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The TACI Receptor Regulates T-Cell-Independent Marginal Zone B Cell Responses through Innate Activation-Induced Cell Death

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SUMMARY

Activation-induced cell death (AICD) plays a critical role in immune homeostasis and tolerance. In T-cell-dependent humoral responses, AICD of B cells is initiated by Fas ligand (FasL) on T cells, stimulating the Fas receptor on B cells. In contrast, T-cell-independent B cell responses involve innate-type B lymphocytes, such as marginal zone (MZ) B cells, and little is known about the mechanisms that control AICD during innate B cell responses to Toll-like receptor (TLR) activation. Here, we show that MZ B cells undergo AICD in response to TLR4 activation in vivo. The transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) receptor and TLR4 cooperate to upregulate expression of both FasL and Fas on MZ B cells and also to repress inhibitors of Fas-induced apoptosis signaling. These findings demonstrate an unappreciated role for TACI and its ligands in the regulation of AICD during T-cell-independent B cell responses.

INTRODUCTION

The duration of adaptive immune responses is tightly regulated, and many effector lymphocytes are eventually eliminated via different mechanisms, including activation-induced cell death (AICD) (reviewed in Strasser et al., 2009). In contrast, little is known about what regulates the termination of innate immune responses to innate signals. This is particularly important for innate-type MZ and B1 B cell subsets, known to respond highly efficiently to innate activation but which contain potentially self-reactive B cells (Chen et al., 1997). Therefore, mechanisms of regulation must exist to control their response to unspecific innate activation.

The Fas ligand (FasL)-Fas receptor pair from the tumor necrosis factor (TNF) ligand-receptor super-families triggers apoptosis. Fas signals play a central role in AICD and maintenance of peripheral T and B cell tolerance and homeostasis (reviewed in Mizuno et al., 2003; Strasser et al., 2009). Accordingly, humans and mice with mutations in Fas (autoimmune lymphoproliferative syndrome patients; *lpr* mice) or its ligand, FasL (*gld* and *Fasl*$^{\Delta m/\Delta m}$ mice that lack functional membrane-bound FasL, mFasL), develop severe autoimmune disorders linked to the expansion of hyperactivated effector B and T cells as a direct result of defective lymphocyte apoptosis (Mizuno et al., 2003; O' Reilly et al., 2009; Rieux-Laucat et al., 1995). B cells are key drivers of autoimmunity in mice lacking functional FasL or Fas, as shown by the fact that B cell ablation or restoration of Fas expression selectively in B cells prevented autoimmunity in Fas-deficient *lpr* mice (Shlomchik et al., 1994).

Naive B cells are Fas^{lo} and therefore show relatively low sensitivity to FasL-induced apoptosis (Mizuno et al., 2003). In the case of T-cell-dependent (TD) B cell activation, effector T cells express CD40L, which binds to CD40 on activated B cells inducing upregulation of Fas expression (Schattner et al., 1995). However, B cell receptor (BCR) stimulation by antigens inhibits Fas-mediated apoptosis (Rathmell et al., 1996). Such resistance to Fas-mediated killing appears to be achieved via induction of antiapoptotic regulators, such as FLICE-inhibitory protein (cFLIP), X-linked inhibitor of apoptosis protein (XIAP), and Bcl-x_L (reviewed in Mizuno et al., 2003; Strasser et al., 2009). Upon pathogen clearance, antigen-driven BCR signals stop, allowing Fas signaling and B cell death.

In contrast, little is known about mechanisms regulating B cell apoptosis after T-cell-independent (TI) antigen activation. B cell responses to TI antigens are normally short lasting (Liu et al., 1991). This suggests the existence of mechanisms terminating TI B cell responses. Interestingly, although the majority of B cells proliferate in response to the Toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS), ~10% of B cells will rapidly undergo apoptosis (Acosta-Rodríguez et al., 2007; Zhang et al., 2009), an effect possibly resulting from LPS-induced Fas expression on B cells (Acosta-Rodríguez et al., 2007; Mizuno et al., 2003).

