

Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells

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Toll-like receptor (TLR), a ligand for single-stranded RNA, has been implicated in the development of pathogenic anti-RNA autoantibodies both in systemic lupus erythematosus (SLE) patients and in murine models of lupus. It is still unclear, however, where and how TLR7-mediated interactions affect the development of autoreactive B cells. We found that overexpression of TLR7 in transgenic mice (TLR7.1Tg) leads to marked alterations of transitional (T1) B cells, associated with their expansion and proliferation within the splenic red pulp (RP). This phenotype was intrinsic to the T1 subset of B cells and occurred independently of type 1 IFN signals. Overexpression of RNase in TLR7.1Tg mice significantly limited the expansion and proliferation of T1 cells, indicating that endogenous RNA complexes are driving their activation. TLR7.1Tg T1 cells were hyper-responsive to anti-IgM and TLR7 ligand stimulation in vitro and produced high concentrations of class-switched IgG2b and IgG2c, including anti-RNA antibodies. Our results demonstrate that initial TLR7 stimulation of B cells occurs at the T1 stage of differentiation in the splenic RP and suggest that dysregulation of TLR7 expression in T1 cells can result in production of autoantibodies.

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Abbreviations used: AID, activation-induced deaminase; FO, follicular; HEL, hen egg lysozyme; MZ, marginal zone; RNP, ribonucleoprotein; RP, red pulp; SLE, systemic lupus erythematosus; ssRNA, single-stranded RNA; T1, Transitional 1; TLR, toll-like receptor.

The generation of diverse BCR specificities in developing B cell precursors occurs through random V(D)J gene recombination, which can result in high levels of autoreactive B cells (Nemazee, 2006; Tiller et al., 2007; Meffre and Wardemann, 2008). If not properly eliminated or tolerized, autoreactive B cells can become activated and promote the development of autoimmune diseases, such as systemic lupus erythematosus (SLE). Nuclear antigens, including DNA, histones, RNA, and ribonucleoproteins (RNPs), are dominant targets of autoantibodies in SLE patients and murine models of lupus (Green and Marshak-Rothstein, 2011). While the etiology of SLE is multifaceted, recent studies have implicated the important contribution of innate pattern recognition receptors, such as TLRs in the development of SLE (Leadbetter et al., 2002; Viglianti et al., 2003; Lau et al., 2005). Toll-like receptor (TLR) 7 is an intracellular TLR, specialized in the recognition of single-stranded RNA (ssRNA), and highly

expressed by plasmacytoid DCs and B cells (Diebold et al., 2004; Flygare et al., 2005). Deletion of a single TLR7 allele in lupus-prone MRL.Fas/lpr mice leads to elimination of anti-RNA autoantibodies and significant reduction of disease symptoms, suggesting a critical role for TLR7 in the development of murine lupus (Christensen et al., 2006; Santiago-Raber et al., 2010b). Furthermore, changing the level of TLR7 expression by increasing *Tlr7* gene dosage has been implicated in the development of autoimmune disease. For example BXSB/MpJ mice, which carry the Yaa (Y-linked autoimmune acceleration) translocation of the locus encoding *Tlr7* from the X chromosome onto the Y chromosome, have one extra copy of *Tlr7* and develop an SLE-like disease (Pisitkun et al., 2006;

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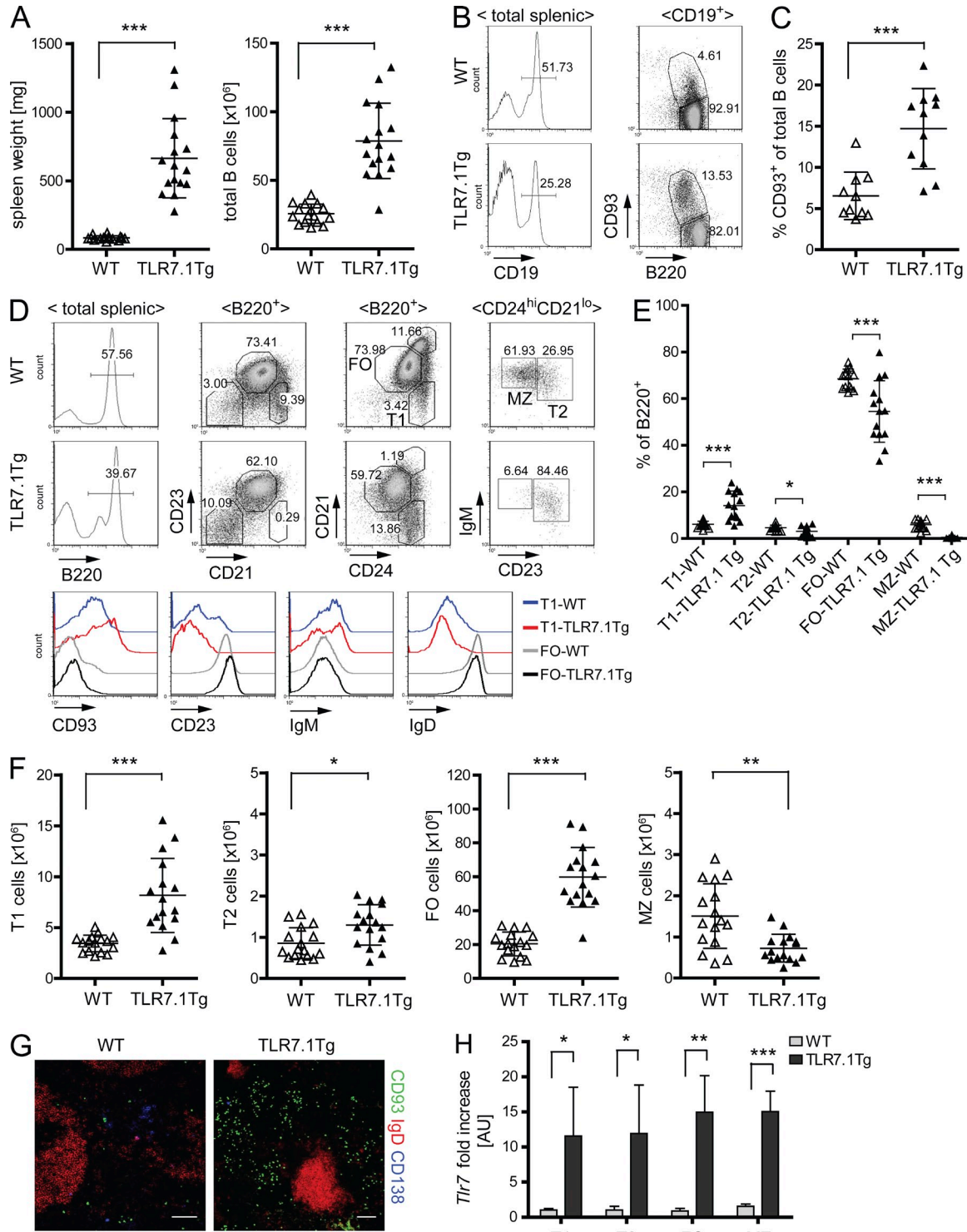


Figure 1. TLR7 overexpression promotes the expansion of T1 and FO B cell subsets. (A) Spleens from 8–14-wk-old WT and TLR7.1Tg mice were analyzed for splenomegaly and the frequencies of B220⁺ cells B cells were assessed by flow cytometry. Cell numbers were calculated based on the total cell number per spleen. (B and C) Splenocytes from WT and TLR7.1Tg mice were gated on CD19⁺ B cells and the frequencies of immature (B220⁺CD93⁺) B cells were assessed by flow cytometry. (D) Representative flow cytometric plots and gating strategy used to identify splenic B cell subsets. The following markers were used to discriminate T1 (B220⁺CD24^{hi}CD21^{lo}), T2 (B220⁺CD24^{hi}CD21^{int/hi}CD23^{hi}), FO (B220⁺CD24^{int}CD21^{int}), and MZ (B220⁺CD24^{hi}CD23[−]CD21^{hi}) B cell subsets. Histograms below show the expression of CD93, CD23, IgM, and IgD of T1 and FO B cells from WT and TLR7.1Tg mice. (E and F) The frequencies of different splenic B cell subsets were assessed by flow cytometry and cell numbers were calculated based on the total cell number per spleen. In A, C, E, and F, each dot represents an individual animal with the mean indicated for each group (horizontal bars). Presented data are from five or more

Subramanian et al., 2006). The Yaa mutation also greatly accelerates the development of SLE in lupus-prone FcγRIIB^{-/-} mice (Bolland et al., 2002; Pisitkun et al., 2006). Directly increasing *Tlr7* gene dosage by creating BAC-TLR7Tg mice leads to an acute systemic autoimmune disease characterized by glomerulonephritis, production of anti-RNA autoantibodies, and myeloproliferative syndrome (Deane et al., 2007). Genetic studies in humans have further supported a link between copy number variations or polymorphisms at the TLR7 locus and susceptibility to SLE (García-Ortiz et al., 2010; Shen et al., 2010; Kawasaki et al., 2011; Lee et al., 2012; Tian et al., 2012). Furthermore, genetic variations of IRF7, a transcription factor expressed downstream of TLR7, have been implicated in the development of pathogenic anti-RNA Abs in SLE (Salloum et al., 2010).

Despite the pivotal role of TLR7 in murine lupus and strong evidence for its key role in both susceptibility to and expression of the disease, surprisingly little is known about the intrinsic effects of TLR7 overexpression on the B cell lineage. Yaa mice develop a “hyperactive” B cell phenotype and have a marked reduction of the marginal zone (MZ) B cell compartment (Amano et al., 2003; Pisitkun et al., 2006). The underlying mechanism for the loss of MZ B cells in these mice and its relevance to the development of pathogenic autoantibodies remains unclear (Subramanian et al., 2006; Santiago-Raber et al., 2010a). TLR7Tg mice with a modest increase in *Tlr7* gene dosage recapitulate the B cell phenotype observed in Yaa mice, including loss of MZ B cells (Deane et al., 2007; Hwang et al., 2012). It remains unknown, however, where and how RNA-TLR7-mediated interactions might affect the development of peripheral B cells and promote the activation of autoreactive B cells. In this study, we found that overexpression of TLR7 in TLR7.1Tg mice had a profound, cell-intrinsic effect on transitional 1 (T1) splenic B cells associated with their expansion and RNA-driven proliferation. The activation of T1 B cells occurred in the splenic red pulp (RP), suggesting that this might be an important site for activation of anti-RNA-specific B cells. The hyper-proliferative phenotype of TLR7.1Tg T1 B cells was associated with increased expression of activation-induced deaminase (AID) and T-bet and production of class-switched IgG antibodies, including IgG anti-RNA. Our results reveal that in the spleen the TLR7 ligand ssRNA engages autoreactive cells first at the stage of T1 B cells, leading to activation, expansion, and the potential to produce anti-RNA autoantibodies.

