Consequences of the recurrent *MYD88^{L265P}* somatic mutation for B cell tolerance

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***MYD88^{L265P}* has recently been discovered as an extraordinarily frequent somatic mutation in benign monoclonal IgM gammopathy, Waldenström's macroglobulinemia, and diffuse large B cell lymphoma. In this study, we analyze the consequences for antigen-activated primary B cells of acquiring *MYD88^{L265P}*. The mutation induced rapid B cell division in the absence of exogenous TLR ligands and was inhibited by *Unc93b1^{3d}* mutation and chloroquine or TLR9 deficiency, indicating continued dependence on upstream TLR9 activation. Proliferation and NF-κB activation induced by *MYD88^{L265P}* were nevertheless rapidly countered by the induction of *TNFAIP3*, an NF-κB inhibitor frequently inactivated in *MYD88^{L265P}*–bearing lymphomas, and extinguished by Bim-dependent apoptosis. *MYD88^{L265P}* caused self-reactive B cells to accumulate in vivo only when apoptosis was opposed by *Bcl2* overexpression. These results reveal checkpoints that fortify TLR responses against aberrant B cell proliferation in response to ubiquitous TLR and BCR self-ligands and suggest that tolerance failure requires the accumulation of multiple somatic mutations.**

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Abbreviations used: ABC-DLBCL, activated B cell type diffuse large B cell lymphoma; BCR, B cell receptor; CLL, chronic lymphocytic leukemia; CTV, cell trace violet; DD, death domain; GCB-DLBCL, germinal center B cell type diffuse large B cell lymphoma; IRAK, interleukin receptor activated kinase; MYD88, myeloid differentiation primary response 88; TIR, Toll/interleukin-1 receptor; TNFAIP3, tumour necrotic factor alpha-induced protein 3.

B cell lymphoproliferative diseases represent natural mutagenesis experiments that shed light on normal B cell regulatory mechanisms (Rui et al., 2011) in addition to being major causes of human morbidity and mortality. These take numerous forms, encompassing non-Hodgkin and Hodgkin lymphomas, chronic lymphocytic leukemia, Waldenström's macroglobulinemia, myeloma, and clinical or subclinical monoclonal gammopathies (Shaffer et al., 2002). Learning about normal B cell regulation from malignant B cells is confounded, however, by the accumulation of 20 or more protein-altering somatic mutations in malignant B cell clones (Morin et al., 2011; Pasqualucci et al., 2011; Puente et al., 2011). The drive toward malignancy must begin with individual mutations, but aside from a few well-studied mutations like *MYC* and *BCL2* translocations (ar-Rushdi et al., 1983; Tsujimoto et al., 1985; Vaux et al., 1988), little is known about the consequences of recurring lymphoma mutations individually or combinatorially for the behavior of otherwise normal mature B cells.

MYD88 mutations have emerged as one of the most frequently recurring mutations in mature B cell lymphoproliferative disease. Somatic missense mutations in *MYD88* were discovered by Ngo et al. (2011) in 39% of cases of a common form of non-Hodgkin's lymphoma, activated B cell type diffuse large B cell lymphoma (ABC-DLBCL), with a single L265P substitution accounting for 75% of the mutations. The L265P mutation occurs in almost 100% of cases of Waldenström's macroglobulinemia (Treon et al., 2012; Xu et al., 2013), at least 47% of cases of IgM monoclonal gammopathy of undetermined significance (Xu et al., 2013), 3–10% of cases of chronic lymphocytic leukemia (Puente et al., 2011; Wang et al., 2011), and 13% of splenic marginal zone lymphoma (Trøen et al., 2013). Other TIR domain mutations, such as S219C, predominate in germinal center B cell type diffuse large B cell lymphoma (GCB-DLBCL; Ngo et al., 2011).

MYD88 is an important adaptor protein that bridges TLR and the IL-1 receptor to the activation of downstream IL receptor–activated kinases (IRAKs) and NF-κB transcription factor

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activation (Akira and Takeda, 2004). MYD88 has two distinct domains, the Toll/IL-1R like domain (TIR), via which MYD88 proteins homodimerize upon activation, and the death domain (DD), which recruits IRAKs to form the signaling complex (Akira and Takeda, 2004). Interestingly, all *MYD88* lymphoma mutations are found in the TIR domain and result in uncontrolled formation of the MYD88–IRAK signaling complex (Ngo et al., 2011). An ABC-DLBCL cell line with the *MYD88^{L265P}* mutation showed hyperphosphorylation of IRAK1 and elevated NF-κB activity, whereas shRNA studies established that the dysregulated MYD88 to NF-κB signaling was necessary for the survival of this cell line (Ngo et al., 2011). Similarly evidence for this mutation driving exaggerated NF-κB activity has been obtained in malignant cells from Waldenström's macroglobulinemia (Treon et al., 2012) and CLL (Wang et al., 2011). However, it remains unclear whether *MYD88^{L265P}* mutation actively drives the proliferation of these malignant B cells or only maintains their survival, and the consequences of *MYD88^{L265P}* mutation in the precursors of malignant B cells that do not carry numerous other somatic mutations are unknown.

Discrimination between chemical components of infecting microbes and self-tissues is the central problem for normal B cell regulation. B cells express multiple TLRs, each serving as a sensor for infection by binding evolutionarily conserved molecules that differ between microbes and self (Akira and Takeda, 2004; Beutler, 2004). TLR3, TLR7, and TLR9 bind features of RNA or DNA that are enriched in microbial as opposed to mammalian nucleic acids, such as unmethylated CpG-rich DNA sequences or double-stranded RNA (Krieg, 2002). Because these features are also present at lower abundance in self-nucleic acids, the nucleic acid–sensing TLRs must use additional mechanisms to ensure they tolerate and do not trigger immune responses to self-nucleic acids. The mechanisms for TLR self-tolerance are nevertheless not well understood. One important mechanism is restriction of the activity of TLR3, TLR7, and TLR9 to acidified endosomes, where microbes are frequently trafficked by endocytosis after being captured by cell surface immunoglobulin (B cell antigen receptors [BCRs]). Restriction is achieved by Unc93b1-mediated TLR3, TLR7, and TLR9 trafficking to endosomes (Tabeta et al., 2006; Kim et al., 2008), and by requirement for proteolytic activation of the TLR ectodomain by endosomal proteases active only at low pH (Ewald et al., 2008). Because self-binding BCRs are negatively selected through processes of central and peripheral B cell deletion, editing, and anergy (Goodnow and Ohashi, 2013), these BCR tolerance mechanisms assist self–nonself discrimination with nucleic acid–sensing TLRs in B cells.

TLR tolerance to self-nucleic acids breaks down when BCR tolerance mechanisms fail, allowing BCRs to capture self-nucleic acids directly or indirectly and deliver them to acidified endosomes (Leadbetter et al., 2002). TLR and BCR signaling converges on NF-κB activation through separate adapter proteins, MYD88 and CARD11, respectively. BCR tolerance involves an uncoupling of CARD11 signals to NF-κB, and is disrupted by a range of activating somatic mutations in CARD11 frequently found in non-Hodgkin lymphoma (Lenz et al., 2008; Jeelall et al., 2012). It is conceivable that BCR tolerance mechanisms might also be disrupted by uncontrolled activity of nucleic acid sensing TLRs or gain-of-function *MYD88* mutations, potentially initiating a vicious cycle of cross-activation by self-sensing BCRs and TLRs, but this theoretical possibility has yet to be examined.

Here, we analyze the effect of *MYD88* mutations on the behavior of normal antigen-activated B cells, including B cells bearing self-reactive BCRs, using a retroviral gene transfer system in mice. We show that *MYD88^{L265P}* initially breaks tolerance to RNA- or DNA-sensing TLRs, resulting in *Unc93b1* and TLR9-dependent B cell proliferation in the absence of foreign ligands for TLR9. However, activation of NF-κB signaling and proliferation by *MYD88^{L265P}* is rapidly terminated through inhibition of NF-κB signaling by *Tnfaip3* and by *Bcl2/Bim*-regulated apoptosis. These results reveal a multilayered mechanism for B cell tolerance to self-ligands of nucleic acid–sensing TLRs, whose progressive disruption by mutations often found in human B cells cross-interferes with tolerance to self-ligands of the BCR.

RESULTS

MYD88^{L265P} mutation drives B cell proliferation independent of foreign TLR ligand in vitro

To investigate the consequences of *MYD88* mutations arising in mature, antigen-activated B cells that lack other lymphoma mutations, we used retroviral gene transfer to introduce normal or mutated mouse *Myd88* alleles into activated B cells from mice that bear a transgenic BCR to a known antigen, hen egg lysozyme (HEL; Goodnow et al., 1988; Fig. 1 A). B cells were activated by antigen, and then stimulated by CD40; retrovirally transduced with mutant *Myd88*, WT *Myd88*, or EGFP-only control reporter vectors; washed; and placed back into tissue culture in the absence of mitogen stimulation to compare the fate of EGFP$^+$ cells over time (Fig. 1, A and B). We initially tested the effects of six MYD88 TIR domain mutations (Ngo et al., 2011): L265P, S219C, S222R, M232T, S243N, and T294P (Fig. 1 A). Four of the six, L265P (Mut1), S219C (Mut2), M232T (Mut4), and S243N (Mut5), promoted mitogen-independent B cell proliferation in culture that was not observed when wild-type MYD88 was expressed, whereas S222R (Mut3) and T294P (Mut6) showed no proliferation. The wild-type and mutant Myd88 proteins were expressed at comparable levels, demonstrating that proliferation resulted from specific mutations and not simply from overexpression of the protein (Fig. 1, B–D). B cells transduced with the *MYD88^{L265P}:EGFP* vector spontaneously increased their number in the first 3 d of culture to 4–5 times the starting culture number, despite the absence of antigen or anti-CD40 antibody (Fig. 1 B). In contrast, over the same period the *MYD88^{WT}:EGFP* and *empty:EGFP* vector-transduced B cells did not increase in number (Fig. 1 B). EGFP$^-$ nontransduced B cells served as an internal control and all declined in number over the course of 3 d, indicating that