Moreover, loss of cFLIP, a key inhibitor of Fas signaling, enhances the death of LPS-stimulated B cells (Zhang et al., 2009). Interestingly, LPS-induced Fas upregulation on B cells is further enhanced by costimulation with B cell activating factor from the TNF family (BAFF), indicating that BAFF may prepare B cells for Fas-mediated killing in the context of TLR4 activation (Acosta-Rodríguez et al., 2007).

BAFF is essential for B cell survival (reviewed in Mackay and Schneider, 2009). BAFF binds to BAFF receptor (BAFF-R or BR3), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) (Mackay and Schneider, 2009). BAFF-R and BCMA trigger survival and maturation of various B cell subpopulations (Mackay and Schneider, 2009). In contrast, TACI is essential for immunoglobulin (Ig) isotype switching and plasma cell differentiation in response to TI antigens (Ozcan et al., 2009; von Bülow et al., 2001). TACI is highly expressed on MZ and B1 B cells (Groom et al., 2007). Loss of TACI in mice leads to B cell hyperplasia, abnormal expansion of the MZ B cell subset (Yan et al., 2001), and autoimmunity and lymphomas (Seshasayee et al., 2003). Therefore, TACI normally plays a negative regulatory role in B cells, possibly involving B cell apoptosis (Seshasayee et al., 2003). Interestingly, activation of TLR4 strongly upregulates TACI expression on B cells (Treml et al., 2007). The ability of BAFF stimulation to further upregulate Fas expression on LPS-activated B cells and the regulation of TACI on MZ B cells may mediate this effect and thereby promote the death of TLR4-activated MZ B cells to limit TI innate B cell responses.

Here, we show that TACI is required for the efficient upregulation of FasL and Fas expression on TLR4-activated MZ B cells and their apoptosis. This work describes a mechanism regulating innate activation of MZ B cells.

RESULTS

TLR4 Activation Upregulates TACI Expression on B Cells In Vivo

LPS-activated B cells upregulate TACI expression in vitro (Treml et al., 2007). We confirmed this in vivo: substantial upregulation of TACI expression was detected on splenic B cells from mice treated with a sublethal dose of LPS (Figure 1A). Furthermore, although LPS upregulated TACI expression on all splenic B cell subsets, this was most pronounced on MZ B cells and their precursors, the transitional type 2 (T2)-MZ B cells (Figure 1A).

BAFF is a potent stimulator of TLR7 and TLR9 expression in B cells (Groom et al., 2007). In contrast, BAFF did not upregulate TLR4 expression on B cells and did not augment the proportions of $TLR4^+$ follicular (Fo) and MZ B cells (Figure S1A available online). Moreover, TLR4 expression was unaffected by loss of TACI (Figure S1A). Collectively, these data show that in vivo TLR4 activation strongly upregulates TACI expression on MZ B cells and their precursors, whereas TLR4 expression on B cells is unaffected by BAFF or loss of TACI expression.

LPS-Induced Fas Expression on B Cells Requires TACI Signaling In Vitro

Given that BAFF potentiates TLR4-mediated Fas upregulation on B cells (Acosta-Rodríguez et al., 2007), we tested the role of TACI in mediating this effect, with particular attention to MZ B cells. We isolated splenic B cell subsets from WT and TACI-deficient mice (*Tnfrsf13b*$^{-/-}$ mice) and compared their ability to upregulate Fas in response to LPS in vitro. LPS upregulated Fas expression on WT B cells and costimulation with BAFF further augmented Fas expression on MZ B cells (Figure 1B) as previously described (Acosta-Rodríguez et al., 2007). LPS was able to increase Fas expression on MZ B cells from *Tnfrsf13b*$^{-/-}$ mice, although the magnitude of this increase was significantly reduced compared to LPS-treated WT MZ B cells (Figure 1B, $p < 0.05$). This difference is probably explained by the fact that B cells express BAFF in response to LPS activation (Chu et al., 2007). Indeed, LPS stimulation of B cells in vitro augmented BAFF expression and reduced that of APRIL (Figure S1B), suggesting that endogenous BAFF production by LPS-activated splenic B cells can signal through TACI and synergize with TLR4 signaling to further upregulate Fas expression on WT MZ B cells but not *Tnfrsf13b*$^{-/-}$ MZ B cells.

