Dual signaling by innate and adaptive immune receptors is required for TLR7-induced B-cell–mediated autoimmunity

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***Toll-like receptor 7* (*Tlr7*) has been linked to systemic lupus disease incidence in humans and mice, but how TLR7 potentiates autoimmunity is unclear. We used a *Tlr7* transgenic (tg) mouse model to investigate the cellular and molecular events required to induce spontaneous autoimmunity through increased TLR7 activity. We determined that *Tlr7* exerts B-cell–intrinsic effects in promoting spontaneous germinal center (GC) and plasmablast B-cell development, and that these B-cell subsets are dependent on T-cell–derived signals through CD40L and SLAM-associated protein (SAP), but not IL-17. Antigen specificity also factored into TLR7-induced disease, as both a restricted T cell receptor (TCR) specificity and MHC haplotype H2$^{k/k}$ protected *Tlr7*tg mice from spontaneous lymphocyte activation and autoantibody production. Inflammatory myeloid cell expansion and autoimmunity did not develop in *Tlr7*tgIgH$^{-/-}$ mice, suggesting either that spontaneous TLR7 activation does not occur in dendritic cells, or, if it does occur, cannot drive these events in the absence of B-cell aid. These data indicate that autoimmune disease in *Tlr7*tg mice is contingent upon B cells receiving stimulation both through innate pathways and T-cell–derived signals and suggest a codependent relationship between B cells and T cells in the development of autoimmunity.**

inflammation | SLE | T follicular helper cells

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease with multiple causal factors that induce breakdown in immune tolerance and promote autoimmunity development. It is increasingly apparent that innate pathways, such as Toll-like receptor (TLR) signaling, contribute to spontaneous autoimmunity (1). TLR7 is an intracellular receptor that is expressed in B cells and plasmacytoid dendritic cells (pDCs) and recognizes pathogen-associated molecular patterns in the context of single-stranded viral RNA (2). *Tlr7* gene duplication in autoimmune-prone mice potentiates disease incidence and accelerates lupus-like disease development, implicating *Tlr7* as a probable susceptibility gene in the generation of autoimmune responses (3, 4). Expression of multiple copies of *Tlr7* in an autoimmune-resistant mouse strain also results in spontaneous inflammation and autoimmunity (5), whereas deletion of this gene promotes disease resistance in autoimmune-prone strains (6). Importantly, peripheral blood mononuclear cells from SLE patients display higher levels of *Tlr7* expression compared with healthy controls, and a genetic study detected *Tlr7* gene duplication in a subset of lupus patients (7, 8). These clinical data highlight the necessity of determining the underlying contribution of TLR7 activation to cellular interactions and signals in SLE development.

Multiple potential mechanisms exist by which TLR7 could exert disease-promoting influence through either pDC- or B-cell–intrinsic signals. TLR7-activated pDCs produce large amounts of IFN-α, a cytokine known to have pathologic consequences in autoimmunity, and could drive disease in *Tlr7* transgenic (tg) mice (1). However, extensive B-cell and T-cell activation and autoantibody production in *Tlr7*tg mice suggest a putative role for B cells and maybe for T cells in disease development, possibly through a mechanism involving GCs.

During a conventional adaptive immune response against an invading pathogen, GCs form under the impetus of coordinated interactions between B- and T cells. A specialized subset of CD4$^+$ CXCR5$^+$ T cells called T follicular helper cells (T_{FH}) supports B-cell differentiation in the GC (9). T_{FH} cells provide essential costimulatory signals through CD40L and signaling lymphocytic activation molecule (SLAM) family receptors that promote B-cell survival and class switch recombination (CSR) to IgG antibody isotypes (9). However, conflicting evidence exists as to whether these interactions are vital to the development of an autoimmune response, or whether B cells activated through innate pathways are able to cause autoimmunity independently of T-cell help (10–12).

In some cases of particular relevance to our *Tlr7*tg system, lupus-like disease occurs in the absence of T-cell–derived signals. A mouse strain tg for B cell activating factor (BAFF), a survival molecule and growth factor for B cells triggered by TLR7 activation, develops activated B cells and autoimmunity independently of T cells (13). Activation, expansion, and differentiation of autoimmune rheumatoid factor-specific B cells from AM14tg mice on the Mrl-*lpr*/*lpr* background also occur in the absence of T cells, but are dependent on innate-derived TLR7 and TLR9 signals (14).

With these concerns in mind, in this study we examine the cellular requirements underlying the development of spontaneous autoimmunity in mice with excessive *Tlr7* gene expression. We found that excessive *Tlr7* preferentially expands GC B cells and plasmablasts, but that these events are dependent on T-cell–derived signals through CD40L and SAP. T-cell antigen specificity and the context of MHC presentation also played a role in the development of autoimmunity. Furthermore, inflammation did not develop in *Tlr7*tgIgH$^{-/-}$ mice, indicating that inflammatory myeloid cell expansion in *Tlr7*tg mice occurred downstream of B-cell–derived events. Together, these data suggest that B-cell signaling through innate and adaptive immune receptors works in conjunction with T-cell–B-cell interactions to induce autoimmunity.

