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Xinrui Li *et al.*

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Editor's Summary

B Cell Balancing Act

Immune cells walk a tight rope as defenders of the body: If there's too little activation, an infection will go unchecked, whereas if there's too much, the immune cell may attack the body's own cells. Hence, these cells are highly regulated through negative and positive signals. For B cells, some of these signals come through Fc receptors, which bind the Fc tail of antibodies. Now, Li *et al.* report the activating receptor FcγRIIc on B cells.

B cells had been thought to express only inhibitory Fcγ receptor, FcγRIIb, which serves as feedback inhibition for immunoglobulin G production. The authors report that human B cells may also express Fc γRIIc, which counterbalances negative signaling through Fc γRIIb. FcγRIIc enhanced humoral immune responses to vaccination both in transgenic mice and in humans in an anthrax vaccine trial. What's more, the *FCGR2C*-ORF allele is associated with autoimmunity risk in humans. By determining which individuals express this allele, these data will help guide more precise antibody-based therapy.

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IMMUNOLOGY

Allelic-Dependent Expression of an Activating Fc Receptor on B Cells Enhances Humoral Immune Responses

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B cells are pivotal regulators of acquired immune responses, and recent work in both experimental murine models and humans has demonstrated that subtle changes in the regulation of B cell function can substantially alter immunological responses. The balance of negative and positive signals in maintaining an appropriate B cell activation threshold is critical in B lymphocyte immune tolerance and autoreactivity. FcγRIIb (CD32B), the only recognized Fcγ receptor on B cells, provides immunoglobulin G (IgG)–mediated negative modulation through a tyrosine-based inhibition motif, which down-regulates B cell receptor–initiated signaling. These properties make FcγRIIb a promising target for antibody-based therapy. We report the discovery of allele-dependent expression of the activating FcγRIIc on B cells. Identical to FcγRIIb in the extracellular domain, FcγRIIc has a tyrosine-based activation motif in its cytoplasmic domain. In both human B cells and B cells from mice transgenic for human FcγRIIc, FcγRIIc expression counterbalances the negative feedback of FcγRIIb and enhances humoral responses to immunization in mice and to BioThrax vaccination in a human anthrax vaccine trial. Moreover, the *FCGR2C*-ORF allele is associated with the risk of development of autoimmunity in humans. FcγRIIc expression on B cells challenges the prevailing paradigm of unidirectional negative feedback by IgG immune complexes via the inhibitory FcγRIIb, is a previously unrecognized determinant in human antibody/autoantibody responses, and opens the opportunity for more precise personalized use of B cell–targeted antibody-based therapy.

INTRODUCTION

Tight control of B cell receptor (BCR) signaling maintains the normal shape of humoral immunity. In both negative and positive selection, the BCR signal strength serves as a critical element in cell fate because only those cells with appropriate tonic BCR signaling successfully mature and differentiate. Thus, regulation of the B cell signaling pathway is critical in controlling immune responsiveness and the balance between tolerance and autoimmunity, making such regulation a hotspot for autoimmunity risk genes (*1*). A series of co-receptors and adaptors modulate BCR signaling (*2*), and immune complexes may suppress BCR-mediated activation through engagement of FcγRIIb (*3*). The importance of this regulation has been elegantly studied in mouse models, in which FcγRIIb serves as a switch between immune tolerance and autoimmunity. In such studies, a 30 to 40% restoration of FcγRIIb on B cells in *FCGR2B*$^{-/-}$ B6/129 mice decreased the autoantibody production and reversed the lupus-like disease progression (*4*). In human, decreased expression of FcγRIIb on memory B cells occurs in systemic lupus erythematosus (SLE) (*5*, *6*). Several functional polymorphisms in both the promoter and the transmembrane (TM) domains, which alter FcγRIIb expression and signaling, respectively, are associated with autoimmune diseases (*7*–*10*). Because of its key role in B cell fate, FcγRIIb is a promising therapeutic target for treating B cell–related immune diseases, including SLE and B cell lymphoma (*3*, *11*–*16*).

The human FcγRII (CD32) family also contains two activating receptors: FcγRIIa and FcγRIIc. Highly homologous in the extracellular (EC) domains, FcγRII family receptors diverge in their cytoplasmic (CY) domains with an ITIM-bearing sequence in FcγRIIb and identical ITAM-bearing sequences in FcγRIIa and FcγRIIc (*17*–*19*). *FCGR2C* is often regarded as a pseudogene because of a translation termination codon at codon 13 in its first EC domain. However, a nonsynonymous coding region single-nucleotide polymorphism (SNP) (rs10917661, nt202 T>C) in 7 to 15% of healthy individuals changes the stop codon (TAG) to an open reading frame (ORF) encoding glutamine (CAG) (Fig. 1A). Previous studies have indicated that FcγRIIc is expressed on natural killer (NK) cells from those individuals carrying the ORF allele and is associated with more severe rheumatoid arthritis (*20*, *21*). *FCGR2C*-ORF alleles may also be associated with idiopathic thrombocytopenic purpura (ITP) (*22*).

Using a pair of receptor-specific antibodies, we find full-length FcγRIIc protein in human B cells carrying the ORF polymorphism but not the homozygous stop codon. In multiple in vitro and ex vivo systems, the co–cross-linking of FcγRIIc and BCR leads to FcγRIIc tyrosine phosphorylation and enhanced BCR signaling. In a B cell–specific transgenic (TG) mouse model, expression of FcγRIIc enhanced responses to immunization. Similarly, in a human vaccine trial, healthy individuals with homozygous ORF alleles showed a 2.5-fold increase in the primary antibody response. Furthermore, The *FCGR2C*-ORF allele is also associated with the risk of developing systemic lupus in both

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Caucasians and African Americans. These data suggest that FcγRIIc expression on B cells contravenes FcγRIIb-mediated negative feedback and that the ORF allele is one important determinant in the human antibody responses.

RESULTS

FcγRIIc protein is expressed on B cells from ORF allele–positive individuals but not homozygous stop codon individuals

To study the expression profile of the *FCGR2* family genes, we performed reverse transcription polymerase chain reaction (RT-PCR) using RNA from B cells homozygous for either the ORF or the STP allele of *FCGR2C* (Fig. 1A). Consistent with current understanding, we found the expression of mRNA for the inhibitory *FCGR2B* but not mRNA for *FCGR2A* in human B cells (*23*). Surprisingly, we also found abundant mRNA for the activating *FCGR2C* (Fig. 1B). In contrast, using RNA from the human myeloid cell line U937, *FCGR2A* and *FCGR2B*, but not *FCGR2C*, transcripts were amplified. This observation prompted us to explore the potential expression of FcγRIIc receptor in B cells.

For more than 30 years, immunoglobulin G (IgG)–mediated feedback regulation of B cell activity has been ascribed to the inhibitory FcγRIIb (*3*). We sought FcγRIIc protein in Epstein-Barr virus (EBV)–transformed human B cells and primary B cells from genotyped healthy donors, each confirmed by sequencing of full-length *FCGR2C*. Cells were lysed and receptor protein was immunoprecipitated with our recently developed FcγRIIb/c-EC domain–specific monoclonal antibody (mAb) 4F5 (*6*). The CY domain of FcγRIIa/c was found in B cells from ORF$^+$ individuals, whereas the absence of the FcγRIIa/c-CY corresponded with the homozygous STP genotype (Fig. 1C). Probing with a pAb against the FcγRIIb-CY tail demonstrated FcγRIIb protein in all subjects.

