Analysis of a wild mouse promoter variant reveals a novel role for FcγRIIb in the control of the germinal center and autoimmunity

Marion Espéli,[1,2] Menna R. Clatworthy,[1,2] Susanne Bökers,[1,2] Kate E. Lawlor,[1,2] Antony J. Cutler,[1,2] Frank Köntgen,[3] Paul A. Lyons,[1,2] and Kenneth G.C. Smith[1,2]

[1]Cambridge Institute for Medical Research and [2]Department of Medicine, School of Clinical Medicine, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 0XY, England, UK
[3]Ozgene Pty. Ltd., Perth, Western Australia 6102, Australia

Genetic variants of the inhibitory Fc receptor FcγRIIb have been associated with systemic lupus erythematosus in humans and mice. The mechanism by which *Fcgr2b* variants contribute to the development of autoimmunity is unknown and was investigated by knocking in the most commonly conserved wild mouse *Fcgr2b* promoter haplotype, also associated with autoimmune-prone mouse strains, into the C57BL/6 background. We found that in the absence of an AP-1–binding site in its promoter, FcγRIIb failed to be up-regulated on activated and germinal center (GC) B cells. This resulted in enhanced GC responses, increased affinity maturation, and autoantibody production. Accordingly, in the absence of FcγRIIb activation–induced up-regulation, mice developed more severe collagen-induced arthritis and spontaneous glomerular immune complex deposition. Our data highlight how natural variation in *Fcgr2b* drives the development of autoimmune disease. They also show how the study of such variants using a knockin approach can provide insight into immune mechanisms not possible using conventional genetic manipulation, in this case demonstrating an unexpected critical role for the activation-induced up-regulation of FcγRIIb in controlling affinity maturation, autoantibody production, and autoimmunity.

CORRESPONDENCE
Kenneth G.C. Smith:
kgcs2@cam.ac.uk

Abbreviations used: AFC, antibody-forming cell; ChIP, chromatin immunoprecipitation; dsDNA, double-stranded DNA; ES, embryonic stem; GC, germinal center; KI, knockin; PC, plasma cell; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; T_{FH} cell, T follicular helper cell.

The exact mechanisms by which natural noncoding variants contribute to autoimmune diseases have proven very difficult to dissect. We used a knockin (KI) approach to address this for the inhibitory receptor FcγRIIb, uncovering novel mechanisms of immune regulation and demonstrating that this method provides insights into normal immune function that conventional genetic manipulation models do not.

FcγRIIb binds to the Fc portion of IgG and negatively regulates immune complex–mediated signaling, including BCR signaling on B cells, on which it is the only Fcγ receptor expressed (Nimmerjahn and Ravetch, 2008; Smith and Clatworthy, 2010). The low-affinity Fc receptor family is found in a complex in a systemic lupus erythematosus (SLE)–associated region on chromosome 1 in both humans and mice (Vyse et al., 1997; Morel et al., 2001; Bolland et al., 2002), and dysregulation of FcγRIIb expression and function has been associated with autoimmunity in both species. In humans, a single nucleotide polymorphism (SNP) in *FCGR2B* results in reduced inhibitory

S. Bökers' present address is Division of Genetics, Dept. of Biology, University of Erlangen-Nuremburg, 91058 Erlangen, Germany.

K.E. Lawlor's present address is Cell Signalling and Cell Death Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia.

A.J. Cutler's present address is Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 OXY, England, UK.

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function (Floto et al., 2005; Kono et al., 2005) and has been associated with SLE (Kyogoku et al., 2002; Siriboonrit et al., 2003; Chu et al., 2004; Willcocks et al., 2010) but protection against malaria (Clatworthy et al., 2007; Willcocks et al., 2010), an effect independent of variation in neighboring FcγRs (Niederer et al., 2010). More recently Baerenwaldt et al. (2011) showed using humanized mice that this polymorphism affects human B cell development and is associated with autoantibody production in vivo. Naturally occurring variations have also been described in the promoter of human *FCGR2B*, and these too may be implicated in SLE predisposition (Su et al., 2004; Blank et al., 2005). Consistent with human data, FcγRIIb-deficient mice have an enhanced immune response (Takai et al., 1996), are prone to inducible (Yuasa et al., 1999) and spontaneous (Bolland and Ravetch, 2000) autoimmunity, and have an increased frequency of potentially autoreactive B cell clones in their germinal centers (GCs; Tiller et al., 2010). In addition, FcγRIIb balances the risk of pneumococcal bacteremia with that of septic shock (Clatworthy and Smith, 2004) and determines the outcome of malarial infection (Clatworthy et al., 2007). The original KO mouse was generated using a 129/Sv embryonic stem (ES) cell, and the presence of polymorphisms in genes neighboring *Fcgr2b* in this strain may account for part of its phenotype (Bygrave et al., 2004). The specific effect of FcγRIIb in SLE pathogenesis in MRL/Lpr mice has been confirmed, however, by lentiviral (McGaha et al., 2005) and cell-specific transgenic approaches (Brownlie et al., 2008). More recently, the careful analysis of FcγRIIb-deficient mice made on the C57BL/6 background was consistent with FcγRIIb contributing to SLE in a polygenic fashion (Boross et al., 2011). Variation in demethylated regions of the *Fcgr2b* promoter and intron 3 was described in several autoimmune-prone mouse strains, including NOD, NZB, NZW, and 129/Sv (Luan et al., 1996; Jiang et al., 2000; Pritchard et al., 2000), in which it was associated with reduced FcγRIIb expression and inhibitory function (Pritchard et al., 2000). Analyses of congenic strains have shown that mice bearing the SLE susceptibility loci *Sle1* or *Nba2* (derived from the NZW and NZB strains, respectively) show reduced FcγRIIb expression on GC B cells and plasma cells (PCs; Rahman and Manser, 2005; Lin et al., 2006; Vuyyuru et al., 2009; Jørgensen et al., 2010) and enhanced B cell immune responses (Vuyyuru et al., 2009; Jørgensen et al., 2010). However, these congenic strains carry large regions of chromosome 1 of NZB or NZW origin encompassing many genes involved in the control of the immune response, and thus *Fcgr2b* variants cannot be conclusively implicated in the phenotype observed in them. Moreover, the mechanism by which natural *Fcgr2b* variation contributes to autoimmunity in mouse and human is not known. Studies of natural genetic variants of *Fcgr2b* might allow dissection of these mechanisms in a way that models involving absolute deficiency, constitutive overexpression, or large congenic regions may not.

After analyzing the variants of *Fcgr2b* present in wild mice, we used a KI approach to show that a naturally occurring variant found in wild mice and in autoimmune strains is associated with an impaired up-regulation of FcγRIIb on GC B cells, as the result of differential binding of the activation-induced transcription factor complex AP-1. This stage-specific change in FcγRIIb expression was associated with enhanced GC formation and affinity maturation, but also with the spontaneous production of autoantibodies and autoreactive memory B cells and with enhanced severity of collagen-induced arthritis. These data highlight a previously uncharacterized role for FcγRIIb up-regulation in the control of the survival, selection, and affinity maturation of GC B cells.

RESULTS

Conservation of autoimmunity-associated polymorphisms in *Fcgr2b* in wild mice

Genetic variation found in the regulatory regions of *Fcgr2b* in inbred mice (Luan et al., 1996; Jiang et al., 2000; Pritchard et al., 2000) results in three distinct haplotypes (Fig. 1 A). We confirmed that these were the only haplotypes present by sequencing and examined their distribution within inbred strains using the genealogy generated by Beck et al. (2000; Fig. 1 B). Haplotype III (which lacks all deletions) was found in the majority of commonly used strains such as BALB/c and C57BL/6. Haplotype II (comprised of deletions 3 and 4) was found only in NZW, SJL/J, and SWR/J mice. Haplotype I (which includes deletions 1–3) was found in many autoimmune-prone strains such as NZB, NOD, and MRL/MpJ and sometimes appeared to arise at points of the inbred mouse genealogy where strains had been introduced from external sources (Fig. 1 B).