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Results

Excess *Tlr7* Promotes Germinal Center B-Cell and Plasmablast Production in a B-Cell–Intrinsic Manner. Previous data determined that *Tlr7* gene multiplicity alone is able to induce autoimmune pathology in mice (5). *Tlr7* is most commonly expressed in B cells and plasmacytoid dendritic cells (pDCs), but it is unknown whether *Tlr7* exerts culpable influence in the development of autoimmunity in one or both of these cell types. To address this question, we generated mixed bone marrow (BM) chimeras composed of equal proportions of WT and *Tlr7*tg cells. We found that B cells and pDCs had equivalent total numbers of cells derived from WT and *Tlr7*tg origin (Fig. 1*A*). However, *Tlr7*tg B cells had increased cell surface MHC Class II expression, indicating a more activated phenotype compared with WT cells, whereas *Tlr7*tg and WT CD11c$^+$ cells expressed similar levels of this marker (Fig. 1*B*). We also found that GC and CD138$^+$ plasmablast B-cell subsets skewed preferentially to being of *Tlr7*tg origin (Fig. 1*A*). All other B-cell populations examined were equally likely to be derived from WT as *Tlr7*tg BM (Fig. 1*A*), indicating that *Tlr7* causes a B-cell–intrinsic effect by promoting GC B-cell and plasmablast development.

Because we found an increase in the number of GC B cells and plasmablasts in *Tlr7*tg mice, we next studied gene expression differences in *Tlr7*tg B cells compared with WT B cells. To do this, we performed microarray analysis on CD9$^-$CD43$^-$ follicular B cells from *Tlr7*tg and WT mice and found several striking changes, including up-regulation of type-1 IFN signature genes as well as antigen processing and presentation genes (Fig. 1*C*, Table S1). Interestingly, Ig genes comprised 54% of the total number of genes up-regulated more than twofold (Fig. 1*C*). Ig gene transcription precedes CSR or somatic hypermutation in activated B cells, so *Tlr7*tg B cells could have enhanced basal or postclass switch transcription of these genes. To investigate this, we analyzed transcription of both germline and class-switched Ig genes and found that *Tlr7*tg follicular B cells had increased transcription of IgG2b and IgG1, but not IgG2a genes, before and after CSR compared with WT B cells (Fig. 1*D*).

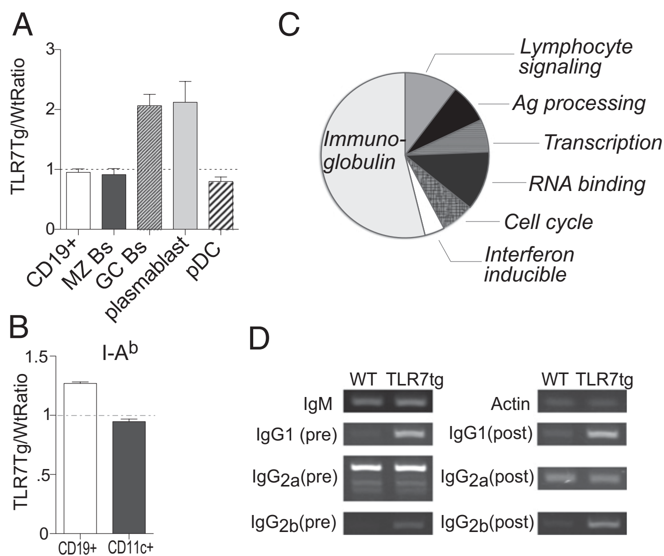

Fig. 1. *Tlr7* promotes the generation of germinal center and plasmablast B cells through B-cell–intrinsic mechanisms. (*A* and *B*) 50:50 mix of *Tlr7*tg and WT BM was injected into irradiated WT mice; eight weeks later splenic cell populations were examined by flow cytometry. (*A*) Number of *Tlr7*tg-derived cells for cell types was expressed as a ratio between *Tlr7*tg and WT cells. (*B*) The ratio of *Tlr7*tg to WT expression of I-A^b on B cells and CD11c+ cells. Dotted line indicates equal proportions of WT and *Tlr7*tg cells. $n = 3$ mice/group. (*C* and *D*) cDNA derived from follicular B cells of WT or *Tlr7*tg mice was analyzed by (*C*) microarray (pie chart segments represent percentages of gene categories that contain genes up-regulated more than twofold in at least three mice) or (*D*) RT-PCR for Ig transcripts of preisotype switched and postisotype switched Ig genes.