BAFF failed to further increase LPS-induced Fas expression on *Tnfrsf13b*$^{-/-}$ MZ B cells, in contrast to WT MZ B cells (Figure 1B), indicating that TACI mediates this effect. Moreover, the specificity of this effect for MZ B cells is probably related to the high levels of TACI expression on these cells, which are further augmented upon LPS activation (Figure 1A). Indeed, Fas expression levels correlated with that of TACI on LPS-treated splenic B cells (Figure S1C). Interestingly, circulating human memory B cells expressing higher TACI levels also expressed higher levels of Fas (Figure S1D).

Of note, activation of TLR9, TLR1-2, TLR2-6, and TLR3 augmented Fas expression on WT and *Tnfrsf13b*$^{-/-}$ MZ B cells, but BAFF did not stimulate this further (Figures 1B and S1E–S1G). Moreover, CD40L augmented Fas expression similarly on *Tnfrsf13b*$^{-/-}$ and WT MZ B cells (Figure S1F), suggesting that our observation is specific to LPS and TACI and not due to an intrinsic dominant (possibly developmentally imposed) defect in Fas expression in *Tnfrsf13b*$^{-/-}$ MZ B cells.

BAFF binds to BAFFR and TACI whereas APRIL binds only to TACI (Mackay and Schneider, 2009). Both BAFF and APRIL enhanced LPS-induced Fas mRNA and protein expression in MZ B cells (Figures 1C and 1D). Controls with boiled BAFF and APRIL ruled out possible endotoxin contaminants. Results obtained with APRIL, in particular, indicate that signaling via TACI alone (BCMA is not expressed on MZ B cells) (Stadanlick et al., 2008) enhances LPS-mediated Fas expression on MZ B cells (Figure 1D). Collectively, these results demonstrate a strong relationship between TACI and TLR4 signaling, leading to upregulation of Fas expression on MZ B cells.

LPS-Induced Fas and FasL Expression on B Cells Requires TACI Signaling In Vivo

To test whether the effects described above operate in vivo, we challenged WT and *Tnfrsf13b*$^{-/-}$ mice with LPS. Interestingly, $TACI^{lo}$ Fo B cells weakly upregulated Fas expression in response to LPS injection (Figure 2A), whereas Fas upregulation was prominent on ($TACI^{hi}$) WT T2, T2-MZ, and MZ B cells (Figures 2A and S2A). Upregulation of Fas expression was substantially impaired in MZ and T2-MZ B cells from LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figure 2A). Remarkably, we also observed dramatic upregulation of FasL expression on the surface of T2-MZ and MZ B cells but only minimally on Fo B cells (Figure 2B