Expression of FcγRIIc on the cell surface as a mature receptor was confirmed using fluorescence microscopy. Positive staining for both the FcγRIIb/c-EC and the FcγRIIc-CY with domain-specific mAbs (fig. S1) was established in ORF-bearing B cells (Fig. 2A) with >90% of FcγRIIb/c-EC–positive cells also positive for FcγRIIc-CY. Upon receptor cross-linking, clustering of both FcγRIIb/c-EC and FcγRIIc-CY was clearly evident in the ORF cells (Fig. 2A), whereas STP homozygous B cells showed no staining for FcγRIIc-CY (Fig. 2, C and F; $P < 0.001$). Both the ORF and STP cells stained positive for FcγRIIb-CY (Fig. 2, B and D), with >85 and >90% of the STP and the ORF cells positive for both FcγRIIb/c-EC and FcγRIIb-CY. No difference in clustering of anti-FcγRIIb mAbs was observed between B cells from STP- and ORF-positive cells (Fig. 2F). The absence of FcγRIIa-EC staining in ORF$^+$ cells further confirmed that there is no FcγRIIa protein expressed by B cells (Fig. 2E). These data demonstrate that FcγRIIc protein is expressed on the surface of B lymphocytes from individuals with the ORF allele. Furthermore, the total FcγRII expression on B cells increases with increasing numbers of *FCGR2C*-ORF alleles in EBV-transformed cell lines, primary B cells, and, most strikingly, primary memory B cells ($P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively; Fig. 3).

FcγRIIc enhanced BCR-induced signaling in in vitro and ex vivo systems

To evaluate the signaling potential of FcγRIIc, we retrovirally transduced human *FCGR2C* or *FCGR2B* complementary DNAs (cDNAs) in the FcR-deficient, surface IgG BCR–expressing A20-IIA1.6 mouse B cell line (fig. S2). Coligation of transduced receptor with BCR was compared to engagement of BCR alone by using equimolar amount of either intact or F(ab′)$_2$ fragments of anti-Ig antibody. Coligation of FcγRIIc to BCR greatly enhanced total whole-cell tyrosine phosphorylation compared with BCR engagement alone (Fig. 4A), whereas, in contrast, FcγRIIb/BCR coligation recapitulated the known inhibitory effect of FcγRIIb (Fig. 4B). FcγRIIc/BCR coligation also caused rapid tyrosine phosphorylation of FcγRIIc itself, reaching maximal level in 1 to 3 min (Fig. 4C). This coligation also resulted in enhanced and more sustained tyrosine phosphorylation of the key B cell signaling

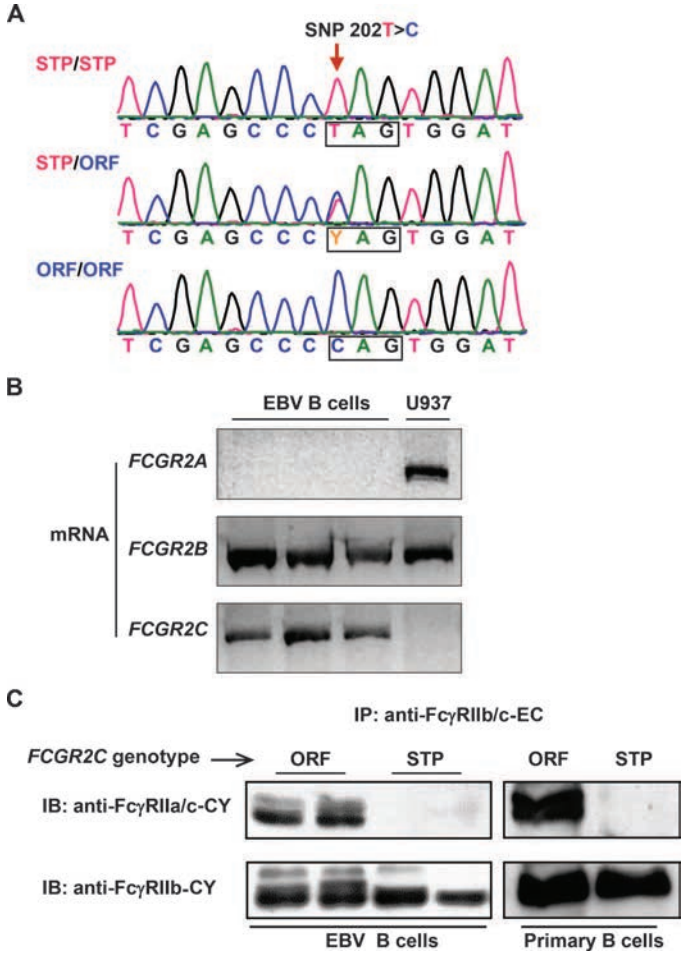

Fig. 1. Expression of FcγRIIc protein in B cells. (**A**) Chromatograms showing the rs10917661 (nt202 T>C) polymorphism (amino acid position 13) in the first EC domain of *FCGR2C*. (**B**) RT-PCR detection of *FCGR2A*, *FCGR2B*, and *FCGR2C* mRNA in EBV B cells containing either the *FCGR2C*-ORF (lanes 1 and 2 from left) or *FCGR2C*-STP allele (lane 3). RT-PCR analysis of human myeloid cell line U937 cells using the same primers is used as a control for *FCGR2A/B/C* message (lane 4). Data are representative of two independent experiments. (**C**) Lysates of EBV B cells or primary human CD19$^+$ cells were immunoprecipitated with mAb 4F5 recognizing the FcγRIIb/c-EC domain and then blotted with either anti–FcγRIIc-CY polyclonal antibody (pAb) or anti–FcγRIIb-CY pAb. FcγRIIc protein was detected as a protein with the FcγRIIb-EC domain and FcγRIIc-CY tail only in ORF B cells but not in STP B cells. Data are representative of three independent experiments.