CD40L or SAP Deficiency Abrogates the Development of Spontaneous Germinal Centers and Autoimmunity in *Tlr7*tg Mice. Because TLR7 signaling selectively supported GC B-cell differentiation, we wanted to determine whether GC formation is integral to spontaneous autoimmunity in *Tlr7*tg mice. To do this, we analyzed *Tlr7*tg*Cd40lg*$^{-/y}$ mice for disease and found a drastic reduction in spontaneous GC B-cell and plasmablast numbers in these mice compared with *Tlr7*tg mice by flow cytometry (Fig. 2*A*) and immunohistochemistry (Fig. 2*C*). We analyzed B-cell activation markers MHC Class II and SLAM on *Tlr7*tg*Cd40lg*$^{-/y}$ B cells and found that expression of these molecules was reduced compared with *Tlr7*tg B cells (Fig. 2*B*). We also found an absence of two lupus disease cardinal features: antinuclear serum antibodies by antinuclear antibody (ANA) staining, and glomerulonephritis development by kidney section H&E staining (Fig. 2*C*).

Dendritic cells can induce pathogenic inflammation in the development of lupus disease, particularly pDCs that are capable of producing copious quantities of IFN-α (15). Other data suggest that myeloid dendritic cells (mDCs) may also trigger autoimmunity by presenting self-antigens to self-reactive T cells (16, 17). Both of these cell types are known to interact with B- and T cells via CD40–CD40L signaling; thus, we enumerated both mDCs and pDCs in *Tlr7*tg*Cd40lg*$^{-/y}$ mice and found that the absence of CD40–CD40L signaling eliminated the expansion of both of these dendritic cell (DC) subsets (Fig. 2*D*).

Because it is possible that lupus-like disease induction in *Tlr7*tg mice requires either B cells or DCs or both of these subsets to interact with T cells via CD40, we wanted to specifically pinpoint whether B-cell interactions with T cells are required. To do this, we crossed SAP$^{-/y}$ mice with *Tlr7*tg mice. T-cell SAP signaling downstream of SLAM receptors is essential for productive B-cell activation and prolonged adhesion to B cells, whereas it is dispensable for T-cell–DC interactions (18). Similar to our findings in *Tlr7*tg*Cd40lg*$^{-/y}$ mice, we saw very few GC B cells, plasmablasts, mDCs, and pDCs (Fig. 2 *A*, *C*, and *D*), ablated production of antinuclear antibodies, and no development of glomerulonephritis in *Tlr7*tgSAP$^{-/y}$ mice (Fig. 2*C*). *Tlr7*tgSAP$^{-/y}$ B cells had even lower MHC Class II expression and comparably low B-cell SLAM expression compared with *Tlr7*tg*Cd40lg*$^{-/y}$ B cells (Fig. 2*B*). Collectively, these data indicate that B-cell–T-cell interactions are integral to the development of lupus-like disease in *Tlr7*tg mice.

Germinal Center Formation and Autoimmunity in *Tlr7*tg Mice Require Antigen-Specific T-Cell Signals but Not T-Cell–Derived IL-17 Production. To determine whether T cells provide CD40L-dependent help to B cells, we generated 1:1 mixed BM chimeras by transferring *Tlr7*tg*Cd40lg*$^{-/y}$ + TCRβ$^{-/-}$ BM into irradiated WT hosts. In this scenario, TCRβ$^{-/-}$ BM restores all costimulatory signals to *Tlr7*tg*Cd40lg*$^{-/y}$ mice except those that are T-cell–derived because TCRβ$^{-/-}$ mice have a developmental block at the CD4$^-$CD8$^-$ double negative thymocyte stage (19). When we examined these chimeras we observed that the spleens weighed significantly less than spleens from *Tlr7*tg*Cd40lg*$^{-/y}$ + WT mixed BM chimeras (Fig. 3*A*). The number of GC B cells and activated B-cell subsets expressing high levels of CD69 and SLAM were also reduced (Fig. 3 *B* and *C*). *Tlr7*tg mice display diminution of the marginal zone (MZ) B-cell subset (5), but *Tlr7*tg*Cd40lg*$^{-/y}$ or *Tlr7*tg*Cd40lg*$^{-/y}$ + TCRβ$^{-/-}$ BM reconstituted this population in WT mice (Fig. 3*C*), indicating that T-cell–derived signals may contribute to contraction of the MZ B-cell compartment as well.