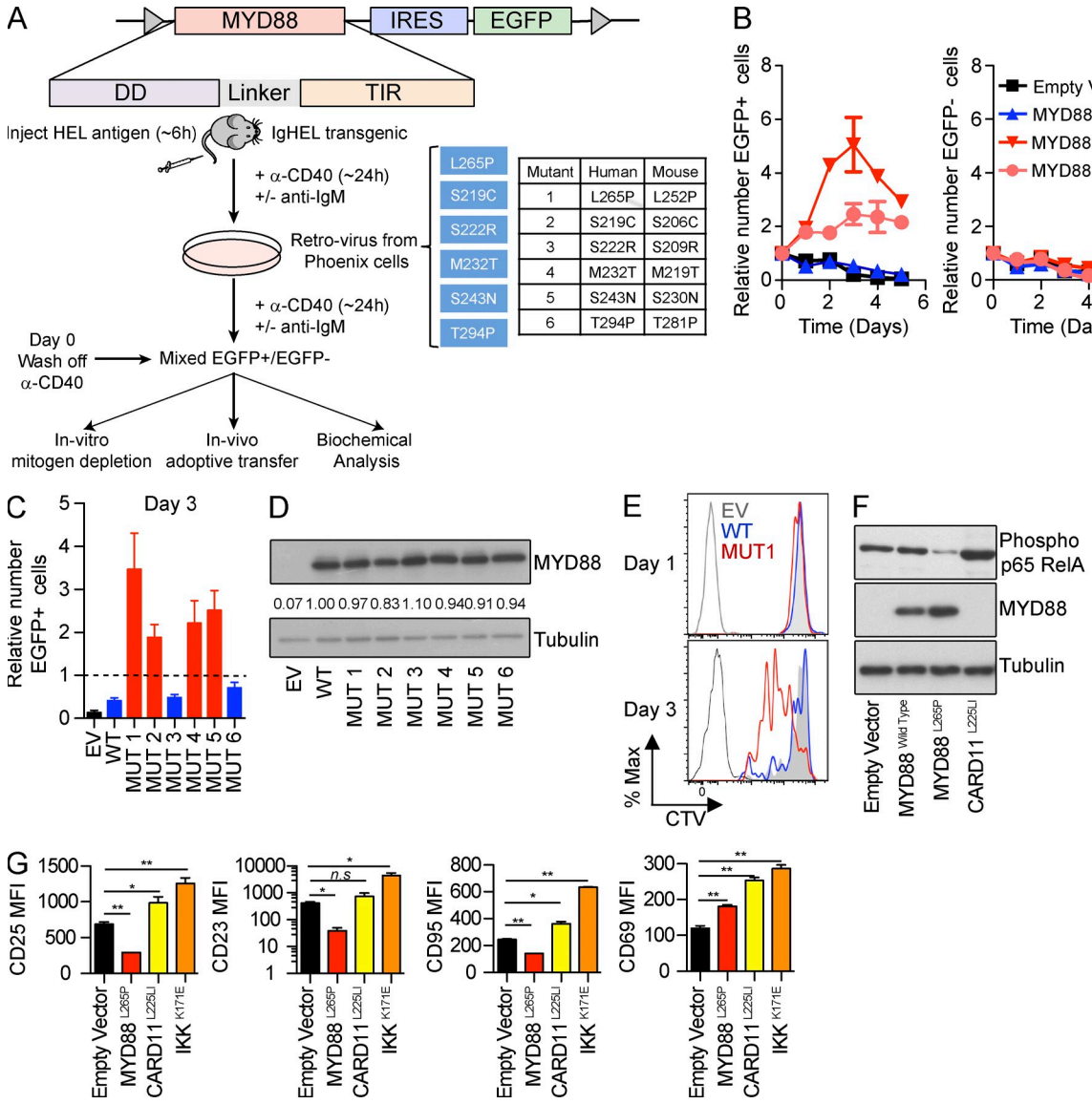

Figure 1. ***MYD88*** **mutations promote mitogen-independent B cell proliferation in vitro but, paradoxically, shut down NF-κB.** (A) Experimental strategy for retrovirally introducing mutant *MYD88* into activated mature splenic B cells. HEL-specific B cells from IgHEL-transgenic mice were activated by HEL antigen in vivo and polyclonal B cells from nontransgenic mice were activated by including anti-IgM during culture with anti-CD40. After transduction, the cells were washed and cultured without these mitogens (day 0) or transplanted into syngeneic mice. Table shows the corresponding human and mouse amino acid substitutions tested. (B) Activated HEL antigen-specific B cells transduced with the indicated vectors were placed into fresh triplicate cultures in the absence of antigen or CD40 stimuli for 5 d. Mean and SD of EGFP$^+$ cells expressing each vector were compared with the starting amount on day 0 of the cultures. Data are representative of at least three independent experiments. (C) Mean and SD of EGFP$^+$ B cells transduced with the indicated vectors after 3 d in triplicate cultures without antigen or CD40 stimulation, relative to the number at the start of the cultures (dashed line) are representative of at least three independent experiments. (D) EGFP$^+$ cells expressing the indicated vectors were sorted on day 1 of culture without antigen or CD40 stimuli, and lysates analyzed by SDS-PAGE and Western blot for MYD88 protein and tubulin as loading control. Numbers show densitometric measurements of MYD88 in each sample, expressed as relative to cells expressing the MYD88^{WT} vector. (E) Cell division measured by CTV dilution on days 1 and 3 of culture without antigen or CD40 stimulation, gated on *MYD88^{L265P}:EGFP* (MUT1) or *MYD88^{WT}:EGFP*-transduced (WT) B cells or in nondividing *empty:EGFP* vector transduced B cells (EV). Unlabeled cells are shown by the open gray histogram. Data are representative of at least three independent experiments. (F) EGFP$^+$ cells expressing the indicated vectors were sorted on day 1 of culture without antigen or CD40 stimuli, and lysates were analyzed by SDS-PAGE and Western blot for phosphorylation of the p65 subunit of NF-κB, with the blot sequentially reprobed with antibodies to MYD88. Tubulin was used as a loading control. (G) Mean fluorescent intensity (MFI) of cell surface expression of proteins encoded by NF-κB–inducible genes, measured in independent triplicate cultures (mean and SD) by flow cytometry on day 1 of culture without antigen or CD40 stimuli, gated on EGFP$^+$ cells transduced with the indicated vectors. Statistical analysis by Student's *t* test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Data are representative of at least three independent experiments.

proliferation induced by *MYD88*^{L265P} was limited to the B cells that expressed the mutation and not the result of a secreted or cell–cell interaction factor (Fig. 1 B). However, the *MYD88*^{L265P}-induced proliferation was short lived and the numbers of live EGFP$^+$ B cells dropped sharply after day 3 in culture (Fig. 1 B).

Cell division was measured by labeling the transduced B cells with cell trace violet (CTV), a dye that is progressively diluted with each cell division. *MYD88*^{L265P}*:EGFP*$^+$ B cells diluted the CTV dye over the course of 3 d as a result of multiple cell divisions (Fig. 1 E). Most B cells expressing comparable levels of WT *MYD88*^{wildtype}*:EGFP* or empty*:EGFP* vector did not divide over this time course, although a subset of *MYD88*^{WT}–transduced B cells expressing very high levels of EGFP did persist and divide (Fig. 1 E and not depicted). In contrast, the accumulating B cells with *MYD88*^{L265P} expressed the bicistronic *MYD88*^{L265P}*:EGFP* vector over a broad range that mirrored the starting distribution and the distribution in cells transduced with empty:*EGFP*.

We next looked at the downstream pathways that drove the proliferation of *MYD88*^{L265P} B cells. Since MYD88 and CARD11 are adaptor proteins that connect TLRs and the B cell antigen receptor, respectively, to NF-κB activation, we compared the effects of activated mutant *MYD88* and *CARD11* alleles on NF-κB activity in primary B cells, alongside a mutant *IKBKB* allele (K171E; Rossi et al., 2011) that directly activates NF-κB (Mercurio et al., 1997). As described previously (Jeelall et al., 2012), B cells transduced with *CARD11*^{L232LI}*:EGFP* vector displayed increased NF-κB p65 phosphorylation (Fig. 1 F). *CARD11*^{L232LI} or *IKK*^{K171E} increased expression of CD25, CD95, and CD69, whereas the latter also induced CD23, each encoded by an NF-κB–induced gene (Fig. 1 G). Paradoxically, *MYD88*^{L265P}-transduced B cells had markedly diminished NF-κB p65 phosphorylation and decreased CD25, CD95, and CD23 expression relative to control B cells transduced with empty vector (Fig. 1, F and G).

TNFAIP3 mediates MYD88L265P-induced shutdown of proliferation and NF-κB activation

The aforementioned experiments showed that B cell dysregulation caused by *MYD88*^{L265P} differs from other NF-κB–activating lymphoma mutations in multiple respects: *MYD88*^{L265P}-induced cell division in vitro was rapidly self-limiting, and *MYD88*^{L265P} triggered a decrease instead of induction of NF-κB–activated genes encoding CD23, CD25, and CD95. These results, together with the frequent co-occurrence of *MYD88* and *TNFAIP3* mutations in ABC-DLBCL (Ngo et al., 2011), raised the possibility that *MYD88*^{L265P} might have an intrinsically self-limiting effect on B cells by inducing a negative feedback loop through induction of *TNFAIP3* (also designated A20). *TNFAIP3* mRNA and protein are rapidly induced by NF-κB and form a critical negative feedback loop to diminish NF-κB activity by adding K48-linked ubiquitin chains, removing K63-linked ubiquitin chains, and inhibiting the addition of linear ubiquitin chains to key molecules in the NF-κB signaling axis (Harhaj and Dixit, 2012; Tokunaga et al., 2012; Verhelst et al., 2012).

To test the involvement of *TNFAIP3*, we first measured the mRNA level of *TNFAIP3* in B cells transduced with MYD88^{L265P} and found it was increased 300% compared with B cells transduced with *empty:EGFP* vector (Fig. 2 A). We then took advantage of a recently discovered ENU mutant mouse strain, *lasvegas*, with a partial loss of function *Tnfaip3* mutation in the OTU domain that abolishes deubiquitinating activity (*Tnfaip3*^{lsv}; unpublished data). When B cells with homozygous *Tnfaip3*^{lsv} mutation were transduced with *MYD88*^{L265P} and cultured without mitogens, the number of EGFP$^+$ B cells continued to increase between days 3 and 5, in contrast to the rapid drop that occurred in WT B cells expressing *MYD88*^{L265P} (Fig. 2, B and E). WT and *Tnfaip3*^{lsv} mutant B cells had diluted the cell division tracking dye, CTV, to comparable levels on day 3 but by day 5 the mutant B cells had greater dilution (Fig. 2 F). The increased division of mutant B cells between days 3 and 5 corresponded to less than one additional division (Fig. 2 F), consistent with less than a doubling of the numbers of mutant cells over this time (Fig. 2 E), and is insufficient to account for the large difference in numbers of mutant and wild-type B cells at day 5. The main effect of *Tnfaip3* mutation in B cells expressing *MYD88*^{L265P} appears to be a delayed induction of apoptosis.

EGFP$^+$ WT or *Tnfaip3*^{lsv} B cells expressing *MYD88*^{L265P} or control vectors were sorted on day 1 of culture without mitogens, and cell lysates were analyzed by Western blotting for phosphorylation of NF-κB p65. *MYD88*^{L265P} decreased p65 phosphorylation in WT cells, as observed above, but this effect was negated in *Tnfaip3*^{lsv} B cells (Fig. 2 C). Likewise, in WT B cells *MYD88*^{L265P} decreased the expression of CD23, CD25, CD95 and CD86 on EGFP$^+$ cells relative to EGFP$^-$ internal control B cells, but this was abolished in *Tnfaip3*^{lsv} B cells (Fig. 2 D). These results established that the rapid shut down of *MYD88*^{L265P}-driven B cell proliferation and NF-κB activity is partially *Tnfaip3* dependent.