This observation prompted us to examine genetic variation in *Fcgr2b* in wild mice. Most of the *Mus musculus* subspecies tested were found to be of haplotype I. The few wild mice bearing haplotype III were mainly found in *Mus musculus molossinus* in Japan, which made important genetic contributions to inbred strains (Fig. 1 C; Beck et al., 2000; Guénet and Bonhomme, 2003). The predominance of the autoimmunity-associated haplotype I in the wild suggests it might be under selection pressure by infection in a manner analogous to the MHC and KIR loci (Espeli et al., 2010) and to FcγRIIb in humans (Willcocks et al., 2010) and thus may be functionally significant.

Naturally occurring variants of *Fcgr2b* abrogate the activation-induced up-regulation of FcγRIIb

Because haplotype I is the most commonly observed variant in wild mice, is found in SLE-associated loci in NZB and MRL/MpJ mice, and may be associated with altered expression of FcγRIIb in B cells and macrophages (Pritchard et al., 2000), we decided to examine its functional implications using a KI approach. We generated a mouse model in which the promoter and the first three exons of *Fcgr2b* from a haplotype I (H1) mouse were knocked in to a C57BL/6 ES cell ($FcγRIIb^{wild/H1}$ KI mouse; Fig. 2 A).

The $FcγRIIb^{wild/H1}$ KI mice were viable, bred normally, and had no abnormalities in hematopoietic cell numbers (not depicted). FcγRIIb expression was assessed before and after immunization with 4-hydroxy-3-nitrophenylacetyl

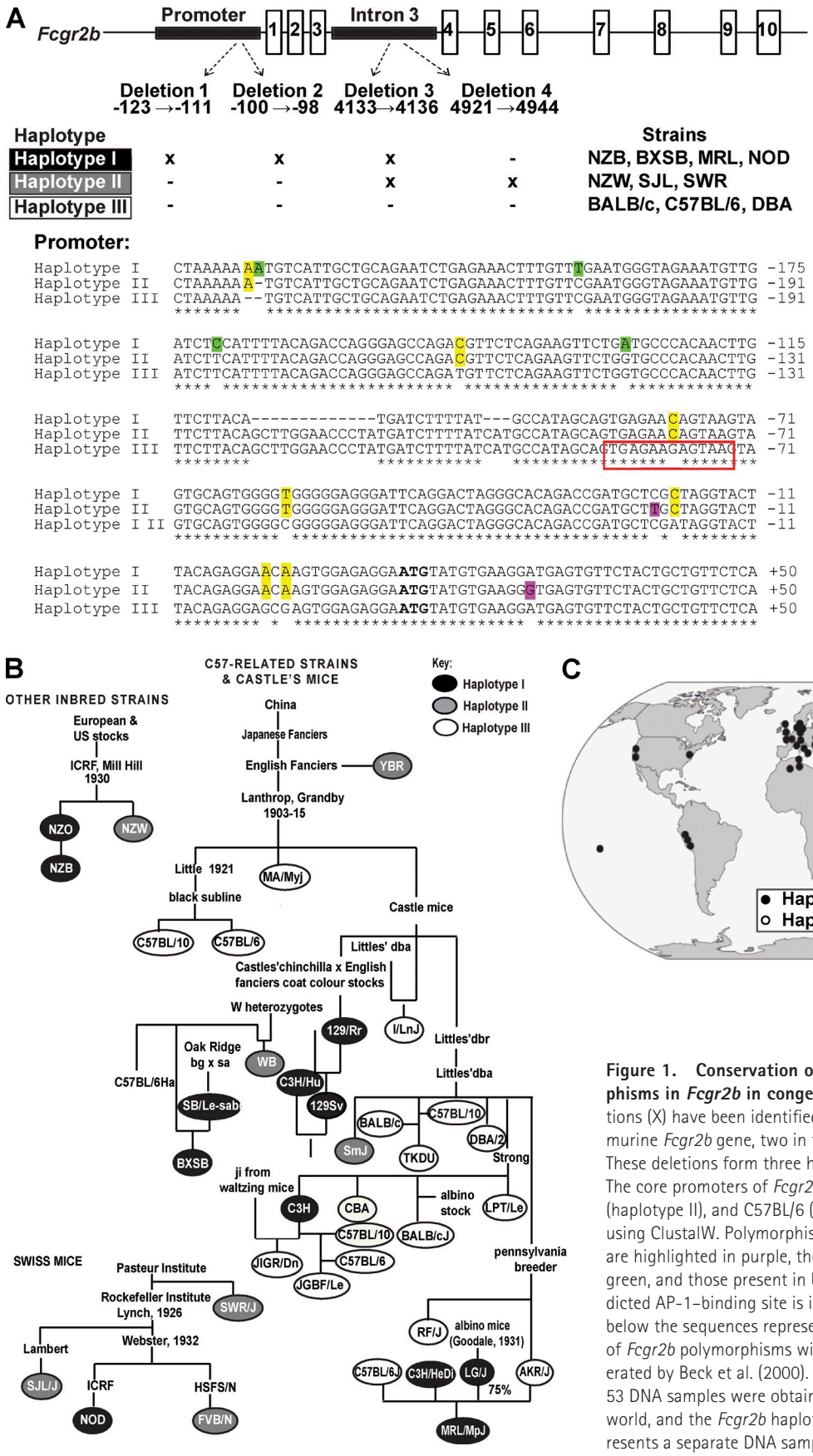

Figure 1. Conservation of naturally occurring polymorphisms in *Fcgr2b* in congenic and wild mice. (A) Four deletions (X) have been identified within regulatory regions of the murine *Fcgr2b* gene, two in the promoter and two in intron 3. These deletions form three haplotypes in inbred strains of mice. The core promoters of *Fcgr2b* from the NZB (haplotype I), NZW (haplotype II), and C57BL/6 (haplotype III) strains were aligned using ClustalW. Polymorphisms present only in the NZW strain are highlighted in purple, those present only in the NZB strain in green, and those present in both NZB and NZW in yellow. A predicted AP-1–binding site is indicated by a red box. The asterisks below the sequences represent sequence identity. (B) Distribution of *Fcgr2b* polymorphisms within the inbred strain genealogy generated by Beck et al. (2000). (C) *Fcgr2b* haplotypes in wild mice. 53 DNA samples were obtained from wild mice from around the world, and the *Fcgr2b* haplotype was determined. Each circle represents a separate DNA sample.