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Fig. 2. CD40L and SAP signals are required for the generation of spontaneous autoimmunity in *Tlr7*tg mice. At eight weeks of age, spleens, kidneys, and serum were harvested from *Tlr7*tg, *Tlr7*tg*Cd40lg*$^{-/y}$, and *Tlr7*tgSAP$^{-/y}$ mice. (*A*) Representative FACS plots showing GC B-cell percentages (*Top*) or plasmablasts (*Bottom*) in *Tlr7*tg (*Left*), *Tlr7*tg*Cd40lg*$^{-/y}$ (*Center*), and *Tlr7*tgSAP$^{-/y}$ mice (*Right*). (*B*) Representative histograms of surface expression of I-A^b (*Left*) and SLAM (*Right*) on B cells from *Tlr7*tg (black line), *Tlr7*tg*Cd40lg*$^{-/y}$ (dotted line), and *Tlr7*tgSAP$^{-/y}$ (gray fill) mice. (*C*) (*Top*) Frozen spleen sections stained with B220 (red) and PNA (green) in *Tlr7*tg (*Left*), *Tlr7*tg*Cd40lg*$^{-/y}$ (*Center*), and *Tlr7*tgSAP$^{-/y}$ mice (*Right*). Bars = 50 μM. (*Middle*) Serum ANA tests. Bars = 50 μM. (*Bottom*) Histological analysis of representative kidney sections stained with H&E. Bars = 50 μM. (*D*) Absolute numbers of CD11c$^+$B220$^+$ pDCs and CD11c$^+$CD11b$^+$ mDCs. Experiments are representative of n = 3 mice per group repeated two times. *$P < 0.05$.

*Tlr7*tg*Cd40lg*$^{-/y}$ + TCRβ$^{-/-}$ BM chimeras also had fewer activated CD4$^+$ T cells expressing CD44 or ICOS and fewer B cells and DCs expressing high levels of I-A^b than *Tlr7*tg*Cd40lg*$^{-/y}$ + WT BM reconstituted mice (Fig. 3 *D* and *E*). Additionally, WT mice transplanted with *Tlr7*tg*Cd40lg*$^{-/y}$ + TCRβ$^{-/-}$ BM tested negative for serum autoantibodies by ANA staining, similar to *Tlr7*tg*Cd40lg*$^{-/y}$ BM reconstituted mice, whereas serum from *Tlr7*tg*Cd40lg*$^{-/y}$ + WT BM chimeras indicated the presence of both cytoplasmic and nucleolar autoantibodies (Fig. 3*F*). Collectively these data suggest an integral role for T cells in the development of autoimmunity in *Tlr7*tg mice.

IL-17 is a primarily CD4$^+$ T-cell–derived cytokine implicated in autoimmune disease development, including lupus, in some mouse models and humans (20, 21). Because our previous microarray from C57BL/6^{Yaa} mice showed IL-17 family member transcriptional up-regulation, we wanted to determine whether IL-17 played a role in *Tlr7*tg autoimmunity by generating 1:1 *Tlr7tgCd40lg*$^{-/y}$ + IL-17$^{-/-}$ BM transplants (3). We found equivalent spleen weights, spontaneous activation of B cells and T cells, and serum ANA staining in these mice compared with *Tlr7*tg*Cd40lg*$^{-/y}$ + WT BM chimeras (Fig. 3 *A–F*), indicating that IL-17 was dispensable for disease incidence in *Tlr7*tg mice.

Fig. 3. Spontaneous autoimmunity in TLR7tg mice requires antigen-specific T-cell help but not IL-17. Irradiated WT mice received BM from *Tlr7*tg*Cd40lg*$^{-/y}$, or an equal mix of BM from *Tlr7*tg*Cd40lg*$^{-/y}$ and the indicated mouse strain, except for OT-II cells, which were used at a 4:1 ratio. (*A*) Measured spleen weight. Percentages of (*B*) FAS$^+$GL7$^+$ B cells; (*C*) splenic CD21^{hi}CD23^{low} MZ B cells, activated CD69$^+$ B cells, and activated SLAM$^+$ B cells; (*D*) ICOS$^+$ or CD44$^+$ CD4$^+$ T cells; (*E*) mean fluorescence intensity (MFI) of I-A^b, SLAM$^+$ B cells or DCs; and (*F*) ANA$^+$ serum autoantibodies.

Self-antigen-dependent activation of T cells is purported to be one of the driving forces behind autoimmune responses (10, 22, 23). If T-cell antigen specificity is important for spontaneous autoimmunity induction, then restricting the repertoire to an irrelevant antigen would prevent disease occurrence in *Tlr7*tg mice. To explore this possibility, we created *Tlr7*tg*Cd40lg*$^{-/y}$ + OT-II (an ovalbumin-specific TCR tg) mixed BM chimeras. We examined the splenic populations of these mice, compared them with *Tlr7*tg*Cd40lg*$^{-/y}$ + WT BM chimeras, and found significantly lower percentages of CD69$^+$ and SLAM$^+$ activated B-cell populations (Fig. 3*C*) and CD4$^+$ICOS$^+$ T cells (Fig. 3*D*). *Tlr7*tg*Cd40lg*$^{-/y}$ + OT-II BM chimeras also had reduced I-A^b MFI on B cells and DCs, negative serum ANA staining (Fig. 3 *E* and *F*), as well as lower percentages of GC B cells and CD4$^+$CD44^{hi} cells compared with *Tlr7*tg*Cd40lg*$^{-/y}$ + WT BM chimeras (Fig. 3 *B* and *D*). These data indicate a requirement for T-cell receptor antigen specificity in *Tlr7*tg autoimmune responses.

