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# Allelic Dependent Expression of an Activating Fc receptor on B cells Enhances Humoral Immune Responses

**Xinrui Li**[1], **Jianming Wu**[1,†], **Travis Ptacek**[1], **David T Redden**[2], **Elizabeth E Brown**[3], **Graciela S Alarcón**[1], **Rosalind Ramsey-Goldman**[4], **Michelle A Petri**[5], **John D. Reveille**[6], **Richard A Kaslow**[1,3,‡], **Robert P Kimberly**[1,*], and **Jeffrey C Edberg**[1,*]

[1]Department of Medicine, University of Alabama at Birmingham, Birmingham Alabama 35294, USA

[2]Department of Biostatistics, University of Alabama at Birmingham, Birmingham Alabama 35294, USA

[3]Department of Epidemiology, University of Alabama at Birmingham, Birmingham Alabama 35294, USA

[4]Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611 USA

[5]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, USA

[6]Department of Medicine, University of Texas Health Science Center at Houston, Houston, Texas 77030, USA

## Abstract

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 significantly 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 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. Here 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 in 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 uni-directional negative feedback by IgG immune complexes via the inhibitory FcγRIIb, is a previously unrecognized determinant in human

*Corresponding authors. rpk@uab.edu or jedberg@uab.edu.
†Current affiliation for J.W. is the Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN 55108.
‡Current affiliation for R.A.K is the the United States Department of Veterans Affairs, Washington, DC.

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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 antigen 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 since 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 30–40% 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 SLE (5, 6). Several functional polymorphisms in both the promoter and the transmembrane 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 domains (EC), FcγRII family receptors diverge in their cytoplasmic 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 extracellular domain. However, a non-synonymous coding region SNP (rs10917661, nt202T>C) in 7–15% 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 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 unique 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-crosslinking of FcγRIIc and BCR leads to FcγRIIc tyrosine phosphorylation and enhanced BCR signaling. In a B cell-specific transgenic 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 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.



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## 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, reverse-transcription (RT)-PCR was performed using RNA from B cells homozygous for either the ORF or 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, IgG-mediated feedback regulation of B cell activity has been ascribed to the inhibitory FcγRIIb (3). We sought FcγRIIc protein in 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-extracellular (EC) domain specific mAb4F5 (6). The cytoplasmic (CY) domain of FcγRIIa/c was found in B cells from $ORF^+$ individuals, while the absence of the FcγRIIa/c CY corresponded with the homozygous STP genotype (Fig. 1C). Probing with a polyclonal Ab 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) while STP homozygous B cells showed no staining for FcγRIIc-CY (Fig. 2C and 2F, $p<0.001$). Both the ORF and STP cells stained positive for FcγRIIb-CY (Fig. 2B and 2D), 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 in 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, human *FCGR2C* or *FCGR2B* cDNAs were retro virally transduced 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 equi-molar 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), while 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 1–3 min (Fig. 4C). This coligation also resulted in enhanced and more sustained tyrosine phosphorylation of the key B cells signaling 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).

Given 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 co-engaged. 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 co-expressed 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 further enhanced the level of both Syk phosphorylation by nearly 2-fold (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 sIg signaling, consistent with prior studies in primary human B cells (5).

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

In order 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 transgenic mice on C57BL/6 background, driven by $V_H$ promoter combined with μ and κ enhancers, as employed 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 transgenic (TG) mice. The transgene expression did not affect endogenous mouse FcγRIIb expression (Fig. 5B). Major B cell subsets in young mice (6-week-old) were normal in proportion compared to wild type (WT) littermates (fig. S4). Examination of naïve IgG and IgM levels in 10-week-old mice showed no significant difference between TG mice and their non-transgenic 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 with either anti-IgM $F(ab')_2$ to crosslink BCR alone or with intact anti-IgM IgG antibody to simultaneously co-engage human FcγRIIc, mouse FcγRIIb and BCR. First, when coligated with BCR, the transgene itself was activated (Fig. 5D). Secondly, as expected, in NTG littermates, mFcγRIIb/BCR coligation 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 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).