MYD88^{L265P}-driven B cell proliferation is chloroquine sensitive and *Unc93b1* and IRAK dependent

To test if normal B cell proliferation induced by *MYD88*^{L265P} required upstream signaling by the RNA/DNA-sensing receptors TLR3, TLR7, and TLR9, as has been observed in malignant ABC-DLBCL cell lines (unpublished data), we repeated the retroviral transduction experiments in B cells where RNA/DNA-sensing TLRs were inhibited. Trafficking of these TLRs to endosomal compartments needed for active signaling was blocked by transducing B cells homozygous for the *Unc93b1*^{3d} mutation (Tabeta et al., 2006; Kim et al., 2008). Endosomal acidification needed for proteolytic activation of the TLR7 and TLR9 ectodomains was inhibited by treating the B cells with chloroquine (Ewald et al., 2008), and TLR9 was selectively eliminated by transducing B cells homozygous for a *Tlr9*-null mutation (Hemmi et al., 2000). These genetic (Fig. 3, A–F) or pharmacological (Fig. 3, C and D) interventions specifically inhibited proliferation of *MYD88*^{L265P}*:EGFP*-expressing B cells, but had no effect on proliferation of *CARD11*^{L232LI}*:EGFP*-expressing B cells tested in parallel. Slow residual division nevertheless consistently occurred in

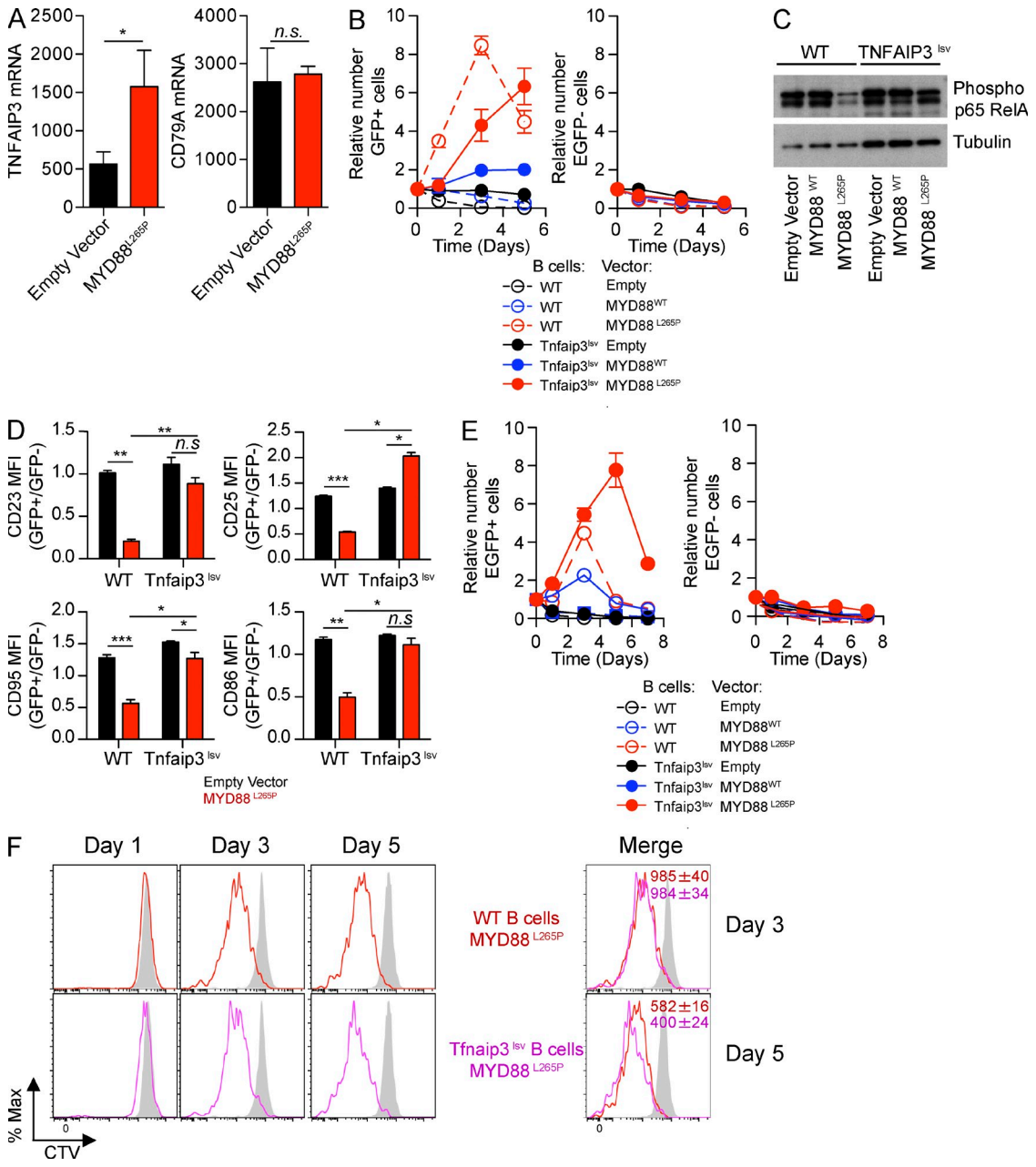

Figure 2. ***Tnfaip3*** **curtails the effects of** ***MYD88^{L265P}*** **on B cells.** (A) EGFP$^+$ B cells expressing the indicated vectors were sorted from three independent cultures on day 1, and mRNA from each cell was converted to cDNA and analyzed on microarrays. Shown are the mean and SD arbitrary units of *Tnfaip3* mRNA and a constitutively expressed B cell transcript, *Cd79a*, from each sample. *, $P < 0.05$. (B) WT or *Tnfaip3^{lsv}* homozygous mutant polyclonal B cells were transduced with the indicated vectors and cultured in triplicates in the absence of mitogenic stimuli for 5 d. The number of EGFP$^+$ transduced B cells and EGFP$^-$ nontransduced control B cells in each culture was compared with the starting number on day 0 (mean and SD). Data are representative of three independent experiments. (C) Western blot analysis of EGFP$^+$ WT or *Tnfaip3^{lsv}* polyclonal B cells expressing the indicated vectors, sorted on day 1 of culture without mitogenic stimuli, measuring phosphorylated p65 NF-κB. Tubulin was used as a loading control. (D) Relative expression of CD23, CD25, CD95, and CD86 on EGFP$^+$ WT or *Tnfaip3^{lsv}* polyclonal B cells expressing *MYD88^{L265P}* or empty vector compared with nontransduced EGFP$^-$ B cells in the same culture. Mean and SD for triplicate cultures per group. Statistical analysis by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of two independent experiments. (E) WT or *Tnfaip3^{lsv}* homozygous HEL-specific B cells were activated by HEL in vivo and anti-CD40 in vitro, transduced with indicated vectors, and cultured in triplicates in the absence of mitogenic stimuli for 7 d, and the number of EGFP$^+$ transduced B cells and EGFP$^-$ nontransduced B cells in each culture compared with starting number on day 0 (mean and SD). Data are representative of two independent experiments. (F) Division of HEL-specific B cells measured by CTV dilution on days 1, 3, and 5 of triplicate cultures in the absence of antigen or anti-CD40 stimuli. Cells were gated on WT or *Tnfaip3^{lsv}* transduced with MYD88^{L265P} or nondividing B cells transduced with *empty:EGFP* vector (gray histogram) showing the median and SD CTV intensity. Data are representative of two independent experiments.

Unc93b1^{3d} or *Tlr9* mutant B cells bearing *MYD88*^{L265P}, indicating either weak activation by other *Unc93b1*-independent receptors or low-level, receptor-independent signaling by the MYD88 mutant protein. Thus, mutations in *MYD88* induce transient B cell proliferation in the absence of normal B cell mitogens that nevertheless depends for the most part on an intact DNA-sensing TLR9 signaling apparatus.

We tested whether downstream IRAK activity was also required for proliferation by treating transduced B cells with an IRAK1/4 inhibitor, *N*-acyl 2-aminobenzimidazole (Powers et al., 2006). *MYD88*^{L265P} transduced B cells incubated with 5 µM IRAK1/4 inhibitor showed an 80% reduction in proliferation (Fig. 3 G). This inhibitory effect was specific for B cells carrying mutant *MYD88*, as the IRAK inhibitor had little inhibitory effect on proliferation of B cells transduced with vectors encoding gain-of-function alleles of *CARD11* or *IKK* analyzed in parallel cultures (Fig. 3 G).

MYD88^{L265P} induces T cell–independent B cell proliferation in vivo

To examine the consequences of *MYD88*^{L265P} mutation for normal B cells in vivo, we tracked its effect on mature retrovirally transduced B cells transplanted into *Rag1*$^{-/-}$ recipient mice. After in vitro transduction with *MYD88*^{L265P}*:EGFP*, *MYD88*^{WT}*:EGFP*, or empty:*EGFP* vector, as above, the activated B cells were loaded with the cell division dye CTV and transplanted by intravenous injection into syngeneic *Rag1*$^{-/-}$-recipient mice (Fig. 4 A). Groups of recipients were analyzed 5, 7, 11 and 14 d after transplantation for the number of EGFP$^+$ donor B cells in the spleen. Flow cytometry was used to identify donor B cells by their surface expression of B220 and IgM, and to enumerate the percentage and absolute number that expressed EGFP before and after transplantation (Fig. 4, A and B). While similar percentages and numbers of EGFP$^+$ cells expressing each vector were injected into the mice (Fig. 4, C and G), the percentage and total number of EGFP$^+$ cells expressing *MYD88*^{L265P} increased 8 to 12 fold by day 5 after transplantation whereas no increase occurred in control B cells expressing *MYD88*^{WT} (Fig. 4, D–H). The increase in *MYD88*^{L265P}-transduced B cells was accompanied by extensive dilution of CTV, demonstrating that the mutation induced at least eight rounds of cell division in many of the EGFP$^+$ B cells in vivo (Fig. 4, B and F). When later times after transplantation were analyzed, there only a slight additional increase or decrease in the numbers of *MYD88*^{L265P}-expressing B cells (Fig. 4 I). Thus, *MYD88*^{L265P} was sufficient to initiate spontaneous proliferation of mature B cells both in vitro and in vivo, although in both cases the aberrant clonal growth was rapidly self-limiting.