H2^k Haplotype and B-Cell Deficiency Render *Tlr7*tg Mice Resistant to Spontaneous Lymphocyte Activation and Autoimmune Disease. Because we found T-cell receptor antigen specificity made a difference to autoimmunity development in *Tlr7*tg mice, we wanted to determine whether there were also MHC restrictions for antigen presentation to T cells. We engineered C57BL/6 *Tlr7*tg mice homozygous or heterozygous for H2^k MHC alleles to determine whether MHC haplotypes change the autoimmune susceptibility of *Tlr7*tg mice. Overall, we found an H2^k allele dose-dependent decrease in spontaneous autoimmune phenotype in *Tlr7*tg mice. *Tlr7*tgH2$^{b/b}$ mice had ~4.5-fold higher average splenocyte numbers than *Tlr7*tgH2$^{k/k}$ mice, whereas *Tlr7*tgH2$^{k/b}$ mice displayed an intermediate phenotype (Fig. 4*A*). *Tlr7*tgH2$^{k/k}$ mice had drastically reduced CD11c$^+$ cell populations compared with *Tlr7*tgH2$^{b/b}$ mice, whereas *Tlr7*tgH2$^{k/b}$ again exhibited an intermediate phenotype (Fig. 4*B*). We determined the cell numbers in different B- and T-cell splenic populations in the three groups of mice, and the same trends held true for GC B cells, plasmablasts, and activated CD69$^+$ B-cell subsets, as well as CD69$^+$, CD44$^+$, and ICOS$^+$ activated CD4$^+$ T-cell subsets (Fig. 4*B*). Lastly, *Tlr7*tgH2$^{b/b}$ mouse serum exhibited a higher prevalence of ANAs, whereas fewer *Tlr7*tgH2$^{k/b}$ mice had ANA positive serum, and *Tlr7*tgH2$^{k/k}$ serum displayed little presence of ANAs (Fig. 4*C*). These data suggest that MHC molecules do influence lupus disease susceptibility in *Tlr7*tg mice.

B-cell autoreactivity correlates to the development of autoimmunity in humans and mouse models, and most likely is a control element of disease. However, in some murine disease models, other cell types drive autoimmunity (10, 24, 25). Thus, we wanted to determine whether cells other than B cells are able to induce inflammation and pathology in *Tlr7*tg mice. We intercrossed IgH$^{-/-}$ mice with *Tlr7*tg mice to render them deficient in B cells and examined splenic immune cell populations as well as kidney and liver pathology. We found a dramatic reduction in spleen cellularity of *Tlr7*tgIgH$^{-/-}$ mice compared with both WT and *Tlr7*tgIgH$^{+/-}$ littermate control spleens (Fig. 4*D*).

Monocyte, granulocyte, and CD11b$^+$ precursor cell population expansion is associated with many autoimmune models as well as human disease (15). We examined these populations in *Tlr7*tgIgH$^{-/-}$ mice to determine whether myeloid cells still expand in the absence of B cells. Whereas *Tlr7*tgIgH$^{+/-}$ mice maintained a much larger population of CD11b$^+$ cells in contrast with WT mice, *Tlr7*tgIgH$^{-/-}$ animals had twofold fewer CD11b$^+$ cells than WT and 10-fold fewer cells than *Tlr7*tgIgH$^{+/-}$ mice (Fig. 4*E*). *Tlr7*tgIgH$^{-/-}$ mice also had dramatically reduced numbers of mDCs and pDCs compared with *Tlr7*tgIgH$^{+/-}$ mice (Fig. 4*E*).

We determined the cell surface phenotype of CD4$^+$ T cells in *Tlr7*tgIgH$^{-/-}$ mice and found similar CD4$^+$ T-cell expression of PD-1, CD69, CD44, and ICOS compared with WT CD4$^+$ T cells, which indicated a more naive phenotype than *Tlr7*tgIgH$^{+/-}$ CD4$^+$ T cells (Fig. 4*E*). In addition, whereas glomerulonephritis and liver inflammation were readily evident in H&E stained sections of *Tlr7*tgIgH$^{+/-}$ mice, *Tlr7*tgIgH$^{-/-}$ exhibited no pathology in either tissue (Fig. 4*F*). Collectively, these data indicate that B cells are required for development of spontaneous lupus-like disease in *Tlr7*tg mice, as well as for concomitant expansion of inflammatory monocytes, granulocytes, and pDCs.