## Enhanced immune responses to vaccination in B cell-specific FcγRIIc transgenic mice

In order to examine the biological effect of FcγRIIc expression on B cells *in vivo*, TG mice and their WT littermates were immunized 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



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comparable in magnitude to the changes observed in FcγRIIb deficient mice (27). While 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 2-fold increase in antibody production in TG mice compared to NTG littermates (*P*=0.006) (Fig. 6D). Relative affinity maturation was assessed by $TNP_2$ ELISA after secondary immunization. The high affinity IgG1 titers at Day 40 and Day 51 were 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 Analysis of anti-TNP reactivity at the Day40 time point in TG compared to NTG littermates (*P*=0.041, fig. S7).

## Enhanced antibody responses 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 CDC (clinicaltrials.gov identifier NCT00119067) (28, 29). This study provided a unique paradigm for a naive 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 4 immunizations (the 4 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 non-synonymous 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* transmembrane allelic variant (TM) (rs1050501) on AbPA responses, the association of *FCGR2C*-ORF on response was analyzed stratifying by 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/SQ group (n=366, *P*=0.046). Furthermore, this association was replicated in an independent vaccine cohort comprised of the group of AVA vaccine participants that received 3 immunizations (the 3IM group) (n=525, *P*=0.0012). These results demonstrated the in vivo biological consequences of the *FCRG2C*-ORF variant in humoral responses in humans.

## FCGR2C-ORF allele is associated with autoimmunity in humans

Given 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 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

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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% CI: 1.04–1.39) 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 (IC) (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 extracellular 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 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 extracellular domain, it has been technically difficult to distinguish FcγRIIa/b/c. For this reason, expression and function of FcγRIIc has been explored in NK cells, given 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 pro-inflammatory functions and has been reported to influence disease severity in rheumatoid arthritis (21). Furthermore, CNV of *FCGR2C*-ORF is associated with idiopathic thrombocytopenic purpura (ITP) (22), supporting the idea of FcγRIIc involvement in autoimmunity.

In our *FCGR2C* transgenic mice, B-cell restricted expression of FcγRIIc was sufficient to prompt a 2-fold 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. Interestingly, 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-protective antigen IgG titers in the very early stage of vaccination. There

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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 Americans and European Americans, the frequency of ORF allele copies, ranging from 1 copy to 3 and 4 copies in the AA and EA 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 cells types. Indeed, expression of FcγRIIc has been demonstrated on NK cells (35, 37) and it is likely that other cells 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 extracellular 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, that 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 Anthrax Vaccine Absorbed 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 comprised 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 transgenic mouse model in which cDNA from the human *FCGR2C*-ORF allele is expressed on murine CD19 positive B cells. Cell for ex vivo studies were coded to the experimenter and were repeated at least three times. Experimental immunization studies were performed in

randomly chosen tg and control littermates, with 8–12 animals in each group, and serum was analyzed in a blinded manner.

### Participants

For the analysis of *FCGR2C* genotype in the Anthrax Vaccine Absorbed trial (clinicaltrials.gov identifier NCT00119067) (29), DNA from 366 individuals enrolled into the 4-dose vaccination groups (4-IM and 4-SQ) and from 525 individuals enrolled into the 3-dose vaccination group (3-IM) 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 Supplemental table 1. 1194 patients with SLE were participants in the longitudinal PROFILE cohort as previously described (38, 40). Patients and 1656 controls were from 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 Supplemental table 2. For in vitro experiments, PBMCs were isolated by Ficoll-Hypaque gradient centrifugation as previously described (43). $CD19^+$ cell were further purified using Magnetic Micro Beads (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 A20IIA1.6 B cell lines (kindly provided by Dr. T. Wade, Dartmouth Medical Center) were maintained in DMEM (Invitrogen). All cell lines were tested negative for mycoplasma contamination. Medium was supplemented with 10% FBS, 25mM HEPES, 100U/ml penicillin and 100μg/ml streptomycin. For stable transductants, FcγRIIc and FcγRIIb cDNAs were cloned into pMX-PIE retroviral vector with enhanced green fluorescent protein (eGFP). Retroviral transduction was done as described previously (44). Thanks to the generous 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 previous described (24, 25). Transgenic mice were established at UAB Transgenic Mouse Facility by injecting the DNA fragment into fertilized oocytes from C57BL/6 mice (Jackson Laboratories). The presence of transgene was identified by genotyping of tail DNA (primers are listed in Supplemental table 3). All studies were performed with approval from the UAB Institutional Animal Care and Use Committee.