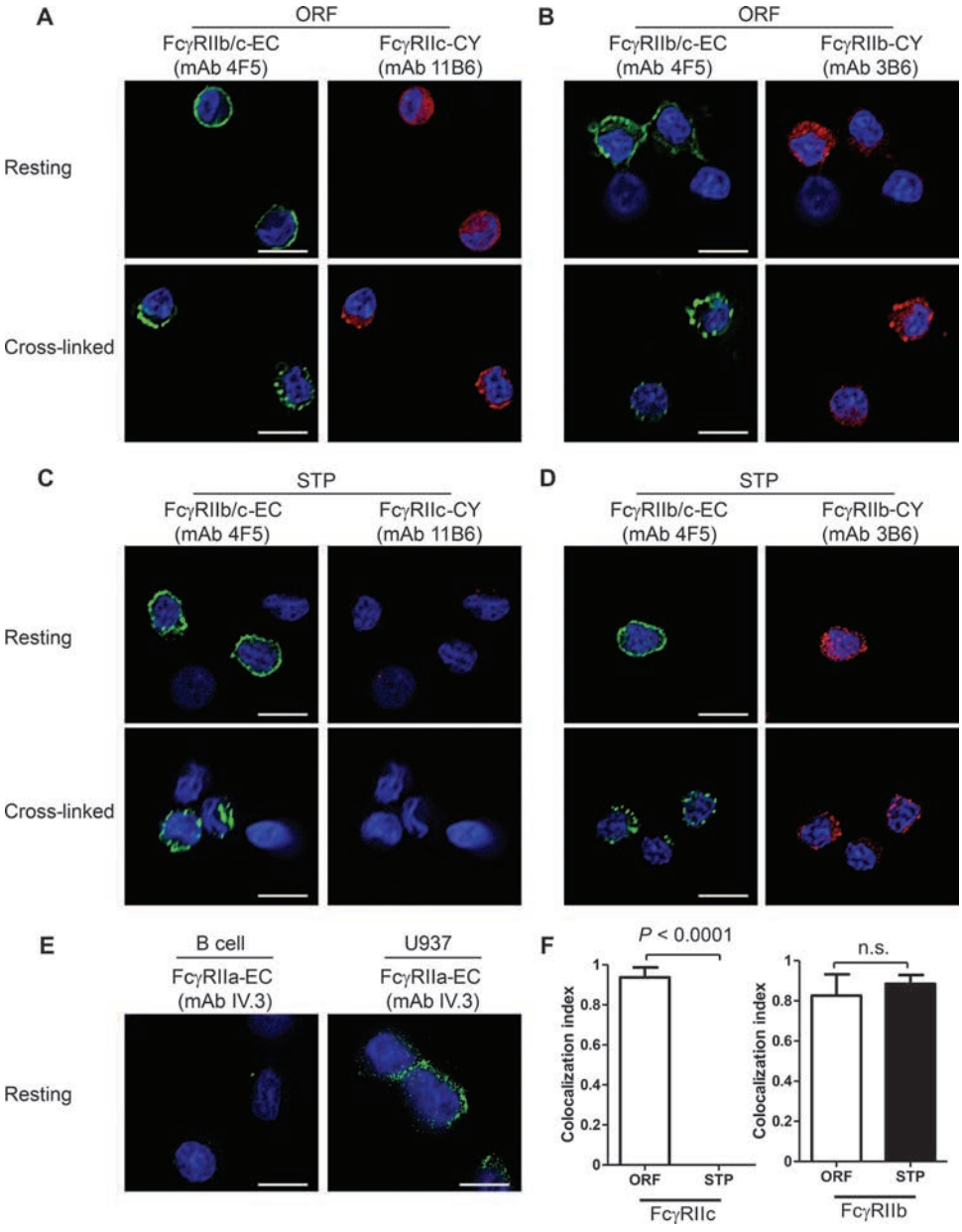

Fig. 2. Surface expression of FcγRIIc protein on human B cells. (**A** to **D**) EBV-transformed human B cells from individuals containing the *FCGR2C*-ORF allele (A and B) or that are homozygous for the *FCGR2C*-STP allele (C and D) were opsonized with mAb 4F5 (green) either alone or with cross-linking using a secondary antibody and then intracellularly stained for the FcγRIIc-CY domain (red). (**E**) *FCGR2C*-ORF allele–positive EBV-transformed human B cells were incubated with a mAb specific to FcγRIIa EC and showed no staining, comparing to the positive staining of FcγRIIa EC in U937 cells. Results are representative of three independent experiments. Scale bars, 10 μm. (**F**) Quantification of the EC (green) and the CY (red) staining of FcγRIIc or FcγRIIb colocalization after cross-linking, calculated by MetaMorph software from six to eight randomly chosen cells.

components Syk and BLNK (Fig. 4E). In contrast, FcγRIIb engagement with BCR and its activation (Fig. 4D) caused a reduced level of Syk and BLNK phosphorylation (Fig. 4F).

Considering the potent effect of the FcγRIIc on BCR-induced tyrosine phosphorylation, we next examined the effects of FcγRIIc on BCR-induced calcium flux. BCR engagement with $F(ab')_2$ resulted in a typical calcium flux (Fig. 4G, blue tracing), and this response was enhanced when FcγRIIc was coengaged. In contrast, FcγRIIb/BCR co–cross-linking resulted in a reduced calcium flux. In control experiments with cells transduced with empty vector, both stimuli elicited same levels of calcium mobilization (Fig. 4G).

In primary human B cells, both FcγRIIb and FcγRIIc are coexpressed in ORF^+ individuals, and this dual expression could alter the net magnitude of B cell activation. Indeed, in B cells from the ORF donors, the participation of FcγRIIc not only offset the inhibitory effect of FcγRIIb on BCR signaling but also further enhanced the level of both Syk phosphorylation by nearly twofold ($n = 6$, $P = 0.024$) (Fig. 4H) and quantitative rinse in intracellular Ca^{2+} ($n = 5$, $P = 0.032$) (Fig. 4I). As expected, B cells from homozygous STP donors stimulated with intact anti-Ig showed a modestly decreased level of surface Ig signaling, consistent with previous studies in primary human B cells (*5*).

Expression of human FcγRIIc on B cells from TG mice enhances B cell functions

To directly assess whether B cell–specific expression of the activating FcγRIIc would alter quantitative B cell responses in vivo, we generated B cell–specific FcγRIIc-expressing TG mice on C57BL/6 background, driven by V_H promoter combined with μ and κ enhancers, as used in previous studies (*24*, *25*) (Fig. 5A). Demonstrated by both flow cytometric analysis (Fig. 5B and fig. S3) and Western blot (Fig. 5C), the transgene was expressed in B cells but no other cell types in TG mice. The transgene expression did not affect endogenous mouse FcγRIIb expression (Fig. 5B). Major B cell subsets in young mice (6 weeks old) were normal in proportion compared to wild-type littermates (fig. S4). Examination of naïve IgG and IgM levels in 10-week-old mice showed no significant difference between TG mice and their nontransgenic (NTG) littermates (fig. S5).

The functional consequences of B cell FcγRIIc expression were tested in a series of ex vivo assays. Purified splenic B cells were stimulated either with anti-IgM $F(ab')_2$ to cross-link BCR alone or with intact anti-IgM IgG antibody to simultaneously coengage human FcγRIIc, mouse FcγRIIb, and BCR. First, when coligated with BCR, the transgene itself was activated (Fig. 5D). Second, as expected, in NTG littermates, mFcγRIIb/BCR coligation

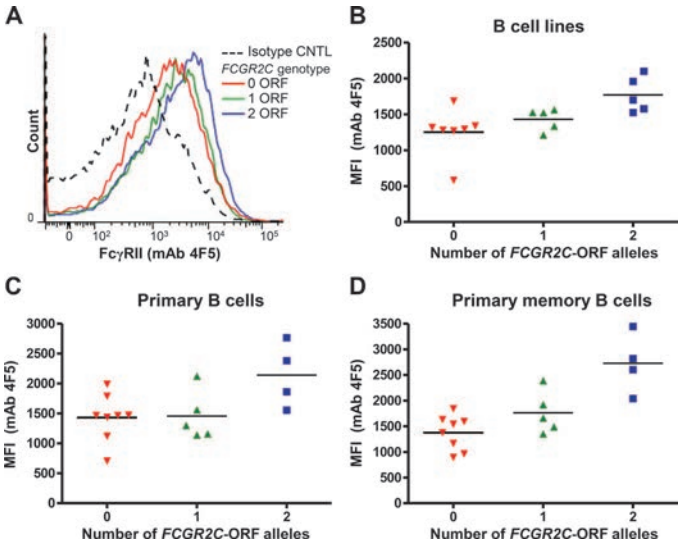

Fig. 3. Higher total FcγRII expression on B cells with increasing numbers of the *FCGR2C*-ORF allele. (**A**) Representative results of mAb 4F5 staining on EBV B cell lines derived from donors with different numbers of *FCGR2C*-ORF alleles. (**B**) A summary of increasing mAb 4F5 staining on EBV B cell lines derived from donors with different numbers of *FCGR2C*-ORF alleles [$P = 0.017$, analysis of variance (ANOVA)]. (**C** and **D**) Summary of increasing mAb 4F5 staining of total FcγRII (FcγRIIb + FcγRIIc) expression on primary $CD19^+$ B cells (C) and $CD19^+CD27^+$ memory B cells (D) from donors with different numbers of *FCGR2C*-ORF alleles ($P = 0.042$ and 0.0005, respectively, ANOVA).

suppressed B cell activation (Fig. 5E). In contrast, in TG mice, the involvement of FcγRIIc not only completely neutralized the inhibitory effect of mouse FcγRIIb but also resulted in amplified B cell activation signals as depicted by increased Syk phosphorylation. Downstream of the BCR signaling, calcium mobilization was also enhanced in the presence of FcγRIIc (Fig. 5F). A higher level of B cell activation was also evident by increased expression of CD69 and proliferation (fig. S6). Notably, the expression of BR3 (*TNFRSF13C*), the receptor for B cell–activating factor (BLyS/BAFF), is down-regulated by FcγRIIb/BCR interaction (*26*). In our TG mice, however, this down-regulation was also attenuated, suggesting the possibility that FcγRIIc might regulate B cell maturation, development, and the ability to produce antibodies on multiple levels (Fig. 5G).