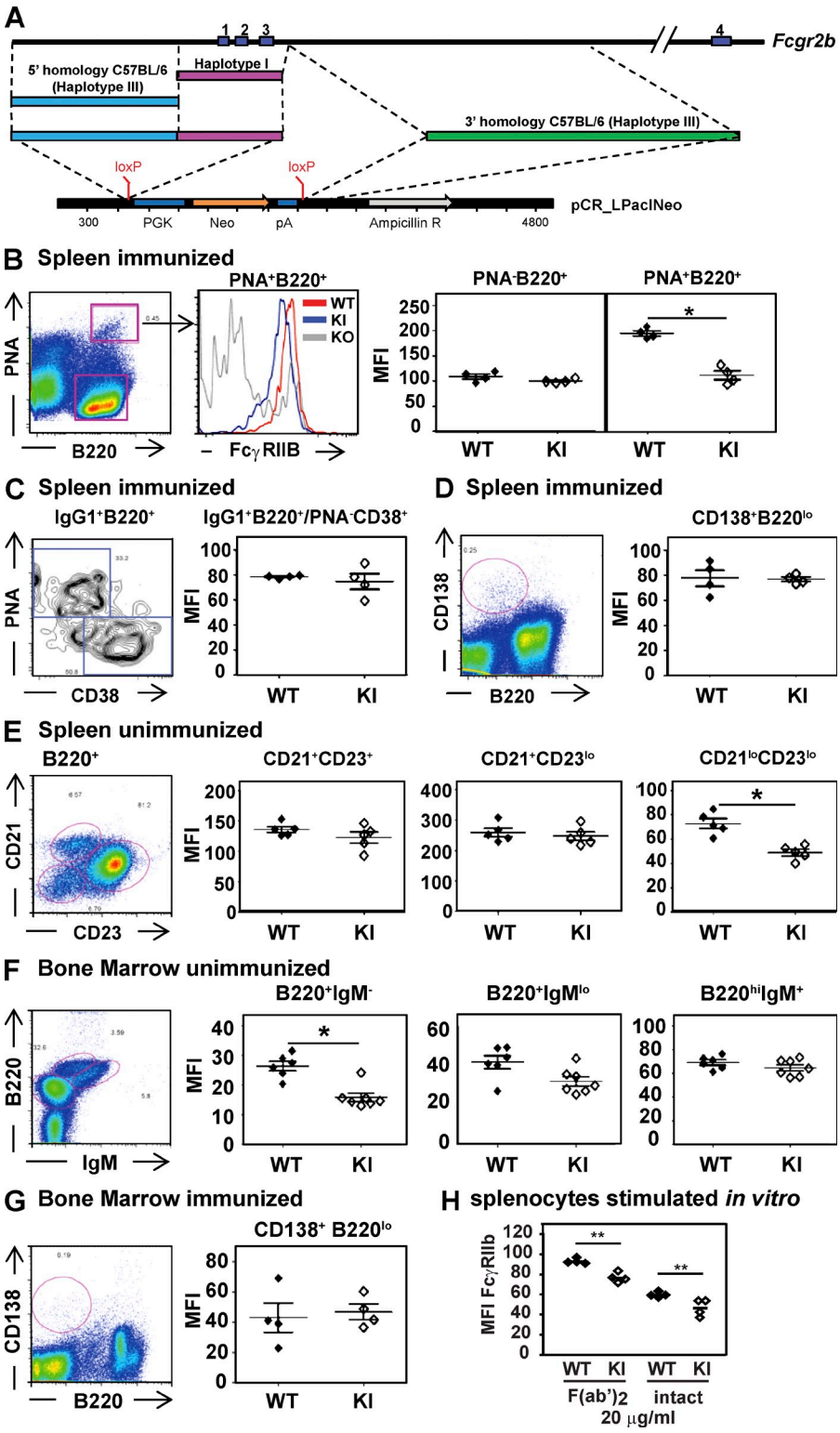

Figure 2. Defective up-regulation of FcγRIIb expression on GC B cells in FcγRIIB$^{wild/H1}$ KI mice. (A) Cloning strategy for the generation of the FcγRIIB$^{wild/H1}$ KI mice. A PCR product corresponding to the promoter and three first exons from *Fcgr2b* from haplotype I (1,271 bp) was first fused with the 5′ homology arm (1,996 bp from haplotype III). The fusion product was then cloned into the targeting vector (pCR_LPacI-Neo; Ozgene). The 3′ homology arm (3,905 bp from haplotype III) was then amplified and cloned in the targeting vector. (B–G) Surface expression of FcγRIIb was assessed by flow cytometry 14 d after NP-KLH immunization on naive (PNA$^-$B220$^+$) and GC B cells (PNA$^+$B220$^+$; B), memory B cells (IgG1$^+$/PNA$^-$CD38$^+$; C), splenic PCs (CD138$^+$B220^{lo}; D), and BM PCs (CD138$^+$B220^{lo}; G). Surface FcγRIIb expression was also assessed by flow cytometry on unimmunized mice for splenic follicular B cells (CD21$^+$CD23$^+$), marginal zone B cells (CD21$^+$CD23^{lo}), and transitional B cells (CD21^{lo}CD23^{lo}; E) and for BM pre-B cells (IgM$^-$B220$^+$), immature B cells (IgM$^+$B220$^+$), and recirculating mature B cells (IgM^{hi}B220$^+$; F). (left) Representative dot plot of the gating strategy used to determine FcγRIIb expression on each subset. (middle) Representative overlay of FcγRIIb expression by FcγRIIb$^{wild/H1}$ KI, WT, and FcγRIIb KO mice is shown for PNA$^+$B220$^+$ GC B cells. (right) Geometric mean fluorescence intensity (MFI) of FcγRIIb expression on the different cell subsets. (H) Splenocytes were stimulated with 20 μg/ml goat anti-mouse IgM F(ab′)2 or intact goat anti-mouse IgM for 72 h at 37°C. The expression of FcγRIIb on activated B cells was assessed by flow cytometry. The mean fluorescence intensity of FcγRIIb on activated B cells was plotted for WT and FcγRIIb$^{wild/H1}$ KI mice. For all experiments, $n \geq 4$ mice per group, and data are representative of at least three independent experiments. Error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5% (*, $P < 0.05$; **, $P < 0.01$).

conjugated to KLH (NP-KLH) in alum. FcγRIIb expression was identical on naive B cells (B220$^+$PNA$^-$), but the increase in FcγRIIb expression observed on control GC B cells was not seen in FcγRIIb$^{wild/H1}$ mice (Fig. 2 B). FcγRIIb expression was normal on memory B cells (Fig. 2 C) and on splenic and BM PCs, in contrast to the reduction previously noted in NZB (Xiang et al., 2007) and Sle1.B6 congenic (Jørgensen et al., 2010) PCs (Fig. 2, D and G).

FcγRIIb expression was also normal on all myeloid subpopulations tested (splenic dendritic cells, monocytes, and