MYD88^{L265P} cooperates with dysregulated Bcl2 or Bim deficiency

Bcl2 mRNA and protein are frequently elevated in malignant B cells and correlate with poor clinical outcome (Shen et al., 2004; Iqbal et al., 2006). Because Bcl2 inhibits B cell apoptosis induced by the Bim, Bak, and Bax proteins (Cory, 1995), we

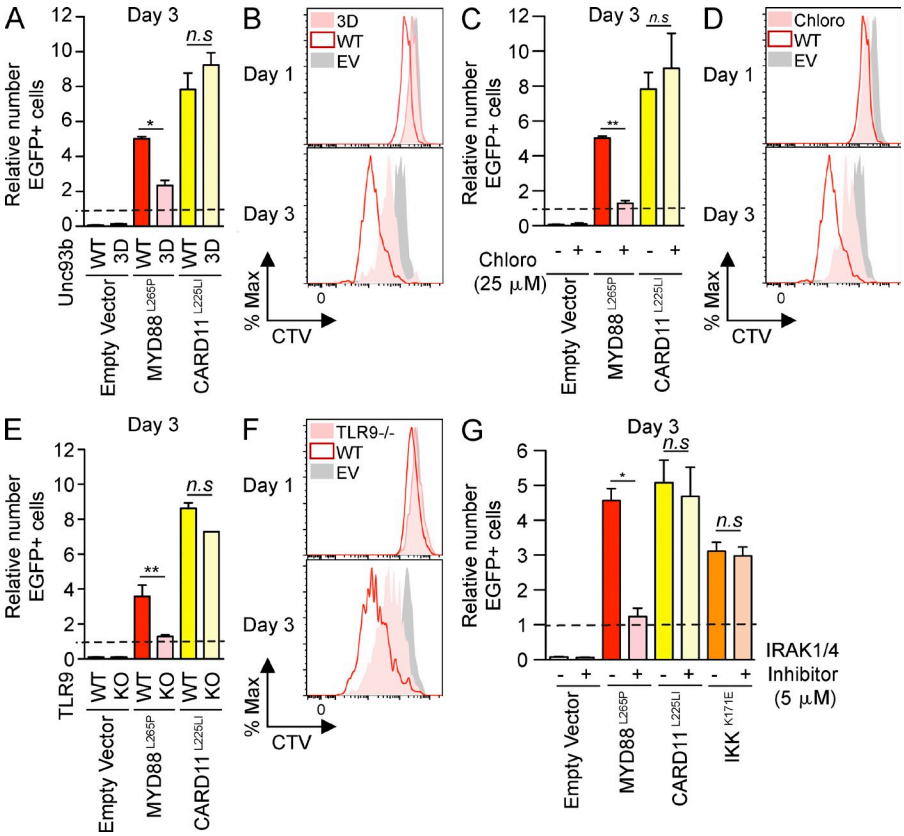

Figure 3. Role of nucleotide-sensing TLRs and downstream IRAKs in *MYD88*^{L265P}–driven B cell proliferation. (A, C, E, and G) Mean and SD of EGFP$^+$ polyclonal B cells on day 3 relative to the starting cell numbers in triplicate cultures transduced with activated *MYD88:EGFP*, *CARD11:EGFP*, *IKKβ:EGFP*, or empty:EGFP vectors. Relative growth is compared in WT or *Unc93b1*$^{3d/3d}$ (3D) mutant B cells (A); WT B cells cultured from day 0 with 0 or 25 µM chloroquine (Chloro; C); WT or *Tlr9*$^{-/-}$ (KO) B cells (E); and WT B cells in the in the presence or absence of 5 µM IRAK1/4 inhibitor (G). Statistical analysis by Student's *t* test: ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments. (B, D, and F) Cell division after 1 or 3 d of culture measured by CTV dilution in *MYD88*^{L265P}*:EGFP*$^+$ B cells, comparing WT (red histogram) or *Unc93b1*$^{3d/3d}$ (filled pink) B cells (B); WT B cells in either 0 (red histogram) or 25 µM chloroquine (filled pink; D); or WT (red histogram) or *Tlr9*$^{-/-}$ (filled pink) B cells (D). As a reference for nondividing viable B cells, parallel cultures were established of CTV-labeled *Vav-Bcl2* B cells transduced with empty:*EGFP* vector (gray shaded histogram). Data are representative of two independent experiments.

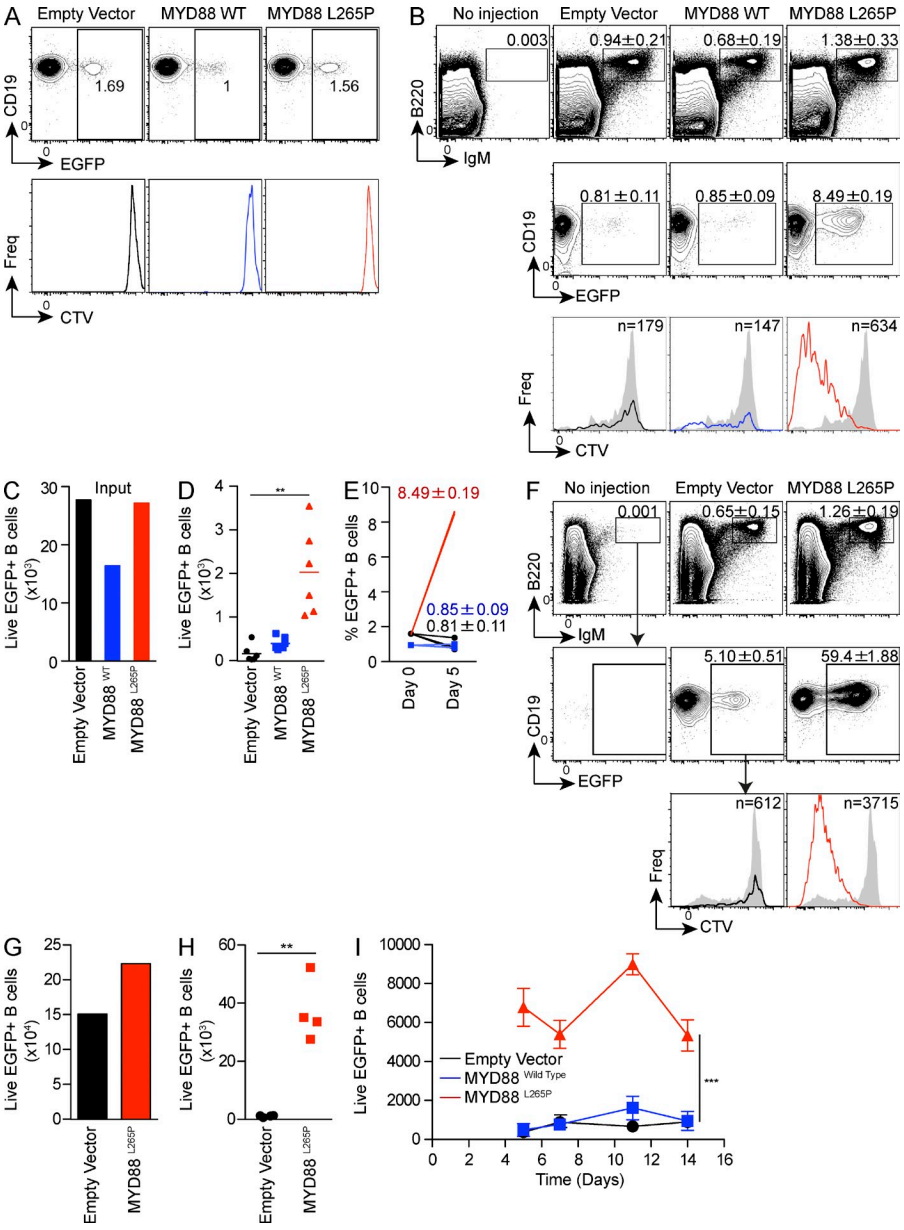

Figure 4. *MYD88*^{L265P} mutation promotes T cell–independent B cell proliferation in vivo. (A) Antigen plus CD40-activated HEL-specific B cells were transduced with the indicated vectors, labeled with CTV, and analyzed at the time of injection into *Rag1*$^{-/-}$ mice for the percentage EGFP$^+$ (top) and CTV intensity (bottom). (B) Flow cytometric analysis of the spleen of *Rag1*$^{-/-}$ recipient mice 5 d after transplantation of antigen-activated B cells transduced with the indicator vectors. Plots show concatenated data from three recipients per treatment. The top row shows the mean percentage and SD of B220$^+$IgM$^+$ transferred B cells. The middle row, gated on B220$^+$IgM$^+$ cells, shows the mean percentage and SD EGFP$^+$ cells and expression of CD19. The bottom row, gated on B220$^+$IgM$^+$EGFP$^+$ cells, displays dilution of CTV as a measure of cell division and the total number of B220$^+$IgM$^+$EGFP$^+$ events in each concatenated dataset. Data are representative of at least three independent experiments. (C) Number of EGFP$^+$ B cells transduced with the indicated vectors injected into each recipient *Rag1*$^{-/-}$ mouse analyzed in B. (D) Number of EGFP$^+$ B cells in the spleen of each recipient mouse 5 d after transplantation. Results show pooled data from two independent experiments, each with $n = 3$ per group. Statistical analysis by Student's *t* test: **, $P < 0.01$. (E) Percentage of EGFP$^+$ cells among transferred B cells at time of injection (day 0) and 5 d later in independent recipient animals ($n = 3$ per group) numbers depict mean and SD of day 5 (F–H). Independent experiment performed and analyzed as in B–D. Statistical analysis by Student's *t* test: **, $P < 0.01$. (I) Number of EGFP$^+$ B cells expressing the indicated vectors in the spleen of recipient mice on days 5, 7, 11, and 14 after injection. Statistical analysis by ANOVA: ***, $P < 0.001$.

tested the possibility that the short-lived proliferative burst induced by *MYD88*^{L265P} was curtailed by activation of this apoptotic pathway. Myd88^{WT}:*EGFP*-, Myd88^{L265P}:*EGFP*-, or *empty:EGFP*-transduced B cells washed off CD40 were placed in culture for either 1 or 3 d and stained intracellularly for Bim protein levels. Compared with empty:EGFP vector, B cells transduced with Myd88^{L265P} showed elevated Bim protein on day 1 and day 3 (Fig. 5 A). The increased Bim in MYD88^{L265P}-transduced cells contrasts with our previous finding that Bim is decreased in B cells expressing activated *Card11* or *Ikk* (Jeelall et al., 2012). To test whether Bcl2 overexpression could overcome the effects of Bim, activated B cells were obtained from *Vav-Bcl2* transgenic mice that constitutively express Bcl2 in hematopoietic cells (Egle et al., 2004) and from WT control mice. These were transduced with *MYD88*^{L265P}:*EGFP* or empty:*EGFP* vector, and cultured without mitogenic stimuli to enumerate EGFP$^+$ cells. The number of WT control B cells expressing *MYD88*^{L265P} increased until day 3, and then declined sharply by day 5 as observed above, whereas the corresponding *Vav-Bcl2* B cells increased to greater numbers on day 3 and continued to increase by day 5 (Fig. 5 B). *Vav-Bcl2* B cells transduced with the empty:*EGFP*-only vector did not proliferate, but survived better in culture than corresponding WT cells, as expected, because of resistance to apoptosis (Fig. 5 B). To distinguish if the increased accumulation of *Vav-Bcl2* B cells expressing *MYD88*^{L265P} was caused by enhanced cell division or better survival of the progeny, the transduced B cells were labeled with CTV. Despite much higher accumulation of *Vav-Bcl2* than WT control B cells, there was no difference in the dilution of CTV within the