Immune responses to vaccination are enhanced in B cell–specific FcγRIIc TG mice

To examine the biological effect of FcγRIIc expression on B cells in vivo, we immunized TG mice and their wild-type littermates with TNP-Ficoll (Fig. 6, A and B) as a model T-independent antigen or TNP-CGG/alum as a model T-dependent antigen (Fig. 6, C and D). In both cases, FcγRIIc TG mice showed a significant increase in IgM-specific antibody titers comparable in magnitude to the changes observed in FcγRIIb-deficient mice (*27*). Although not statistically significant, the IgG3 antibody response to TNF-Ficoll was also increased in mice TG for human *FCGR2C*-ORF compared to NTG littermates (Fig. 6B). Most striking is the increase in the T-dependent IgG1 response, with TNP-CGG/alum eliciting a twofold increase in antibody production in TG mice compared to NTG littermates ($P = 0.006$) (Fig. 6D). Relative affinity maturation was assessed by TNP_2 enzyme-linked immunosorbent assay (ELISA) after secondary immunization. The high-affinity IgG1 titers at days 40 and 51were enhanced in TG mice compared to NTG littermates ($P = 0.0097$ and 0.027 respectively) (Fig. 6E), and this increased affinity was corroborated by surface plasmon resonance (SPR) analysis of anti-TNP reactivity at the day 40 time point in TG compared to NTG littermates ($P = 0.041$; fig. S7).

Antibody responses are enhanced in a human vaccine trial

The significance of FcγRIIc expression in humoral responses was explored in a human trial of the anthrax vaccine adsorbed (AVA) vaccine initiated by the Centers for Disease Control and Prevention (CDC) (clinicaltrials.gov identifier NCT00119067) (*28*, *29*). This study provided a paradigm for a naïve primary response to antigen and allowed us to assess the role of the *FCGR2C*-ORF in an early human humoral immune response (Fig. 7A and fig. S8, demographics summarized in table S1). In the analysis of antibody against protective antigen (AbPA) levels in 366 vaccine recipients at the earliest time point assessed in the trial (4 weeks), *FCGR2C*-ORF^+ subjects in the groups that received four immunizations [the four intramuscular (4IM) or subcutaneous (4SQ) vaccine administrations] were more likely to respond to vaccination (fig. S8) and, among responders, displayed a 2.5-fold higher AbPA production (4 weeks, $P < 0.02$) (Fig. 7A). The magnitude of the effect is comparable to that established for other genetic variants on former human vaccine responses (*30*). Higher quantitative AbPA levels were also noted at the 8-week time point but did not reach statistical significance (Fig. 7A). Functional nonsynonymous variants in other Fcγ receptors (*FCGR2A*, *FCGR3A*, and *FCGR3B*) did not show association with AbPA levels in these studies.

Recognizing the potential role for FcγRIIb and its functionally important *FCGR2B*-TM allelic variant (rs1050501) on AbPA responses, the association of *FCGR2C*-ORF on response was analyzed by stratifying the *FCGR2B*-TM allelic status. With this integrated *FCGR2B-FCGR2C* analytical approach in the subpopulation of participants with the *FCGR2B*-TM common variant, the association of *FCGR2C*-ORF with AbPA responses was significant in the 4IM/4SQ group ($n = 366$, $P = 0.046$). Furthermore, this association was replicated in an independent vaccine cohort composed of the group of AVA vaccine participants that received three immunizations (the 3IM group) ($n = 525$, $P = 0.0012$). These results demonstrated the in vivo biological consequences of the *FCGR2C*-ORF variant in humoral responses in humans.

FCGR2C-ORF allele is associated with autoimmunity in humans

Considering the impact of FcγRIIc on B cell function, we speculated that there might be an association between the *FCGR2C*-ORF and humoral autoimmunity in humans. SLE is a prototypic immune complex–mediated autoimmune disease, and functionally important genetic variants in *FCGR2A*, *FCGR2B*, *FCGR3A*, and *FCGR3B*, including both SNPs and CNVs (copy number variations), are associated with the development and progression of SLE (*19*, *31*). We hypothesized that not only the presence of the *FCGR2C*-ORF allele but also the number of ORF alleles would be important in the development of autoimmunity. Accordingly, we assessed the frequency of each *FCGR2C*-ORF copy number variant ($n = 1$ through $n = 4$ copies of *FCGR2C*-ORF) in