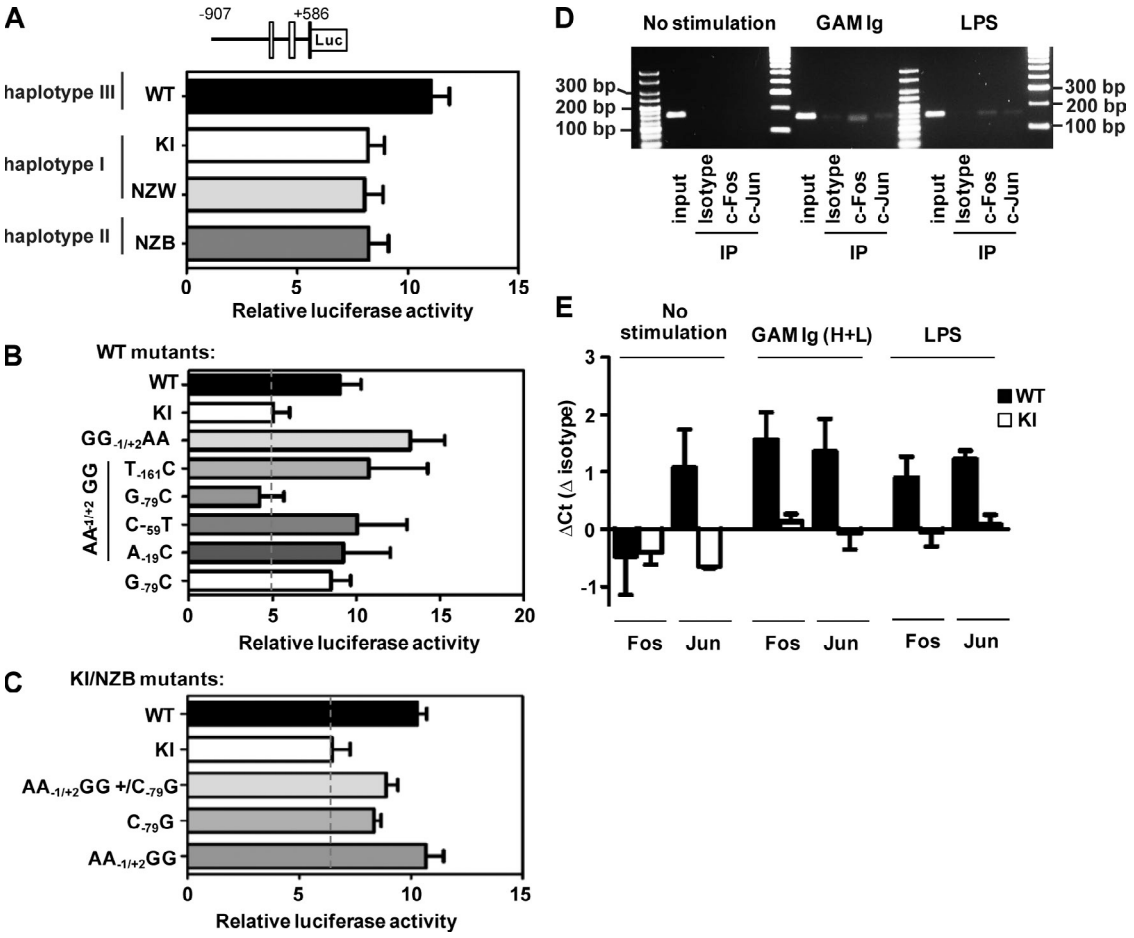

Figure 3. The reduced transcriptional activity of the haplotype I *Fcgr2b* promoter is caused by three single nucleotide substitutions leading to defective AP-1 binding. (A) The transcriptional activity of the WT, KI, NZB, and NZW promoter of *Fcgr2b* was determined in the Bal17 B cell line stimulated with LPS for 48 h. WT versus: KI, P = 0.015; NZW, P = 0.037; and NZB, P = 0.034. (B) The transcriptional activity of the WT promoter mutated at the indicated positions was determined as in A. WT versus: KI/NZB, P = 0.02; WT $GG_{-1/+2}AA$, P = 0.1; WT $GG_{-1/+2}AA/T_{-161}C$, P = 0.38; WT $GG_{-1/+2}AA/G_{-79}C$, P = 0.0025; WT $GG_{-1/+2}AA/C_{-59}T$, P = 0.88; WT $GG_{-1/+2}AA/A_{-19}C$, P = 0.5; and WT $G_{-79}C$, P = 0.3. (C) The transcriptional activity of the KI/NZB promoter mutated at the indicated positions was determined as in A. WT versus: KI/NZB, P = 0.001; KI/NZB $AA_{-1/+2}GG/C_{-79}G$, P = 0.07; KI/NZB $C_{-79}G$, P = 0.02; and KI/NZB $AA_{-1/+2}GG$, P = 0.94. KI/NZB versus: KI/NZB $AA_{-1/+2}GG/C_{-79}G$, P = 0.03; KI/NZB $C_{-79}G$, P = 0.06; and KI/NZB $AA_{-1/+2}GG$, P = 0.001. (B and C) The dashed lines represent the luciferase activity of the *Fcgr2b* KI promoter. (D) Bal17 cells were stimulated for 24 h with anti-Ig or LPS before ChIP with anti–c-Fos or –c-Jun or an isotype control. The region of the *Fcgr2b* promoter encompassing position −79 was amplified by PCR from input and coimmunoprecipitated DNA. (E) WT and $FcγRIIb^{wild/H1}$ KI splenic $CD19^+$ B cells were stimulated for 8 h with anti-Ig or LPS and processed as in D. The region of the *Fcgr2b* promoter encompassing position −79 was amplified by SYBR green quantitative PCR, and the ΔCT of isotype and c-Fos or c-Jun was plotted for each condition. In all panels, error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%.

neutrophils), on follicular dendritic cells, on resting follicular and marginal zone splenic B cells, and on BM mature recirculating B cells (Fig. 2, E and F; and not depicted). In addition to the changes seen on GC B cells, FcγRIIb expression was also lower on $FcγRIIb^{wild/H1}$ KI splenic transitional B cells and BM pre-B cells (Fig. 2, E and F). After in vitro stimulation, expression of FcγRIIb was reduced on splenic B cells from $FcγRIIb^{wild/H1}$ KI compared with controls, suggesting that it is the activation-induced up-regulation of FcγRIIb which is abnormal in these mice (Fig. 2 H). Thus, naturally occurring variants in the *Fcgr2b* promoter abrogate activation-induced up-regulation of FcγRIIb, an effect most prominently seen on GC B cells.

Transcriptional control of FcγRIIb up-regulation

The effect of *Fcgr2b* promoter variants on transcription was analyzed by luciferase assay. The polymorphisms present in the *Fcgr2b* promoter in haplotypes I (NZB, KI) and II (NZW) were associated with lower transcriptional activity compared with the control haplotype III (C57BL/6) promoter after B cell activation (Fig. 3 A). Consistent with the equivalent effects of haplotypes I and II in reducing *Fcgr2b* transcriptional activity, congenic mice incorporating distal chromosome 1 from either NZB or NZW backgrounds demonstrate reduced FcγRIIb expression on GC B cells (Fig. 3 A; Xiu et al., 2002; Rahman et al., 2007). As this suggests that the genetic variants driving the failure of activation-induced up-regulation are

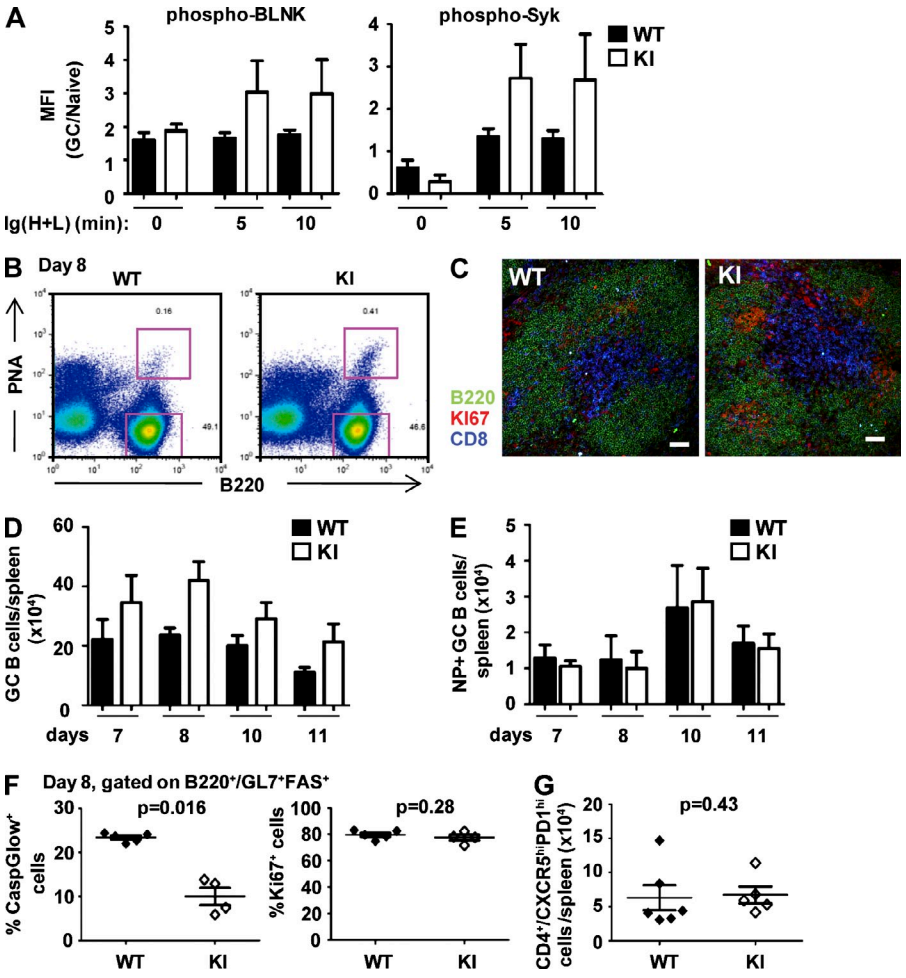

Figure 4. Increased GC reaction in FcγRIIb$^{wild/H1}$ KI mice. (A) 8 d after immunization with NP-KLH in alum, splenocytes were stimulated ex vivo, and the mean fluorescence intensity (MFI) of phospho-Syk and phospho-BLNK in GC B cells gated as B220$^+$/GL7^{hi}FAS^{hi} and naive B cells gated as B220$^+$/GL7$^-$FAS$^-$ was determined by flow cytometry. The ratio of naive and GC B cell mean fluorescence intensity is plotted. Data are representative of two or three independent experiments depending on the time points. $n = 4$ mice per group. (B) Splenic GC B cell numbers were determined by flow cytometry at day 8. Representative dot plots of WT and KI are shown. (C) GC formation in the spleen 8 d after immunization was assessed by immunohistology. Staining: anti-B220, B cell follicle; anti-CD8, T cell zone; and anti-KI67, GCs. Representative images of at least four mice per group are shown. Bars, 50 µm. (D and E) GC B cells (D) and NP-specific GC B cells (E) were enumerated at days 7, 8, 10, and 11 after immunization. $n \geq 4$ mice, and data are representative of at least two experiments per time point. (F) 8 d after immunization, apoptosis and proliferation of GC B cells were assessed by staining apoptotic cells with CaspGLOW (left) and cells in cycle with anti-KI67 (right). $n = 4$ mice for the KI and 5 mice for the WT. Data are representative of at least two independent experiments. (G) The number of T_{FH} cells (CD4$^+$/CXCR5^{hi}PD1^{hi}) per spleen was determined 11 d after immunization with NP-KLH in alum. $n = 6$ for the WT and 5 for the KI. Data are representative of three independent experiments. In all panels, error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%.