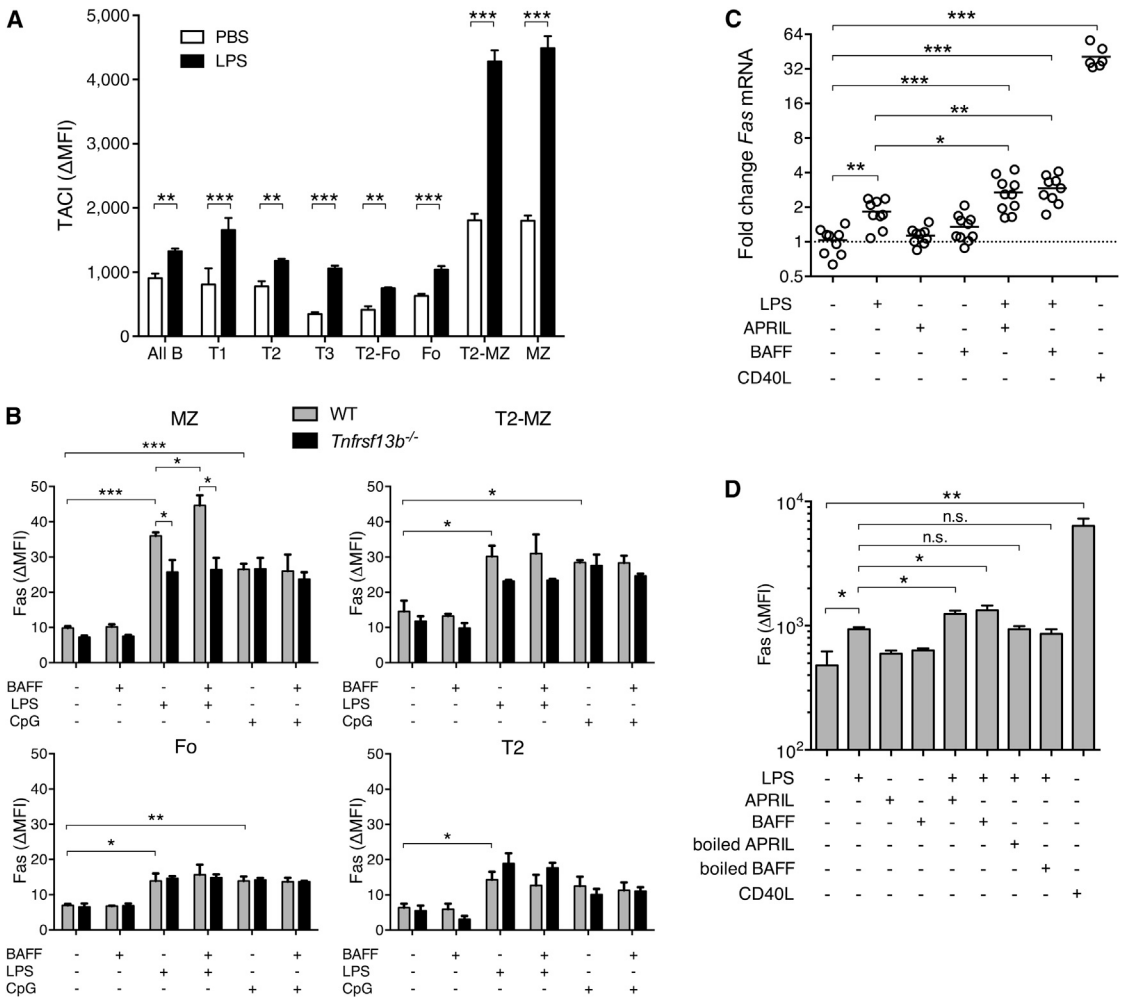

Figure 1. TLR4 Activation Upregulated TACI Expression on B Cells In Vivo and *Tnfrsf13b*$^{-/-}$ B Cells Failed to Fully Upregulate Fas Expression upon LPS Activation In Vitro

(A) TACI expression on WT splenic B cell subsets from four mice as indicated, 48 hr after LPS (black bars) or PBS (white bars) injection.

(B) Fas expression on splenic B cell subsets from five WT (gray bars) or five *Tnfrsf13b*$^{-/-}$ (black bars) mice 24 hr in vitro with BAFF and/or LPS or CpG as indicated.

(C) Splenic B cells (prepared from six to ten WT mice) were stimulated for 30 hr with LPS, BAFF, APRIL, or CD40L as indicated. Isolated mRNA samples were analyzed for Fas expression by RT-PCR. Results are shown as fold-change compared to Fas mRNA expression in unstimulated B cells.

(D) Splenic B cells (prepared from ten mice) were stimulated for 48 hr with LPS, ±BAFF or ±APRIL, or with denatured boiled BAFF or APRIL, or with CD40L, as indicated. Surface mean Fas expression measured by FACS on gated MZ B cells is shown.

In all panels except (C) data are shown as the mean +1 SEM. In (C) horizontal bars indicate the mean. Data in all panels are representative of three to five independent experiments. See also Figure S1.

and S2A). Similar to Fas, upregulation of FasL expression was impaired on MZ and T2-MZ B cells from LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figures 2B and S2A). The proportions of Fas- and FasL-expressing T2-MZ and MZ B cells increased in LPS-treated mice and this effect was TACI dependent (Figures 2C and 2D). These results were confirmed at the mRNA level (Figures 2E and 2F). Importantly, BAFF-R expression was unaffected on B cells from LPS-treated WT and *Tnfrsf13b*$^{-/-}$ mice (Figure S2B).