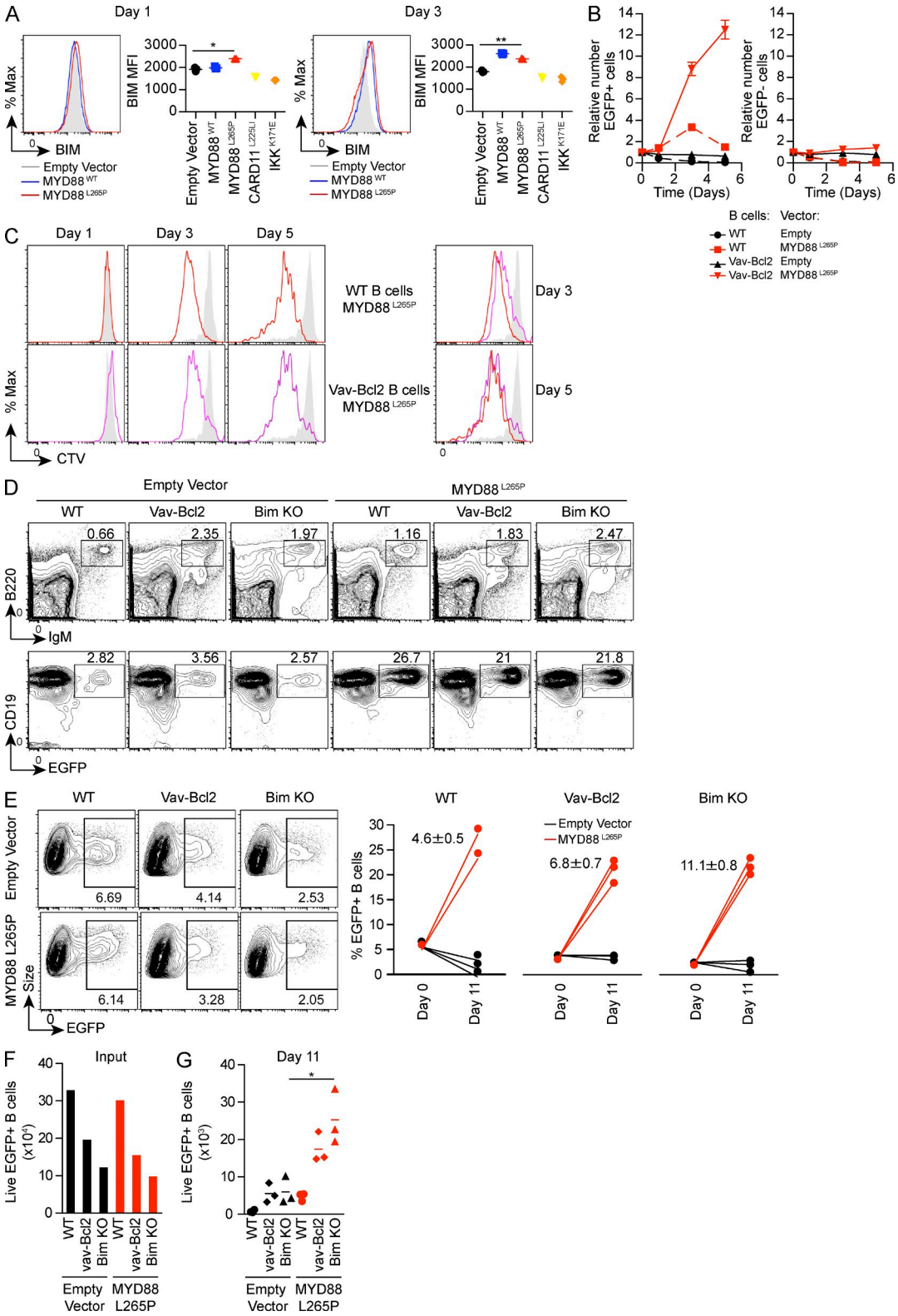

Figure 5. Bcl2- and Bim-regulated apoptosis limits *MYD88^{L265P}*-induced accumulation of mature B cells. (A) Intracellular BIM mean fluorescent intensity (MFI) gated on EGFP$^+$ HEL-specific B cells transduced with indicated vectors and cultured without anti-CD40 for 1 or 3 d. Statistical comparison of independent cultures with empty vector or MyD88^{L265P} by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. (B and C) Proliferation of polyclonal WT or *Vav-Bcl2* transgenic B cells transduced with the indicated vectors, labeled with CTV, and then cultured without exogenous stimuli for 1, 3, or 5 d. Data are representative of two independent experiments. (B) Mean and SD of EGFP$^+$ or EGFP$^-$ B cells in triplicate cultures relative to day 0. (C) Dilution of CTV in

MYD88^{L265P}*:EGFP*$^{+}$ population on days 3 or 5 (Fig. 5 C). Thus, the *Vav-Bcl2* transgene did not alter the rate of cell division induced by *MYD88*^{L265P} but enhanced the accumulation of progeny at each division. Despite this, the modal CTV fluorescence only decreased from 1/8 to 1/16 of the nondividing empty-vector control cells between days 3 and 5 (Fig. 5 C), indicating that *MYD88*^{L265P}-induced B cell proliferation arrested after 4 divisions independent of apoptotic loss.

We then asked whether mutations that inhibit B cell apoptosis either through Bcl2 overexpression or inactivation of the proapoptotic Bim protein (*Bcl2l11*) also enhanced the accumulation of *MYD88*^{L265P}-expressing B cells in vivo, where the prosurvival cytokine BAFF/Blys is available (Woodland et al., 2006). Transduced B cells from *Vav-Bcl2* transgenic, Bim-deficient (BimKO; Bouillet et al., 1999), or WT control mice were injected into *Rag1*$^{-/-}$ recipient mice and spleen cells analyzed 11 d after injection (Fig. 5 D). In control groups that received B cells transduced with empty vector, three to four times more B220^{+}IgM^{+} B cells remained in recipients that received *Vav-Bcl2* or BimKO B cells compared with WT B cells, which is consistent with enhanced survival of nontransduced and transduced B cells in vivo (Fig. 5 D). However the percentage of transferred B cells expressing the empty EGFP vector did not increase between the time of transfer and 11 d after transfer, regardless of *Bcl2* or *Bim* genotype (black lines in Fig. 5 E). In contrast, the percentage of B cells expressing *MYD88*^{L265P}*:EGFP* increased 4-fold by day 11 in the case of wild-type B cells, and increased 7-fold and 11-fold in *Vav-Bcl2* transgenic or Bim-deficient B cells, respectively (red lines in Fig. 5 E). Measured as total number of EGFP^{+} B cells, half as many EGFP^{+} cells were injected in the groups receiving *Vav-Bcl2* or BimKO B cells (Fig. 5 D) due to lower transduced percentages on day 0 (Fig. 5 E). There was nevertheless a multiplicative effect of combining *MYD88*^{L265P} with either *Vav-Bcl2* or BimKO on the numbers 11 d later: alone, each mutation increased the numbers of EGFP^{+} B cells by ∼6-fold relative to the number of empty-vector–expressing WT B cell controls, whereas combined they increased the number of EGFP^{+} B cells 25–35-fold (Fig. 5, E and G).

MYD88^{L265P} blocks self-antigen–induced elimination only when combined with dysregulated *Bcl2*

All lymphoma *CARD11* mutations tested so far have the property of switching B cells from self-antigen–induced death into self-antigen–induced proliferation and plasma cell differentiation (Jeelall et al., 2012). To test if lymphoma mutations in *MYD88* have the same activity, HEL antigen–specific B cells transduced with *MYD88*^{L265P}*:EGFP* or EGFP-only empty vectors were transplanted into *Rag1*$^{-/-}$ mice that express HEL in their circulation as a self-antigen (Fig. 6 A). When analyzed 13 d after transfer, EGFP^{+} B cells expressing *MYD88*^{L265P} proliferated and persisted in the absence of antigen in nontransgenic *Rag1*$^{-/-}$ control mice as observed previously, but were eliminated in HEL-transgenic recipients (Fig. 6, A–C). The unimpeded deletion of *MYD88*^{L265P}-expressing B cells contrasts with the protection from elimination and induction of plasma cell differentiation induced by *CARD11* mutations under the same experimental conditions (Jeelall et al., 2012).

Whereas *MYD88*^{L265P} alone was insufficient to block antigen-induced death, we next asked if it could do so in cooperation with dysregulated *Bcl2*, given the cooperation observed above in the absence of antigenic stimulation and the capacity of dysregulated *Bcl2* alone to delay elimination of mature self-reactive B cells (Cyster et al., 1994). HEL-specific B cells transduced with *MYD88*^{L265P} or empty vector were transferred into *Rag1*$^{-/-}$ HEL-transgenic mice as above, except that half of the mice received B cells from *Vav-Bcl2*-Tg donors (Fig. 6 D). WT B cells transduced with the *empty:EGFP* or *MYD88*^{L265P}*:EGFP* vectors were eliminated by day 13, whereas small numbers of *Vav-Bcl2*-Tg cells carrying empty vector survived; however, their frequency did not increase relative to the percentage EGFP^{+} on day 0 (Fig. 6, D–G). However the combination of *MYD88*^{L265P} and *Vav-Bcl2* resulted in 10 times more EGFP^{+} B cells accumulating in HEL-transgenic recipients compared with *Vav-Bcl2* alone and 200 times more EGPF^{+} B cells than empty vector alone (Fig. 6, D–F). In contrast to *CARD11* mutations (Jeelall et al., 2012), the combination of *MYD88*^{L265P} and *Vav-Bcl2* mutations remained insufficient to drive spontaneous differentiation of self-antigen–stimulated B cells into CD19^{low} plasmablasts, as the majority of accumulating EGFP^{+} B cells remained CD19^{high} (Fig. 6 D). Thus *MYD88*^{L265P} was insufficient to interfere with BCR-induced deletion on its own, but exhibited a highly cooperative interaction with dysregulated *Bcl2* to block elimination of self-reactive B cells and allow their accumulation.