RESULTS

Expansion of T1 and follicular (FO) B cell subsets in TLR7.1Tg mice

To identify which stages of splenic B cell development are affected by TLR7 overexpression, we first compared B cell

subset distribution between WT B6 mice and TLR7.1Tg mice, which express 8–16-fold higher TLR7 mRNA compared with WT (Deane et al., 2007). 8–14-wk-old TLR7.1Tg mice had a significant (approximately threefold) increase in the total number of splenic B cells compared with control mice (Fig. 1 A), although the frequency of B220⁺ B cells was decreased due to the expansion of myeloid cells (Deane et al., 2007; Buechler et al., 2013; Sun et al., 2013). Further flow cytometric analyses showed a marked increase in the percentage of CD19⁺B220⁺CD93⁺ (AA4.1) B cells, suggesting an expansion of the immature B cells (Fig. 1, B and C). Using a panel of surface markers, we confirmed that the expanded cells were in fact B220⁺CD24^{hi}CD21^{lo} T1 B cells (Fig. 1 D). The percentage of T1 cells in TLR7.1Tg mice increased nearly threefold, whereas the percentages of T2 (B220⁺CD24^{hi}CD21^{hi/int}CD23^{hi}) and FO (B220⁺CD24^{int}CD21^{int}) subsets of total splenic B cells were significantly decreased (Fig. 1 E). Actual numbers of T1, T2, and FO B cells increased, with the most significant increases in T1 and FO B cells (Fig. 1 F). In accordance with Deane et al. (2007), we also found that the proportion and number of MZ B cells (defined as B220⁺CD24^{hi}CD23⁻CD21^{hi}) were significantly reduced in TLR7.1Tg mice (Fig. 1, D–F). Consistent with the flow cytometric data, immunohistological analyses showed significant accumulation of CD93^{bright}IgD^{low}CD138^{neg}T1-like B cells in the RP areas of the TLR7.1Tg but not the WT mouse spleens (Fig. 1 G).

To determine whether peripheral B cells in TLR7.1Tg mice differed in their *Tlr7* expression levels, we performed RT-PCR analyses of sorted B cell T1, T2, FO, and MZ B cell populations. We found that *Tlr7* mRNA levels were significantly elevated (9–18-fold) in all B cell subsets of TLR7.1Tg mice, as compared with WT controls, but there was no statistically significant difference between the subsets (Fig. 1 H).

Together, these results showed that *Tlr7* is overexpressed in all splenic B cell subsets of TLR7.1Tg mice. T1 and FO B cell subset numbers were the most significantly expanded, whereas MZ B cells decreased significantly. The expansion of T1 cells was associated with marked accumulation of these cells in the splenic RP.

TLR7 overexpression promotes cell-intrinsic proliferation of T1 B cells in the splenic RP

To further understand the basis for the accumulation of T1, T2, and FO B cells in TLR7.1Tg mice, we assessed cell proliferation by a BrdU pulse in vivo, using relatively young (8–12 wk old) TLR7.1Tg and B6 control mice. We found a strikingly higher percentage of BrdU⁺ cells within the T1 B cell subset (≥15%) of TLR7.1Tg mice compared with control B6 mice (≤4.5%; Fig. 2, A and B). A small increase of the

independent experiments. (G) Splenic sections were prepared from frozen spleens of WT and TLR7.1Tg mice and stained with CD93, IgD, and CD138 antibodies. Data show an accumulation of (CD93^{bright}IgD^{low}CD138^{neg}) T1 cells in the splenic RP of TLR7.1Tg mice and are representative of more than six sections analyzed from two mice per genotype. Bars, 100 μm. (H) T1, T2, FO, and MZ cell subsets from WT and TLR7.1Tg mice were sorted and the expression of *Tlr7* mRNA was quantified by RT-PCR. Data are presented as fold change of mRNA levels relative to WT T1 cells. Data are from an average of four mice per genotype (8–12 wk old). *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by two-tailed, unpaired Student's *t* test.

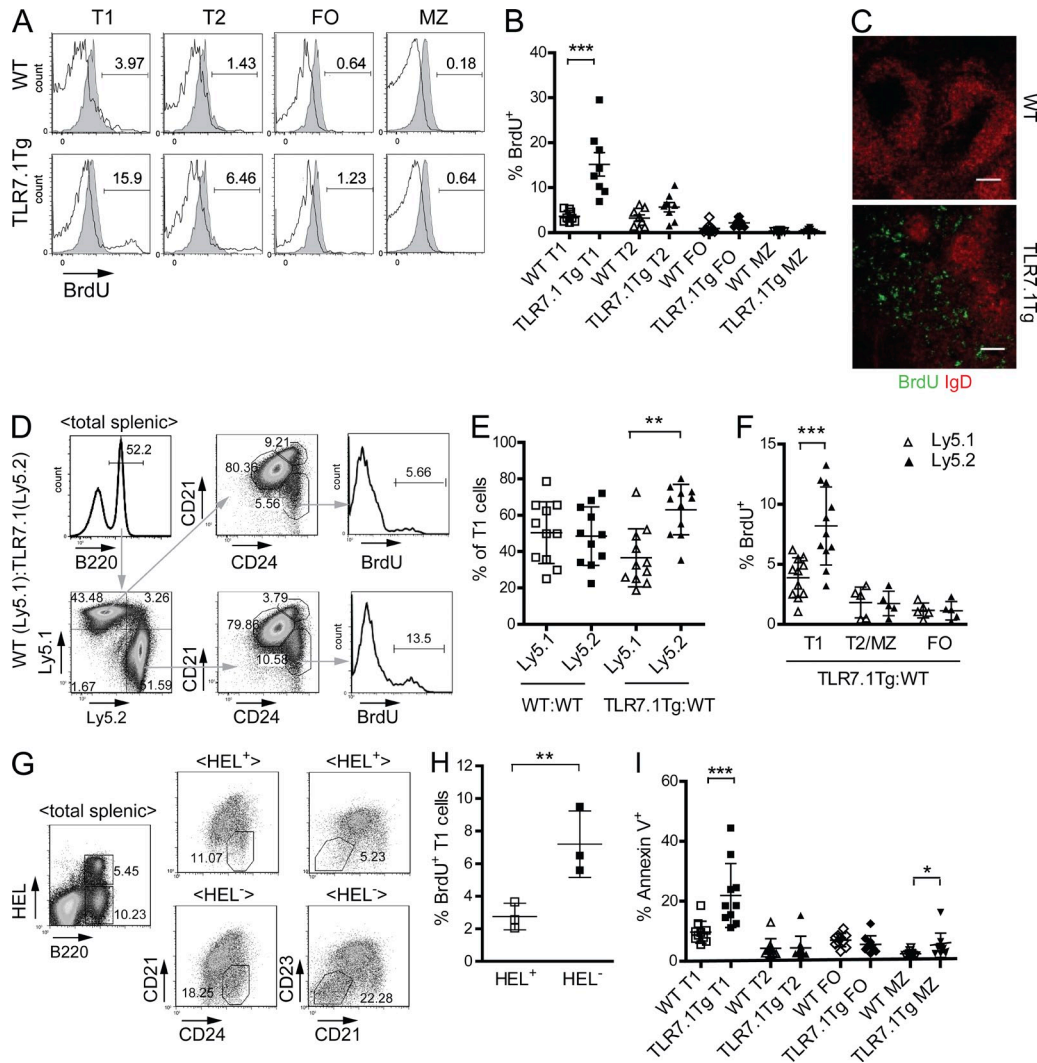


Figure 2. Increased proliferation and increased apoptosis of T1 B cells in TLR7.1Tg mice. (A and B) 8–12-wk-old WT and TLR7.Tg mice were injected with 1 mg BrdU 1 h before sacrifice, and BrdU incorporation in different B cell subsets was analyzed by flow cytometry. Representative histograms in A show the percentage of BrdU⁺ cells in each B cell subset. (B) Summarized data from individual mice as in A. Each dot represents an individual animal with the mean indicated for each group (horizontal bars). Presented data are from three independent experiments. (C) Splenic sections were prepared from frozen spleens of WT and TLR7.1Tg mice injected with BrdU 1 h before sacrifice and stained with anti-IgD and anti-BrdU Abs. Data show the presence of proliferating BrdU⁺ cells (green) outside of IgD⁺ B cell follicles (red). Data are representative data of more than six sections analyzed from three mice per genotype. Bars, 100 μ m. (D–F) Lethally irradiated B6 mice were reconstituted with an equal mix of congenic-labeled WT (Ly5.1⁺) and TLR7.1Tg (Ly5.2⁺) BM cells. Mice were injected with BrdU 1 h before sacrifice and the percentages of T1, FO, and T2/MZ B cell populations within gated Ly5.1⁺B220⁺ or Ly5.2⁺B220⁺ cells and the frequency of BrdU⁺ cells within each subset were analyzed by flow cytometry. (D) Gating strategy for detecting BrdU⁺ B cells and representative flow cytometric plots. (E and F) Summarized data from three independent experiments showing the percentage of Ly5.1⁺ or Ly5.2⁺ cells with the T1 cells (E) or the percentages of BrdU⁺ T1, T2/MZ, or FO cells (F) in WT: WT or TLR7.1Tg:WT chimeric mice. Each dot represents an individual animal with the mean indicated for each group (horizontal bars). (G and H) TLR7.1Tg \times HEL mice were injected with BrdU 1 h before sacrifice and the percentages of T1 cells (defined as CD24^{hi} CD21^{lo} CD23^{lo}) and the frequencies of BrdU⁺ T1 cells within the HEL⁺ and HEL⁻ cell population were analyzed by flow cytometry. HEL⁺ B cells were identified using biotinylated HEL. (G) Gating strategy and representative flow cytometric plots. (H) Summarized data showing the percentage of BrdU⁺ T1 cells within gated HEL⁺ and HEL⁻ cell populations in TLR7.1Tg \times HELTg mice. (I) Annexin V binding within individual B cell subsets from WT and TLR7.1Tg mice was assessed by flow cytometry. Presented data are from three independent experiments. Each dot represents an individual animal with the mean indicated for each group (horizontal bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as determined by two-tailed, unpaired Student's *t* test or one-way ANOVA with Tukey's post-test, where appropriate.

percentage of BrdU⁺ cells was also noticeable within the T2 B cell subset; however, little or no BrdU⁺ cells were detectable within the FO or MZ B cell populations (Fig. 2 B). The proliferation of T1 B cells was also supported by histological analyses, which revealed a significant accumulation of BrdU⁺ cells

within the splenic RP of TLR7.1Tg mice (Fig. 2 C) where T1 B cells reside (Fig. 1 G; Loder et al., 1999).