In LPS-treated mice, FasL, Fas, and TACI expression levels peaked on WT MZ B cells after ~30 hr and declined thereafter, in contrast to *Tnfrsf13b*$^{-/-}$ MZ B cells where this effect was markedly slower and of lower magnitude (Figure S2C). FasL expression was also upregulated on memory CD4^{+} T cells from LPS-injected mice (Figure S2D). LPS-treated *Tlr4*$^{-/-}$ mice failed to upregulate Fas and FasL expression on MZ B cells, confirming the role of TLR4 in this process (Figures 2G and 2H). *Tnfsf13b* transgenic (BAFF Tg) mice have abnormally high numbers of MZ B cells (Batten et al., 2000). Interestingly, LPS-treated BAFF Tg mice failed to upregulate expression of FasL but not Fas on MZ B cells (Figures 2G and 2H). This observation may explain the high numbers of MZ B cells in these mice.

TLR4 signals via two main signaling pathways: one that is dependent on both TIRAP (also known as Mal) and MyD88, which is important for NF-κB activation and inflammation, and the alternate pathway that is MyD88 independent but dependent

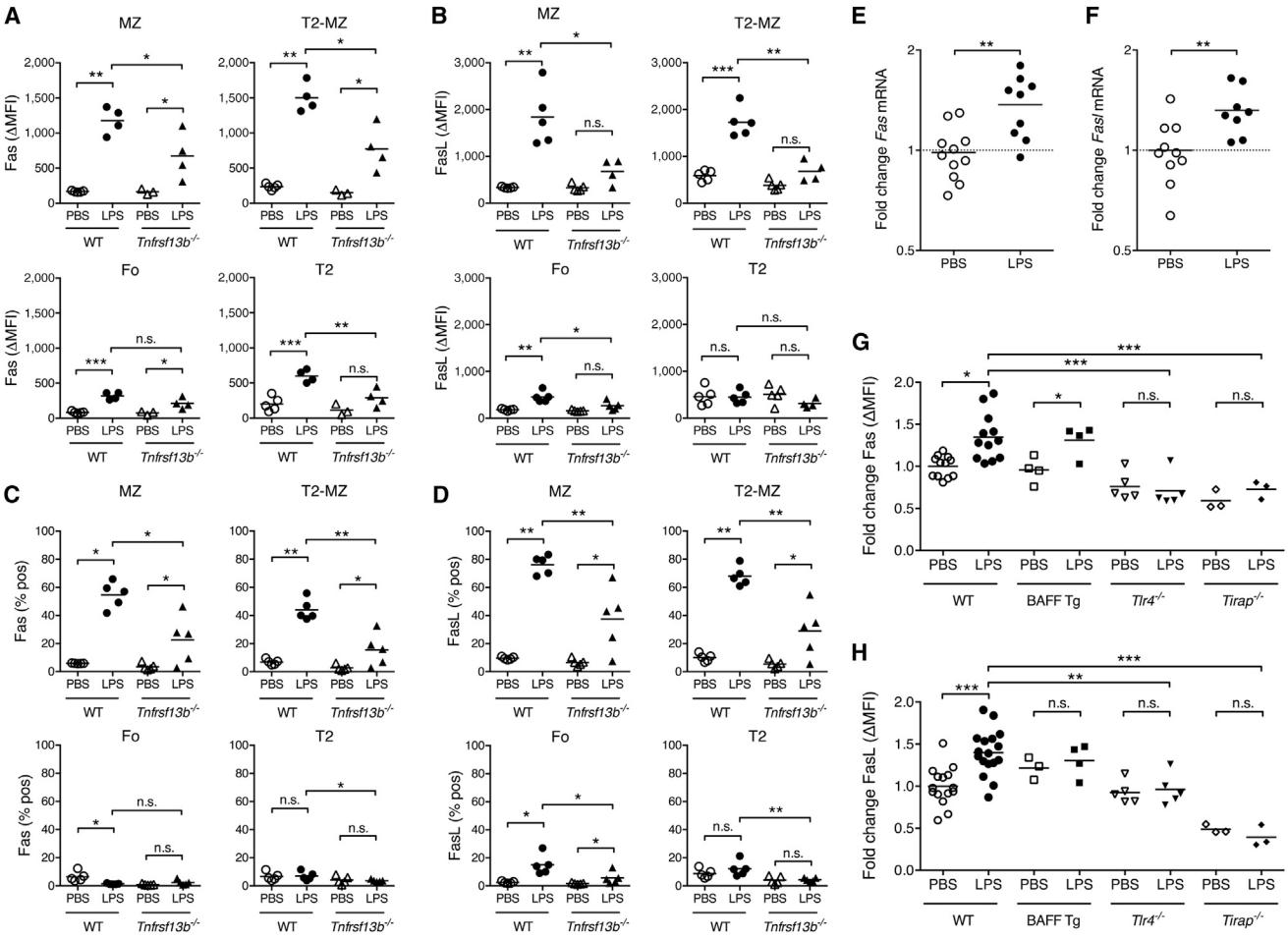

Figure 2. TACI Is Required for FasL and Fas Expression upon LPS Activation In Vivo, in a TLR4- and TIRAP-Dependent Manner

(A) Fas expression on gated splenic B cell subsets from WT or *Tnfrsf13b*$^{-/-}$ mice, 48 hr after LPS injection or PBS, as indicated.

(B) FasL expression on B cells from (A).

(C) Percent of Fas-positive B cells from (A).

(D) Percent of FasL-positive B cells from (A).

(E) mRNA samples from splenic B cells cultured for 48 hr with PBS or LPS (as labeled) and tested for *Fas* expression by RT-PCR.

(F) RT-PCR analysis of *Fasl* mRNA levels in samples from (E).

In (E) and (F), results are shown as fold-change compared to *Fas* expression in B cells from PBS-treated mice.