### Antibodies

Polyclonal anti-FcγRIIb/c antibody (pAb) was obtained from Santa Cruz Biotech (#sc-12815, Santa Cruz, CA). Monoclonal anti-FcγRIIb mAb 4F5 was made in our lab as previously described (6). Anti-FcγRIIa mAb IV.3 hybridoma were purchased from 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) Abs were purchased from Cell Signaling Technologies. Phospho-Syk (Y352)/Zap70 (Y319) (#557881), CD20 CY (clone H1) (#555677) Abs were obtained from BD Pharmingen. Monoclonal anti-FcγRIIb-CY mAb 3B6 and monoclonal anti-FcγRIIa/c-CY mAb 11B6 were generated at the UAB Epitope Recognition and



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Immunoreagent Core facility by immunizing BALB/C mice with FcγRIIb or FcγRIIc polypeptides (fig. S1).

### RT-PCR

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-Alexa488 (10 μg/ml) for 30 min on ice, goat anti-mouse $F(ab')_2$ (15 μg/ml) was added then cells were incubated at 4 °C 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) abs for various time 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) abs (Jackson ImmunoResearch Laboratories) for indicated time 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-PAGE 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 $5 \times 10^6$/ml in modified HBSS (1.1mM $CaCl_2$, 1.6mM $MgCl_2$). Calcium concentration was detected by flow cytometry as Fluo-4/SNARF-1 ratio. Data were analyzed using FlowJo (Treestar).

### 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 $2 \times 10^6$ 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 pre-loaded with 5 μM CFSE (Invitrogen) before culturing.

### T-independent and T-dependent immunization

8–12 weeks old, gender and age matched mice received intraperitoneal injection of 25μg TNP-Ficoll alone for T-independent immunization, or 50μg 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 50μg TNP-CGG/alum on day 28.

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Littermates were screened for expression of transgene, 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).

## ELISA

Antibody responses to TNF-Ficoll and TNP-CGG in mice were assessed by ELISA. 96-well plates (Costar 9018; Thermo Fisher Scientific) were coated overnight at 4 °C with 5 μg/ml of TNP conjugated BSA (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 h at 37°C. Serum samples were serially diluted, incubated on coated plates for 2h at room temperature and Ab detected with HRP–conjugated goat anti-murine IgM, IgG1 or IgG3 (SouthernBiotech) developed with BD OptEIA TMB Substrate and stopped with 2M $H_2SO_4$. Absorbance at 450 nm was determined and antibody concentration was calculated based on 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 Anthrax Vaccine Absorbed trial (clinicaltrials.gov identifier NCT00119067) (28, 29). As the anthrax protective antigen (PA) is known to be 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 immunoglobulin G in comparison to reference samples of known AbPA concentrations in a quantitative enzyme-linked immunosorbent assay, with an empirical reactivity threshold of 3.7 μg ml −1 (29, 47).

## Surface Plasmon Resonance Analysis

To further characterize the serum antibody binding properties SPR technology was utilized using a Biacore T200 system (GE Healthcare). The ligand (biotinylated BSA-TNP) was captured on a Sensor chip SA (GE Healthcare), pre-coated with streptavidin, according to the manufacturer’s instructions. The ligand was captured by injection of 100 μg/ml in PBS with 0.1% Surfactant P20 (PBS-P) (GE Healthcare) at 5 μl/min for 2–6 min resulting in ~2000 resonance units (RU). 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 post immunization diluted in PBS-P and injected over both surfaces at a flow rate of 30μl/min at 20°C in various concentrations, and binding was monitored. At 120 sec, injection of serum was stopped to monitor antibody dissociation for 600 sec. The surface of the chip was regenerated by injection with 10 mM Glycine, pH 2.0, for 60 sec. A non-immune 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 due to 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 PCR reactions