common to both NZB and NZW strains, we focused on the seven SNPs found in both of these strains but not in C57BL/6 (Fig. 1 A). None of the individual polymorphisms significantly reduced the transcriptional activity of *Fcgr2b* promoter when mutated alone, but the combination of the $GG_{-1/+2}AA$ and $G_{-79}C$ SNPs reduced the transcriptional activity to a level similar to that of the KI/NZB promoter (Fig. 3 B). When these variants were corrected in the NZB promoter, expression was restored to the C57BL/6 level (Fig. 3 C). These results suggest that these two substitutions are sufficient to disrupt the activation-induced up-regulation of FcγRIIb. Several transcription factor binding site prediction algorithms (see Materials and methods for details) predicted the existence of an AP-1–binding site overlapping with position −79 of the *Fcgr2b* promoter from haplotype III (C57BL/6) but not from haplotypes I or II (NZB/KI and NZW; Fig. 1 A, red box). AP-1 is a heterodimer composed of the transactivators c-Fos and c-Jun, and its expression is induced by BCR or TLR cross-linking on B cells (Yi et al., 2003; de Gorter et al., 2007). We confirmed that after activation, AP-1 binds to the *Fcgr2b* promoter at position −79 by performing c-Fos and c-Jun chromatin immunoprecipitation (ChIP) on a B cell line of haplotype III (Fig. 3 D). In primary B cells, the binding of c-Fos and c-Jun to position −79 of *Fcgr2b* promoter was up-regulated on C57BL/6 B cells after stimulation, but no binding was detected for either c-Fos or c-Jun in resting and activated FcγRIIb$^{wild/H1}$ KI B cells (Fig. 3 E). These results suggest that AP-1 drives the activation-induced up-regulation of FcγRIIb and that natural variants of the *Fcgr2b* promoter disrupt this process.

FcγRIIb up-regulation controls GC B cell number and affinity maturation

Reduced expression of FcγRIIb would be expected to enhance BCR-mediated signaling and the phosphorylation of downstream kinases. After immunization and ex vivo stimulation, both phospho-BLNK and phospho-Syk were increased in FcγRIIb$^{wild/H1}$ KI GC B cells compared with naive B cells (Fig. 4 A), consistent with the lower expression

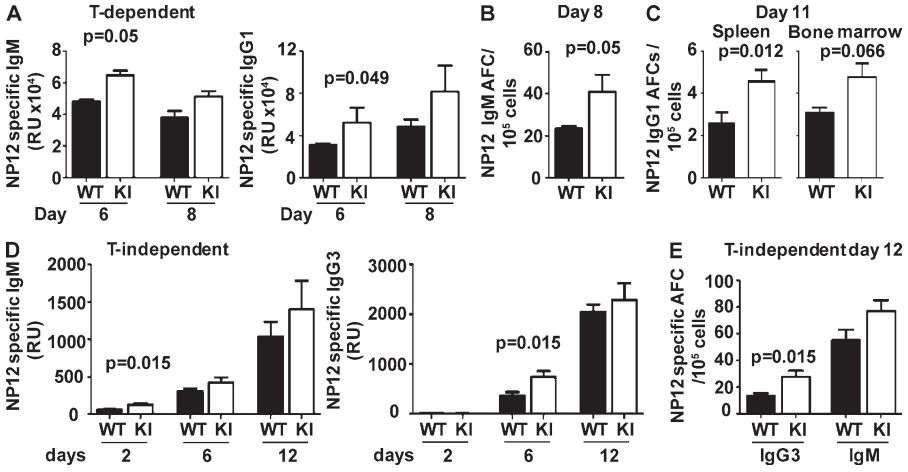

Figure 5. The early immune response is increased in FcγRIIb$^{wild/H1}$ KI mice. (A) NP-specific IgM and IgG1 antibody serum titers were determined by ELISA 6 and 8 d after immunization with NP-KLH. (B) NP-specific IgM and IgG1 AFCs were enumerated by ELISPOT at day 8 in the spleen. Data are representative of at least two independent experiments. (C) NP-specific IgG1 AFCs were enumerated by ELISPOT at day 11 in the spleen and the BM. Two experiments have been pooled. (D) NP-specific IgM and IgG3 antibody serum titers were determined by ELISA 2, 6, and 12 d after immunization with NP-Ficoll. (E) Splenic NP-specific IgG3 and IgM AFCs were enumerated by ELISPOT 12 d after immunization. In all panels, error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%. RU, relative units.

of FcγRIIb on GC B cells reducing the BCR activation threshold of these cells. As early as 7 d after immunization, an increase in the number of total GC B cells in FcγRIIb$^{wild/H1}$ KI mice was observed by flow cytometry and immunohistochemistry and found to hold true throughout the GC response (Fig. 4, B–D). Surprisingly, no difference was observed in the number of NP-specific GC B cells (Fig. 4 E). We found that half as many FcγRIIb$^{wild/H1}$ KI GC B cells contained active caspases (as determined by CaspGLOW staining; Fig. 4 F) and were TUNEL positive (not depicted) compared with controls, consistent with reduced apoptosis, whereas similar proportions were in cell cycle as determined by KI67 expression (Fig. 4 E). Interestingly, despite clear evidence of increased GC number, the number of T follicular helper cells (T_{FH} cells) was normal (Fig. 4 G). These results suggest that in the absence of FcγRIIb up-regulation, the BCR activation threshold is decreased, leading to enhanced signaling and survival of GC B cells.

This dysregulation of the GC led us to examine antibody production, selection, and affinity maturation. FcγRIIb$^{wild/H1}$ KI mice had an increase in the titer of NP-specific IgM and IgG1 that was particularly prominent early after immunization, and the increased number of NP-specific IgM and IgG1 secreting cells in the spleen and BM was consistent with this (Fig. 5, A–C). T-independent type 2 immune responses to NP-Ficoll also showed a modest increase in NP-specific IgM and IgG3 early after immunization (Fig. 5, D and E). Although total anti-NP IgG1 was only significantly raised in the first week after NP-KLH immunization (Figs. 5 and 6 A), the titer of high-affinity NP_2-specific IgG1 was higher in the KI mice at all time points analyzed (Fig. 6 B). Consistent with this, the NP_2/NP_{12} ratio was higher in FcγRIIb$^{wild/H1}$ KI mice from day 14, indicating enhanced affinity maturation (Fig. 6 C). To confirm this, we performed single cell sorting of NP-specific IgG1$^+$ GC B cells. We sequenced the Ig heavy chain V_H186.2, which is preferentially used by clones specific for NP, and analyzed somatic hypermutation (Smith et al., 1997). NP-specific GC B cells from FcγRIIb$^{wild/H1}$ KI mice had a similar number of mutations per V gene as controls, but 56.4% bore the high-affinity mutation W33L versus 37% in controls (Fig. 6, D and E).

B cell–deficient μMT mice were reconstituted with FcγRIIb$^{wild/H1}$ KI or control BM. The FcγRIIb$^{wild/H1}$ KI B cell–specific chimeras showed reduction in FcγRIIb expression on GC B cells and increased GC B cell number compared with WT chimeras (not depicted). They also had a higher anti-NP_2/NP_{12} serum IgG1 ratio compared with controls (Fig. 6 F) and enhanced accumulation of the high-affinity W33L mutation in V_H186.2 sequences from sorted single NP-specific GC B cells (13.3% in FcγRIIb$^{wild/H1}$ KI vs. 7% in control chimeras; Fig. 6 G), demonstrating that the increased affinity maturation observed in FcγRIIb$^{wild/H1}$ KI mice is B cell intrinsic and further demonstrating that FcγRIIb expression on GC B cells plays an important role in controlling affinity maturation and selection in the GC.