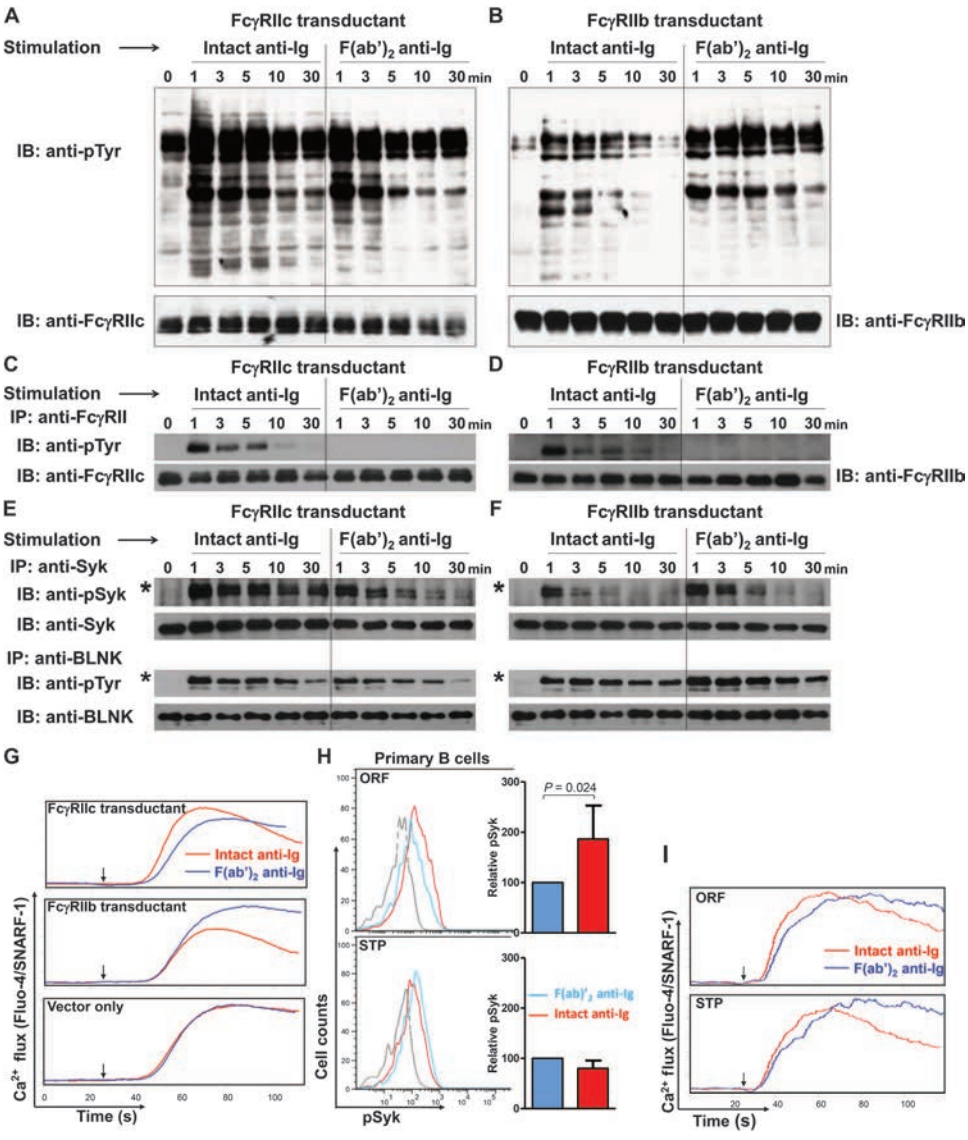

Fig. 4. Activating properties of FcγRIIc in transduced A20-IIA1.6 cells and primary human B cells. (**A** and **B**) Cellular tyrosine phosphorylation in FcγRIIc (A)– or FcγRIIb (B)–transduced IIA1.6 cells upon coligation with BCR. Cells were stimulated with equal molar amount of intact (25 μg/ml) or $F(ab')_2$ fragment (16.6 μg/ml) of goat anti-mouse IgG for indicated times, affording BCR/FcγRIIc coligation or BCR cross-linking alone. Whole-cell lysates were also reprobed for FcγRIIc (A) or FcγRIIb (B) to verify receptor expression and comparable protein loading. (**C** and **D**) Tyrosine phosphorylation of FcγRIIc (C) or FcγRIIb (D) upon co–cross-linking with BCR. (**E** and **F**) Tyrosine phosphorylation of Syk and BLNK detected by immunoprecipitation. Normalized intensities of both phospho-Syk (pSyk) and phospho-BLNK (pBLNK) are significantly increased with FcγRIIc/BCR coligation and decreased with FcγRIIb/BCR coligation comparing to BCR ligation alone [*P = 0.002 (pSyk) and 0.003 (pBLNK), ANOVA (E); *P = 0.033 (pSyk) and 0.0097 (pBLNK), ANOVA (F)]. Data are representative of two replicate experiments. (**G**) Ca^{2+} flux in different transductants evaluated by flow cytometry as Fluo-4/SNARF-1 ratio. Arrows indicate time points of adding indicated stimuli. Data are representative of three independent experiments. (**H**) Human peripheral blood mononuclear cells (PBMCs) were stimulated as indicated, and the level of Syk phosphorylation was quantitated in $CD20^+$ B cells by fluorescence-activated cell sorting (FACS). Gray line indicates baseline of pSyk in unstimulated cells. Net effect of FcγRIIc is analyzed by comparing mean fluorescence intensity (MFI) of pSyk with equal molar amount of intact (15 μg/ml) versus $F(ab')_2$ (10 μg/ml) of goat anti-human (IgG + IgM) stimulation (mean ± SEM; n = 6; P = 0.024, t test). (**I**) Ca^{2+} flux in primary B cells evaluated by flow cytometry. Data are representative of five independent experiments (P = 0.032, t test).

African American (Fig. 7B) and European American (Fig. 7C) SLE cases and healthy controls. The cumulative frequency of the *FCGR2C*-ORF allele is enriched in patients with SLE in both groups. Our combined analysis of 2850 cases and controls from the two different populations (demographics summarized in table S2) demonstrates that the *FCGR2C*-ORF allele contributes to the predisposition to development of SLE (P = 0.018, Fig. 7D). The magnitude of this association (OR, 1.20; 95% confidence interval, 1.04 to 1.39) is comparable to the magnitude of other well-established genetic effects in SLE (*32*).

DISCUSSION

The balance between negative and positive regulatory elements is important in maintaining an appropriate B cell activation threshold and self-tolerance. Many autoimmunity risk alleles have been identified in B cell signaling pathways, including FcγRIIb (*1*), which is the only recognized classical Fc receptor expressed on B cells and can function as a brake when the BCR interacts with immune complexes (*33*). The balance of positive and negative regulation of BCR signaling cascades is tightly regulated, and subtle changes in the balance of this system can lead to either B cell immune tolerance or autoimmunity (*1*, *2*, *4*, *34*).

We demonstrate the unexpected finding of the expression of the activating CD32C Fc receptor for IgG on B cells. Individuals with the *FCGR2C*-ORF allele express FcγRIIc protein on B cells. FcγRIIc is activated upon engagement with BCR, and its presence enhances BCR responsiveness on multiple levels, from upstream signaling events including phosphorylation of Syk and increases in intracellular Ca^{2+} to antibody production. We also provide evidence that in human, the *FCGR2C*-ORF allele is part of the portfolio of regulatory genes associated with lupus.

With nearly identical EC sequence and with complete identity in the ligand-binding site, FcγRIIc competes with FcγRIIb for ligand binding and counterbalances the negative feedback mediated by FcγRIIb, thus revising the classical paradigm of antibody-mediated feedback inhibition of B cell activation. The presence of FcγRIIc

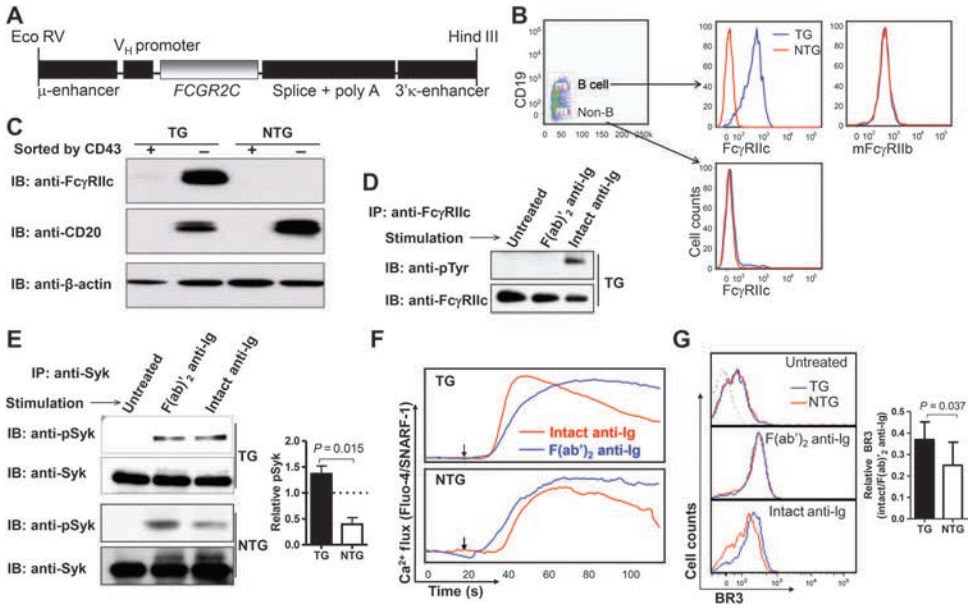

Fig. 5. Generation and characterization of B cell–specific FcγRIIc TG mice. (**A**) Schematic illustration of the fragment used to inject fertilized mouse eggs. (**B**) FACS analysis of FcγRIIc transgene and endogenous mouse FcγRIIb expression in splenic B cells from TG and NTG littermates. Data are representative of three independent experiments. (**C**) Demonstration of FcγRIIc protein in CD43$^-$ splenocytes (CD43$^-$ = B cells, CD43$^+$ = non–B cells) by Western blot. CD20 and β-actin blots are shown as controls. (**D**) Tyrosine phosphorylation of FcγRIIc after coengagement with intact anti-Ig. Data are representative of two independent experiments. (**E**) Syk phosphorylation in splenic B cells stimulated as indicated. Relative pSyk is analyzed by normalizing intensity of pSyk bands to corresponding Syk bands and then comparing intact (15 μg/ml) versus F(ab')$_2$ (10 μg/ml) goat anti-mouse IgM stimulation (mean ± SEM; n = 2; P = 0.015, t test). (**F**) Ca^{2+} flux in splenic B220$^+$ cells was evaluated by flow cytometry as Fluo-4/SNARF-1 ratio. Arrows indicate time points of adding indicated stimuli. Data are representative of three independent experiments. (**G**) Surface BR3 expression on splenic B cells after 72 hours of treatment with indicated stimuli. Net effect of FcγRIIc is analyzed by comparing MFI of BR3 with equal molar amount of intact (15 μg/ml) versus F(ab')$_2$ (10 μg/ml) goat anti-mouse IgM stimulation (mean ± SEM; n = 3; P = 0.024, t test).

protein on B cells in ORF-positive donors may impinge on efforts to target FcγRIIb therapeutically in B cell–related diseases such as autoimmunity and B cell lymphoma. Such targeting might result in engagement of activating FcγRIIc in individuals with the *FCGR2C*-ORF allele and change the ability of therapeutic mAbs to alter B cell functions. Thus, targeting of B cell Fc receptors may provide a paradigm for personalized therapeutic approach based on an understanding of *FCGR2C* genotype (*16*).