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were performed with High Fidelity PCR (primers are shown in Supplemental table 3). *FCGR2C* copy number was determined by a quantitative Pyrosequencing approach. In brief, using primers listed in Supplemental table 3, 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 (standard error of the mean). For in vitro/ex vivo studies, data are represented as Mean ± s.e.m. Statistical significance was calculated by Student's 2-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. Analysis of covariance (ANCOVA) was utilized to test whether log-base 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 Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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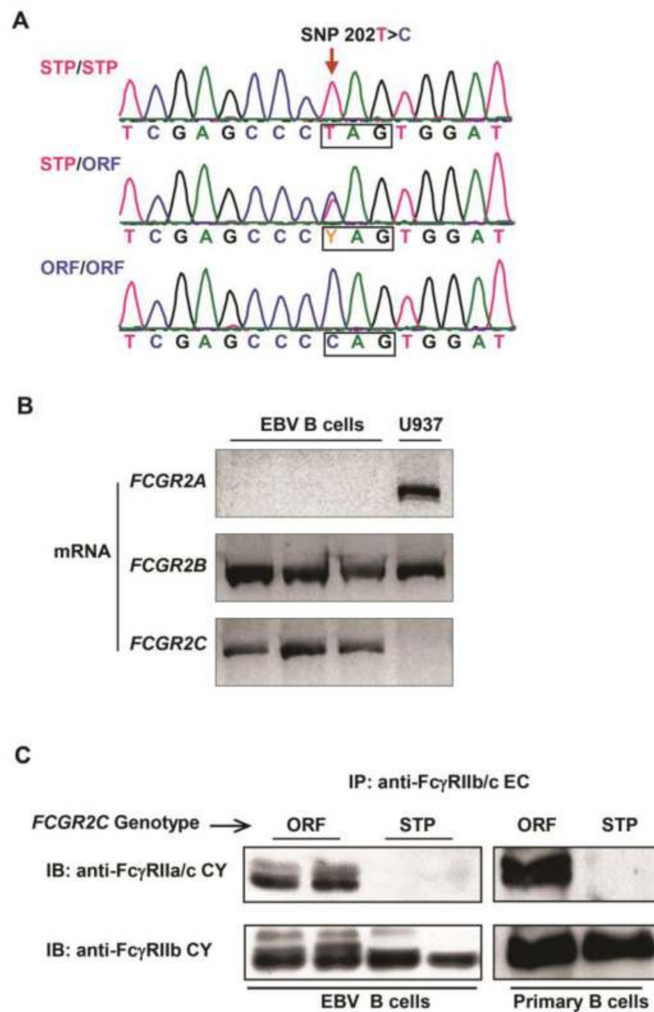


**Fig. 1. Expression of FcγRIIc protein in B cells**

(**A**) Chromatograms showing the rs10917661 (nt202 T>C) polymorphism (amino acid position 13) in the first extracellular domain of *FCGR2C*. (**B**) RT-PCR detection of *FCGR2A*, *2B* and *2C* mRNA in EBV B cells containing either the *FCGR2C*-ORF (lane 1 and 2 from left) or -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 2 independent experiments. (**C**) Lysates of EBV B cells or primary human $CD19^+$ cells were immunoprecipitated with mAb4F5 recognizing the FcγRIIb/c EC domain and then blotted with either anti-FcγRIIc CY 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 STP B cells. Data are representative of 3 independent experiments.