To test whether the increased cell proliferation was due to an intrinsic change in T1 B cells overexpressing TLR7, we generated mixed BM chimeric mice by reconstituting lethally irradiated

B6 × B6.SJL F1 mice with an equal mix of congenically marked B6.SJL (Ly5.1⁺) and TLR7.1Tg (Ly5.2⁺) donor BM cells. 3 mo after reconstitution, TLR7.1:B6.SJL chimeric mice displayed many of the characteristics of TLR7.1Tg mice, including the development of splenomegaly and myeloid cell expansion (Buechler et al., 2013). Flow cytometric analyses of B cell subsets in mixed BM chimeras revealed a preferential expansion of TLR7.1Tg-derived T1 cells (Fig. 2, D and E). Furthermore, TLR7.1:B6.SJL chimeric mice showed a two- to threefold increase in the percentage of proliferating (BrdU⁺) TLR7.1Tg T1 cells, compared with B6.SJL T1 cells. No significant differences in cell proliferation were observed in either Ly5.1⁺ or Ly5.2⁺ T1 cells in the B6:B6.SJL control chimeras (unpublished data). The effects of TLR7 overexpression in promoting B cell proliferation was selective to the TLR7.1Tg T1 B cells, as we detected no significant differences in proliferation within the T2/MZ or FO B cell subsets from Ly5.2 TLR7.1Tg donors (Fig. 2 F).

To determine whether the proliferation of T1 B cells in TLR7.1Tg was a consequence of TLR7 overexpression by itself or interaction with self-antigens, we crossed TLR7.1Tg mice with hen egg lysozyme (HEL)-specific VDJ9κ5 mice to create TLR7.1Tg mice in which a high proportion of B cells (15–35%) express BCRs specific for HEL (HEL⁺ cells). Analyses of TLR7.1Tg × HEL mice showed lower percentages of T1 cells within the HEL-binding B220⁺ B cell population as compared with B cells that did not bind HEL (HEL⁻ cells; Fig. 2 G). Furthermore, HEL⁺ T1 B cells displayed significantly less BrdU uptake compared with HEL⁻ T1 B cells (Fig. 2 H). Therefore, TLR7 overexpression did not affect the expansion of B cells specific for exogenous antigen. These results suggest that BCR-mediated signaling, presumably via endogenous auto-Ags, is required for T1 B cell expansion.

As TLR signaling has been also implicated in negative selection of autoreactive B cells (Isnardi et al., 2008), we next sought to determine if TLR7 overexpression affected the percentage of cell death in different B cell subsets. Consistent with the notion that the transition from T1 to T2/FO stage represents an important checkpoint for the elimination of potentially autoreactive B cells (Yurasov et al., 2005; Giltiay et al., 2012), we observed an increase in the percentage of Annexin V⁺ T1 cells in both WT and TLR7.1Tg mice compared with other peripheral B cell subsets (Fig. 2 H). The rate of apoptosis was significantly higher in TLR7.1Tg T1 cells compared with the WT T1 cells.

Collectively, these data show that TLR7 overexpression results in a striking increase in both proliferation and cell death of T1 B cells in the RP of the spleen. Because there is an overall expansion of these cells, the rate of proliferation and/or survival must exceed the rate of cell death. This hyper-proliferative phenotype is intrinsic to TLR7Tg T1 B cells but is not present in HEL-specific T1 B cells, strongly supporting the hypothesis that an endogenous antigen is driving their activation.

TLR7.1Tg T1 B cells are hyper-responsive to TLR7 and to BCR engagement

Because we observed that RP T1 B cells in TLR7.1Tg mice proliferate at high rates in vivo, we asked whether T1 cell

subpopulation also contains activated cells with characteristics of plasmablasts. Flow cytometric analysis revealed an increase in the percentage of B220⁺CD138⁺CD44^{hi} cells, suggesting the presence of Ag-activated (plasmablast) cells. However, these cells constituted a small proportion (~1%) of the T1 B cells in the TLR7.1Tg mice. Similar percentages of CD138⁺CD44^{hi} cells were also present within the TLR7.1 FO B cell subset (Fig. 3, A and B).

To further test if TLR7.1Tg T1 and FO cells can proliferate and differentiate into Ab-producing cells, we purified T1 and FO cells from WT or TLR7.1Tg mice (Fig. S1), loaded them with CFSE, and stimulated in vitro with TLR7 agonist (R848), anti-IgM, or SmRNP, a natural RNA-containing ligand. Consistent with the observation that RNA antigens drive the excessive proliferation of T1 cells in vivo, TLR7.1Tg T1 B cells activated by R848 showed a significant increase in the percentage of live cell (scattered gated) and proliferating (CFSE^{lo}) cells compared with WT T1 cells (Fig. 3 C). We also observed an increased survival and proliferation of TLR7.1Tg T1 cells in response to anti-IgM, whereas less striking differences in survival and proliferation between TLR7.1Tg and WT T1 cell cultures were seen when stimulated by the combination of anti-IgM and R848. Similar to TLR7.1Tg T1 cells, TLR7.1Tg FO B cells were also more responsive to R848 stimulation (Fig. 3 D) but actually responded less to anti-IgM stimulation. Importantly, we found that a portion of TLR7.1Tg T1 cells, but not WT T1 B cells, proliferated in response to stimulation with SmRNP. No responses to SmRNP stimulation were evident in either TLR7.1Tg or WT FO B cell samples.

Because TLR7 and/or BCR stimulation led to cell proliferation rather than death in TLR7.1Tg T1 B cells, we asked whether the in vitro activation of TLR7.1 T1 B cells was also associated with increased immunoglobulin secretion. We observed higher concentrations of both IgM and IgG in non-stimulated cultures of TLR7.1Tg T1 B cells and an approximately twofold increase in IgG concentration after activation with R848 or anti-IgM plus R848 (Fig. 4 A). Small but detectable levels of IgG were also found in cultures from TLR7.1Tg T1 cells stimulated with SmRNP. Under the same experimental conditions, sorted FO B cells from TLR7.1Tg mice also produced increased levels of IgG, but at significantly lower levels compared with T1 B cells (Fig. 4 A). Furthermore, although the T1 and FO populations contained similar percentages of CD44^{hi}CD138⁺ cells (Fig. 3 B), the stimulated T1 cells produced significantly more IgG than the FO cells (Fig. 4 A). In addition, sorted TLR7 Tg T1 cells, from which we removed CD138⁺ cells from the T1 gate, still proliferated at higher rates and produced higher titers of IgG than did the WT T1 cells (unpublished data). Together, these results indicate that the high IgG production by T1 cells was not simply due to CD138⁺ cells.

Further analysis showed that T1 cells predominantly produced IgG2c and IgG2b subclasses (Fig. 4 B). Consistent with these in vitro findings, we detected increased frequencies of IgG2b⁺ and IgG2c⁺ cells within the splenic RP in TLR7.1Tg

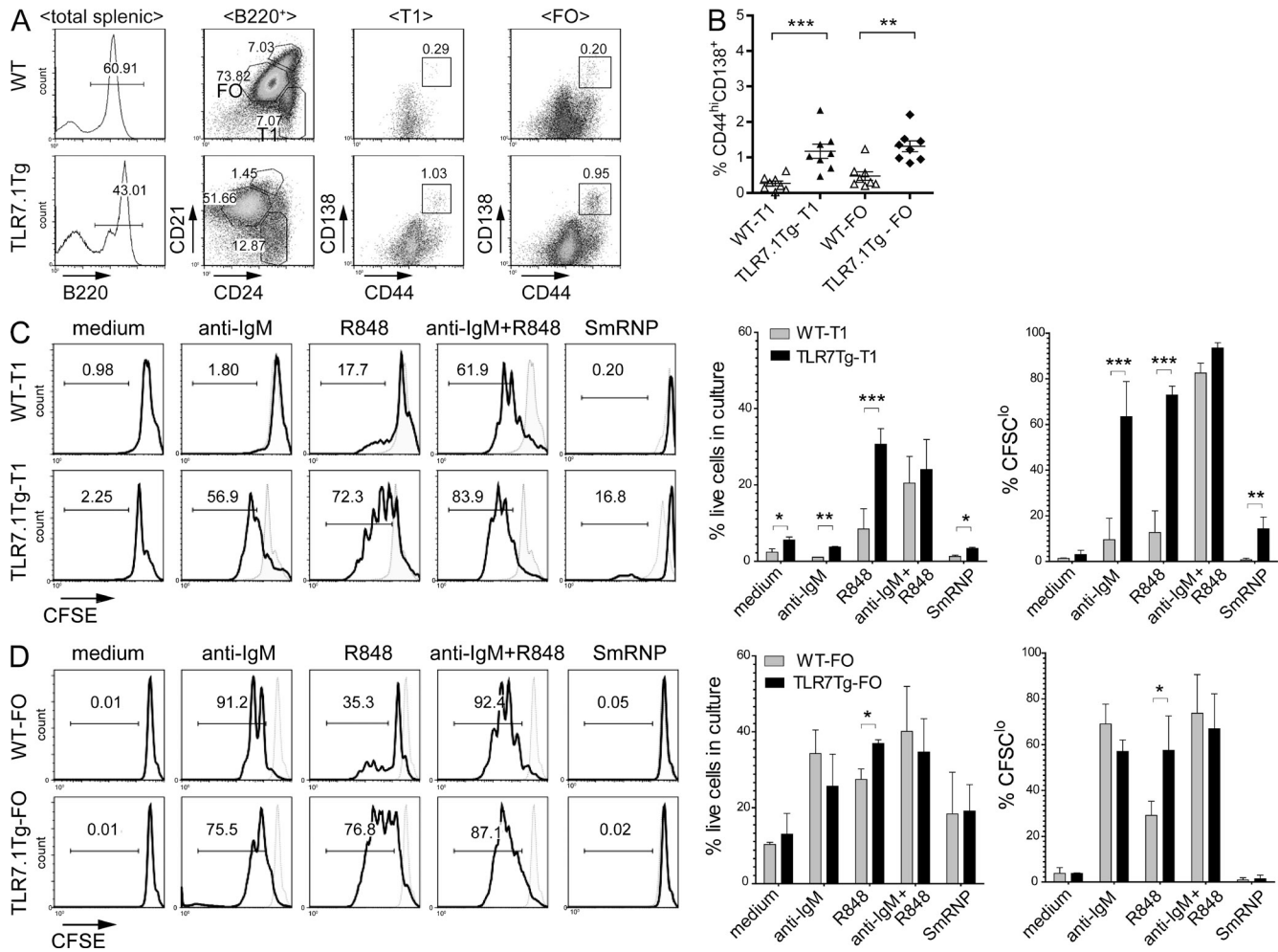


Figure 3. TLR7.1Tg T1 B cells are hyper-responsive to in vitro TLR7 ligand stimulation. (A and B) Splens from 8–14-wk-old WT and TLR7.1Tg mice were analyzed for the frequencies of CD44^{bright}CD138⁺ cells within gated T1 or FO B cell subsets by flow cytometry. (A) Gating strategy and representative flow cytometric plots. (B) Summarized data from individual WT and TLR7.1Tg mice. Purified T1 cells (C) or FO cells (D) from WT or TLR7.1Tg mice were loaded with CFSE and cultured for 72 h in RPMI medium or stimulated with anti-IgM (10 μg/ml), R848 (50 ng/ml), a combination of anti-IgM plus R848, or SmRNP (10 ng/ml). Representative histograms show the percentage of proliferating (CFSE⁰) cells after the indicated treatments. Bar graphs summarize the percentage of live cells (defined based on FSC/SSC gating) and the percentage of proliferating cells. Shown are the combined means ± SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by two-tailed, unpaired Student's *t* test or one-way ANOVA with Tukey's post-test, where appropriate.

mice localized in the same area as the T1 cells (Fig. 4 C). Importantly, analysis of IgG produced by TLR7.1Tg T1 cells treated with anti-IgM, R848, or SmRNP also showed reactivity against RNA (Fig. 4 D). ANA test further confirmed the presence of anti-nuclear antibodies in culture supernatants from TLR7Tg T1 cells, stimulated with R848 or anti-IgM plus R848 (Fig. 4 E). The above results indicate that a portion of TLR7.1Tg T1 cells are hyper-responsive to both TLR7 and BCR engagement, resulting in increased survival, proliferation, and secretion of class-switched Abs, including the production of anti-RNA Abs.