FcγRIIc is often thought to be a pseudogene because of the high prevalence of the STP allele. With more than 95% identity of the EC domain, it has been technically difficult to distinguish FcγRIIa/b/c. For this reason, expression and function of FcγRIIc have been explored in NK cells, considering that NK cells typically carry message for *FCGR2C* but not *FCGR2A* or *FCGR2B* (*20*, *21*, *35*, *36*). The expression of FcγRIIc on NK cells may enhance NK cell proinflammatory functions and has been reported to influence disease severity in rheumatoid arthritis (*21*). Furthermore, CNV of *FCGR2C*-ORF is associated with ITP (*22*), supporting the idea of FcγRIIc involvement in autoimmunity.

In our *FCGR2C* TG mice, B cell–restricted expression of FcγRIIc was sufficient to prompt a twofold increase in titers of TNP-specific IgM and IgG3 in T-independent responses and TNP-specific IgM and IgG1 in T-dependent responses throughout the course of measurement. The effect was more noticeable in the primary stage after immunization. The effect of Fc receptors on the early immune response has also been shown in a recent study in mouse models of a wild mouse *FCGR2B* promoter variant (*34*). Similarly, in our human anthrax vaccine trials, we noticed an association of *FCGR2C*-ORF with higher anti-PA IgG titers in the very early stage of vaccination. There was no demonstrable difference in the extended period of vaccination, and we detected no signal from variants of other Fcγ receptors, including *FCGR2A*, *FCGR3A*, and *FCGR3B*.

We also observed the involvement of *FCGR2C*-ORF allele in the predisposition of humoral autoimmunity in human. In both African

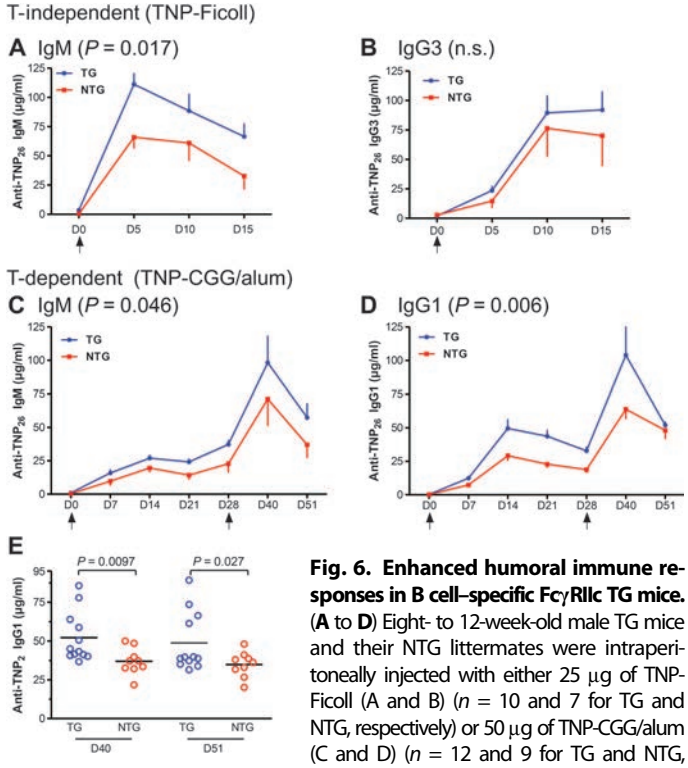

Fig. 6. Enhanced humoral immune responses in B cell–specific FcγRIIc TG mice. (**A** to **D**) Eight- to 12-week-old male TG mice and their NTG littermates were intraperitoneally injected with either 25 μg of TNP-Ficoll (A and B) (n = 10 and 7 for TG and NTG, respectively) or 50 μg of TNP-CGG/alum (C and D) (n = 12 and 9 for TG and NTG, respectively) on day 0 and bled at various time points as indicated. For T-dependent experiments, animals received a secondary immunization on day 28. Serum antibody titers were measured by ELISA using TNP$_{26}$-coated plates. Data are presented as means ± SEM, and group effects were analyzed by repeated-measures ANOVA. (**E**) Production of high-affinity antibody after boost was measured by ELISA using TNP$_2$-coated plates (P = 0.0097 for day 40 and 0.027 for day 51, t test).

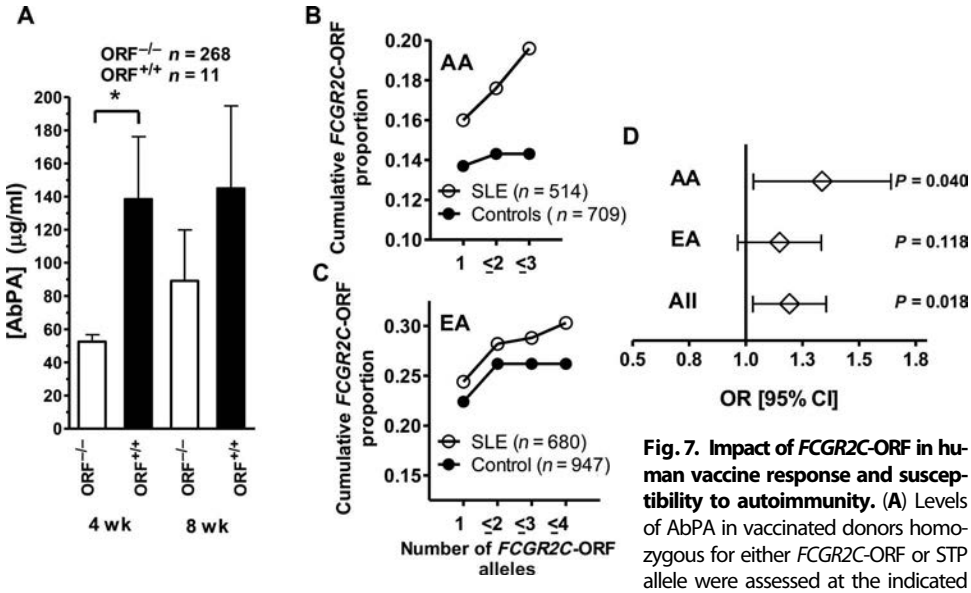

Fig. 7. Impact of *FCGR2C*-ORF in human vaccine response and susceptibility to autoimmunity. (**A**) Levels of AbPA in vaccinated donors homozygous for either *FCGR2C*-ORF or STP allele were assessed at the indicated time points. AbPA levels determined by ELISA were analyzed by analysis of covariance (ANCOVA), and AbPA levels at the earliest time points in the vaccine study are higher in donors homozygous for the ORF allele with significance reached at the 4-week time point ($P < 0.02$). (**B** and **C**) The cumulative proportion of *FCGR2C*-ORF–positive participants with increasing gene copy number in (B) African Americans (AA) and in (C) European Americans (EA) in patients with SLE and healthy controls. (**D**) Determination of the effect size of the *FCGR2C*-ORF allelic association with the risk of development of SLE. The odds ratio (OR) and *P* values, determined by logistic regression, between the number of *FCGR2C*-ORF alleles and the lupus phenotype are shown. CI, confidence interval.