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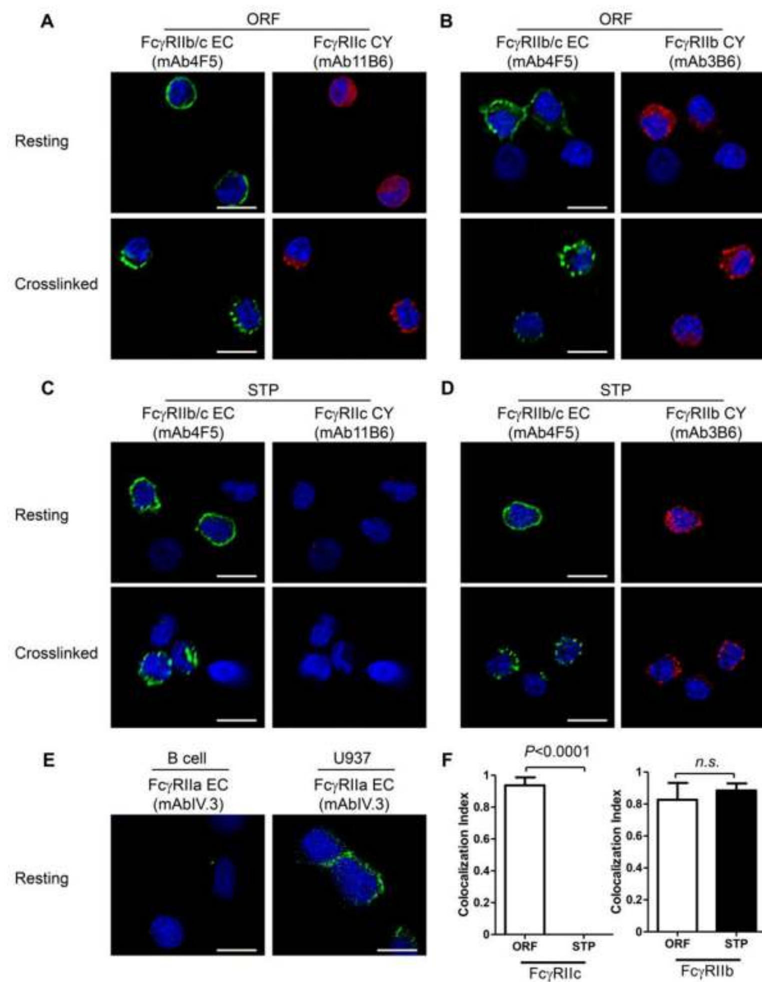


**Fig. 2. Surface expression of FcγRIIc protein on human B cells**

(**A–D**) EBV transformed human B cells from individuals containing the *FCGR2C*-ORF allele (**A**,**B**) or that are homozygous for the *FCGR2C*-STP allele (**C**,**D**) were opsonized with mAb4F5 (green) either alone or with crosslinking using a secondary antibody, 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 3 independent experiments (Scale bar: 10μm). (**F**) Quantification of the EC (green) and the CY (red) staining of FcγRIIc or FcγRIIb colocalization after crosslinking, calculated by Metamorph software from 6–8 randomly chosen cells.

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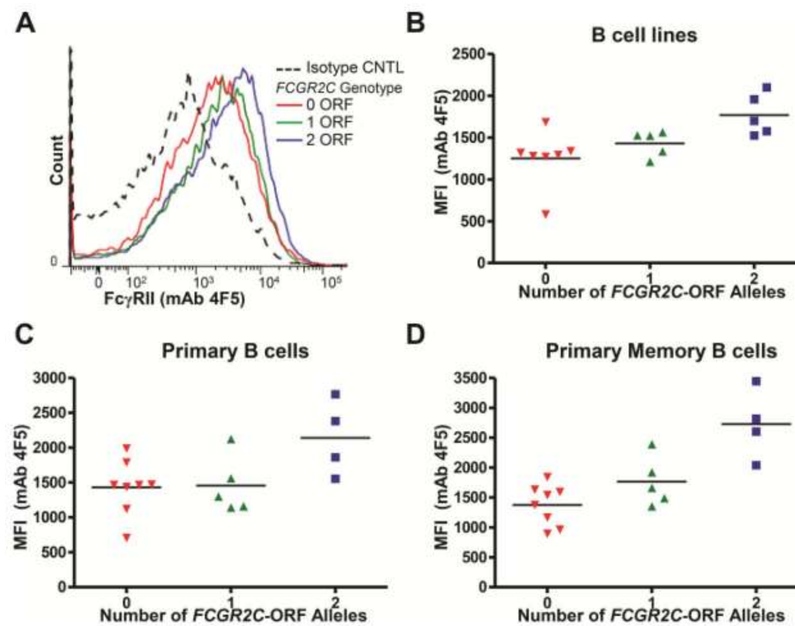