TLR7.1Tg T1 B cells display elevated expression of AID and T-bet involved in Ig class switching

In previous studies, T1 B cells were shown to constitutively express AID and respond rapidly to TLR9 and TLR3 stimulation

(Han et al., 2007; Ueda et al., 2007). In addition, the transcription factor T-bet is induced in B cells upon activation through TLR7 or TLR9 and mediates IgG2a class-switch recombination (Peng et al., 2002; Berland et al., 2006). Given the surprising predominance of IgG2c and IgG2b Abs produced by purified TLR7.1Tg T1 cells, we tested if AID and/or T-bet might be induced downstream of TLR7. Expression levels of AID encoding gene (*Aicda*) in freshly isolated TLR7.1Tg T1 was somewhat higher compared with B6 T1 cells, but differences were not statistically significant (Fig. 4 F). Importantly, consistent with Ueda et al. (2007) and Han et al. (2007), T1 cells were the only B cell subset with measurable levels of *Aicda* mRNA, suggesting that unstimulated T1 cells have the capacity to produce class-switched Abs. Further analysis of TLR7.1Tg and WT T1, T2, FO, and MZ B cells revealed that TLR7.1Tg B cells have a 2.5–5-fold increase in

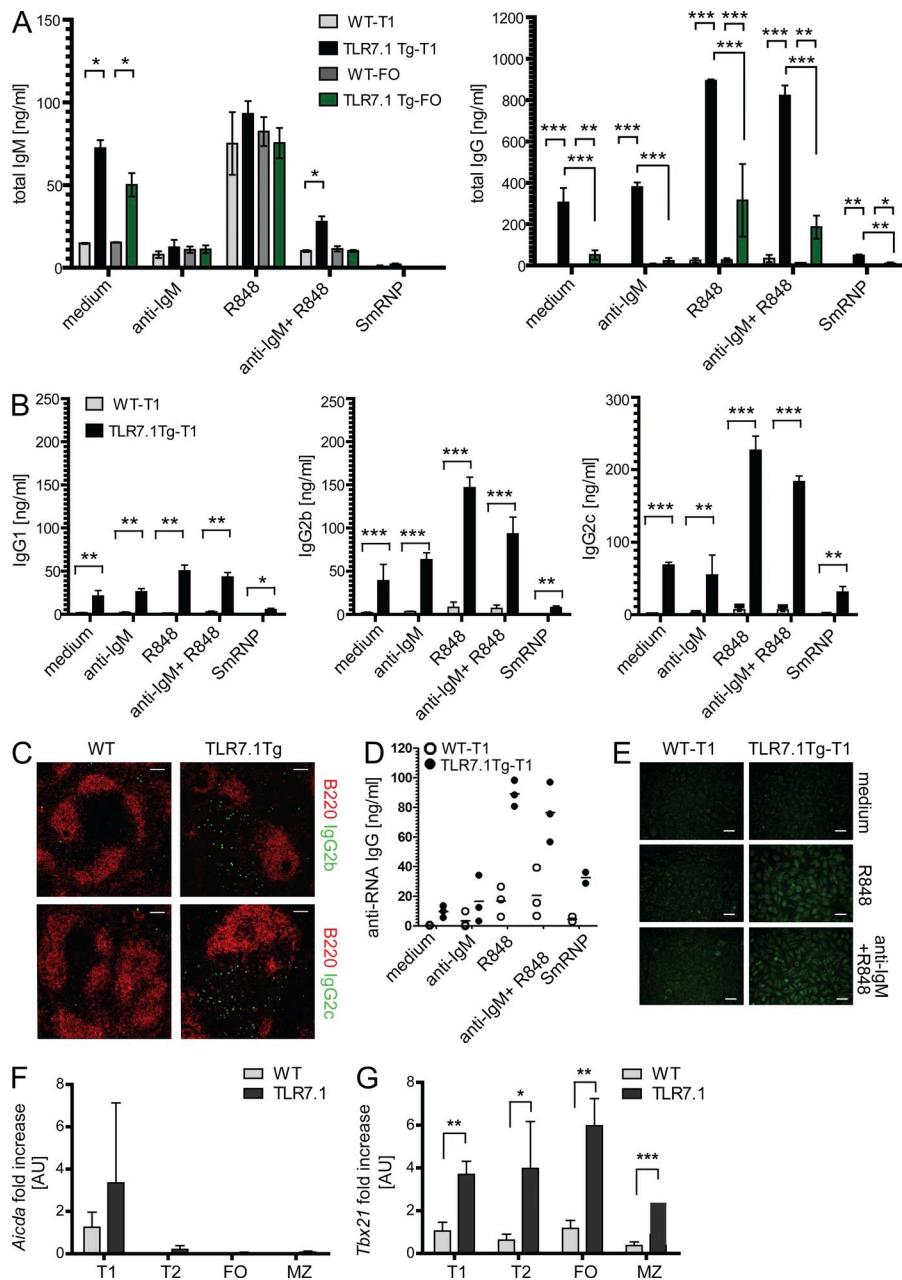


Figure 4. TLR7.1Tg T1 B cells produce class-switched IgG. (A and B) Sorted T1 and FO B cells from WT and TLR7.1Tg mice were cultured for 72 h in RPMI medium or stimulated with anti-IgM (10 μ g/ml), R848 (50 ng/ml), or a combination of anti-IgM plus R848 or SmRNP (10 ng/ml). Ab production in cell culture supernatants were analyzed by ELISA. (A) Bar graphs show quantities of total IgM and IgG produced by T1 and FO B cells in response to different stimuli. (B) Bar graphs show quantities of IgG1, IgG2b, and IgG2c produced by T1 B cells in response to different stimuli. The data are presented as mean \pm SD from three independent experiments. (C) Splenic sections were prepared from frozen spleens from 5-mo-old WT and TLR7.1Tg mice and stained with anti-B220 and anti-IgG2b and anti-IgG2c Abs. Data show presence of IgG2b⁺ and IgG2c⁺ cells in the splenic RP of TLR7.1Tg mice and are representative of more than four sections analyzed from two mice per genotype. Bars, 100 μ m. (D) Sorted T1 B cells from WT and TLR7.1Tg mice were cultured for 72 h in RPMI medium or stimulated with anti-IgM (10 μ g/ml), R848 (50 ng/ml), or a combination of anti-IgM plus R848 or SmRNP (10 ng/ml). Anti-RNA IgG titers in cell culture supernatants were analyzed by ELISA. Individual dots represent data from two independent experiments. (E) Representative Hep-2 ANA staining of undiluted culture supernatants from WT or TLR7Tg T1 cells cultured for 72 h in RPMI medium or stimulated with R848, or a combination of anti-IgM plus R848. Data are representative of three independent experiments. Bars, 50 μ m. (F and G) T1, T2, FO, and MZ cell subsets from WT and TLR7.1Tg mice were isolated by cell sorting and the expression of *Aicda* (F) and *Tbx21* (G) mRNA were quantified by RT-PCR. Data are presented as fold change of mRNA levels relative to WT T1 cells. Data are average of four mice per genotype (8–12 wk old) and show mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as determined by two-tailed, unpaired Student's *t* test.

the expression of T-bet encoding gene (*Tbx21*) compared with WT counterparts (Fig. 4 G). Together, these results demonstrate that additional copies of TLR7 in T1 B cells lead to enhanced expression of AID and T-bet and increased production of IgG2b and IgG2c in response to TLR7 stimulation in vitro (Fig. 4 B).

Overexpression of RNase A limits the expansion and proliferation of T1 B cells in TLR7.1Tg mice

ssRNA is a ligand for TLR7, yet it is not known where in the spleen B cells encounter RNA that might be free or part of RNPs released from dying cells. To test the role of extracellular RNA as the ligand stimulating TLR7.1Tg B cells, we crossed

TLR7.1Tg mice with mice that express bovine RNase A under the human albumin promoter (RNaseTg mice). RNaseTg mice display 5–10-fold higher serum RNase A activity (Sun et al., 2013) but show no alterations in their spleen weight and B cell numbers in the spleen compared with WT mice (Fig. 5 A).

Overexpression of RNase in TLR7.1 \times RNase dTg mice led to a reduction in spleen size, which was associated with a modest but not statistically significant reduction in the number of total B cells (Fig. 5 A). However, flow cytometric analyses of individual B cell subsets in TLR7.1 \times RNase dTg mice revealed a significant (2–2.5-fold) reduction in the numbers of T1 B cells in the TLR7.1 \times RNase dTg mice