Americans and European Americans, the frequency of ORF allele copies, ranging from one copy to three and four copies in the African American and European American patient population, respectively, is enriched in patients with SLE, a prototypic immune complex disease. SLE, as with many other complex autoimmune diseases, is influenced by many genetic and environmental factors. For many multigenic diseases, risk genes likely contribute in an additive manner, with each variant conferring relatively small increment in risk (*32*). In the family of Fcγ receptors, CNV and multiple SNPs, affecting either expression level, signaling capacity, or binding affinity, contribute to the susceptibility of autoimmune/inflammatory diseases. *FCGR2C*-ORF provides an additional risk allele to the genetic diversity in the Fcγ receptor cluster, which, in turn, may collectively impact the balance between proactive and inhibitory edges in different immune cells and determine the overall predisposition to autoimmunity.

Our studies have delineated the unappreciated expression of the activating FcγRIIc on circulating B cells in individuals with the *FCGR2C*-ORF allele. However, there are still many questions that are unexplored, including the role of FcγRIIc on B cell maturation and maintenance of immunologic tolerance. Our data also do not address the potential for FcγRIIc expression on other cell types. Indeed, the expression of FcγRIIc has been demonstrated on NK cells (*35*, *37*), and it is likely that other cell types such as myeloid cells will also express this protein. Future studies will be required to determine the functional importance of this receptor in other cell lineages.

In conclusion, we identified the activating FcγRIIc as a second classical FcγR on human B cells. Its expression is determined by an ORF/STP SNP in its first EC domain. Through modulation of BCR signaling, FcγRIIc can determine the net magnitude of stimulatory effects, set new thresholds for humoral immune responses to vaccines and autoantigens, and potentially remodel the biology of B cell responses.

MATERIALS AND METHODS

Study design

To determine the expression and functional properties of FcγRIIc in human B cells, we performed studies with B cells isolated from peripheral blood from genotyped healthy participants and from cell lines. All experiments were repeated at least three times. The importance of the *FCGR2C*-ORF allele, which encodes the FcγRIIc protein, was assessed in two separate human population-based studies. The influence of FcγRIIc on vaccine response in humans was assessed using DNA and quantitative antibody response data in the AVA trial (clinicaltrials.gov identifier NCT00119067) (*29*). This study was a multisite, randomized, double-blind, noninferiority, phase 4 human clinical trial to assess serologic responses and injection adverse events using different routes and schedules of vaccine immunization. The genetic association of the *FCGR2C*-ORF allele with SLE was assessed in an existing collection of DNAs from the PROFILE cohort composed of patients with SLE and healthy controls (*38*, *39*). Genotyping of all samples was performed in a blinded manner.

To further explore the role of B cell expression of FcγRIIc, we developed a TG mouse model in which cDNA from the human *FCGR2C*-ORF allele is expressed on murine CD19-positive B cells. The origin of cells isolated for ex vivo studies was coded to the experimenter and was repeated at least three times. Experimental immunization studies were performed in randomly chosen TG and control littermates, with 8 to 12 animals in each group, and serum was analyzed in a blinded manner.

Participants

For the analysis of *FCGR2C* genotype in the AVA trial (clinicaltrials.gov identifier NCT00119067) (*29*), DNA from 366 individuals enrolled into the four-dose vaccination groups (4IM and 4SQ) and from 525 individuals enrolled into the three-dose vaccination group (3IM) was available. Individuals were of European American or African American ancestry as determined by self-report and principal components analysis (*28*). Participant demographics are shown in table S1. One thousand one hundred ninety-four patients with SLE were participants in the longitudinal PROFILE cohort as previously described (*38*, *40*). Patients and 1656 controls were from the University of Alabama at Birmingham (UAB), Johns Hopkins University, Northwestern University, and University of Texas Health Science Center. All patients were seen by a study physician and met the revised American College of Rheumatology criteria for the diagnosis of SLE (*41*, *42*). The clinical characteristics of patients in the PROFILE study have been published (*39*). Baseline demographic features

for the PROFILE study participants are summarized in table S2. For in vitro experiments, PBMCs were isolated by Ficoll-Hypaque gradient centrifugation as previously described (*43*). $CD19^+$ cells were further purified using Magnetic MicroBeads (Miltenyi Biotec). All participants provided written informed consent, and these studies were approved by the Institutional Review Board for Human Use.

Cell lines and animals

EBV-transformed human B cell lines from various *FCGR2C*-genotyped donors were maintained in RPMI 1640 medium (Invitrogen Life Technologies). The mouse A20-IIA1.6 B cell lines (provided by T. Wade, Dartmouth Medical Center) were maintained in Dulbecco's modified Eagle's medium (Invitrogen). All cell lines were tested negative for *Mycoplasma* contamination. Medium was supplemented with 10% fetal bovine serum (FBS), 25 mM Hepes, penicillin (100 U/ml), and streptomycin (100 μg/ml). For stable transductants, FcγRIIc and FcγRIIb cDNAs were cloned into pMX-PIE retroviral vector with enhanced green fluorescent protein. Retroviral transduction was done as described previously (*44*). Thanks to the gift from T. Tsubata (Tokyo Medical and Dental University, Tokyo, Japan), we constructed a transgene sequence with *FCGR2C* cDNA, human V_H promoter, and both μ and κ enhancers, as previously described (*24*, *25*). TG mice were established at UAB Transgenic Mouse Facility by injecting the DNA fragment into fertilized oocytes from C57BL/6 mice (The Jackson Laboratory). The presence of transgene was identified by genotyping of tail DNA (primers are listed in table S3). All studies were performed with approval from the UAB Institutional Animal Care and Use Committee.

Antibodies

Anti-FcγRIIb/c pAb was obtained from Santa Cruz Biotechnology (sc-12815). Anti-FcγRIIb mAb 4F5 was made in our laboratory as previously described (*6*). Anti-FcγRIIa mAb IV.3 hybridoma was purchased from the American Type Culture Collection. Goat anti-human IgG and $F(ab')_2$ were obtained from Jackson ImmunoResearch Laboratories. IgG isotype controls were from Sigma. Anti–phosphotyrosine 4G10 was from Upstate Biotechnologies (05-321). Anti-pSyk (2710), Syk (12358), and BLNK (12168) antibodies were purchased from Cell Signaling Technology. Phospho-Syk (Y352)/Zap70 (Y319) (557881) and CD20 CY (clone H1) (555677) antibodies were obtained from BD Pharmingen. Anti–FcγRIIb-CY mAb 3B6 and anti–FcγRIIa/c-CY mAb 11B6 were generated at the UAB Epitope Recognition and Immunoreagent Core facility by immunizing BALB/c mice with FcγRIIb or FcγRIIc polypeptides (fig. S1).

Reverse transcription polymerase chain reaction

Total RNA was prepared from cells using TRIzol Reagents (Invitrogen Life Technologies). The RT-PCR for *FCGR2A*, *FCGR2B*, and *FCGR2C* genes was performed using the SuperScript III First-Strand kit (Invitrogen Life Technologies) followed by PCR with gene-specific primers as listed in table S3.

Immunocytochemistry

Cells were labeled with mAb 4F5–Alexa 488 (10 μg/ml) for 30 min on ice, goat anti-mouse $F(ab')_2$ (15 μg/ml) was added, and then cells were incubated at 4° or 37°C for 15 min. Cells were then fixed with 4% paraformaldehyde for 15 min at room temperature. After centrifuging onto glass slides, cells were permeabilized with 0.1% Triton, blocked with 5% FBS/10% goat serum, and stained with mAb 3B6 or mAb 11B6 labeled with Zenon Alexa 594 (Molecular Probes). Images were acquired with Nikon Eclipse TE-2000U inverted high-resolution digital microscope and MetaMorph software.

In vitro stimulation

Stable transductants were stimulated with equal molar amount of intact (25 μg/ml) or $F(ab')_2$ fragment (16.6 μg/ml) of goat anti-mouse IgG (γ chain–specific) antibodies for various times at 37°C. Primary human cells were stimulated with IgG (15 μg/ml) or $F(ab')_2$ fragments (10 μg/ml) of goat anti-human (IgG + IgM) (μ + γ) antibodies (Jackson ImmunoResearch Laboratories) for indicated times at 37°C.

