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

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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.

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function (Floto et al., 2005; Kono et al., 2005) and has been associated with SLE (Kyogoku et al., 2002; Sirboonrit 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 (Yusa 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 FcγR2b 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 FcγR2b 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; Jörgensen et al., 2010) and enhanced B cell immune responses (Vuyyuru et al., 2009; Jörgensen 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 FcγR2b variants cannot be conclusively implicated in the phenotype observed in them. Moreover, the mechanism by which natural FcγR2b variation contributes to autoimmunity in mouse and human is not known. Studies of natural genetic variants of FcγR2b 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 FcγR2b 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). Haplo-type III (which lacks all deletions) was found in the majority of commonly used strains such as BALB/c and C57BL/6. Haplo-type II (comprised of deletions 3 and 4) was found only in NZW, SJL/J, and SWR/J mice. Haplotype 1 (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 FcγR2b 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 molosinus in Japan, which made important genetic contributions to inbred strains (Fig. 1 C; Beck et al., 2000; Gueñot 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 FcγR2b (not depicted). FcγR2bwild/H1 KI mouse; Fig. 2 A).

The FcγRIIbwild/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-nitrophenylacetil
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.
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<sup>wild/H1</sup> KI 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 (Jorgensen et al., 2010) PCs (Fig. 2, D and G).

FcγRIIb expression was also normal on all myeloid subpopulations tested (splenic dendritic cells, monocytes, and...
Transcriptional control of FcγRIIb up-regulation

The effect of FcγRIIb promoter variants on transcription was analyzed by luciferase assay. The polymorphisms present in the FcγRIIb 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. 3A). Consistent with the equivalent effects of haplotypes I and II in reducing FcγRIIb transcriptional activity, congenic mice incorporating distal chromosome 1 from either NZB or NZW backgrounds demonstrate reduced FcγRIIb expression on GC B cells (Fig. 3A; Xiu et al., 2002; Rahman et al., 2007). As this suggests that the genetic variants driving the failure of activation-induced up-regulation are 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 FcγRIIbwild/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 FcγRIIbwild/H1 KI compared with controls, suggesting that it is the activation-induced up-regulation of FcγRIIb which is abnormal in these mice (Fig. 2H). Thus, naturally occurring variants in the FcγRIIb promoter abrogate activation-induced up-regulation of FcγRIIb, an effect most prominently seen on GC B cells.

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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 Fgr2b promoter when mutated alone, but the combination of the GG\textsubscript{-1}/+2AA and G\textsubscript{-79}C SNPs reduced the transcriptional activity to a level similar to that of the KI/NZB promoter (Fig. 3 C). These results suggest that these two substitutions are sufficient to disrupt the activation-induced up-regulation of Fc\(\gamma\)RIIb.

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 Fgr2b 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 Fgr2b 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 Fgr2b 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 Fc\(\gamma\)RIIbwild/H1 KI B cells (Fig. 3 E). These results suggest that AP-1 drives the activation-induced up-regulation of Fc\(\gamma\)RIIb and that natural variants of the Fgr2b promoter disrupt this process.