**Fig. 3. Higher total FcγRII expression on B cells with increasing numbers of the *FCGR2C*-ORF allele**

(**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 number of *FCGR2C*-ORF alleles ($p = 0.017$, ANOVA). (**C**,**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 number of *FCGR2C*-ORF alleles ($p = 0.042$ and 0.0005 respectively, ANOVA).

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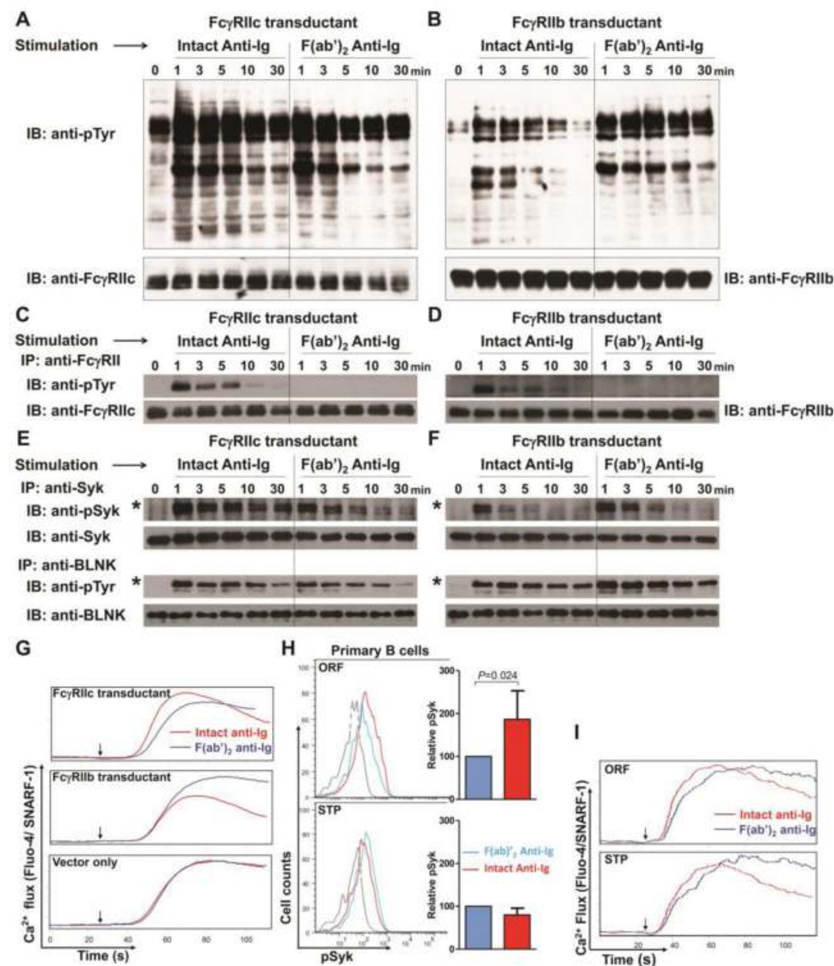