FcγRIIb up-regulation alone can control the development of autoreactive antibodies and memory after immunization

Control of the GC reaction has long been proposed to constitute a crucial checkpoint preventing the breakdown of immune tolerance (Goodnow et al., 2005). As total GC B cells, but not those specific for NP, were increased in FcγRIIb$^{wild/H1}$ KI mice, we wondered whether immunization might lead to the bystander activation of autoreactive clones in these mice, a phenomenon observed after viral or bacterial infection in humans and mice (Zinkernagel et al., 1990; Brás and Aguas, 1995; Hunziker et al., 2003). 8 d after NP-KLH immunization, higher titers of antichromatin and anti–double-stranded DNA (dsDNA) IgM and IgG1 were observed in FcγRIIb$^{wild/H1}$ KI mice. These autoreactive antibodies were not detectable 11 d after immunization, consistent with a transient production of short-lived autoreactive PCs. After a secondary immunization, however, a more substantial increase in antichromatin and anti-dsDNA antibodies was seen (Fig. 7 A), suggesting that autoreactive B cell

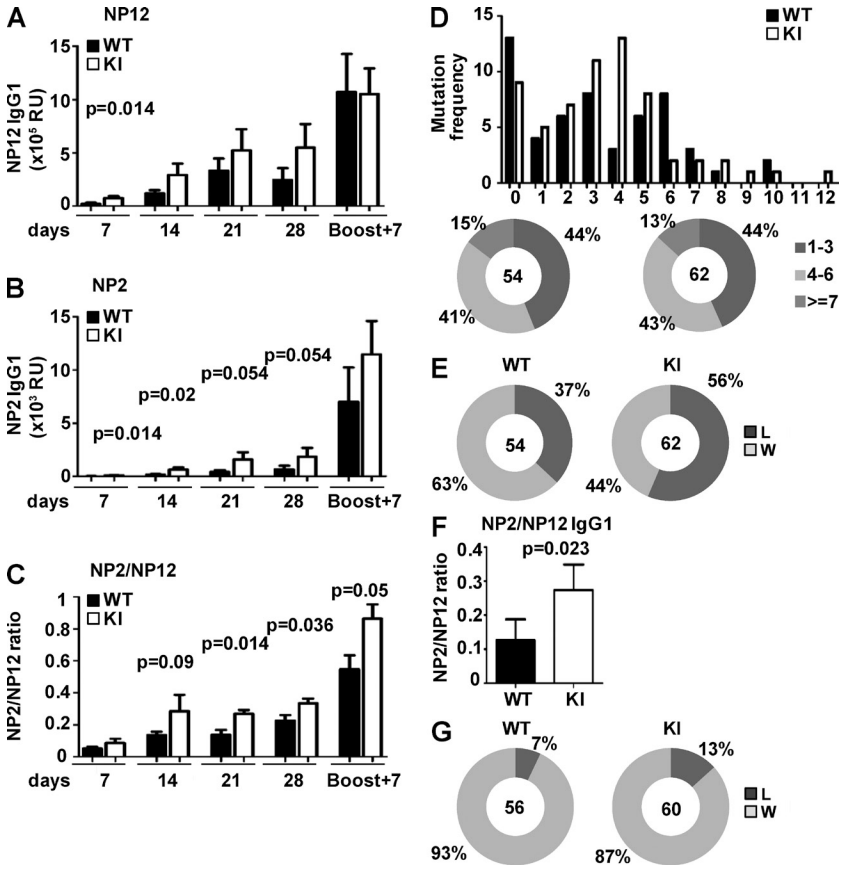

Figure 6. B cell–specific increased affinity maturation in FcγRIIb$^{wild/H1}$ KI mice. (A and B) Total NP_{12}-specific IgG1 antibody titer (A) and high-affinity NP_2-specific IgG1 antibody titer (B) were determined by ELISA 7, 14, 21, and 28 d after immunization with NP-KLH and 7 d after secondary immunization (day 35). Concentrations are expressed in relative units (RU). (C) The ratio of high-affinity anti-NP_2 and total anti-NP_{12} IgG1 titer at each time point was determined. $n = 8$ mice per group, and data are representative of at least two independent experiments. (D) V_H mutation analysis of single cell sorted NP-specific GC B cells 11 d after immunization with NP-KLH (three pooled mice for each group). $n = 54$ sequences for the WT, and $n = 62$ sequences for the KI mice. Data are representative of three independent experiments. (E) The frequency of the high-affinity mutation W33L in V_H186.2 CDR1 was assessed after nested PCR and sequencing. (F) WT and FcγRIIb$^{wild/H1}$ KI μMT chimera mice were immunized with NP-KLH, and NP_2/NP_{12} IgG1 ratio was determined by ELISA after 11 d. $n = 12$ mice for the WT, and $n = 14$ mice for the KI from three experiments pooled. (G) μMT chimera were immunized with NP-KLH, and GC B cells were sorted 11 d later from three pooled WT and three pooled KI chimera mice. The frequency of the high-affinity mutation W33L in V_H186.2 CDR1 was assessed after nested PCR and sequencing of each clone. $n = 56$ sequences for the WT, and $n = 60$ sequences for the KI chimera mice. Data are representative of two independent experiments. In all panels, error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%.

memory was generated during the primary immunization in FcγRIIb$^{wild/H1}$ KI mice. Given the dysregulation apparent in FcγRIIb$^{wild/H1}$ KI mice, we examined predisposition to spontaneous autoantibody production in these mice. By 14 mo of age, FcγRIIb$^{wild/H1}$ KI mice produced significantly more antichromatin and anti-dsDNA autoantibodies than sex- and age-matched control littermates (Fig. 7 B), and females had evidence of pronounced glomerular immune complex deposition (Fig. 7 C), although they had no evidence of proteinuria nor impairment of kidney function (not depicted).

This production of switched autoantibodies in response to immunization and the spontaneous development of autoantibodies with age suggest a breakdown in tolerance that could predispose to immune-mediated disease. This was tested in the collagen-induced arthritis model. Arthritis developed in 75% of both genotypes, but this incidence was reached in 6 d in FcγRIIb$^{wild/H1}$ KI mice against 14 d in controls. The severity of the disease was also increased in the FcγRIIb$^{wild/H1}$ KI mice (Fig. 7 D), further supporting a role for naturally occurring polymorphisms of *Fcgr2b* in increasing susceptibility to autoimmune disease.

DISCUSSION

Analysis of the effect of naturally occurring polymorphisms on the immune response has proven difficult. Human studies are restrained by the genetic complexity inherent to an outbred population and by the fact that limited experimental manipulation of the immune response is possible. Absolute deficiencies or overexpression models do not replicate the complex and potentially subtle effects on gene regulation that common natural variants might confer. Congenic approaches are also rarely able to prove associations, as they invariably contain genetic material that extends beyond the immediate regions of interest. We have used a mouse KI approach to overcome these limitations and study the impact of genetic variation in *Fcgr2b* promoter on immune regulation. This has revealed the potential importance of subtle regulatory variations seen in wild mouse populations and in doing so has provided novel insight into GC regulation by the inhibitory receptor FcγRIIb.

