effect of different mutations on the affinity for the different human FcγR classes and identified variants that exhibit differential binding affinities for the various human FcγR classes. These Fc domain variants had the capacity for enhanced engagement of either activating FcγRs, such as FcγRIIa and FcγRIIIa (G236A/S239D/A330L/I332E), or the inhibitory FcγRIIb (S267E/L328F) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI72676DS1). Additionally, an Fc domain variant exhibiting minimal binding (N297A) for all classes of human FcγRs was generated (Supplemental Table 1).

Wild-type hIgG1 and Fc domain variants of 19D9 were expressed in mammalian cells, and their specificity and affinity to the antigen, protective antigen (PA) of *Bacillus anthracis*, were determined by ELISA (Figure 1B) and surface plasmon resonance (Figure 1C and Supplemental Table 2). While these Fc domain variants exhibited differential binding capacity for human FcγRs, no differences in their affinity and specificity for PA were evident. This observation is consistent with a prior observation that isotype switching of the mouse IgG1-producing hybridoma 19D9 to produce mIgG2a and mIgG2b had no effect on specificity, implying that the 19D9 V regions are not susceptible to the Fc-mediated effects on affinity that have been described for other mAbs (20).

The protective efficacy of mouse-human chimeric 19D9 mAb was assessed using mice expressing all classes of human FcγRs (FcγRI, FcγRIIa^{R131}, FcγRIIb, FcγRIIIa^{F158}, and FcγRIIIb) (19). To determine the contribution of FcγR-mediated pathways to the activity of the chimeric 19D9 hIgG1 mAb, bone marrow–derived macrophages (BMDMs), the main target of the anthrax lethal toxin (LeTx) during anthrax infection, were obtained from mice lacking all genes encoding for FcγRs (FcγRα^{null}) and from FcγRα^{null} mice expressing human FcγRs (FcγR-humanized mice). Comparison of the in vitro neutralization activity of the chimeric hIgG1 19D9 mAb in FcγRα^{null} and FcγR-humanized cells revealed significantly decreased protective activity in the absence of FcγRs, as evidenced by enhanced anthrax LeTx-mediated cytotoxicity in FcγRα^{null} BMDMs compared with that in FcγR-humanized BMDMs (Figure 2A) (hIgG1 19D9 IC_{50} in FcγR-humanized BMDMs, 0.21 μg ml^{-1}; hIgG1 19D9 IC_{50} in FcγRα^{null}, 21.45 μg ml^{-1}). Analysis of the expression of human FcγRs of BMDMs obtained from FcγR-humanized mice showed an expression pattern similar to that of primary human macrophages (18). In particular, FcγR-humanized BMDMs expressed FcγRIIa (CD32a), FcγRIIb (CD32b), and FcγRIIIa (CD16a), while they lacked FcγRI (CD64) and FcγRIIIb (CD16b) (Figure 2B). We next determined the protective activity of the mouse-human chimeric hIgG1 19D9 mAb in vivo using FcγR-humanized mice and FcγRα^{null} mice. While the hIgG1 19D9 mAb (750 μg; i.p. 3 hours prior to bacterial challenge) was protective in FcγR-humanized mice following the i.v. challenge