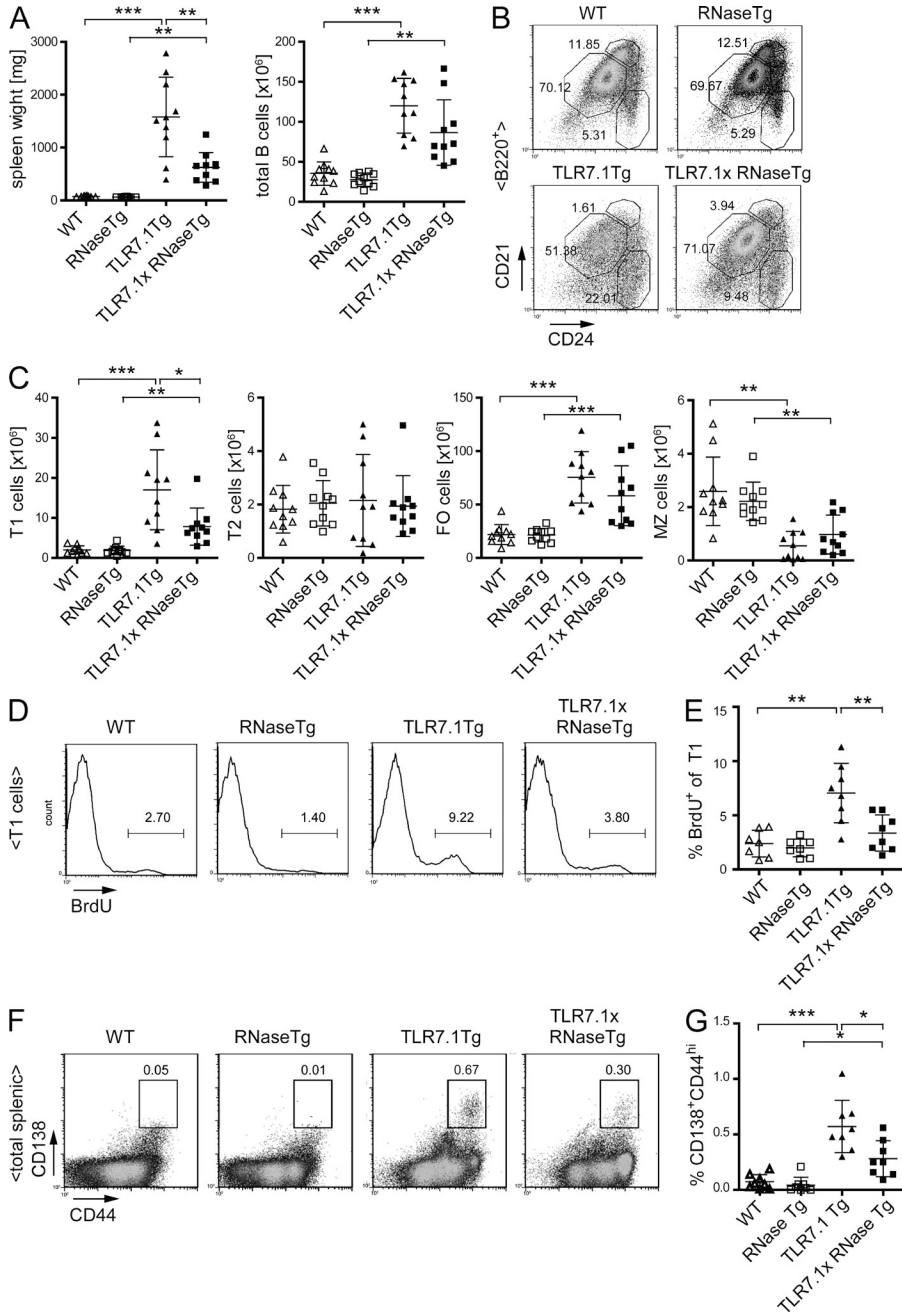


Figure 5. Overexpression of RNase limits the expansion of T1 cells in TLR7.1Tg mice. (A–C) 4–6-mo-old WT, RNase Tg, TLR7.1Tg, and TLR7.1 \times RNase Tg mice were analyzed for splenomegaly (A) and the frequencies of B220⁺, T1, T2, FO, and MZ B cells were assessed by flow cytometry. (B) Representative flow cytometric plots showing the frequencies of T1, T2/MZ, and FO B cells of gated B220⁺ cells. Cell numbers were calculated based on the total cell number per spleen. (C) Summarized data showing the number of T1, T2, FO, and MZ B cells. Data in A–C are from three independent experiments. (D and E) Mice were injected with 1 mg BrdU 1 h before sacrifice, and BrdU incorporation in gated T1 cells was analyzed flow cytometry. Representative histograms in D show the percentages of BrdU⁺ T1 cells in different mice genotypes. Data from two independent experiments are summarized in E. (F and G) Splenocytes from WT, RNase Tg, TLR7.1Tg, and TLR7.1 \times RNase Tg mice were stained with anti-B220, anti-CD138, and anti-CD44 mAb to assess the frequencies of plasma cells. (F) Representative flow cytometry plots show the gating strategy and frequency of plasma cells (CD138⁺CD44^{hi}) within total splenic cells from mice with different genotypes. Data from three independent experiments are summarized in G. In A, C, E, and G, each dot represents an individual animal with the mean indicated for each group (horizontal bars). *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by two-tailed, unpaired Student's *t* test or one-way ANOVA with Tukey's post-test, where appropriate.

compared with TLR7.1Tg controls (Fig. 5, B and C). The number of FO B cells was also decreased in TLR7.1 \times RNase dTg mice but not as much as the decrease in T1 B cells. In contrast, RNase A overexpression had little effect on MZ B cell numbers, as both TLR7.1 and TLR7.1 \times RNase dTg mice had reduced levels of MZ B cells.

The increased expression of RNase A not only reduced TLR7.1Tg T1 B cell numbers but also affected their ability to proliferate in vivo. WT, RNaseTg, TLR7.1Tg, and TLR7.1 \times RNase dTg mice were pulsed with BrdU, and BrdU incorporation in different B cell subsets was analyzed as in Fig. 2. The percentage of BrdU⁺ T1 cells in TLR7.1 \times RNase dTg mice was significantly reduced compared with the TLR7.1Tg

mice (Fig. 5, D and E). No substantial cell proliferation was observed in other B cell subsets. The overexpression of RNase A also significantly limited the accumulation of plasma cells in the TLR7.1Tg mice (Fig. 5, F and G), suggesting that endogenous RNA is required for the generation of Ab-producing cells in TLR7.1Tg mice.

Based on these results, we conclude that the in vivo proliferation and expansion of TLR7.1Tg T1 B cells is driven by endogenous, RNA-containing Ag. In contrast, the reduction of MZ B cells in TLR7.1Tg mice was not significantly affected by the presence of RNase, suggesting that T1 B cell and MZ B cell subsets in TLR7.1Tg mice are regulated by different mechanisms.

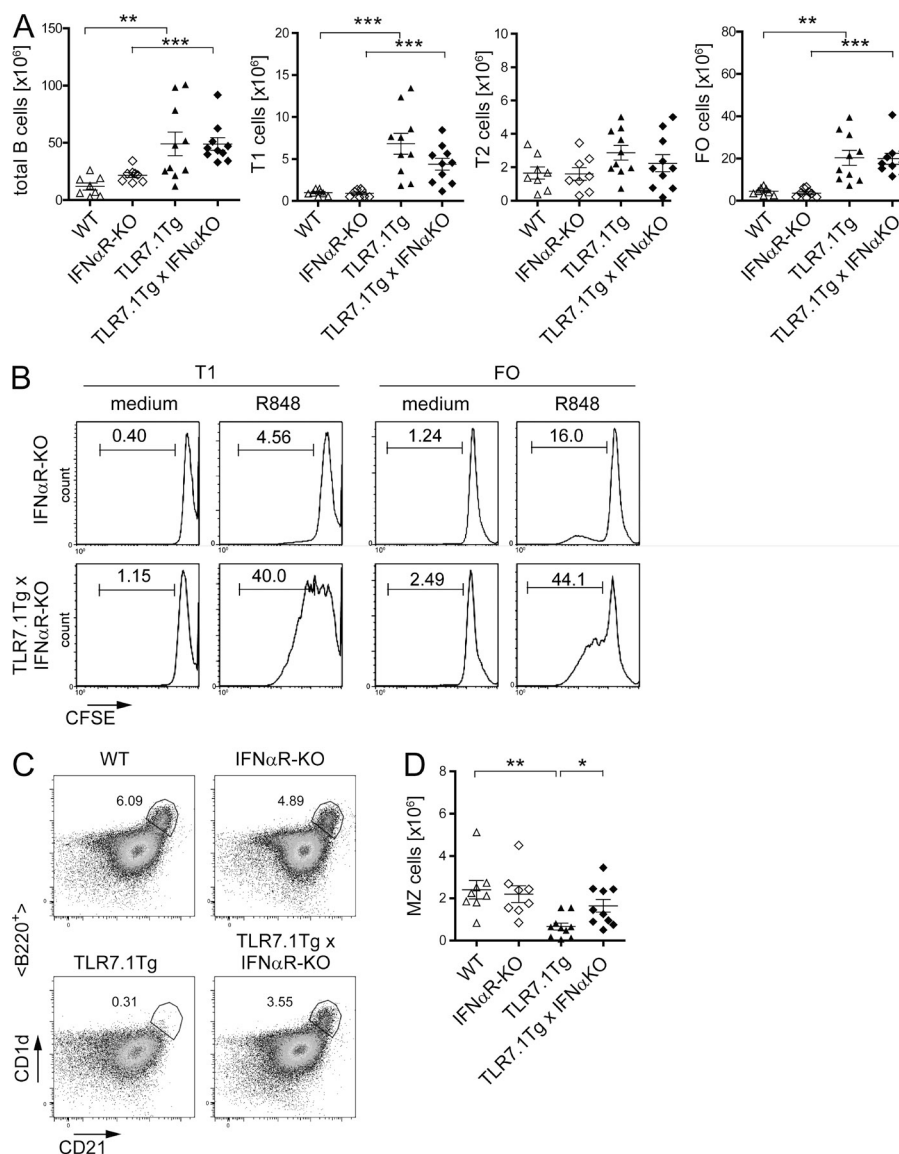


Figure 6. Type I IFN signaling is not required for the expansion of T1 and FO B cells but is required for depletion of the MZ B cell population in TLR7.1Tg mice. (A) Spleens from 3–6-mo-old WT, IFN α R-KO, TLR7.1Tg, and TLR7.1Tg \times IFN α R-KO mice were analyzed by flow cytometry to assess the frequencies of total B cells, T1, T2, and FO B cells. Cell numbers were calculated based on the total cell number per spleen. Each dot represents an individual animal with the mean indicated (horizontal bars). (B) Purified T1 cells or FO B cells from IFN α R-KO and TLR7.1Tg \times IFN α R-KO mice were loaded with CFSE and cultured for 72 h in RPMI medium or stimulated with 50 ng/ml R848. Histograms show the percentage of proliferating (CFSE^{lo}) cells after the indicated treatments. (C and D) MZ B cells populations in WT, IFN α R-KO, TLR7.1Tg, and TLR7.1Tg \times IFN α R-KO mice were analyzed by flow cytometry. (C) Representative flow plots showing the frequencies of MZ B cells, defined as B220⁺CD21^{bright}CD1d^{bright} cells. (D) Summary of the flow cytometric data showing the number of MZ B cells in mice of the indicated genotypes. Data in A, C, and D are the summary of three independent experiments, and data presented in B are representative of two independent experiments. In A and D, each dot represents an individual animal with the mean indicated for each group (horizontal bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as determined by one-way ANOVA with Tukey's post-test.