The *Fcgr2b* promoter variant found in most wild mice and in several autoimmune-prone strains was associated with a failure of B cells to up-regulate expression of FcγRIIb upon activation. This increase in FcγRIIb expression upon activation has been noted before (Rudge et al., 2002), but its regulation and functional significance were unknown. This promoter variant was not associated with FcγRIIb expression changes previously described on myeloid-derived cells (Pritchard et al., 2000) or PCs (Xiang et al., 2007; Jørgensen et al., 2010) from NZB or NZW mice, suggesting the latter are governed by

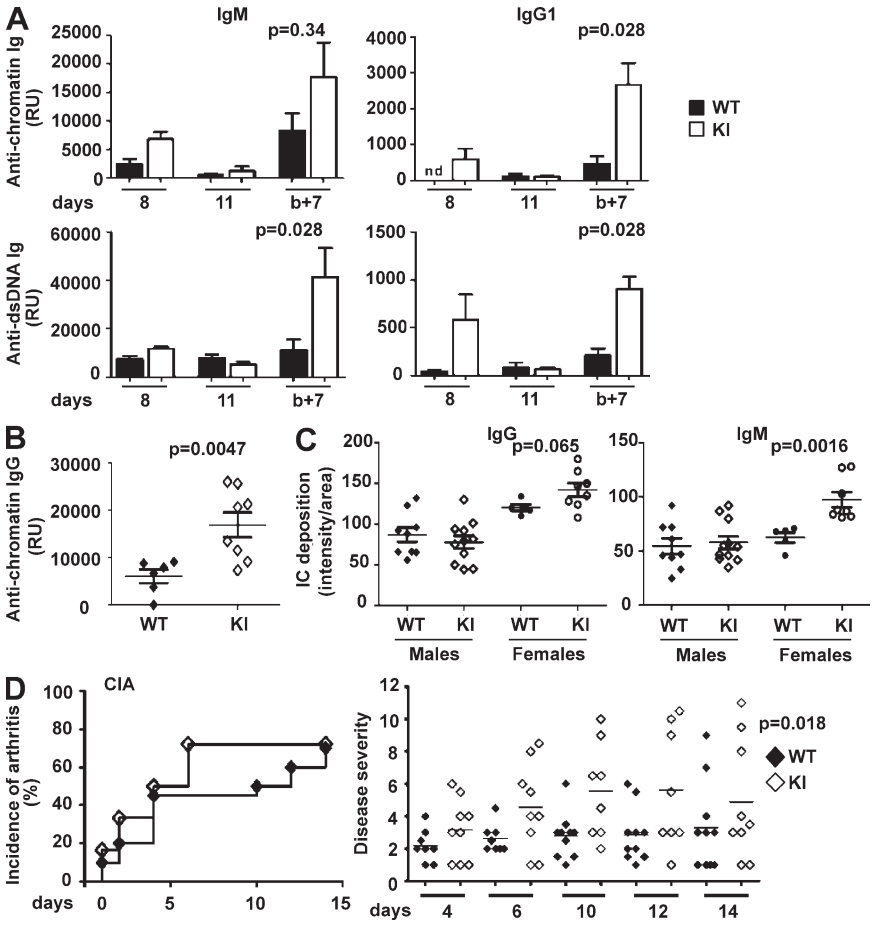

Figure 7. FcγRIIb$^{wild/H1}$ KI mice demonstrate increased autoantibody production and are more susceptible to collagen-induced arthritis. (A) After immunization with NP-KLH, anti-chromatin (top) and anti-dsDNA (bottom) IgM and IgG1 titers were determined in WT and FcγRIIb$^{wild/H1}$ KI mice at days 8 and 11 and 7 d after secondary immunization. $n \geq 4$. Data are representative of at least two independent experiments. (B) Antichromatin IgG titer was determined in sex- and age-matched mice between 11 and 18 mo old. $n = 6$ mice for the WT, and $n = 8$ mice for the FcγRIIb$^{wild/H1}$ KI. (C) IgG and IgM immune complex deposition in the kidney glomeruli of ageing mice was determined by immunohistofluorescence and quantified using Volocity. The relative intensity was normalized to the size of the glomeruli. The results obtained from two different ageing cohorts have been pooled. Mice were all between 14.5 and 15.5 mo old at time of sacrifice. (D) Representative incidence of arthritis after collagen injection (left). Disease severity in mice developing arthritis was scored from 1–3 per paw for a total of 1–12 per animal (right). Day 0 corresponds to the onset of disease. Data from three independent experiments were pooled. In all panels, error bars represent SEM, and p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%, with the exception of D (right), in which the p-value was determined using the Kruskal–Wallis test. RU, relative units.

genetic variation not included in the FcγRIIb$^{wild/H1}$ KI mice, but likely in either intron 3 or more distantly. Our results have revealed that this increased expression on GC B cells is likely to be driven by the transcription factor AP-1, itself induced by activation through the BCR or TLRs (Yi et al., 2003; de Gorter et al., 2007). It is interesting to note that AP-1 also binds to the human *FCGR2B* promoter (Blank et al., 2005; Olferiev et al., 2007). The *FCGR2B* promoter haplotype 2B4, for which an association with SLE has been proposed, is also associated with reduced binding of AP-1 and lower expression of FcγRIIB on activated cells (Blank et al., 2005; Olferiev et al., 2007). This suggests that natural variants affecting the same pathway may have been conserved in both human and mouse. Moreover, AP-1 is predicted to bind to the promoters of several other inhibitory receptors such as CD22 (Andersson et al., 1996), PIR-B (Alley et al., 1998), and CD72 (unpublished data). Activation-induced expression of inhibitory receptors may thus represent an important general mechanism of immune regulation.

This failed up-regulation of FcγRIIb expression on GC B cells has a significant impact on the immune response. Lower FcγRIIb expression was associated with a reduced BCR activation threshold on GC B cells. This result fits with a recent study showing that GC B cell BCR signaling is dampened by increased expression and activation of the phosphatases SHIP-1 and SHP-1 (Khalil et al., 2012), which are under the control of several inhibitory receptors, including FcγRIIb. Accordingly, we observed an enhanced early GC reaction and humoral immune response in the absence of FcγRIIb up-regulation. Surprisingly, however, we observed an early and sustained increase in affinity maturation of antigen-specific GC B cells in these mice. Previous models suggested that reduction of the BCR activation threshold might, on the contrary, result in less stringent selection of B cells on the basis of affinity, and thus less affinity maturation (Tarlinton and Smith, 2000). Our results allow us to propose a model explaining this unexpected finding. The FcγRIIb$^{wild/H1}$ KI mice display reduced apoptosis and increased number of total GC B cells but normal antigen-specific GC B and T_{FH} cell numbers compared with WT mice, suggesting that FcγRIIb up-regulation limits the survival of antigen nonspecific (or bystander) GC B cells. Competition for T_{FH} help has been shown to be a limiting factor for B cell affinity maturation (Schwickert et al., 2011), and it is therefore possible that competition between NP-specific B cells and non-NP B cells for limited T_{FH} help is responsible for the increased affinity maturation seen in FcγRIIb$^{wild/H1}$ KI mice. This could constitute a new model for selection in the GC, where B cell activation threshold controls survival of bystander B cells, whereas affinity maturation of antigen-specific B cells is controlled by competition for T_{FH} and/or antigen.

Another striking feature of the FcγRIIb$^{wild/H1}$ KI mice is the transient production of autoantibodies after immunization. Together with the age-related spontaneous autoantibody production, increased immune complex deposition in the renal glomeruli, and the enhanced severity of collagen-induced arthritis observed in our KI mice, these findings demonstrate that naturally occurring variants of *Fcgr2b* can contribute to the development of autoimmune disease but, like all contributors to polygenic disease, need the additional influence of other genetic variants to achieve full disease penetrance. These data provide insight into the mechanistic details of this contribution, suggesting that FcγRIIb up-regulation plays a critical role in the counter-selection of autoreactive clones and contributes to the GC checkpoint long proposed to be important for maintaining peripheral tolerance (Goodnow et al., 1990; Nossal and Karvelas, 1990). The FcγRIIb contribution to that checkpoint appears to be to promote the apoptosis of bystander GC B cells by increasing the BCR threshold of activation, thus leading to the elimination of clones that could otherwise differentiate into autoreactive plasmablasts and/or memory B cells. The early onset of IgM autoantibodies after T-dependent and -independent immunization may also suggest that FcγRIIb up-regulation on activated B cells plays a role in the control of autoreactive plasmablasts before the GC reaction. Thus, FcγRIIb may influence both affinity maturation and tolerance by controlling the survival of bystander B cells, a possibility which warrants confirmation in other models.

In summary, the study of a specific naturally occurring wild mouse promoter haplotype has directly implicated this variant in controlling GC formation, affinity maturation, and immunological tolerance and has also demonstrated the importance of activation-induced up-regulation of the inhibitory FcγRIIb in controlling immune responses and self-reactivity. It provides a definitive, controlled demonstration that a promoter polymorphism that affects only stage-specific changes in the expression of a BCR signaling modulator is sufficient to contribute to autoimmunity.