Discussion

In an autoimmune response it is likely that synergy between innate immune receptors, such as TLR7, and antigen receptors,

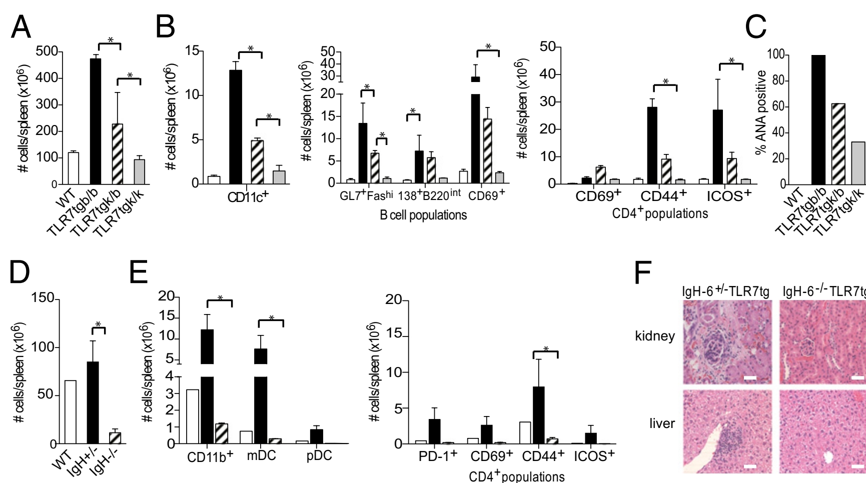

Fig. 4. H-2$^{k/k}$ haplotype or B-cell deficiency in *Tlr7*tg mice confers protection against spontaneous autoimmunity. At 12 weeks of age, livers, kidneys, spleens, and serum were harvested from denoted groups of mice. (*A*) Total spleen cell counts in WT (white bars), *Tlr7*tgH2$^{b/b}$ (black bars), *Tlr7*tgH2$^{k/b}$ (hatched bars), and *Tlr7*tgH2$^{k/k}$ mice (gray bars). (*B*) Total numbers of indicated cell types from groups designated in (*A*): CD11c$^+$ cells (*Left*), indicated B-cell populations (center panel), and CD4$^+$ T-cell populations (right panel), $n = 3$ mice/group. (*C*) Percentage of ANA positive serum, $n = 3$ mice/group. (*D*) Total splenocyte cell counts in WT (white bars), *Tlr7*tgIgH$^{+/-}$ (black bars), and *Tlr7*tgIgH$^{-/-}$ mice (hatched bars). (*E*) Total numbers of CD11b$^+$ cells, CD11b$^+$CD11c$^+$ mDCs, and CD11c$^+$B220$^+$ pDCs (*Left*), and indicated CD4$^+$ cell populations (*Right*) from groups described in *D*, $n = 2$ mice/group, repeated two times. (*F*) Representative H&E stained kidney and liver sections. (Scale bar, 50 μm.)

such as B-cell receptors, is pivotal to reach an activation threshold that overcomes tolerance. A B-cell–intrinsic role for TLR7 signaling in the development of systemic autoimmunity is unproven up to this point, although evidence from *Tlr7* deficient mice and *Tlr7*tg mice suggests that triggering of this innate receptor in B cells may significantly contribute to disease incidence (3, 4, 6, 14). Our current studies confirm the need for duality in B-cell receptor signaling through both innate pathways and T-cell–derived costimulatory signals to cause autoimmunity in *Tlr7*tg mice.

Expression of multiple copies of *Tlr7* in mixed BM chimeras led to preferential expansion of GC B cells and plasmablasts in *Tlr7*tg-derived B cells (Fig. 1*A*), indicating that spontaneous TLR7 signaling exerted a B-cell–intrinsic effect. It is possible that DCs expressing multiple copies of *Tlr7* may present antigen to T cells and preferentially skew the T-cell repertoire to differentiate into T_{FH} cells that promote GC and plasmablast B cells. However, $CD11c^+$ cells did not expand in our mixed BM chimera system (Fig. 1*A*), nor did they spontaneously up-regulate MHC II (Fig. 1*B*), suggesting that DC functions, important for initiation of inflammatory responses in general, are dependent on humoral immune activation and may play a secondary role in our spontaneous *Tlr7*tg model of autoimmunity. Previous research suggests that pDC IFN-α production up-regulates TLR7 and promotes B-cell plasmablast differentiation and class switch to IgG2a (1, 26). However, *Tlr7*tg B cells had increased transcription of IgG1 and IgG2b genes rather than IgG2a, suggesting that this phenotype is IFN-α independent (Fig. 1*C*). Additionally, multiple copies of *Tlr7* in mice enhanced expression of genes associated with antigen processing and presentation in B cells, perhaps increasing the likelihood of autoreactive B cells to present self-antigen to T cells and to garner T-cell support in the GC (Fig. 1*B*). Together, these data suggest that *Tlr7* exerts B-cell–specific effects that influence B-cell fate and development of spontaneous autoimmunity.