Regulation of B cell subsets by TLR7-driven type I IFN

The data above revealed that the activation and expansion of T1 B cells is due to cell-intrinsic expression of TLR7 and is driven by endogenous RNA. This activation of T1 cells results in cell proliferation and production of class-switched Abs. Previous studies in mice have postulated the requirement of IFN receptor (IFN α R) for B cell responsiveness to TLR

agonists (Green et al., 2009). To test the potential role of type I IFN signaling in the activation of T1 cells in the TLR7.1Tg mice, we crossed the TLR7.1Tg mice to mice deficient in type I IFN α R, thus generating TLR7.1Tg \times IFN α R-KO mice. 3–6-mo-old TLR7.1Tg \times IFN α R-KO mice had significantly reduced splenomegaly compared with TLR7.1Tg mice; however, the weights of their spleens remained significantly

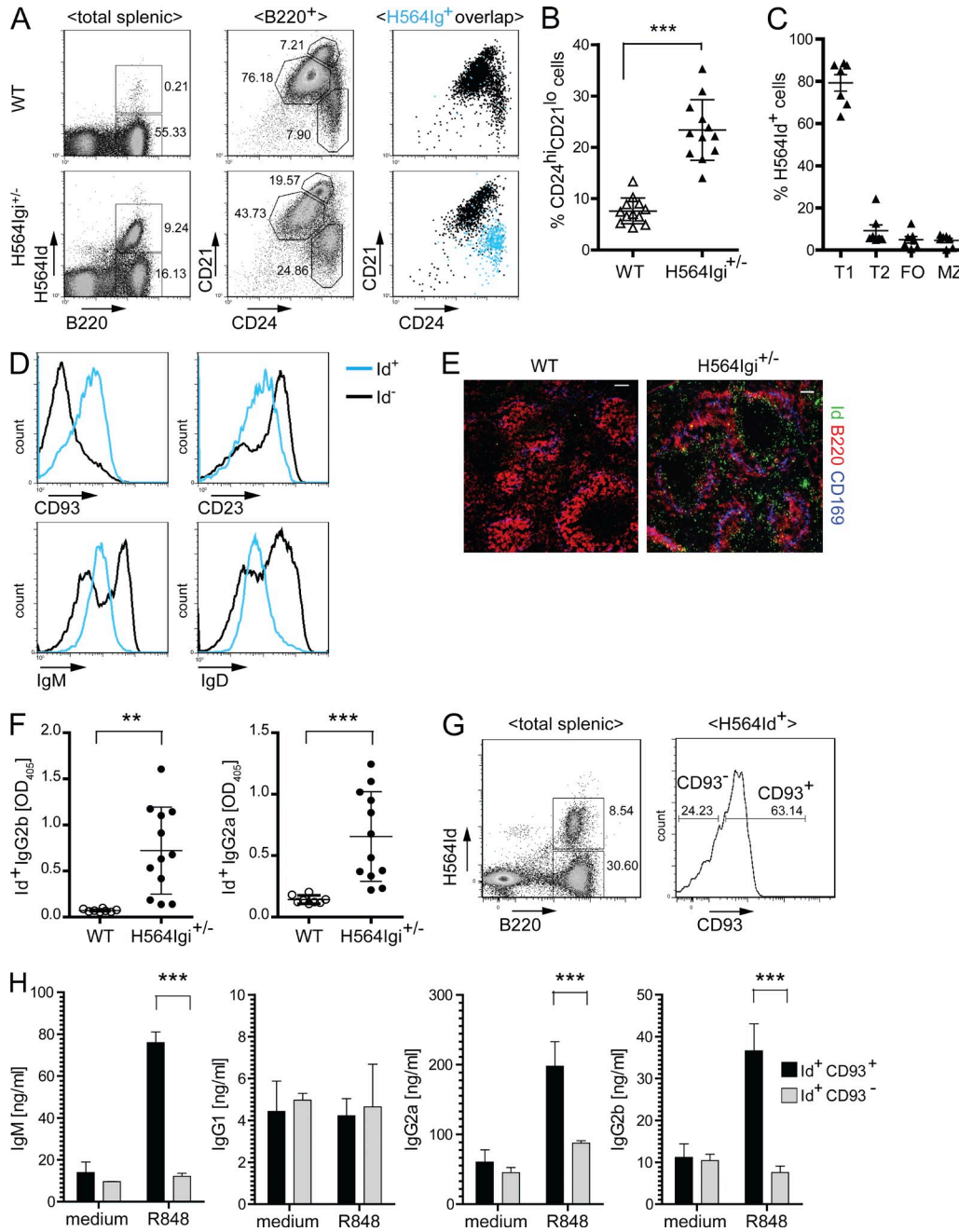


Figure 7. Anti-RNA-specific B cells in H564i Tg mice display an immature phenotype and produce class-switched IgG Abs. (A–D) Splenocytes from 6-mo-old WT and H564Igi^{+/-} mice were analyzed by flow cytometry to assess the frequencies and phenotype of H564Id⁺ B cells. (A) Representative flow plots show staining with anti-H564 Id Ab, used to define Id⁺ cells (right). The middle shows the frequencies of T1, T2, FO, and MZ B cell subsets, as determined by anti-CD24 and anti-CD21 staining. The right shows overlap between Id⁻ and Id⁺ cells (shown in blue). (B) Summarized data from individual WT and H564Igi^{+/-} mice showing the frequencies of CD24^{hi}CD21^{lo} cells of gated B220⁺ cells. (C) Data show the distribution of Id⁺ cells within individual B cell populations in H564Igi^{+/-} mice. (D) Histograms show the expression of CD93, CD23, IgM, and IgD on gated Id⁺ (blue) and Id⁻ (black) B cells in H564Igi^{+/-} mice. (A–D) Data presented are from four independent experiments. In B and C, each dot represents an individual animal with the mean indicated for each group (horizontal bars). (E) Splenic sections were prepared from frozen spleens of WT and H564Igi^{+/-} mice and stained with B220 (red), CD169 (blue), and H564Id (green). Data are representative of more than six sections analyzed from two mice per genotype. Bars, 100 μ m. (F) Serum levels of Id IgG2a and IgG2b in individual WT and H564Igi^{+/-} mice (6–12 mo old) were determined by ELISA. Sera dilutions: 1:15,000 (IgG2a) and 1:3,000 (IgG2b). Each dot represents an individual animal with the mean indicated for each group (horizontal bars). (G and H) Purified Id⁺CD93^{hi} and Id⁺CD93^{lo} cells from H564Igi^{+/-} mice were cultured for 72 h in RPMI medium or stimulated with 50 ng/ml R848, and the production of antibodies in culture supernatants was measured by ELISA. (G) Gating strategy used for purification of Id⁺CD93^{hi} and Id⁺CD93^{lo} cells. (H) Bar graphs show the mean titers of IgM, IgG1, IgG2a, and IgG2b in culture supernatants produced by Id⁺CD93^{hi} and Id⁺CD93^{lo} cells. Data are representative of two independent experiments using

higher than those of WT or IFN α R-KO control mice (Buechler et al., 2013). The reduced splenomegaly in TLR7.1Tg \times IFN α R-KO mice correlated with a decrease in the number of myeloid cells (Buechler et al., 2013); however, IFN α R deficiency had no significant effect on the expansion of splenic B cells driven by TLR7 overexpression (Fig. 6 A). Consistent with the lack of reduction in B cell numbers, TLR7.1Tg \times IFN α R-KO mice showed only a slight but not statistically significant decrease in the numbers of T1, T2, and FO B cells (Fig. 6 A). There was also no significant decrease in the number of plasma cells in TLR7.1Tg \times IFN α R-KO mice compared with TLR7.1Tg mice (unpublished data). Importantly, T1 and FO B cell isolated from TLR7.1Tg \times IFN α R-KO mice proliferated at significantly higher rates in response to R848 stimulation, compared with their IFN α R-KO counterparts (Fig. 6 B).

In spite of the lack of an effect on the numbers of T1 and FO B cell subsets, the absence of IFN α R led to a significant increase in the percentage (four- to fivefold) and the number (two-fold) of MZ (B220⁺CD21^{hi}CD1d^{hi}) B cells in TLR7.1Tg \times IFN α R-KO mice, compared with the TLR7.1Tg mice (Fig. 6, C and D). Thus, IFN α R deficiency did not abolish the effects of TLR7 overexpression on the expansion of T1, FO, and plasma cells; intact IFN α R signals, however, were required for TLR7-mediated loss of MZ B cells.

Anti-RNA-specific B cells in H564i Tg mice display an immature phenotype and produce class-switched IgG Abs

The discovery that TLR7 Tg mice have increased spontaneous proliferation and expansion of T1 B cells in vivo suggested that TLR7 overexpression promoted the activation of a portion of T1 cells (most likely specific for RNA), leading to the production of class-switched Abs. To test whether B cells with specificity for RNA can be stimulated to proliferate and produce Ab at the T1 stage, we used 564Igi^{+/-} knockin mice, which develop an SLE-like disease through a TLR7-dependent process and in which a high proportion of B cells (5–30%) express BCRs specific for ssRNA (Berland et al., 2006). Flow cytometric analyses of spleen cells from 564Igi^{+/-} mice revealed a marked increase in the percentage of B220⁺CD24^{hi}CD21^{lo} T1 B cells, which predominantly consisted of 564Id⁺ B cells (Fig. 7, A–C). Further phenotypic analysis showed that Id⁺ B cells expressed high levels of CD93, low levels of IgD, and low-to-intermediate levels of CD23. As previously reported by Berland et al. (2006), these cells showed relatively low expression of surface IgM (Fig. 7 D). Also consistent with previous reports, we found that H564 Id⁺ cells were excluded from the B cell follicles and MZs and occupy in two major locations: the splenic RP and at the T–B cell borders (Fig. 7 E). Despite the seemingly immature phenotype of Id⁺ cells, 564Igi^{+/-} mice spontaneously developed class-switched Id⁺ Ab (Fig. 7 F; Berland et al., 2006), suggesting

that Id⁺ cells can become activated and differentiate into Ab-producing cells.

To determine whether CD93⁺H564Id⁺ cells can be stimulated to produce Abs in vitro and to compare them with more mature Id⁺ cells, we purified Id⁺CD93^{hi} and Id⁺CD93^{lo} cells (Fig. 7 G), stimulated them with a TLR7 agonist (R848), and then measured antibody production in culture supernatants. The Id⁺CD93^{hi} cells produced significantly higher titer IgG2a and IgG2b Abs compared with Id⁺CD93^{lo} cells (Fig. 7 H). We concluded that the majority of anti-RNA-specific cells in H564Igi mice express a transitional B cell phenotype and are capable of producing class-switched Abs in response to TLR7 stimulation.

DISCUSSION

Although TLR7 is known to play a pivotal role in autoantibody production in murine lupus (Berland et al., 2006; Christensen et al., 2006; Pisitkun et al., 2006; Santiago-Raber et al., 2010b), where and how TLR7 regulates the development of autoreactive B cells in the periphery is not clear. In this study, we found that increased expression of TLR7 led to a cell-intrinsic expansion of T1 B cells, which was driven by RNA in the RP of the spleen. Furthermore, the in vitro production of class-switched IgG antibodies by T1 B cells suggests that they may be important source of anti-RNA autoantibodies.