DISCUSSION

B cells must normally tolerate self-ligands of their pathogen-receptor systems (BCRs and TLRs) and only proliferate in response to foreign ligands for these receptors. The aforementioned experiments reveal that tolerance to nucleic acid-sensing TLRs is disrupted by a somatic mutation in *MYD88* that is very frequently found in the benign disorder, IgM monoclonal

EGFP^{+} cells transduced with *MYD88*^{L265P} (red, WT cells; purple, *Vav-Bcl2* cells) overlayed on the CTV fluorescence of the corresponding cells transduced with empty EGFP vector (gray histograms). (D–H) Polyclonal WT, *Vav-Bcl2* transgenic, or *Bcl2l11*$^{-/-}$ (BimKO) B cells were transduced with the indicated vectors and injected into *Rag1*$^{-/-}$ recipient mice. (D) Representative flow cytometric analysis of spleen 11 d after transplantation. Percentage of cells in the indicated gates is shown. (E) Representative plots showing percentage of EGFP^{+} cells among transferred B cells at time of injection (day 0). Graphs on right compare the percentage on day 0 with day 11 in independent recipient animals, and numbers depict the mean fold increase and SD (n = 3 per group). (F) Total number of EGFP^{+} cells with the indicated vectors injected into each *Rag1*$^{-/-}$ mouse. (G) Number of EGFP^{+} B cells in the spleen of individual recipient mice 11 d after transplantation (n = 3). Statistical analysis by Student's t test: *, P < 0.05.

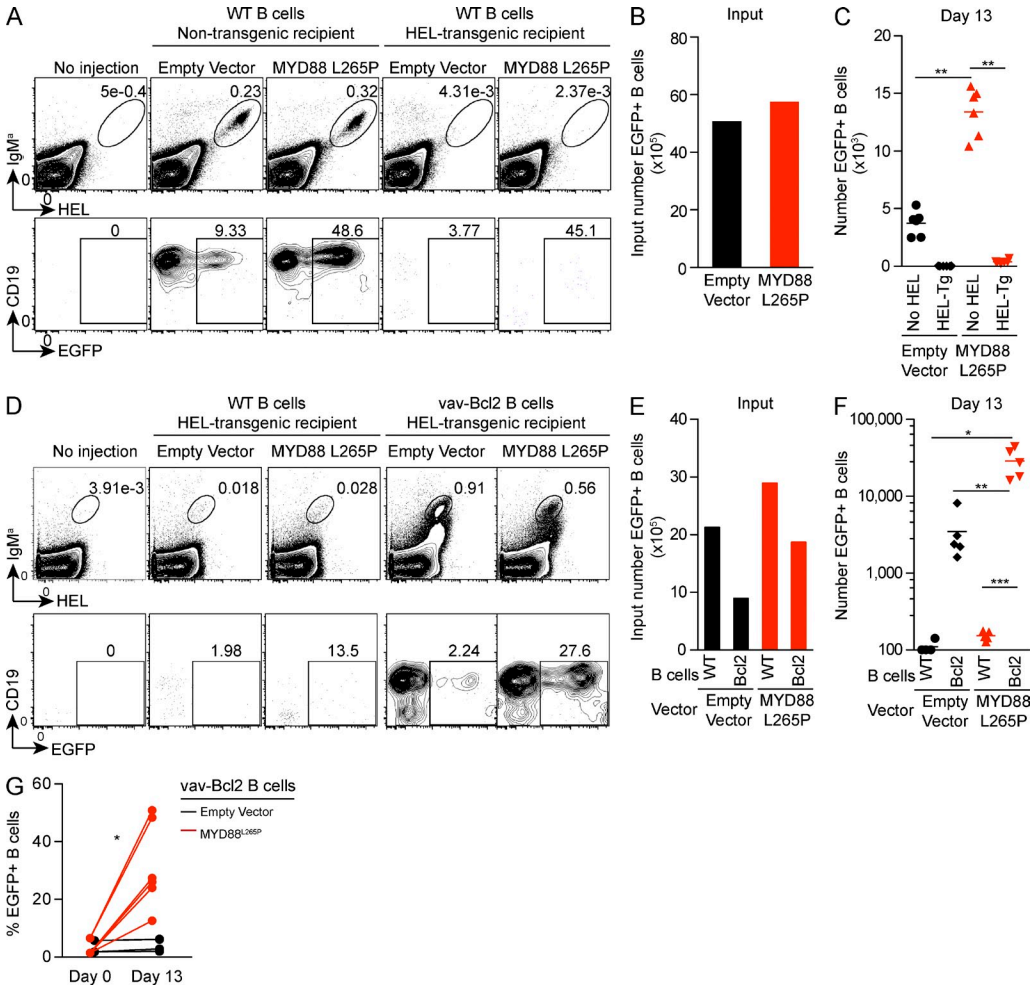

Figure 6. ***MYD88^{L265P}* blocks self-antigen induced B cell death only when combined with dysregulated *Bcl2*.** (A) HEL-specific B cells were transduced with the indicated vectors and injected into *Rag1*$^{-/-}$ mice or into *Rag1*$^{-/-}$ HEL-transgenic mice. Transplanted B cells were defined and enumerated by their IgM^a staining and HEL binding (top row), and the percentage of these B cells that expressed EGFP (bottom row). Data are representative of two independent experiments. (B) Number of EGFP$^+$ B cells with the indicated vectors injected into each recipient mouse. (C) Number of EGFP$^+$ B cells in the spleen of each recipient mouse 13 d after transplantation, results showing pooled data from two independent experiments, each with $n = 3$. Statistical analysis by Student's *t* test: **, $P < 0.01$. (D) As in A, except WT or *Vav-Bcl2*-Tg HEL-specific B-cells were transplanted into *Rag1*$^{-/-}$ HEL-transgenic mice. Data are representative of two independent experiments. (E) Number of WT or *Vav-Bcl2*-Tg EGFP$^+$ B cells injected into each recipient. (F) Number of EGFP$^+$ B cells in the spleen of each recipient mouse 13 d after transplantation. Results showing pooled data from two independent experiments, each with $n = 3$. Statistical analysis by *t* test. *, $P = 0.0019$; **, $P = 0.0024$; ***, $P = 0.0008$. (G) Percentage of EGFP$^+$ B cells among donor B cells on days 0 and 13. Statistical analysis by Student's *t* test. *, $P < 0.05$.

gammopathy of undetermined significance, and in a range of B cell malignancies. In the absence of foreign TLR ligands, *MYD88^{L265P}* was sufficient to drive multiple rounds of B cell division provided the *Unc93b1*-dependent and chloroquine-sensitive steps in TLR9 activation were intact, and provided the B cells were not constantly binding self-antigen. This disruption of normal tolerance to TLR9 has parallels with the effects of weakly activating lymphoma CARD11 mutations, which break normal B cell tolerance to self-ligands of the BCR (Jeelall et al., 2012). However breakdown of TLR tolerance is fortified by more checkpoint mechanisms: *MYD88^{L265P}*-induced proliferation was rapidly curtailed by *Tnfaip3*-mediated shutdown of NF-κB and by Bcl2-inhibited, Bim-dependent apoptosis. When the apoptotic checkpoint was corrupted by a second mutation, *MYD88^{L265P}* promoted much greater B cell accumulation and blocked B cell elimination by self-antigen.

What drives the initial proliferation of *MYD88^{L265P}*-bearing primary B-cells? The inhibition of proliferation by the *Unc93b1^{3d}* mutation, chloroquine, and *Tlr9* deficiency indicates that activation of TLR9 is necessary, and hence the *MYD88* mutation does not simply result in constitutively activated IRAK signaling. This result, in otherwise normal B cells, complements findings (unpublished data) that show malignant ABC-DLBCL cells bearing the *MYD88^{L265P}* mutation remain dependent on active TLR9 for their survival or proliferation. A linkage between dysregulated inflammatory

responses and cancer has long been proposed, starting with Virchow back in 1863 (Balkwill and Mantovani, 2001). Since then, many studies have linked chronic infection and inflammation with B cell malignancy, most notably in gastric MALT lymphoma associated with *Helicobacter pylori* infection (Wotherspoon et al., 1993). The induction of proliferation and resistance to self-antigen–induced elimination in B cells bearing the *MYD88^{L265P}* mutation may represent an unbridled response to endogenous TLR9 ligands, with parallels to the B cell dysregulation observed in mice inheriting either a duplicated *Tlr7* locus (*Y-linked autoimmunity mutation*; *Yaa*) or multiple copies of a *Tlr7* transgene (Pisitkun et al., 2006; Subramanian et al., 2006; Deane et al., 2007). It also parallels the autoimmune B cell proliferation caused when B cell antigen receptors bind and deliver excessive amounts of self-DNA to TLR9 (Leadbetter et al., 2002). Hence the findings here provide a rationale for future studies to test for inherited or somatic *MYD88* mutations in systemic lupus and other autoimmune diseases.

The results also indicate that overexpression of wild-type *MYD88* causes B cell dysregulation. B cells that expressed the highest levels of the *MYD88^{WT}:EGFP* vector were indeed stimulated to divide in culture. In contrast, EGFP^{mid} B cells expressing the different vectors were only stimulated to divide if the vector encoded *MYD88^{L265P}:EGFP*. These results provide experimental evidence to explain the observation that MYD88 overexpression without L265P mutation is seen in 36% of DLBCL cases and is associated with tumor recurrence and shortened disease-free survival (Choi et al., 2013).

The findings here reveal surprising differences between lymphoma *MYD88^{L265P}* and *CARD11* mutations. Both break the tuning of intracellular signals from their respective receptors (TLRs, BCRs) that normally prevents proliferative responses to self. However, based on the findings here TLR signaling appears more heavily fortified against this contingency, and has the potential to cross-interfere with normal tolerance to BCR self-ligands. *MYD88^{L265P}* was sufficient to initiate B cell proliferation much like activated alleles of *CARD11*, but *MYD88^{L265P}* differed from *CARD11* because within 1–2 d, it induced a decrease in NF-κB p65 RelA phosphorylation, a decrease in down-regulation of NF-κB-induced cell surface proteins, and an increase in Bim protein. Cell division was prematurely terminated by apoptosis between 3 and 4 d. These self-extinguishing effects of *MYD88^{L265P}* were negated by a *Tnfaip3* partial loss of function mutation. *Tnfaip3* is induced by NF-κB signaling as an early response gene and encodes the A20 protein, a ubiquitin modifier that inhibits formation of linear and K63-linked ubiquitin chains on key proteins in the NF-κB signaling pathway to provide a negative feedback that terminates further activation of NF-κB (Harhaj and Dixit, 2012; Tokunaga et al., 2012; Verhelst et al., 2012). *Tnfaip3* inactivation accompanies MYD88^{L265P} in 24% of ABC-DLBCL (Ngo et al., 2011) and in 38% of Waldenström's macroglobulinemia (Braggio et al., 2009). The fact that many MYD88^{L265P}-bearing lymphomas have not acquired an inactivating Tnfaip3 mutation raises the possibility that other recurring lymphoma mutations might represent alternative mechanisms to circumvent MYD88's self-extinguishing property. CARD11 mutations may evade this feedback mechanism because CARD11 activates the paracaspase, MALT1, which cleaves and inactivates A20 (Coornaert et al., 2008; Rebeaud et al., 2008). MYD88 and CARD11 mutations also differed in their ability to counter cell death in the context of self-antigen stimulation, and this may reflect the capacity of activated CARD11 to diminish Bim protein levels (Jeelall et al., 2012). In support of that interpretation, we found that deletion could only be blocked by *MYD88^{L265P}* when combined with a mutation that enforced expression of the Bim-inhibitor, Bcl-2.