High levels of *Tlr7* alone could not drive spontaneous B-cell activation, GC formation, and autoantibody production in the absence of costimulatory CD40L or SLAM-family receptor signals (Fig. 2). Interestingly, DC population expansion also depended on costimulatory signals as *Tlr7*tg mice deficient in *Cd40lg* or SAP had significantly reduced numbers of these cells compared with *Tlr7*tg controls. These data are in contrast with those found in an Mrl/Mp-*lpr*/*lpr* mouse model of autoimmunity wherein *Cd40lg* gene deletion results in diminution of kidney disease, pathogenic IgG rheumatoid factors, and anti-dsDNA antibodies, but B cells are still able to class switch and produce snRNP antibodies (12). However, our findings do correlate with data from other autoimmune-prone strains lacking CD40L or SAP—*Cd40lg* deficiency in New Zealand black (NZB) models of spontaneous autoimmunity results in reduced IgG autoantibodies and glomerulonephritis scores (11). Additionally, the autoimmune phenotype in *Sanroque* mice can be reduced when these mice are crossed to an SAP deficient background (10). These data collectively indicate the importance of costimulatory signals delivered to B cells during the development of autoimmunity.

The contribution of T cells to B-cell autoantibody production is the subject of much debate, but it is most likely that T cells, particularly T_{FH} cells, are a source of vital costimulatory signals for GC B cells (9). However, T-cell deletion in a variety of autoimmune-prone mouse strains has met with mixed results. T-cell deficiencies in NZB, MRL/Mp-*lpr*/*lpr*, and B6.56R mice reduce B-cell activation and/or organ disease, but have varying success in preventing autoantibody production (27–29). BAFFtg mice develop autoimmunity independently of T cells and it has been suggested that this occurs through a TLR7-dependent mechanism (13). However, our data from mixed BM chimeras of $TCR\beta^{-/-}$ BM + *Tlr7*tg*Cd40lg*$^{-/y}$ BM support an integral role for T-cell help in the generation of autoimmunity in *Tlr7*tg mice (Fig. 3), indicating that perhaps other pathways along with TLR7 may be involved in the BAFFtg mice. We also determined that IL-17, a cytokine that is attributed with being a key contributor to autoreactive GC development in autoimmune BXD2 mice and ectopic central nervous system GC in a model of experimental autoimmune encephalomyelitis (30, 31), is not essential for lymphocyte activation or autoantibody production in *Tlr7*tg mice. In contrast, restricting the T-cell repertoire to a non-self-antigen, ovalbumin, showed that T-cell antigen specificity was critical to activation of lymphocytes and production of autoantibodies in *Tlr7*tg mice (Fig. 3), adding further weight to the importance of T-cell specificity as a checkpoint in maintaining peripheral tolerance.

In corroboration with our finding that T-cell antigen specificity was crucial to preventing autoimmunity, we found that disease incidence in *Tlr7*tg mice depended on the context of antigen presentation (Fig. 4). Previous studies suggest that some mouse strains expressing $H2^d$ and $H2^k$ MHC haplotypes are less prone to developing autoimmune disease because they express the protective MHC II *I-E* genes contained within the same locus (32–34). This may be the case in *Tlr7*tg mice as well, but there are many possibilities of how MHC restrictions could be affecting disease development in these mice, such as alterations in antigen presentation, changes to thymic repertoire selection, or autoantibody selection. We observed a protective gene dosage effect in *Tlr7*tg mice expressing one versus two alleles of $H2^k$. *Tlr7*tg mice homozygous for $H2^k$ displayed a phenotype very similar to WT mice, indicating a reduced propensity to develop autoimmunity. Whether these results are due to changes to the manner of antigen presentation or the type of antigen that is presented remains to be seen.

Given the results from our current study, we attributed a great deal of importance to the role that B- and T-cell interactions play in autoimmunity in *Tlr7*tg mice. Our data showing a lack of lymphocyte activation and tissue pathology in *Tlr7*tgIg$H^{-/-}$ mice strengthened this conclusion. The pDC compartment paucity was particularly striking, as these cells express *Tlr7* and can produce large quantities of IFN-α. SLE incidence in humans is linked to a strong IFN-α response, and it is thought that IFN-α produced by pDCs drives disease in humans and mice, causing activation of lymphocytes and release of inflammatory mediators (1). However, in the absence of B cells in *Tlr7*tg mice, inflammatory cell populations did not expand, indicating that spontaneous TLR7 activation in pDCs cannot foment inflammation without a corresponding humoral response.