**Fig. 4. Activating properties of FcγRIIc in transduced A20IIA1.6 cells and primary human B cells**

(**A**,**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 time, affording BCR/FcγRIIc coligation or BCR crosslinking alone. Whole cell lysates were also re-probed for FcγRIIc (**A**) or FcγRIIb (**B**) to verify receptor expression and comparable protein loading. (**C,D**) Tyrosine phosphorylation of FcγRIIc (**C**) or FcγRIIb (**D**) upon co-crosslinking with BCR. (**E** and **F**) Tyrosine phosphorylation of Syk and BLNK detected by immunoprecipitation. Normalized intensities of both pSyk and pBLNK are significantly increased with FcγRIIc/BCR colligation, and decreased with FcγRIIb/BCR colligation comparing to BCR ligation alone (***E**, *P*=0.002 (pSyk), 0.003 (pBLNK); **F**, *P*=0.033 (pSyk), 0.0097(pBLNK), ANOVA). Data are representative of 2 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 3 independent experiments. (**H**) Human PBMC were stimulated as indicated and the level of Syk phosphorylation was quantitated in $CD20^+$ B cells by FACS. Grey line indicates baseline of pSyk in unstimulated cells. Net effect of FcγRIIc is analysed by comparing MFI of pSyk with equal molar amount of intact (15 μg/ml) *vs* F(ab′)$_2$ (10 μg/ml) of Goat anti Human-(IgG+IgM) stimulation (mean± s.e.m., *n*=6, *P*=0.024, *t*-test). (**I**) $Ca^{2+}$ flux in

primary B cells evaluated by flow cytometry. Data are representative of 5 independent experiments ($P$=0.032, $t$-test).

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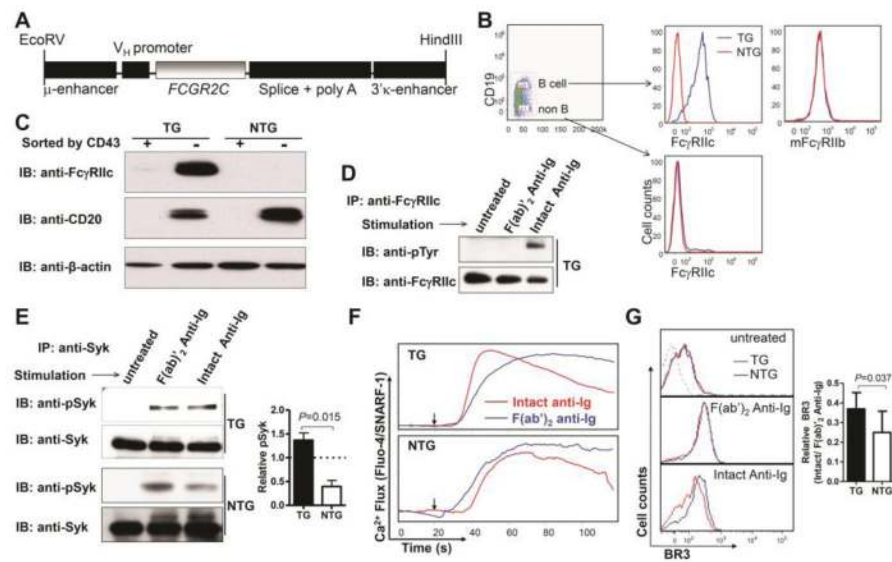


**Fig. 5. Generation and characterization of B-cell specific FcγRIIc transgenic mice**

(**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 3 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 co-engagement with intact anti-Ig. Data are representative of 2 independent experiments. (**E**) Syk phosphorylation in splenic B cells stimulated as indicated. Relative pSyk is analysed by normalizing intensity of pSyk bands to corresponding Syk bands, then compare intact (15 μg/ml) *vs* $F(ab')_2$ (10 μg/ml) goat anti–mouse IgM stimulation (mean± s.e.m. *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 3 independent experiments. (**G**) Surface BR3 expression on splenic B cells after 72 hours of treatment with indicated stimuli. Net effect of FcγRIIc is analysed by comparing MFI of BR3 with equal molar amount of intact (15 μg/ml) *vs* $F(ab')_2$ (10 μg/ml) goat anti–mouse IgM stimulation (mean± s.e.m. *n*=3, *P*=0.024, *t*-test).