(G and H) Fas (G) and FasL (H) expression on MZ B cells from WT, BAFF Tg, *Tlr4*$^{-/-}$, or *Tirap*$^{-/-}$ mice, 48 hr after LPS or PBS injection (as indicated). Results are shown as fold-change surface expression compared to MZ B cells from PBS-treated mice.

In all panels, horizontal bars indicate the mean for each group and 4–11 mice were used per group. Results are representative of four independent experiments. See also Figure S2.

on TRAM and TRIF and critical for type I IFN production (Kenny and O'Neill, 2008). LPS-treated *Tirap*$^{-/-}$ mice were unable to upregulate Fas and FasL on MZ B cells (Figures 2G and 2H), suggesting an essential role for TIRAP downstream of TLR4. Interestingly, proportions (Figure S2E) and absolute numbers (not shown) of MZ B cells were increased in *Tlr4*$^{-/-}$ and *Tirap*$^{-/-}$ mice. Moreover, partial reduction in Fas upregulation was observed on LPS-treated MZ B cells incubated with a blocking anti-IFNAR1 monoclonal antibody (mAb) when compared to mAb control (Figure S2F). Therefore, type I IFN production (dependent on the TRIF signaling pathway) may contribute to the optimal upregulation of Fas on LPS-stimulated MZ B cells. These results show that LPS-mediated upregulation of Fas and FasL on MZ B cells in vivo requires TLR4 with downstream TIRAP-dependent signaling, as well as type I IFN stimulation of IFNAR1.

LPS-Mediated MZ B Cell Death Requires TACI and Membrane-Bound FasL

We tested whether TACI was important for MZ B cell death after LPS treatment and whether MZ B cell killing was dependent on FasL and Fas. For this, we used a flow-cytometry-based TUNEL assay (Figures 3A and S3A). In LPS-treated WT mice, apoptosis of MZ B cells was increased within 48 hr compared to PBS-injected controls; this response was markedly reduced in LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figure 3A).