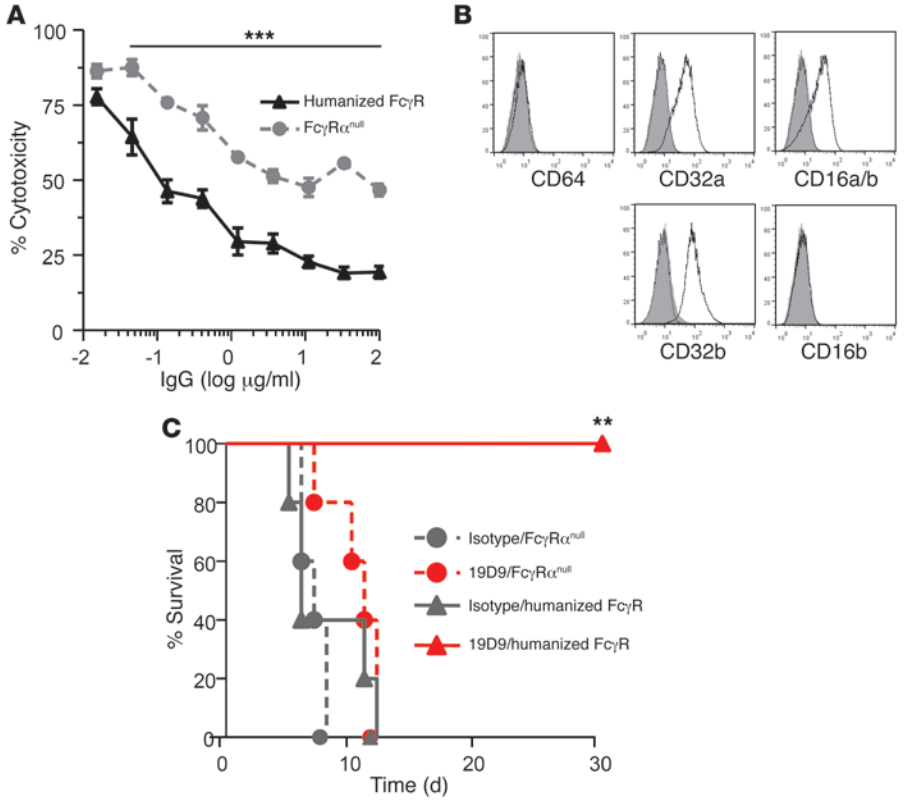

Figure 2

FcγR requirement for the neutralizing activity of the anti-PA mouse-human chimeric mAb. (**A**) The protective activity of 19D9 hIgG1 against anthrax LeTx was compared in BMDMs obtained either from humanized FcγR (wild-type) or FcγR-deficient (FcγRα^{null}) mice. $n = 4$; $^{***}P < 0.0001$; $n = 4$. (**B**) Human FcγR expression profile of BMDMs from humanized FcγR (solid black line) or FcγRα^{null} (solid gray filled) mice (isotype control; dotted line). (**C**) Comparison of the protective activity of 19D9 hIgG1 (750 μg i.p.) in humanized FcγR and FcγRα^{null} mice following challenge with *B. anthracis* Sterne strain. $n = 5$ per group; $^{**}P = 0.005$, FcγRα^{null} vs. humanized FcγR.

with *B. anthracis* Sterne strain (10^4 cells), no activity was observed in FcγR-deficient mice (Figure 2C). These findings clearly suggest the requirement of FcγR engagement in the protective activity of hIgG1 19D9 against anthrax toxin.

Since the protective activity of the mAb 19D9 to LeTx is dependent upon FcγR engagement, we hypothesized that alterations in its capacity to engage particular classes of FcγRs could modulate its neutralizing potency. To test this concept, the in vivo neutralizing activity of different Fc domain variants of hIgG1 19D9 was compared using FcγR-humanized mice. These variants have been characterized in terms of FcγR binding, and they presented enhanced affinity for either activating or inhibitory human FcγRs (Supplemental Table 1). When compared with wild-type hIgG1 19D9, the G236A/S239D/A330L/I332E variant, which has increased affinity for activating FcγRs, including FcγRIIa (13-fold increase) and FcγRIIIa (15-fold increase), demonstrated substantially augmented protective activity in vitro (10-fold improved potency — wild-type hIgG1 IC_{50}, 0.31 μg ml^{-1}; G236A/S239D/A330L/I332E IC_{50}, 0.03 μg ml^{-1}).

To assess whether the G236A/S239D/A330L/I332E variant also displayed augmented activity in vivo, we selected a suboptimal dose (350 μg), at which the wild-type hIgG1 19D9 was ineffective (Figure 3B). As is evident from Figure 3B, >70% survival was achieved with 350 μg G236A/S239D/A330L/I332E 19D9, while no significant protective activity was observed with wild-type hIgG1 19D9 at this particular dose, suggesting that administration of the G236A/S239D/A330L/I332E variant lowered the threshold of mAb required to fully confer in vivo protection.

In contrast, no major difference was observed when we compared the activity of wild-type IgG1 19D9 with that of the S267E/L328F variant, which binds with enhanced affinity to FcγRIIa and the inhibitory FcγRIIb (44-fold and 113-fold increase for FcγRIIa and FcγRIIb, respectively; Supplemental Table 1) (Figure 3, A and C). Minimal in vitro and in vivo neutralizing activity was observed for the FcγR null binding mutant (N297A), further supporting the requirement for FcγR for the mAb-mediated protection against *B. anthracis* infection (Figure 3, A and C).