The discovery that TLR7.1Tg mice have increased spontaneous proliferation and expansion of T1 B cells in vivo suggested that TLR7 overexpression promoted the activation of a portion of T1 cells, which carry BCRs specific for endogenous Ags, most likely ssRNA or Ags associated with ssRNA. This hypothesis is strongly supported by the fact that the removal of RNA-containing material by RNase overexpression significantly limited both the expansion and proliferation of T1 cells. BM chimera experiments revealed that the increased proliferation of T1 cells was cell-intrinsic and the failure of HEL-specific T1 B cells from TLR7.1Tg \times HEL mice to show increased proliferation demonstrates that the results cannot be explained by TLR7 overexpression alone. In addition, even though the TLR7 Tg mice carry a high copy number of TLR7, the fact that Unc93B1 D34A mutant mice phenocopy the TLR7 Tg mice (Fukui et al., 2011) suggest that it is an altered signaling pathway leading to an enhanced response to TLR7 agonists rather than the copy number per se that conveys the immunological abnormalities observed in these two mouse strains.

It was previously shown that the total population of TLR7 Tg splenic B cells was more responsive to TLR7 ligand stimulation in vitro (Deane et al., 2007). We found that the T1 subset in particular proliferated at much higher rates than WT T1 cells, consistent with our in vivo data. Furthermore, stimulation with SmRNP, a natural RNA-containing ligand, also promoted the proliferation of a small but detectable

four individual mice and presented as mean \pm SD. Error bars indicate variation between the individual mice. **, P < 0.01; ***, P < 0.001, as determined by two-tailed, unpaired Student's *t* test or one-way ANOVA with Tukey's post-test, where appropriate.

portion of TLR7.1Tg T1 B cells, strongly suggesting that cells with anti-RNA specificity are being activated. Since neither BAFF nor other survival factors were added to the cultures, TLR7 ligation alone promoted a significant expansion of T1 B cells. This suggests that TLR7 overexpression may promote the survival and proliferation of a limited population of cells, most probably those with low to moderate specificity for RNA. Surprisingly, BCR ligation with anti-IgM alone also stimulated greater survival and proliferation of TLR7.1Tg compared with WT T1 B cells. We speculate that the small proportion of responding cells may have already received a TLR7 signal through RNA-reactive BCR in vivo and that anti-IgM acts as a second signal for these cells. Whereas strong BCR ligation induces cell death and is thought to provide a tolerance checkpoint for T1 B cells (King and Monroe, 2000), it is remarkable to note that when activated through TLR7, anti-IgM induces striking proliferation of WT T1 B cells, further supporting a role for TLR7 in imparting an anti-death/survival signal. The mechanisms by which B cells come in contact with and respond to RNP- and deoxyribonucleoprotein (DNP)-containing antigens after exit from the BM are still poorly understood. We found that the expanded T1 cells in the TLR7.1Tg mice were located predominantly in the splenic RP, as were most of the proliferating (BrdU⁺) cells. These findings suggest that the splenic RP represents a primary site for the activation of anti-RNA-specific B cells in the periphery. Despite the expansion of T1 B cells, they were able to undergo apoptosis, and the increased cell death we observed in TLR7.1Tg T1 B cells may have contributed to the antigenic load stimulating other autoreactive B cells. A likely possibility is that B cells directly bind to RNP-containing antigens exposed on the surface of apoptotic cells (Hall et al., 2004; Radic et al., 2004). Alternatively or in addition, B cells may bind RNP or DNP antigens presented on the surface of other cells such as neutrophils, DCs, or macrophages (Qi et al., 2006; Cyster, 2010; Hakkim et al., 2010; Puga et al., 2012). Histological analysis showed a significant accumulation of T1-like (CD93^{bright}IgD⁻CD138⁻) cells within the splenic RP, a location which would allow interactions with RP macrophages and DCs. The activation of autoreactive T1 B cells in TLR7.1Tg mice may well be driven by these cells, as DCs can directly present Ag to B cells through a recycling pathway following Ag internalization (Bergtold et al., 2005; Qi et al., 2006; Chappell et al., 2012). Recently, Joo et al. (2012) have demonstrated that DCs from SLE patients can efficiently stimulate naive and memory B cells to produce IgG and IgA antibodies, suggesting a direct interplay between myeloid DC and B cells.

Despite their seemingly immature phenotype, recent studies showed that T1 B cells have the potential to become activated and to mount efficient humoral immune responses (Mao et al., 2004; Han et al., 2007; Ueda et al., 2007; Aranburu et al., 2010). Ueda et al. (2007) found that mouse splenic T1 B cells proliferate rapidly in response to TLR ligands, such as CpG DNA (TLR9 ligand) and LPS (TLR4 ligand), and produce IgM and IgG Abs. Notably, the high levels of AID in T1

B cells was associated with class-switch recombination and production of high affinity IgG Abs, which occurred independently of T cell help (Han et al., 2007; Ueda et al., 2007). Human T1-like immature B cells also expand and proliferate in response to bacterial CpG DNA, and these so-called immature B cells acquired a phenotype of IgM⁺ memory B cells, which carry somatic hypermutations; the somatic mutations were mainly in VH1 and VH4/6 genes, which encode for anti-CpG DNA specificity (Aranburu et al., 2010), suggesting possible “cooperation” between the BCR and TLR9 signaling. Recent studies have also reported an increased proliferation of immature (T1 and T2) dual- κ B cells in MRL/*lpr* mice, which might contribute to the increased frequency of autoreactive B cells in these mice (Fournier et al., 2012).

Here, we found that TLR7 stimulation caused very little production of class-switched IgG by WT T1 B cells. In marked contrast, TLR7 stimulation of TLR7.1Tg T1 B cells led to the production of high concentrations of class-switched IgG Abs. Although AID was only modestly increased in TLR7.1Tg T1 B cells compared with WT, the transcription factor T-bet was expressed at significantly higher levels in TLR7 Tg T1 B cells, suggesting that increased expression of this transcription factor may contribute to increased class switching in T1 B cells in TLR7.1Tg mice. Although our in vivo and in vitro findings support a role for class-switched antibody production by T1 B cells in the splenic RP, we cannot rule out the possibility the IgG2b⁺ and IgG2c⁺ cells found in the RP of TLR7.1Tg mice have developed as a result of GC formation, especially as T cell-dependent generation of GCs and plasmablasts in TLR7 Tg mice has recently been demonstrated (Walsh et al., 2012). Both T1 B cells and FO B cell populations in TLR7.1Tg had increased frequencies of CD44^{hi}CD138⁺ cells, supporting a model in which both immature and mature B cell populations contribute to the generation of plasma cells in vivo. However, surprisingly, sorted TLR7.1Tg T1 cells produced significantly higher levels of IgG Abs after TLR7 stimulation as compared with FO B cells. This difference might be due to the fact that TLR7.1Tg T1 B cells, unlike TLR7.1 Tg FO B cells, already express AID and high levels of T-bet so that their activation may not require additional T cell help.

Murine B cells lacking type I IFN receptor (IFN α R-KO) respond only weakly to TLR7 ligands, most likely explained by the requirement for type I IFN signaling to up-regulate TLR7 expression in B cells and for B cells to respond to TLR7 agonists (Bekeredjian-Ding et al., 2005; Thibault et al., 2008; Green et al., 2009). Our results indicate that TLR7 overexpression could compensate for the lack of a type I IFN signal in that TLR7.1 \times IFN α R-KO mice maintained T1 and FO B cell expansion, T1 B cell activation, and the accumulation of plasma cells. In addition, in TLR7.1 \times IFN α R-KO mice, we observed the restoration of MZs and MZ B cell numbers, indicating that the TLR7-mediated loss of MZ B cells requires type I IFN signals. This finding is consistent with previous studies showing that type 1 IFN signals can regulate the MZ B cell population either by promoting MZ B cell egress into the follicles or by influencing their survival

(Rubtsov et al., 2008; Wang et al., 2010). Because the overexpression of RNase in TLR7.1 \times RNase dTg mice limited the expansion and proliferation of T1 cells and the accumulation of plasma cells but did not restore the MZ B cell population, it appears that the T1 and MZ B cell subsets are regulated differently in TLR7.1Tg mice.

Extra-FO immune responses have been implicated in the activation of autoreactive B cells through TLRs (Christensen and Shlomchik, 2007). The mechanisms that regulate these responses remain unknown. Whereas the T1 stage of B cell maturation may be an important checkpoint for autoreactive B cell deletion (Meffre and Wardemann, 2008), our results demonstrate that when TLR7 responsiveness is increased, RNA-containing Ags can promote the activation (rather than deletion) of T1 B cells within the splenic RP, leading to the production of class-switched Abs. Human and mouse studies have implicated that alteration of TLR7 and/or IRF7 expression can be a key factor in the development of pathogenic anti-RNA Ab in SLE (Subramanian et al., 2006; Deane et al., 2007; Salloum et al., 2010; Shen et al., 2010; Kawasaki et al., 2011). An increased frequency of immature T1/T2-like cells has previously been described in patients with autoimmune diseases, including RA, SLE, and Sjogren's Syndrome (Daridon et al., 2006; Landolt-Marticorena et al., 2011; Vossenkämper et al., 2012), suggesting that these cells might represent an important source of autoantibodies. Importantly, the immature B cell compartment in both humans and mice has been shown to contain increased frequencies of polyreactive BCR specificities (Yurasov et al., 2005; Andrews et al., 2013), suggesting that if not properly regulated, B cells derived from this compartment have the potential to produce auto-Abs.

In a second model of lupus-like disease associated with anti-RNA autoantibodies, H564Igi^{-/+} mice, the majority of H564Id⁺ B cells exhibited a classic immature B cell phenotype, similar to that of T1 and T2 B cells, and, as previously described by Berland et al. (2006), they were largely excluded from the B cell follicles and localize within the splenic RP or at the T-B interface of the follicles. Thus, these Id⁺ cells might be competing with nonautoreactive B cells and may become developmentally arrested at an immature stage, similar to what has been described for anti-dsDNA-specific B cells (Mandik-Nayak et al., 1997). Despite their immature phenotype, Berland et al. (2006) found that H564Igi^{-/+} mice spontaneously produced high titers of class-switched Id Abs, indicating that Id⁺ B cells can become activated and produce Abs in vivo. Because very few Id⁺ B cells develop into FO B cells and H564Idi^{-/+} mice did not develop Id⁺ GCs, the activation of H564 Id⁺ B cells and generation of autoantibodies most probably occurs outside of the follicles. Consistent with this idea, our in vitro data showed that CD93⁺H564Id⁺ B cells produced higher titers of class-switched Abs upon TLR7 ligand stimulation when compared with CD93⁻H564Id⁺ cells, suggesting again that in a physiological setting, immature B cells might be a major source of Id Ab. It remains possible that some Id⁺ B cells from the FO B cell compartment contribute to auto-Ab production in vivo. Based on our results, it might

be worth testing if dysregulated expression of TLR7 can bypass normal requirements for T1 cell differentiation and promote activation and Ab production by B cells arrested at the T1 stage caused by deficiency of BAFF, BAFFR, or SPPL2A (signal peptide peptidase-like 2A; Mackay and Schneider, 2009; Beisner et al., 2013; Bergmann et al., 2013; Schneppenheim et al., 2013).