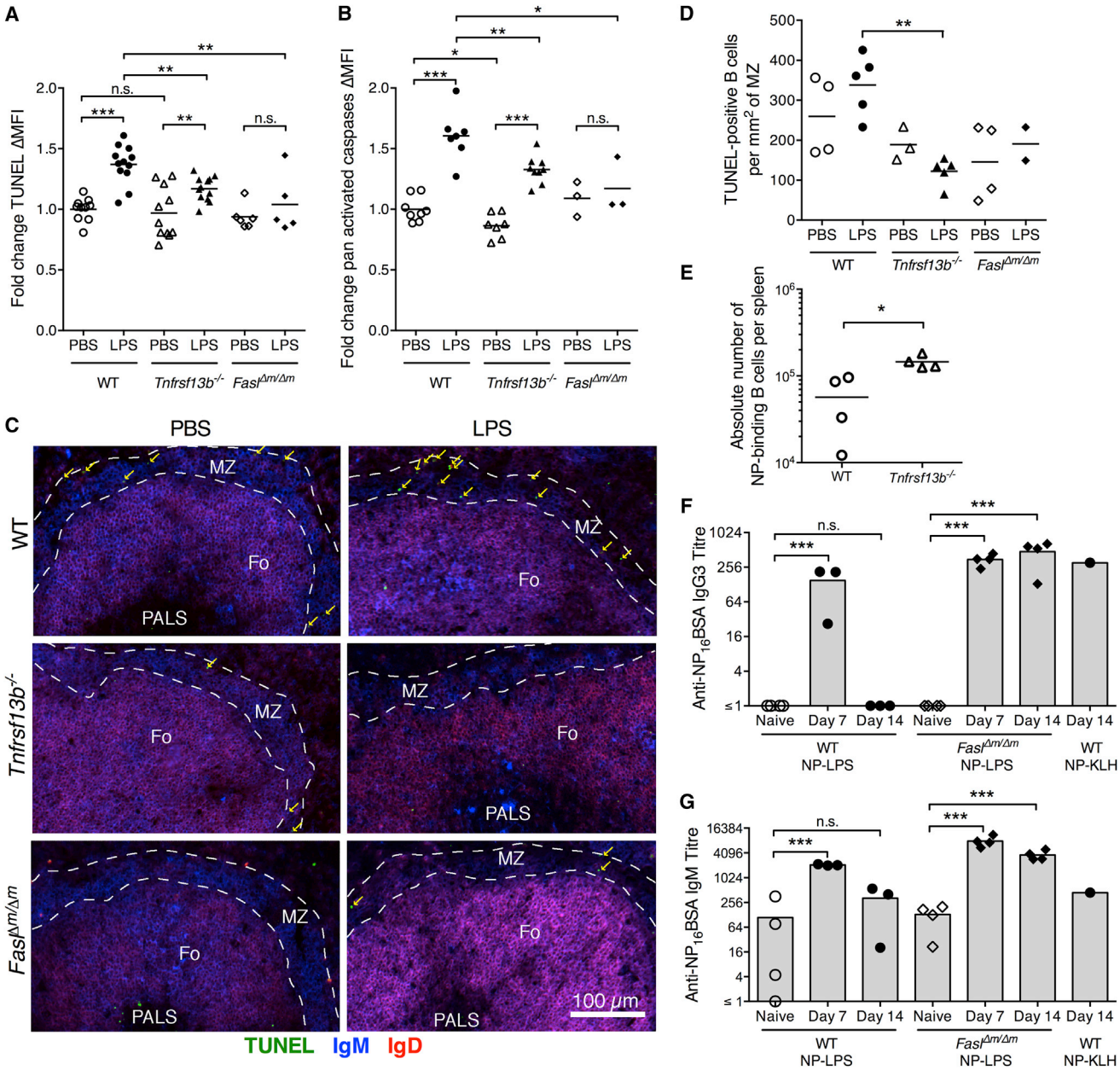

Figure 3. Fas-Mediated LPS-Induced Apoptosis Is Impaired in *Tnfrsf13b*$^{-/-}$ MZ B Lymphocytes

(A) Levels of apoptosis in MZ B cells from WT, *Tnfrsf13b*$^{-/-}$, or *Fasl*$^{\Delta m/\Delta m}$ mice 48 hr after injection with LPS or PBS. DNA fragmentation in MZ B cells was measured with a flow-cytometry-based TUNEL assay.

(B) Pan-activated caspase levels in WT, *Tnfrsf13b*$^{-/-}$, or *Fasl*$^{\Delta m/\Delta m}$ mice injected as in (A) were measured with a flow-cytometry-based (CaspGLOW) staining assay.