MATERIALS AND METHODS

Mice and immunization. All experiments were performed according to the regulations of the UK Home Office Scientific Procedures Act (1986). The FcγRIIb$^{wild/H1}$ KI mice were generated at Ozgene (Köntgen et al., 1993; Koentgen et al., 2010). A fragment of 1,271 bp from the NZB promoter of FcγRIIb was fused to 1,996 bp from the C57BL/6 promoter to form the 5′ homology arm. The 3′ homology arm was composed of 3,905 bp from the C57BL/6 promoter. Bruce 4 C57BL/6J ES cells were used to generate the FcγRIIb$^{wild/H1}$ KI mice. FcγRIIb$^{wild/H1}$ KI mice and WT littermates were immunized with 100 µg NP-KLH (loading 27–29) or NP-Ficoll (loading 41; Biosearch Technologies) in alum (Thermo Fisher Scientific) intraperitoneally. When indicated, mice were immunized a second time 21 or 27 d later with the same antigen.

Genetic analysis of *Fcgr2b* promoter polymorphisms. Genomic DNA was obtained from 53 strains of wild-caught mice or from wild mouse–derived inbred strains from around the world (gift of F. Bonhomme [Institut des Sciences de l'Evolution-Montpellier, Montpellier, France] and J.-L. Guénet [Institut Pasteur, Paris, France]). The promoter was amplified using a nested PCR reaction using primers mGRII53(−384) and mGRII35(87). The product was reamplified with primers mGRII53(−349) and mGRII35(22) for 20 cycles. Sequencing was performed using mGRII53(−349) and mGRII35(22) as primers with the Big Dye terminator sequencing kit (Perkin-Elmer) according to the manufacturer's instructions and analyzed on an ABI Prism 377 automatic DNA sequencer (Applied Biosystems). Primers were purchased from Oswel and are detailed in Table S1.

Flow cytometry. Single cell suspensions of spleen and BM were prepared as previously described (Espeli et al., 2011). For phosphoflow staining, splenocytes from mice immunized with NP-KLH were stimulated at 37°C for 5 or 10 min with 10 µg/ml of a goat anti–mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, Inc.). Cells were then fixed with ice-cold methanol on ice for 30 min before staining with the relevant antibodies. Staining was performed with the antibodies and molecules described in Table S2. FACS analysis was performed on a Cyan analyzer (Dako) or a Fortessa II (BD), and data were analyzed with FlowJo software (Tree Star).

Luciferase assay and mutagenesis. The promoter region and the 5′ untranslated region of *Fcgr2b* (from position −907 to 586) were amplified from WT (C57BL/6), FcγRIIB$^{wild/H1}$ KI, NZB, and NZW genomic DNA and cloned into the pGL4.14 hLuc vector (Promega). The list of the different primers used is provided in Table S1. The *Fcgr2b* WT and KI constructs were mutated using the QuikChange mutagenesis kit (Agilent Technologies) and the indicated primers (Table S1).

Bal17 cells (mature B cell line from BALB/c origin, haplotype III) were stimulated with 10 µg/ml LPS and transiently transfected with the different pGL4.14 hLuc vectors (Promega) and a pCMV Renilla vector (gift of N. Lapaque and T. de Wouter [Institut Micalis, Jouy-en-Josas, France]) as a normalization vector using Lipofectamine 2000 (Invitrogen). Luciferase and Renilla activity were analyzed after 24 h using the Dual-glo kit (Promega) and a Glomax reader (Promega). Relative luciferase activity was calculated by normalizing the luciferase to the Renilla OD.

Transcription factor binding site predictions. The 40 residues surrounding position −1/+2 and the 36 residues surrounding position −79 from WT and FcγRIIb$^{wild/H1}$ KI mice were analyzed for differential transcription factor binding sites with the JASPAR database and the TRANSFAC 6.0–based algorithms TFsearch, Patch, and MATCH. JASPAR, TFsearch, and Patch predicted the existence of an AP-1–binding site in the WT sequence only. Only MATCH predicted the existence of an AP-1–binding site in both the WT and KI sequences.

ChIP. Bal17 cells or MACS-purified CD19$^+$ splenic B cells were left untreated or stimulated for the indicated period with 10 µg/ml goat anti–mouse IgG (H+L; Jackson ImmunoResearch Laboratories, Inc.) or with 10 µg/ml LPS (Sigma-Aldrich). ChIP was performed with the ChIP kit from Abcam and the anti–c-Jun (H-79) and anti–c-Fos (K-25) antibodies from Santa Cruz Biotechnology, Inc. A rabbit anti–mouse IgG (Cell Signaling Technology) was used as an isotype control. Total chromatin (input) and the immunoprecipitated chromatin were used as template, and the −79 locus of the *Fcgr2b* promoter was amplified by conventional or quantitative PCR using the primers indicated in Table S1. Quantitative PCR was performed with the QuantiFast SYBR green PCR kit (QIAGEN).

Immunohistology. Histology was performed as described previously (Espeli et al., 2011). Mouse spleens and kidneys were stained with the antibodies described in Table S2 and mounted in Mowiol supplemented with DAPI. Staining was analyzed on an LSM510 confocal microscope with the LSM analysis software (Carl Zeiss) using a Plan-Neofluar 25× objective at room temperature for the spleen sections and a Plan-Apochromat 20× objective at 37°C for the kidney sections. Immune complex deposition in the kidney glomeruli was quantified using Volocity software (PerkinElmer). The relative intensity of IgG and IgM staining for each glomerulus was divided by the glomerulus area (in square micrometers).

ELISA and ELISPOT. Serum NP-specific and dsDNA-specific Igs were detected by ELISA as previously described (Brownlie et al., 2008; Espeli et al., 2011). This protocol was adapted to detect chromatin-specific Ig by coating ELISA plates with chicken chromatin overnight at 4°C. For detection of NP-specific antibody-forming cells (AFCs), ELISPOT plates (Millipore) were coated with 5 µg/ml NP_{12}-BSA or NP_2-BSA in PBS overnight at 4°C. Single cell suspensions of spleen and BM were added to saturated ELISPOT plates in quadruplicate and incubated overnight at 37°C in 5% CO_2 in a humidified incubator with culture medium. AFCs were detected with goat anti–mouse Ig antibody conjugated to horseradish peroxidase specific for the different isotypes tested (all from SouthernBiotech). Plates were developed using 3-amino-9-ethylcarbazole tablets (Sigma-Aldrich). Plates were read using an AID ELISPOT reader according to the manufacturer's instructions.

Cell sorting and V_H gene analysis. NP-specific $IgG1^+$ GC B cells were sorted as previously described (Smith et al., 1997). 11 d after immunization with NP-KLH, spleen cells from a pool of three mice were prepared and stained to sort ($IgD^-IgM^-Gr1^-CD138^-$), $B220^+$, $IgG1^+$, NP^+ cells. Single cells were directly sorted in lysis buffer (20U RNase inhibitor, 0.3 µg random hexamers, and 1% NP-40). cDNA was prepared using Superscript II (Invitrogen) and used in the first round of two nested PCRs (see Table S1 for primer sequences). PCR products were sequenced using Big Dye terminator version 3.1 (Applied Biosystems). Sequences were analyzed with FinchTV software.

Collagen-induced arthritis. An emulsion of chicken type II collagen (Sigma-Aldrich) in complete Freund's adjuvant was prepared as previously described (Brownlie et al., 2008). Mice were injected intradermally with 100 µl of the collagen/CFA emulsion twice. After the secondary immunization, animals were assessed for redness and swelling of limbs every 2 d. Limbs were scored from 0 (no obvious sign of inflammation) to 3 (severe swelling, erythema, and/or joint ankylosis, stiffness, and distortion), the maximum score per mouse being 12.

Statistical analysis. All p-values were determined using the Mann–Whitney two-tailed test with a risk of 5%, except in Fig. 7 D, where the p-value was determined using the Kruskal–Wallis test.

Online supplemental material. The details of the primers and antibodies used are to be found in Tables S1 and S2, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121752/DC1.

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Author contributions: M. Espéli designed, performed, and analyzed the experiments and wrote the paper. M.R. Clatworthy designed, performed, and analyzed the wild mouse genetics and helped write the paper. S. Bökers, K.E. Lawlor, and A.J. Culter performed and analyzed experiments. F. Köntgen generated the KI mouse. P.A. Lyons contributed to the project design. K.G.C. Smith designed the project, analyzed data, and wrote the paper.

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