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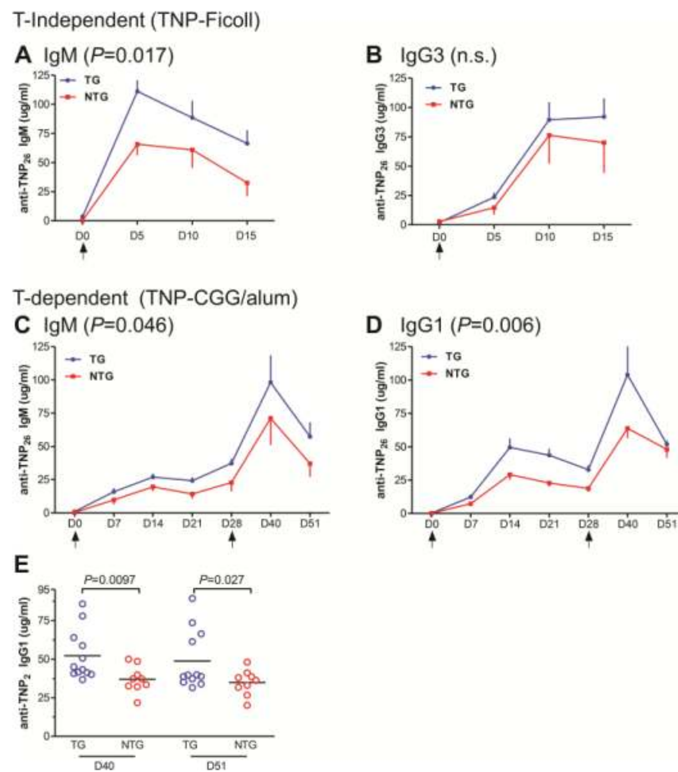


**Fig. 6. Enhanced humoral immune responses in B-cell specific FcγRIIc TG mice**

8–12 week old male TG mice and their NTG littermates were intraperitoneally injected with either 25ug TNP-Ficoll (**A**,**B**, n=10 and 7 for TG and NTG respectively) or 50ug TNP-CGG/alum (**C**,**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 mean ± s.e.m. and group effects were analyzed by repeated measure ANOVA. (**E**) Production of high affinity antibody after boost was measured by ELISA using $TNP_2$-coated plates (*P*=0.0097 for D40 and 0.027 for D51, *t*-test).

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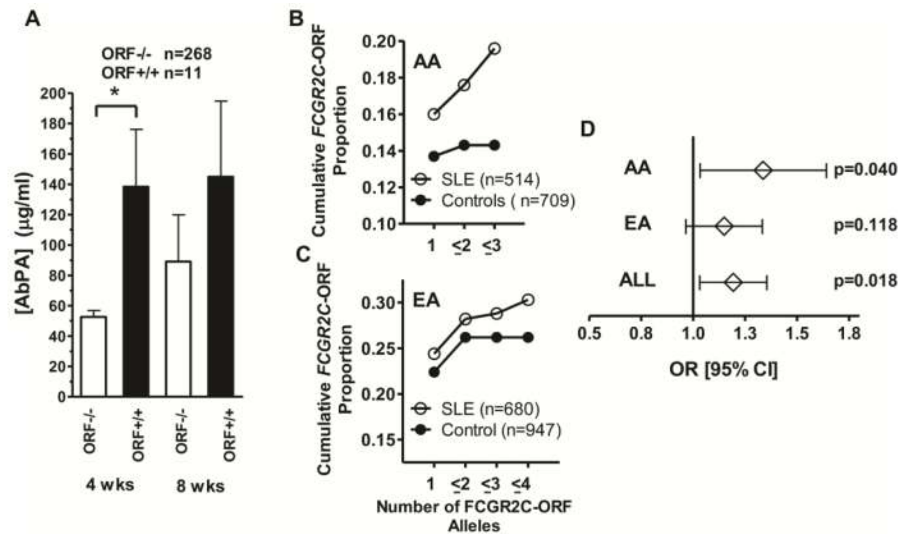


**Fig. 7. Impact of *FCGR2C*-ORF in human vaccine response and susceptibility to autoimmunity**

(**A**) Levels of Ab specific for the Anthrax protective antigen (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 ANCOVA and AbPA levels at the earliest time points in the vaccine study are higher in donors homozygous for the ORF allele with significantly significance reached at the 4 week time point ($p<0.02$). (**B**,**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.