These findings suggest that preferential engagement of activating FcγRs does enhance the neutralization activity of the 19D9 mAb in humans and are consistent with our previous observations in wild-type mice, in which the mouse IgG2a 19D9 isotype variant was more effective compared with mouse IgG1 (1). In contrast, minimal contribution of the inhibitory FcγRIIb to the mAb-mediated toxin neutralization was evident, as the FcγRIIb-enhanced binding Fc domain variant of 19D9 (S267E/L328F) displayed activity comparable to that of wild-type hIgG1. Although this variant (S267E/L328F) also displayed enhanced binding to the activating FcγRIIa, its substantially augmented binding capacity for FcγRIIb is likely to overcome any cellular activation signals following FcγRIIa coengagement, especially given the higher expression levels of FcγRIIb compared with FcγRIIa in all leukocyte types (18, 19). This assumption is further strengthened by our previous observations from mAb-mediated cellular cytotoxicity models in FcγR-humanized mice, in which the S267E/L328F variant failed to display enhanced in vivo cytotoxic activity, despite its capacity to engage FcγRIIa (19).

As we have previously demonstrated (1), mouse 19D9 mAb (irrespective of IgG subclass) failed to protect against *B. anthracis* infection in mice deficient in activating FcγRs (FcR γ-chain knockout [*Fcer1g*$^{-/-}$] mice) or in all classes of FcγRs (*Fcer1g*$^{-/-}$ and *Fcgr2b*$^{-/-}$ mice), further excluding a role of FcγRIIb in the in vivo neutralizing activity. It is likely that increased mAb interactions