BCL2 t(14:18) translocations that cause dysregulated BCL2 expression occur in almost half of apparently healthy adults and increase with age (Schüler et al., 2009). Many lymphomas bearing *MYD88^{L265P}* lack a *BCL2* translocation. In these cases, perhaps other recurring mutations provide alternative mechanisms to counteract Bim-induced apoptosis; e.g., by increasing *BCL2* expression from its normal locus. For example, the delayed apoptosis observed when *MYD88^{L265P}* was combined with *Tnfaip3* mutation may result from increased NF-κB induction of *Bcl2*. The *MYD88^{L265P}* and *Vav-Bcl2* double mutation experiments indicate that additional layers of control exist to counter dysregulation of TLR responses even when *MYD88^{L265P}* becomes paired with Bim inactivation via overexpressed Bcl-2. Although the two mutations cooperate to prevent elimination of self-reactive B cells, few of these rogue self-antigen–binding B cells differentiated into CD19^{low} plasmablasts. This contrasts with lymphoma-associated *CARD11* mutations, where even the weakest alleles caused self-antigen to drive not only proliferation but also extensive differentiation into plasmablasts (Jeelall et al., 2012).

Collectively, the findings in this study reveal checkpoints that normally prevent a single activating mutation in *MYD88* from triggering dysregulated B cell proliferation in response to self-protein, RNA, or DNA. These checkpoints provide insights into the clinical associations observed between *MYD88* somatic mutations and benign monoclonal IgM gammopathy (Xu et al., 2013); between somatic *TNFAIP3* inactivation, *MYD88* activation, and malignant DLBCL (Ngo et al., 2011); between inherited *TNFAIP3* polymorphisms and rheumatoid arthritis or systemic lupus (Plenge et al., 2007; Thomson et al., 2007; Graham et al., 2008). They also provide insight into the association of these autoimmune diseases with development of B cell lymphoma (Goodnow, 2007). Although self-ligands for TLRs and BCRs are a ubiquitous potential stimulus for B cell proliferation and antibody secretion, the TLR signaling pathway appears fortified to ensure this possibility can only arise through accumulation of multiple somatic or inherited mutations.

MATERIALS AND METHODS

Mice. Splenic mature B cells were obtained from (a) MD4 transgenic mice (Goodnow et al., 1988) bearing rearranged transgenic Ig encoding for HEL-specific antibodies (Ig^{HEL} transgenic); (b) *Vav-Bcl2* transgenic mice where Bcl2 expression was driven by the pan-hematopoietic *Vav1* promoter provided by

S. Cory (Walter and Eliza Hall Institute of Mediacal Research, Parkville, VIC, Australia; Egle et al., 2004), either alone or as double-transgenic mice with Ig^{HEL}; (c) Bim (*Bcl2l11*) knockout mice, provided by A. Strasser (Walter and Eliza Hall Institute of Mediacal Research; Bouillet et al., 1999); (d) *Unc93b*^{3D}, an ENU-induced loss of function mutation generated in C57BL/6 mice (Tabeta et al., 2006); and (e) $TLR9^{-/-}$ (Hemmi et al., 2000). All mice were either generated on a C57BL/6 background or backcrossed to that background for >10 generations, and were housed in specific pathogen–free environment at the Australian Phenomics Facility, ANU. Donor mice were 8–16 wk of age. $Rag1^{-/-}$ mice and $Rag1^{-/-}$ ML5-transgenic mice that express soluble HEL (Goodnow et al., 1988) were used as recipients at 8–14 wk of age.

Retroviral vectors and packaging. *Myd88* was amplified by Platinum Pfx DNA polymerase (Invitrogen) from mouse spleen cDNA and cloned into pcDNA 3.1 (+) vector. PCR-based site-directed mutagenesis was used to introduce *Myd88* mutations and the PCR mutagenesis products were sequenced on an AB 3730xl DNA Analyzer. WT and mutant *Myd88* cDNAs were then subcloned in pMXs-IRES-GFP vector (provided by T. Kitamura, University of Tokyo, Tokyo, Japan). Retroviral vectors containing WT or mutant *Myd88* were transfected into Phoenix ecotropic packaging cells (American Type Culture Collection) using calcium phosphate precipitation. Supernatants containing retroviral particles were collected 48 and 72 h after transfection and frozen at −80°C until use.

Retroviral transduction and culture of B cells. B cells from MD4 transgenic mice were activated by in vivo HEL stimulation followed by in vitro anti-CD40 stimulation for 24 h, and then transduced with retroviral vectors as previously described (Jeelall et al., 2012). B cells from nontransgenic mice were activated by in vitro stimulation with anti-IgM (10 µg/ml) and anti-CD40 (10 µg/ml) for 24 h before transduction with the retroviral vectors, as above. The transduced cells were then washed three times with fresh complete RPMI by centrifugation at 300 *g* for 5 min at 8°C, and any remaining media containing trace amount of anti-CD40 antibodies was carefully removed. The number of live cells was determined by hemocytometer counting of Trypan blue–negative cells and the percentage of $EGFP^+$ $7AAD^-$ cells was determined by flow cytometry. Washed, transduced cells were then cultured in 24-well plates at 10^6cells/ml in fresh complete RPMI without any mitogen supplement, with the start of the mitogen-free cultures designated "day 0." The number of $EGFP^+$ cells after different days in culture was determined by harvesting all the cells in a culture well, counting Trypan blue–negative cells in a hemocytometer, and doing flow cytometric analysis of the same cells to measure the percentage $7AAD^-$ cells that were $B220^+$ $EGFP^+$. Flow cytometric analysis was performed as described in (Jeelall et al., 2012). For cell division analysis, transduced cells at day 0 were loaded with 20 µM CTV (Invitrogen) for 30 min before culture.

Adoptive transfer. Retrovirally transduced B cells were cultured in media containing anti-CD40 for 36 h after spin-infection, washed, and then analyzed by hemocytometer and flow cytometry as above. 5×10^6 to 10×10^6 cultured viable B cells were injected into the lateral tail vein of each recipient mouse.

Western blot. $EGFP^+$ transduced splenic B cells were sorted and analyzed by Western blotting as previously described (Jeelall et al., 2012). Myd88 and phospho-p65 NF-κB primary antibodies obtained from Cell Signaling Technology were used at 1:1,000 dilutions. Horseradish peroxidase–conjugated anti–rabbit IgG secondary antibody purchased from Cell Signaling Technology was used at 1:2,500 dilution. Membranes were reprobed with antibody to αβ tubulin obtained from Cell Signaling Technology at 1:5,000 dilution as a loading control.

Gene expression analysis. $EGFP^+$ B cells were harvested on day 1 of culture without anti-CD40, sorted, and resuspended in TRIzol. Phase separation was performed by the addition of chloroform and centrifugation at 12,000 *g* for 15 min, followed by isopropanol RNA precipitation. The air-dried RNA pellet was dissolved in RNase-free water, and mRNA expression was analyzed on Affymetrix mouse ST 1.0 arrays as per the manufacturer's instructions.

Statistical analysis. All experiments were analyzed using Prism version 5 (GraphPad). Statistically significant p-values <0.05, <0.01, and <0.001, respectively are indicated and were determined using a two-tailed unpaired Student's *t* test.

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The authors declare no competing financial interest.

Author contributions: J.Q. Wang, K. Horikawa, and C. Goodnow designed and analyzed the experiments and wrote the paper. J.Q. Wang performed most of the experiments. Y.S. Jeelall assisted with the experiments. B. Beutler provided the Unc93b^{3d} mice and feedback on data analysis.

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REFERENCES

Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4:499–511. http://dx.doi.org/10.1038/nri1391

ar-Rushdi, A., K. Nishikura, J. Erikson, R. Watt, G. Rovera, and C.M. Croce. 1983. Differential expression of the translocated and the untranslocated c-myc oncogene in Burkitt lymphoma. *Science.* 222:390–393.

Balkwill, F., and A. Mantovani. 2001. Inflammation and cancer: back to Virchow? *Lancet.* 357:539–545. http://dx.doi.org/10.1016/S0140-6736(00)04046-0

Beutler, B. 2004. Toll-like receptors and their place in immunology. Where does the immune response to infection begin? *Nat. Rev. Immunol.* 4:498. http://dx.doi.org/10.1038/nri1401

Bouillet, P., D. Metcalf, D.C. Huang, D.M. Tarlinton, T.W. Kay, F. Köntgen, J.M. Adams, and A. Strasser. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science.* 286:1735–1738. http://dx.doi.org/10.1126/science.286.5445.1735

Braggio, E., J.J. Keats, X. Leleu, S. Van Wier, V.H. Jimenez-Zepeda, R. Valdez, R.F. Schop, T. Price-Troska, K. Henderson, A. Sacco, et al. 2009. Identification of copy number abnormalities and inactivating mutations in two negative regulators of nuclear factor-kappaB signaling pathways in Waldenstrom's macroglobulinemia. *Cancer Res.* 69:3579–3588. http://dx.doi.org/10.1158/0008-5472.CAN-08-3701

Choi, J.W., Y. Kim, J.H. Lee, and Y.S. Kim. 2013. MYD88 expression and L265P mutation in diffuse large B-cell lymphoma. *Hum. Pathol.* 44:1375–1381. http://dx.doi.org/10.1016/j.humpath.2012.10.026

Coornaert, B., M. Baens, K. Heyninck, T. Bekaert, M. Haegman, J. Staal, L. Sun, Z.J. Chen, P. Marynen, and R. Beyaert. 2008. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nat. Immunol.* 9:263–271. http://dx.doi.org/10.1038/ni1561

Cory, S. 1995. Regulation of lymphocyte survival by the bcl-2 gene family. *Annu. Rev. Immunol.* 13:513–543. http://dx.doi.org/10.1146/annurev.iy.13.040195.002501

Cyster, J.G., S.B. Hartley, and C.C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature.* 371:389–395. http://dx.doi.org/10.1038/371389a0

Deane, J.A., P. Pisitkun, R.S. Barrett, L. Feigenbaum, T. Town, J.M. Ward, R.A. Flavell, and S. Bolland. 2007. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity.* 27:801–810. http://dx.doi.org/10.1016/j.immuni.2007.09.009

Egle, A., A.W. Harris, M.L. Bath, L. O'Reilly, and S. Cory. 2004. VavP-Bcl2 transgenic mice develop follicular lymphoma preceded by germinal center hyperplasia. *Blood.* 103:2276–2283. http://dx.doi.org/10.1182/blood-2003-07-2469

Ewald, S.E., B.L. Lee, L. Lau, K.E. Wickliffe, G.P. Shi, H.A. Chapman, and G.M. Barton. 2008. The ectodomain of Toll-like receptor 9 is cleaved to

generate a functional receptor. *Nature*. 456:658–662. http://dx.doi.org/10.1038/nature07405

Goodnow, C.C. 2007. Multistep pathogenesis of autoimmune disease. *Cell*. 130:25–35. http://dx.doi.org/10.1016/j.cell.2007.06.033

Goodnow, C.C., and P. Ohashi. 2013. Immunological tolerance. In Fundamental Immunology, W.E. Paul, ed. Philadelphia: Lippincott Williams & Wilkins.

Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 334:676–682. http://dx.doi.org/10.1038/334676a0

Graham, R.R., C. Cotsapas, L. Davies, R. Hackett, C.J. Lessard, J.M. Leon, N.P. Burtt, C. Guiducci, M. Parkin, C. Gates, et al. 2008. Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. *Nat. Genet.* 40:1059–1061. http://dx.doi.org/10.1038/ng.200

Harhaj, E.W., and V.M. Dixit. 2012. Regulation of NF-κB by deubiquitinases. *Immunol. Rev.* 246:107–124. http://dx.doi.org/10.1111/j.1600-065X.2012.01100.x

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*. 408:740–745. http://dx.doi.org/10.1038/35047123

Iqbal, J., V.T. Neppalli, G. Wright, B.J. Dave, D.E. Horsman, A. Rosenwald, J. Lynch, C.P. Hans, D.D. Weisenburger, T.C. Greiner, et al. 2006. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J. Clin. Oncol.* 24:961–968. http://dx.doi.org/10.1200/JCO.2005.03.4264

Jeelall, Y.S., J.Q. Wang, H.D. Law, H. Domaschenz, H.K. Fung, A. Kallies, S.L. Nutt, C.C. Goodnow, and K. Horikawa. 2012. Human lymphoma mutations reveal CARD11 as the switch between self-antigen-induced B cell death or proliferation and autoantibody production. *J. Exp. Med.* 209:1907–1917. http://dx.doi.org/10.1084/jem.20112744

Kim, Y.M., M.M. Brinkmann, M.E. Paquet, and H.L. Ploegh. 2008. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature*. 452:234–238. http://dx.doi.org/10.1038/nature06726

Krieg, A.M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709–760. http://dx.doi.org/10.1146/annurev.immunol.20.100301.064842

Leadbetter, E.A., I.R. Rifkin, A.M. Hohlbaum, B.C. Beaudette, M.J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 416:603–607. http://dx.doi.org/10.1038/416603a

Lenz, G., R.E. Davis, V.N. Ngo, L. Lam, T.C. George, G.W. Wright, S.S. Dave, H. Zhao, W. Xu, A. Rosenwald, et al. 2008. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 319:1676–1679. http://dx.doi.org/10.1126/science.1153629

Mercurio, F., H. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J. Li, D.B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao. 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science*. 278:860–866. http://dx.doi.org/10.1126/science.278.5339.860

Morin, R.D., M. Mendez-Lago, A.J. Mungall, R. Goya, K.L. Mungall, R.D. Corbett, N.A. Johnson, T.M. Severson, R. Chiu, M. Field, et al. 2011. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature*. 476:298–303. http://dx.doi.org/10.1038/nature10351

Ngo, V.N., R.M. Young, R. Schmitz, S. Jhavar, W. Xiao, K.H. Lim, H. Kohlhammer, W. Xu, Y. Yang, H. Zhao, et al. 2011. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 470:115–119. http://dx.doi.org/10.1038/nature09671

Pasqualucci, L., V. Trifonov, G. Fabbri, J. Ma, D. Rossi, A. Chiarenza, V.A. Wells, A. Grunn, M. Messina, O. Elliot, et al. 2011. Analysis of the coding genome of diffuse large B-cell lymphoma. *Nat. Genet.* 43:830–837. http://dx.doi.org/10.1038/ng.892

Pisitkun, P., J.A. Deane, M.J. Difilippantonio, T. Tarasenko, A.B. Satterthwaite, and S. Bolland. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science*. 312:1669–1672. http://dx.doi.org/10.1126/science.1124978

Plenge, R.M., C. Cotsapas, L. Davies, A.L. Price, P.I. de Bakker, J. Maller, I. Pe'er, N.P. Burtt, B. Blumenstiel, M. DeFelice, et al. 2007. Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. *Nat. Genet.* 39:1477–1482. http://dx.doi.org/10.1038/ng.2007.27

Powers, J.P., S. Li, J.C. Jaen, J. Liu, N.P. Walker, Z. Wang, and H. Wesche. 2006. Discovery and initial SAR of inhibitors of interleukin-1 receptor-associated kinase-4. *Bioorg. Med. Chem. Lett.* 16:2842–2845. http://dx.doi.org/10.1016/j.bmcl.2006.03.020

Puente, X.S., M. Pinyol, V. Quesada, L. Conde, G.R. Ordóñez, N. Villamor, G. Escaramis, P. Jares, S. Beà, M. González-Díaz, et al. 2011. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 475:101–105. http://dx.doi.org/10.1038/nature10113

Rebeaud, F., S. Hailfinger, A. Posevitz-Fejfar, M. Tapernoux, R. Moser, D. Rueda, O. Gaide, M. Guzzardi, E.M. Iancu, N. Rufer, et al. 2008. The proteolytic activity of the paracaspase MALT1 is key in T cell activation. *Nat. Immunol.* 9:272–281. http://dx.doi.org/10.1038/ni1568

Rossi, D., S. Deaglio, D. Dominguez-Sola, S. Rasi, T. Vaisitti, C. Agostinelli, V. Spina, A. Bruscaggin, S. Monti, M. Cerri, et al. 2011. Alteration of BIRC3 and multiple other NF-κB pathway genes in splenic marginal zone lymphoma. *Blood*. 118:4930–4934. http://dx.doi.org/10.1182/blood-2011-06-359166

Rui, L., R. Schmitz, M. Ceribelli, and L.M. Staudt. 2011. Malignant pirates of the immune system. *Nat. Immunol.* 12:933–940. http://dx.doi.org/10.1038/ni.2094

Schüler, F., L. Dölken, C. Hirt, T. Kiefer, T. Berg, G. Fusch, K. Weitmann, W. Hoffmann, C. Fusch, S. Janz, et al. 2009. Prevalence and frequency of circulating t(14;18)-MBR translocation carrying cells in healthy individuals. *Int. J. Cancer*. 124:958–963. http://dx.doi.org/10.1002/ijc.23958

Shaffer, A.L., A. Rosenwald, and L.M. Staudt. 2002. Lymphoid malignancies: the dark side of B-cell differentiation. *Nat. Rev. Immunol.* 2:920–932. http://dx.doi.org/10.1038/nri953

Shen, Y., J. Iqbal, J.Z. Huang, G. Zhou, and W.C. Chan. 2004. BCL2 protein expression parallels its mRNA level in normal and malignant B cells. *Blood*. 104:2936–2939. http://dx.doi.org/10.1182/blood-2004-01-0243

Subramanian, S., K. Tus, Q.Z. Li, A. Wang, X.H. Tian, J. Zhou, C. Liang, G. Bartov, L.D. McDaniel, X.J. Zhou, et al. 2006. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc. Natl. Acad. Sci. USA*. 103:9970–9975. http://dx.doi.org/10.1073/pnas.0603912103

Tabeta, K., K. Hoebe, E.M. Janssen, X. Du, P. Georgel, K. Crozat, S. Mudd, N. Mann, S. Sovath, J. Goode, et al. 2006. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat. Immunol.* 7:156–164. http://dx.doi.org/10.1038/ni1297

Thomson, W., A. Barton, X. Ke, S. Eyre, A. Hinks, J. Bowes, R. Donn, D. Symmons, S. Hider, I.N. Bruce, et al; Wellcome Trust Case Control Consortium; YEAR Consortium. 2007. Rheumatoid arthritis association at 6q23. *Nat. Genet.* 39:1431–1433. http://dx.doi.org/10.1038/ng.2007.32

Tokunaga, F., H. Nishimasu, R. Ishitani, E. Goto, T. Noguchi, K. Mio, K. Kamei, A. Ma, K. Iwai, and O. Nureki. 2012. Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation. *EMBO J.* 31:3856–3870. http://dx.doi.org/10.1038/emboj.2012.241

Treon, S.P., L. Xu, G. Yang, Y. Zhou, X. Liu, Y. Cao, P. Sheehy, R.J. Manning, C.J. Patterson, C. Tripsas, et al. 2012. MYD88 L265P somatic mutation in Waldenström's macroglobulinemia. *N. Engl. J. Med.* 367:826–833. http://dx.doi.org/10.1056/NEJMoa1200710

Trøen, G., A. Warsame, and J. Delabie. 2013. CD79B and MYD88 Mutations in Splenic Marginal Zone Lymphoma. *ISRN Oncol.* 2013:252318.

Tsujimoto, Y., J. Cossman, E. Jaffe, and C.M. Croce. 1985. Involvement of the bcl-2 gene in human follicular lymphoma. *Science*. 228:1440–1443. http://dx.doi.org/10.1126/science.3874430

Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. 335:440–442. http://dx.doi.org/10.1038/335440a0

Verhelst, K., I. Carpentier, M. Kreike, L. Meloni, L. Verstrepen, T. Kensche, I. Dikic, and R. Beyaert. 2012. A20 inhibits LUBAC-mediated NF-κB activation by binding linear polyubiquitin chains via its zinc finger 7. *EMBO J.* 31:3845–3855. http://dx.doi.org/10.1038/emboj.2012.240

Wang, L., M.S. Lawrence, Y. Wan, P. Stojanov, C. Sougnez, K. Stevenson, L. Werner, A. Sivachenko, D.S. DeLuca, L. Zhang, et al. 2011. SF3B1 and other novel cancer genes in chronic lymphocytic

leukemia. *N. Engl. J. Med.* 365:2497–2506. http://dx.doi.org/10.1056/NEJMoa1109016

Woodland, R.T., M.R. Schmidt, and C.B. Thompson. 2006. BLyS and B cell homeostasis. *Semin. Immunol.* 18:318–326. http://dx.doi.org/10.1016/j.smim.2006.06.001

Wotherspoon, A.C., C. Doglioni, T.C. Diss, L. Pan, A. Moschini, M. de Boni, and P.G. Isaacson. 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. *Lancet.* 342:575–577. http://dx.doi.org/10.1016/0140-6736(93)91409-F

Xu, L., Z.R. Hunter, G. Yang, Y. Zhou, Y. Cao, X. Liu, E. Morra, A. Trojani, A. Greco, L. Arcaini, et al. 2013. MYD88 L265P in Waldenström macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative disorders using conventional and quantitative allele-specific polymerase chain reaction. *Blood*. 121:2051–2058. http://dx.doi.org/10.1182/blood-2012-09-454355