From the data presented here, we can conclude that spontaneous autoimmunity and inflammation in *Tlr7*tg mice is driven at least in part by B-cell–intrinsic events, but that B cells require costimulatory support from T cells to break tolerance. In turn, T cells require antigen to be presented in a capacity that favors an autoimmune response. These results likely reflect the circumstances that lead to lupus disease in humans, that not one cell type or causal agent induces autoimmunity, but rather that disease results from a complex interplay of inflammatory gene expression, synergistic innate and adaptive immune signals, and cooperative interactions between multiple cell types.

Materials and Methods

Mice. C57BL/6 *Tlr7*tg mice were engineered as previously described (5). *Tlr7*tg mice were crossed with C57BL/6 *Cd40lg*$^{-/y}$ (35), *Ighm*^{tm1Cgn} (Ig$H^{-/-}$) (36), and C57BL/6 $H2^{k/k}$ mice obtained from Jackson Labs, as well as C57BL/6 *Sh2d1a*$^{-/y}$ ($SAP^{-/y}$) mice (37). All animals were kept in specific pathogen-free caging and all experiments were conducted under guidelines from the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

BM Chimeras. BM cells isolated from femur and tibia bones of $CD45.2^+$ *Tlr7*tg and $CD45.1^+$ WT mice were mixed at a 1:1 ratio and injected i.v. via tail vein into 8–10-wk-old C57BL/6 recipient mice that had been irradiated with 940 rad one day before bone marrow transfer (BMT). Eight weeks after BMT, tissues and serum were harvested for analysis. The same scheme was used to

make 1:1 BM chimeras using $CD45.1^{+}CD45.2^{+}$ *Tlr7*tg*Cd40lg*$^{-/y}$ and $CD45.2^{+}$ WT, $TCR\beta^{-/-}$, IL-17$^{-/-}$, or OT-II mice.

Immunohistochemistry. Tissues were embedded in tissue freezing medium and samples frozen at −80 °C. Frozen tissue sections (6 mm thick) were prepared, fixed in acetone for 10 min, and blocked with blocking serum using the Vectastatin ABC-AP kit (Vector Labs). Antibodies were diluted with 1% BSA/PBS and incubated for 1 h. Slides were washed with PBS for 5 min, two times. Slides were mounted with Vectashield (Vector Labs) and visualized using a Zeiss 510 Meta confocal microscope.

Histology. Kidney and liver from selected mice were fixed in neutral buffered formalin, embedded in paraffin, and sections stained with hematoxylin and eosin. Immunofluorescence staining was performed on spleens from *Tlr7*tg, *Tlr7*tg*Cd40lg*$^{-/-}$, and *Tlr7*tgSAP$^{-/-}$ mice. Frozen sections were stained with biotinylated-anti-peanut agglutinin antibody, followed by SA-Alexa 488 and Alexa 647 anti-B220.

Microarray. cDNA synthesis and labeling of follicular B cells purified from mice aged 8–10 wk old was conducted as mentioned previously (3), as were hybridizations (38) using an Mmca probe set from the National Institute of Allergy and Infectious Diseases (NIAID) Microarray Facility. Images were analyzed using GenePix Pro-5.0.1.38 (Molecular Devices) and uploaded data were analyzed using the mAdb program (http://madb.niaid.nih.gov/).

Real-Time PCR. Real-time PCR (RT-PCR) was performed on $CD9^{-}CD43^{-}$ B-cell total RNA using iQ SYBR Green Supermix (Biorad) in a Bio-Rad iCycler. Semiquantitative RT-PCR for Ig gene transcription was conducted using previously reported primers (39).

Flow Cytometry Antibodies. Antibodies against the following antigens were used for flow cytometric analysis of splenocytes: B220, CD19, SLAM, GL7, FAS, IgG2a, IgG1, IgG2b, CD138, CD45.1, CD45.2, CD4, CD69, CD45RB, CD44, CD11b, Ly6C, Gr1, ICOS, CXCR5 from BD Biosciences and CD62L, CD11c, PDCA-1, PD-1, and Fc block (CD16/32) from eBioscience. Flow cytometry was conducted using an FACS Caliber or LSR2 (BD Biosciences) and data were analyzed using FlowJo (Tree Star).

Serological Analysis. Serum was tested for autoantibody production using an ANA test using Hep-2 cells fixed on slides (MBL-Bion) as per manufacturer instructions. Briefly, sera were diluted 1:200 and applied to slide, incubated for 30 min, and after two washes, a goat anti-mouse IgG secondary antibody was used at a 1:1,000 dilution to identify autoreactive sera. Slides were washed two times, and Vectashield mounting medium applied to slide.

Statistical Analyses. Statistical analysis of data by one-way ANOVA coupled with Tukey's multiple comparison posttest was performed using Prism software 5 (GraphPad Software). In some cases a two-tailed paired Student's t test was used. $P < 0.05$ was considered statistically significant.

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