Immunoprecipitation and immunoblot

Cells were lysed, and immunoprecipitation/immunoblotting was performed as described (*6*, *43*). To examine tyrosine phosphorylation, whole-cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose for blotting. Protein abundance was quantified by analyzing bands with ImageJ software.

Analysis of calcium flux

Cells were incubated at 37°C for 30 min with 2 μM Fluo-4 AM and 4 μM SNARF-1 (Molecular Probes). After washing, cells were resuspended at 5×10^6 cells/ml in modified Hanks' balanced salt solution (1.1 mM $CaCl_2$, 1.6 mM $MgCl_2$). Calcium concentration was detected by flow cytometry as Fluo-4/SNARF-1 ratio. Data were analyzed using FlowJo (Tree Star).

Mouse B cell purification and ex vivo stimulation

Splenic B cells were negatively purified using EasySep mouse B cell enrichment kit (Stemcell Technologies) (>97% purity). For ex vivo experiments, purified B cells were cultured at 2×10^6 cells/ml in RPMI 1640/10% FBS in the presence or absence of either $F(ab')_2$ (10 μg/ml) or intact (15 μg/ml) goat anti-mouse IgM (μ chain–specific) (Jackson ImmunoResearch Laboratories). For proliferation assays, cells were preloaded with 5 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen) before culturing.

T-independent and T-dependent immunization

Eight- to 12-week-old, gender- and age-matched mice received intraperitoneal injection of 25 μg of TNP-Ficoll alone for T-independent immunization, or 50 μg of TNP–chicken γ-globulin (CGG) precipitated in alum (Imject, Thermo Scientific) for T-dependent immunization. For the secondary response, mice were immunized with a further 50 μg of TNP-CGG/alum on day 28. Littermates were screened for expression of transgene and then randomly selected to form experimental groups. Sera were collected before initial immunization and after immunization at various time points through submandibular bleeding using the Goldenrod animal bleeding lancet (*45*).

Enzyme-linked immunosorbent assay

Antibody responses to TNF-Ficoll and TNP-CGG in mice were assessed by ELISA. Ninety-six–well plates (Costar 9018; Thermo Fisher Scientific) were coated overnight at 4°C with TNP-conjugated bovine serum albumin (5 μg/ml) (using TNP-BSA conjugated at a molar ratio of either 2 or 26 TNP to BSA) (Biosearch Technologies) and then blocked with 2% gelatin for 1 hour at 37°C. Serum samples were serially diluted and incubated on coated plates for 2 hours at room temperature, and antibody was detected with horseradish peroxidase (HRP)–conjugated goat anti-murine

IgM, IgG1, or IgG3 (SouthernBiotech) developed with BD OptEIA TMB Substrate and stopped with 2 M H_2SO_4. Absorbance at 450 nm was determined, and antibody concentration was calculated on the basis of standard curves constructed with purified anti-TNP isotypes (BD Pharmingen). Naïve IgM and IgG levels were measured with paired capture and HRP-conjugated antibodies (SouthernBiotech). Standard curves were constructed with purified corresponding mouse Ig isotypes (SouthernBiotech).

Antibody responses to anthrax vaccination were previously determined in the AVA trial (clinicaltrials.gov identifier NCT00119067) (*28*, *29*). Because the anthrax PA is an important component of an effective anthrax vaccine, antibodies to PA (AbPA) are commonly used as the primary measure of AVA immunogenicity. Although lethal toxin–neutralizing antibodies may also be relevant, studies in humans and mice have generally indicated a strong correlation between AbPA and toxin-neutralizing antibody levels (*46*). Accordingly, AbPA was measured by ELISA as anti-PA–specific IgG in comparison to reference samples of known AbPA concentrations in a quantitative ELISA, with an empirical reactivity threshold of 3.7 μg/ml (*29*, *47*).

SPR analysis

To further characterize the serum antibody binding properties, SPR technology was used using a Biacore T200 system (GE Healthcare). The ligand (biotinylated BSA-TNP) was captured on a Sensor Chip SA (GE Healthcare), precoated with streptavidin, according to the manufacturer's instructions. The ligand was captured by injection of 100 μg/ml in phosphate-buffered saline (PBS) with 0.1% Surfactant P20 (PBS-P) (GE Healthcare) at 5 μl/min for 2 to 6 min, resulting in ~2000 resonance units. The control surface was similarly injected with buffer only in the absence of biotinylated BSA-TNP ligand. Subsequently, serum from TG and NTG mice 40 days after immunization was diluted in PBS-P and injected over both surfaces at a flow rate of 30 μl/min at 20°C in various concentrations, and binding was monitored. At 120 s, injection of serum was stopped to monitor antibody dissociation for 600 s. The surface of the chip was regenerated by injection with 10 mM glycine (pH 2.0) for 60 s. A nonimmune serum control was subtracted from all sensorgrams to remove bulk shift and nonspecific binding. In addition, response from blank injection was also subtracted to remove response because of change in buffer and other system artifacts. Analysis was performed using Biacore T200 Evaluation Software, version 1.0. The relative stability of antibody binding was evaluated by fitting the dissociation phase data to a model for dissociation from two independent monovalent sites.

Genotyping and CNV measurement

A nested PCR system was designed to specifically amplify *FCGR2C* from genomic DNA, followed by direct sequencing or Pyrosequencing. First-round gene-specific PCRs were performed with High-Fidelity PCR (primers are shown in table S3). *FCGR2C* copy number was determined by a quantitative Pyrosequencing approach. In brief, using primers listed in table S3, a common region with a single-nucleotide difference between *FCGR2A* and *FCGR2C* was amplified, and then the relative levels of each gene were determined through quantitation of the single nucleotide that differs between the genes by Pyrosequencing on the PSQ96HA instrument (Qiagen).

Statistics

Descriptive statistics included measures of central tendency (sample mean, sample frequencies) and measures of dispersion (SEM). For in vitro/ex vivo studies, data are represented as means ± SEM. Statistical significance was calculated by Student's two-sided *t* test using Prism (GraphPad). All other analyses were done using the SAS software. Murine immunization results were analyzed by repeated-measures ANOVA to test whether response to immunization varied over time by receptor status. All distributional assumptions for repeated-measures ANOVA were examined, and no violation of assumptions was observed. ANCOVA was used to test whether $\log_{10}$ mean AbPA levels in the vaccine cohort varied according to the number of *FCGR2*-ORF alleles after adjusting for age, gender, and ethnicity of participants. All distributional assumptions for ANCOVA were examined, and no violation of assumptions was observed. Logistic regression was used to test the association between number of *FCGR2C*-ORF alleles and lupus phenotype.

SUPPLEMENTARY MATERIALS

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Fig. S1. Specificity of anti-FcγRIIc mAb 11B6 and anti-FcγRIIb mAb 3B6.
Fig. S2. Flow cytometric analysis of FcγRIIc and FcγRIIb expression in stable A20-IIA1.6 transductants.
Fig. S3. Absence of transgene expression on macrophages and T cells in TG mice.
Fig. S4. TG mice show normal proportions of B cell subsets.
Fig. S5. No difference in naïve IgM and IgG levels between TG and NTG mice.
Fig. S6. Enhanced B cell proliferation and activation in TG mice.
Fig. S7. Enhanced binding stability of the anti-TNP response in TG compared to NTG mice.
Fig. S8. Decreased frequency of *FCGR2C*-ORF in nonresponders in an anthrax vaccine trial.
Table S1. Demographics of the healthy volunteers participating in the CDC-sponsored NCT00119067 AVA study available for ancillary study on genetic correlates.
Table S2. Demographics of the PROFILE case-control study.
Table S3. List of primers.

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