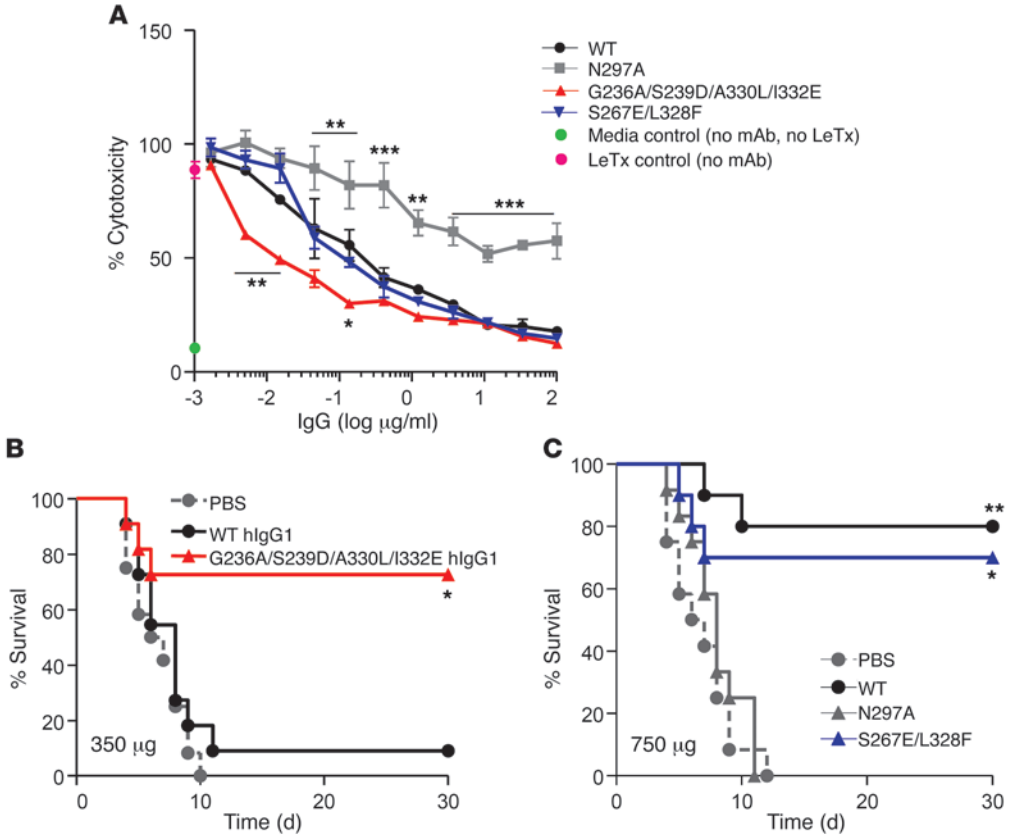

Figure 3

Enhancement of the neutralization activity of anti-PA hIgG1 mAb through Fc domain engineering. Fc domain variants of 19D9 hIgG1 with differential binding capacity for the various classes of human FcγRs were generated and their neutralization activity was assessed both (**A**) in vitro and (**B** and **C**) in vivo. (**A**) LeTx-induced cytotoxicity was assessed in BMDMs in the presence of the different Fc domain variants of 19D9 hIgG1. $n = 2$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. wild-type IgG1. (**B**) Enhanced in vivo protective activity of the G236A/S239D/A330L/I332E 19D9 hIgG1 variant in FcγR-humanized mice challenged with *B. anthracis*. Mice received the indicated mAb variant (350 μg) or PBS i.p. 3 hours prior to challenge. $n = 11–12$ per group; *$P = 0.0094$, compared with wild-type hIgG1 group. (**C**) The neutralization activity of 19D9 hIgG1 Fc domain variants (750 μg i.p.) with differential FcγR binding capacity was compared in humanized FcγR mice following challenge with *B. anthracis*. $n = 10–12$ per group; *$P = 0.005$, **$P = 0.002$, compared with N297A group.

with activating FcγRs lead to substantially enhanced protection during *B. anthracis* infection through modulation of the phagocytic activity of FcγR-expressing effector cells, augmenting the clearance of PA–anti-PA immune complexes and preventing the formation of anthrax LeTx. Additionally, since the G236A/S239D/A330L/I332E IgG1 Fc variant (and particularly the A330L mutation; ref. 21) is deficient in mouse and human C1q binding and complement activation, a role for complement-mediated pathways in the in vivo mAb-mediated neutralization activity of 19D9 could be excluded.

In summary, we have shown that the toxin neutralization activity of a mouse-human antibody against the anthrax LeTx is dependent on interactions with human FcγRs and identified an optimized human FcγR binding profile for toxin-neutralizing efficacy. In the past, the evaluation of the in vivo activity in wild-type mice of human mAbs intended for human therapeutics, like 19D9, was hampered by the substantial interspecies differences both in structure and in the function of FcγRs as well as their expression pattern. In this study, we have circumvented that prior limitation by using FcγR-humanized mice, which faithfully recapitulate the unique pattern of human FcγR expression observed in the various human tissues. So, our findings, obtained with the use of FcγR-humanized mice, are physiologically relevant to the context of human *B. anthracis* infection. Additionally, we showed that the in vivo neutralization activity of mouse-human chimeric 19D9 mAb offers significant advantages over conventional strategies using wild-type mice, thus allowing the precise characterization of the activity of Fc domain variants with altered capacity to engage specific classes of human FcγRs. Most importantly, our findings establish that it is possible to enhance the toxin-neutralizing activity of an antibody by manipulating Fc-mediated interactions. Indeed, increased endocytic uptake of mAb-opsonized PA by augmented engagement of activating FcγRs on effector cells could prevent furin-mediated cleavage of PA and the subsequent assembly of the anthrax LeTx (1, 10). That finding in turn suggests strategies for designing more effective therapeutic antibodies for toxin neutralization and vaccines that elicit superior toxin-neutralizing responses through the production of antibodies that engage activating FcγRs.

Methods

A detailed description of the methods is provided in the Supplemental Methods.

Mice and in vivo protection experiments. Mice deficient for all classes of murine FcγRs (FcγRα^{null} mice) and FcγR-humanized mice have been previously characterized (19). For in vivo protection experiments, 19D9 or isotype antibody was administered i.p. 3 hours prior to i.v. challenge with *B. anthracis* Sterne strain (10^4 cells).

Statistics. Quantitative data from multiple experiments are presented as mean ± SEM. Two-way ANOVA with Bonferroni post-hoc *t* test (2 tailed) was used to test for differences in in vitro protection. For in vivo protection experiments, survival rates were analyzed with the log-rank test. *P* values of less than 0.05 were considered to be statistically significant.

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

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