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

Reduced expression of FcyRIIb 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 FcyRIIbwild/H1 KI GC B cells compared with naive B cells (Fig. 4 A), consistent with the lower expression of phospho–Syk and phospho-BLNK in GC B cells gated as B220\(^{+}\)GL7\(^{+}\)FAS\(^{-}\) 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 immunohistochemistry. 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 ≥ 4 mice, and data are representative of at least two independent 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\(_{\text{H}1}\) cells (CD4\(^{+}\)CXCR5\(^{+}\)PD1\(^{+}\)) 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%.
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<sup>Wld/H1</sup> 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<sup>Wld/H1</sup> 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<sub>FH</sub> 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<sup>Wld/H1</sup> 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-specific IgG1 was higher in the KI mice at all time points analyzed (Fig. 6 B). Consistent with this, the NP<sub>2</sub>/NP<sub>12</sub> serum IgG1 ratio compared with controls (Fig. 6 F) and enhanced accumulation of the high-affinity W33L mutation in V<sub>H</sub>186.2 sequences from sorted single NP-specific GC B cells (13.3% in FcγRIIb<sup>Wld/H1</sup> KI vs. 7% in control chimeras; Fig. 6 G), demonstrating that the increased affinity maturation observed in FcγRIIb<sup>Wld/H1</sup> 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<sup>Wld/H1</sup> 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<sup>Wld/H1</sup> 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
memory was generated during the primary immunization in FcγRIIbwild/H1 KI mice. Given the dysregulation apparent in FcγRIIbwild/H1 KI mice, we examined predisposition to spontaneous autoantibody production in these mice. By 14 mo of age, FcγRIIbwild/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 FcγRIIbwild/H1 KI mice against 14 d in controls. The severity of the disease was also increased in the FcγRIIbwild/H1 KI mice (Fig. 7 D), further supporting a role for naturally occurring polymorphisms of Fgr2b 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 FcγRIIbwild/H1 KI mice. 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 Fgr2b 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...
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 FcγRIIbwild/H1 KI mice display reduced apoptosis and increased number of total GC B cells but normal antigen-specific GC B and TFH cell numbers compared with WT mice, suggesting that FcγRIIb up-regulation limits the survival of antigen nonspecific (or bystander) GC B cells. Competition for TFH 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 TFH help is responsible for the increased affinity maturation seen in FcγRIIbwild/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 TFH help and/or antigen.
Another striking feature of the FcYRIIb\textsuperscript{wild/11} 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 FcYRIIb 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 FcYRIIb 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 FcYRIIb up-regulation on activated B cells plays a role in the control of autoreactive plasmablasts before the GC reaction. Thus, FcYRIIb 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 FcYRIIb 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\textsuperscript{wild/11} KI mice were generated at Ozgene (Köntgen et al., 1993; Köntgen 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 x C57BL/6J ES cells were used to generate the FcγRIIb\textsuperscript{wild/11} KI mice. FcγRIIb\textsuperscript{wild/11} KI mice and WT littermates were immunized with 100 µg NP-KLH (loading 27–29) or NP-Ficoll (loading 41; Biosearch Technologies) in alun (Thermo Fisher Scientific) intraperitoneally. When indicated, mice were immunized a second time 21 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\textsuperscript{wild/11} 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 Wouwer [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\textsuperscript{wild/11} 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 Moviol 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 Iggs were detected by ELISA as previously described (Brownlie et al., 2008; Espel 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$_2$-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-amin-9-ethylcarbazole tablets (Sigma-Aldrich). Plates were read using an AIDS 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 BRD1/BLI4, spleen cells were stained with a mAb to sort IgG1+ (CD138$^+$), B220$, IgG1$, NP$^+$ cells. Single cells were directly sorted in lyse buffer (100 µg/ml RNase inhibitor, 0.3% rhamn 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.

We thank Dr. M. Linterman for helpful discussion, Dr. T. Rayner for assistance with the statistical analysis, Dr. L. Jarvis for overseeing animal husbandry, J. Sowerby and Dr. R. Matthews for technical help, Drs. F. Bonhomme and J.-L. Guénet for wild mouse DNA, and Drs. M. Glennie, N. Lapaque, and T. de Wouter for reagents.

This work was funded by the Wellcome Trust (Programme Grant Number 083650/Z/07/Z to K.G.C. Smith) and the National Institute for Health Research Cambridge Biomedical Research Centre. M. Espêli was supported by a fellowship of European Biomedical Chemical Sciences Long-Term Fellowship, M.R. Clatworthy by a Wellcome Trust Intermediate Fellowship (WT081020), and K.E. Lawlor by a C.J. Martin Training Fellowship (National Health and Medical Council of Australia, 356267). K.G.C. Smith is a Lister Prize fellow.

The authors declare no competing financial interest.

Author contributions: M. Espêli 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. Cutler performed and analyzed experiments. F. Königgen 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.

Submitted: 3 August 2012
Accepted: 1 October 2012

REFERENCES


### Table S1. Primer list

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Point mutations are underlined.
Table S2. List of antibodies and molecules used for flow cytometry and immunohistology

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