The overexpression of TLR7 resulted in increased activation of T1 cells in the RP, associated with production of class-switched Abs. Removal of RNA-containing material in TLR7.1 \times RNase dTg mice significantly limited the expansion and proliferation of T1 cells, suggesting that endogenous RNA-containing Ags drive their activation.

Enhanced TLR7 responses occur in mice that overexpress TLR7 or have mutations in the TLR chaperone Unc93b (Fukui et al., 2009, 2011), as well as in SLE patients with genetic predisposition (García-Ortiz et al., 2010; Shen et al., 2010; Kawasaki et al., 2011; Lee et al., 2012; Tian et al., 2012) or environmental exposure (e.g., altered response to chronic virus infection; Poole et al., 2009). Our data on T1 B cells that overexpress TLR7 suggest that dysregulation of TLR expression and function in T1 B cells may change their response to Ag, which normally induces T1 B cells to undergo cell death (King and Monroe, 2000; Su et al., 2004). Autoreactive T1 cells with altered TLR7 expression or downstream signaling may respond to both RNA and RNA-associated Ags, resulting in the production of IgG auto-Abs. The IgG auto-Abs produced may not initially be pathogenic but, after T cell help either in the GC (Walsh et al., 2012) or in an extra-FO site, could facilitate the subsequent development of high-affinity pathogenic auto-Abs. Thus, activation of autoreactive T1 B cells may play a key role in the initial development of autoantibodies in SLE.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River or The Jackson Laboratory. TLR7.1Tg mice were provided by S. Bolland (National Institutes of Health, Bethesda, MD; Deane et al., 2007); IFN α R-KO mice (Müller et al., 1994) were a gift from D. Stetson (University of Washington, Seattle, WA); TLR7.1Tg \times IFN α R-KO mice were generated by crossing TLR7.1Tg mice to IFN α R-KO mice; RNase Tg mice were generated by expressing bovine RNase under the human albumin promoter (Sun et al., 2013); TLR7.1 \times RNase dTg mice were generated by crossing TLR7.1Tg mice to RNase Tg mice; VDJ9 κ 5 IgH and IgL (HEL-Tg) mice were obtained from J. Cyster (University of San Francisco, San Francisco, CA); and TLR7.1 \times HEL-Tg mice were generated by TLR7.1Tg mice to HEL-Tg mice. H564Igi mice were described previously (Berland et al., 2006). Mice used in the experiments were between 2 and 6 mo, except for H564Igi mice, which were used at 6–12 mo of age. All mice used were maintained under specific pathogen-free conditions at the University of Washington. All animal procedures were approved by the Institute for Animal Care and Use Committee at the University of Washington.

Mixed BM chimeras. Mixed BM chimeric mice were generated by lethally irradiating (1,000 rads) F1 progeny of B6 and B6.SJL mice and reconstituting them with a 1:1 ratio of B6 (Ly5.1) and B6.SJL (Ly5.2) or TLR7.1 (Ly5.2) and B6.SJL (Ly5.1) whole BM cells. Mixed BM chimeric mice were reconstituted for 2–4 mo before analysis.

Antibodies and flow cytometry. For analysis of splenic B cell, RBC-lysed single cell suspensions were stained with the fluorescently labeled mAbs listed in Table S2. Multicolor flow cytometry was performed by staining the

cells with appropriate combinations of mAbs. For BrdU analysis, mice were injected with 1 mg BrdU 1 h before sacrificing. Splenocytes were surface stained with mAbs and BrdU signal was detected using the FITC BrdU Flow kit according to the manufacturer's instructions (BD). HEL-specific B cells that express VDJ9 κ 5 IgH and IgL (HEL⁺) B cells were identified using biotinylated HEL. H564Id⁺ cells were identified using Alexa Fluor 647, biotin, or FITC-conjugated anti-H564 Ab. All data were collected using an LSRII or FACScan flow cytometer (BD) and analyzed with FlowJo software (Tree Star).

Cell sorting. For RT-PCR analyses, single cell suspensions from individual mice were RBC-lysed and stained with anti-B220, anti-IgM (eBioscience), anti-CD24, anti-CD21 (BD), and anti-CD23 (Invitrogen). A minimum of 200,000 cells was sorted from each subset. Cells were washed with cold PBS and cell pellets were kept at -80°C until use. For in vitro cell culturing experiments, spleens from two to four mice per group were used. Single cell suspensions were prepared under sterile conditions and cells were stained with anti-B220, anti-CD24, and anti-CD21. Post-sort analyses were performed to assess the purity of sorted cells. H564 Id⁺ cells from H564Igi^{+/-} mice were stained with anti-B220, anti-H564 Id Ab, and anti-CD93 Abs. All cell sorts were performed using a FACSAria II high-speed cell sorter (BD) at 4°C under sterile conditions.

In vitro cell culturing and CFSE proliferation assay. B cell subsets were sorted as described above and loaded with 2.5 μM CFSE in PBS at 37°C for 10 min, quenched with RPMI 1640 containing 10% FBS (Thermo Fisher Scientific), and washed extensively. Cells were resuspended in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 55 mM 2-ME, and 10 mM HEPES, placed in 96-well plates at a concentration of 0.2×10^6 cells/well, and treated with different stimuli. Reagents used for in vitro stimulation were F(ab')₂ fragment goat anti-mouse μ -chain (Jackson ImmunoResearch Laboratories, Inc.), R848 (InvivoGen), and SmRNP (RNP/Sm; Arotec Diagnostics Limited). Cells were cultured at 37°C and 5% CO₂ for 72 h, harvested, and analyzed for CFSE dilution by flow cytometry. Supernatants from each well were frozen and used to assess the production of antibodies.

Immunohistochemistry. Spleens from C57BL/6, TLR7.1Tg, and H564Igi^{+/-} mice were embedded in OCT medium and frozen at -80°C . 6–8 μm sections were fixed to glass slides and kept at -80°C until use. For primary staining, sections were fixed in ice-cold acetone for 5 min, air-dried, rehydrated in PBS for 5 min, and stained at room temperature with combinations of CD93-FITC, IgD-Biotin, IgD-FITC, B220-eFluor450, CD138-PE (all from eBioscience), CD169-FITC (AbD Serotec), anti-H564-Id-biotin, and IgG2c-biotin or IgG2b-biotin (Jackson ImmunoResearch Laboratories) for 90 min in PBST (PBS containing 0.05% Tween-20). Biotinylated reagents against surface markers were detected by incubation with streptavidin-Alexa Fluor 555 (Invitrogen) or streptavidin-Pacific blue (eBioscience) for 60 min at room temperature in PBST.

For BrdU detection, acetone-fixed slides labeled with IgD-FITC were incubated in Retrieval A solution (BD) at 89°C for 10 min and allowed to cool at room temperature for 20 min before staining with BrdU-biotin (BD). BrdU-biotin was detected using Cyanine3 TSA amplification (PerkinElmer) according to manufacturer's instructions. Sections were washed in PBS and mounted in VectaShield HardSet mounting medium (Vector Laboratories). Images were collected on a confocal microscope (LSM 510 META; Carl Zeiss) with LSM 510 (v 4.2) software using 10 or 20 \times objectives at room temperature. Images were processed using ImageJ (National Institutes of Health) and Photoshop (Adobe) software. The anti-nuclear antibody (ANA) test was performed using HEP-2 antigen substrate slides (MBL Bion). Slides were incubated for 2 h with undiluted culture supernatants, washed, and incubated for 1 h with biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), followed by 1 h incubation with streptavidin-FITC (BioLegend). All incubations were performed at room

temperature. Slides were washed and mounted in mounting medium. Images were captured on a microscope (Inverted DMIRB; Leica) with MetaMorph software using 20 \times magnifications.

RNA preparation and quantitative real-time PCR. Total RNA was isolated from sorted cells using the RNeasy mini kit with on-column DNase treatment (QIAGEN). First-strand cDNA was generated using 25 ng of total RNA with the high-capacity cDNA RT-kit using random primers (Applied Biosystems). cDNA was diluted to an equivalent of 0.1 ng/ μl total RNA and 8 μl were used per reaction. Primers, as indicated in Table S1, were synthesized (IDT) and diluted to the appropriate concentrations for qPCR using molecular grade water. BLAST results of the primers showed specific sequence homology only to the gene of interest. Reactions in duplicate (20 μl) were run on an ABI StepOne Plus system using a 1:1 mix of template and primer to Absolute Blue SYBR green ROX master mix (Thermo Fisher Scientific), using a two-stage cycle of 95°C for 15 s and 60°C for 1 min repeated for 40 cycles, followed by a dissociation stage. Threshold cycle (Ct) values were determined by setting a constant threshold at 0.2, and fold changes in gene expression were then calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001) and relative expression method (Pfaffl et al., 2002).

ELISA. ELISA plates were precoated with 10 $\mu\text{g}/\text{ml}$ goat anti-mouse IgM F(ab')₂ or goat anti-mouse IgG (H+L) mAbs (Jackson ImmunoResearch Laboratories). After washing, plates were blocked with 4% nonfat dry milk in PBS-Tween. Culture supernatant were added in serial dilutions and incubated for 2–4 h at room temperature. Plates were washed and bound Abs were detected using isotype-specific HRP conjugates of anti-IgG1 and anti-IgG2b (ICL); and anti-IgM, anti-IgG2a, and anti-IgG2c (SouthernBiotech). HRP activity was visualized using tetramethylbenzidine peroxidase (Bio-Rad Laboratories). Standard curves were generated using IgM (Jackson ImmunoResearch Laboratories, Inc.), IgG2c, IgG1, IgG2a, and IgG2b (SouthernBiotech) standards and used to calculate the absolute quantities of different antibodies. For detection of anti-RNA Abs, ELISA plates were treated with Poly-L-Lysine (Sigma-Aldrich), coated with 10 $\mu\text{g}/\text{ml}$ yeast RNA (Sigma-Aldrich) diluted in PBS and blocked with 2% BSA (Sigma-Aldrich) in PBS. Culture supernatant were added in serial dilutions and incubated overnight at 4°C . Plates were washed, and bound Abs were detected using biotin-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) and streptavidin-AP (Invitrogen). For measurement of 564Igi-Id⁺ antibodies, ELISA plates were coated with 5 $\mu\text{g}/\text{well}$ of purified mAb B6-256 anti-Id. Bound serum antibody was detected with alkaline phosphatase (AP)-conjugated isotype-specific goat anti-mouse (SouthernBiotech). AP activity was visualized using pNPP-phosphatase substrate (Sigma-Aldrich).

Statistical analyses. Statistical significance between groups was determined by two-tailed, unpaired Student's *t* test or by one-way ANOVA with Turkey post-test. Graphs and statistical analyses were performed using Prism 5.0 software (GraphPad Software). Values are reported as mean \pm SD or \pm SEM.

Online supplemental material. Table S1 shows primer sequences used for qPCR analyses. Table S2 shows antibodies and clones used. Fig. S1 shows gating strategy used for sorting of T1 and FO B populations and post-sort analyses. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122798/DC1>.

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