In (A) and (B), fold-changes are relative to the WT PBS-treated mean.

(C) Apoptotic MZ B cells in the MZ of WT, *Tnfrsf13b*$^{-/-}$, or *Fasl*$^{\Delta m/\Delta m}$ mice treated with LPS or PBS for 24 hr, by a microscopy-based TUNEL assay. Representative images from three replicates per group are shown. Samples were stained for TUNEL (FITC; green), IgD (R-PE; red), and IgM (Cy5; blue). The MZ is delineated with dashed white lines and apoptotic MZ B cells are indicated with arrows.

(D) Quantification of results in (C) via ImageJ software; numbers of TUNEL^{+} cells per mm^2 of MZ are shown.

(E) Absolute numbers of NP-binding B cells per spleen from WT or *Tnfrsf13b*$^{-/-}$ mice 14 days after NP-LPS injection.

(F and G) NP-specific IgG3 (F) and IgM (G) antibodies measured by ELISA in the sera of WT or *Fasl*$^{\Delta m/\Delta m}$ mice 7 and 14 days after immunization with NP-LPS or NP-KLH in alum.

Bars in (A), (B), (D), and (E) represent the mean. The data in all panels are representative of 3–5 experiments and 3–12 individual animals per group were used. See also Figure S3.

Moreover, LPS treatment of *Fasl*$^{\Delta m/\Delta m}$ mice failed to induce MZ B cell apoptosis (Figure 3A). This result demonstrates that mFasL is required for LPS-mediated MZ B cell apoptosis. This specifically affected MZ and T2 MZ B cells (Figure S3B). Similar results were obtained with a flow-cytometry-based analysis of activated caspases as an alternative readout for apoptosis (Figure 3B). The reduction of apoptosis seen in *Tnfrsf13b*$^{-/-}$ mice was in keeping with the reduction in FasL and Fas expression seen on MZ B cells from LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figure 2). Interestingly, proportions of AnnexinV^{+} resting WT MZ B cells were significantly higher than that of WT Fo B cells (Figure S3C; $p < 0.0001$). Importantly, proportions of AnnexinV^{+} resting *Tnfrsf13b*$^{-/-}$ MZ B cells were significantly reduced compared to that of resting WT MZ B cells (Figure S3C; $p < 0.05$). This indicates that MZ B cells normally have greater predisposition to undergo TACI-dependent apoptosis than do Fo B cells.

Previous work has shown that in mice injected with 50 μg of LPS, splenic MZ B cells move away from the MZ as indicated by a change in homing receptor expression (Cinamon et al., 2004). To circumvent this problem, we used less LPS, between 18 and 20 μg per mouse, so that MZ B cell relocation was only partial at 24 hr (Figure 3C). TUNEL staining of spleen tissue sections revealed many apoptotic IgM^{hi} MZ B cells in the MZ of LPS-treated WT mice but drastically fewer in the MZ of LPS-treated *Tnfrsf13b*$^{-/-}$ or *Fasl*$^{\Delta m/\Delta m}$ mice (Figures 3C and 3D). However, we cannot exclude the possibility that in LPS-treated mice, some TUNEL-positive MZ B cells have relocated outside of the MZ.

MZ B cells isolated from LPS-treated WT mice expressed a functional FasL and were more effective at killing A20 target cells than were WT Fo B cells or *Fasl*$^{\Delta m/\Delta m}$ MZ B cells (Figure S3D), confirming that this effect is FasL dependent. This work clarifies the identity of previously described FasL^{+} LPS-activated B cells (Hahne et al., 1996) as being mostly MZ B cells. LPS did not upregulate FasL on B cells in vitro (not shown), allowing us to test the effect of a Fas agonist mAb (clone Jo-2) without any confounding effect from endogenous FasL. LPS-activated *Tnfrsf13b*$^{-/-}$ MZ B cells were substantially less responsive to anti-Fas-mediated apoptosis in comparison to WT B cells (Figure S3E).

We also tested whether reduced LPS-mediated MZ B cell death in *Tnfrsf13b*$^{-/-}$ mice translated into prolonged responses to LPS. The MZ B cell response to LPS in vivo peaks on day 3–4 and is significantly decreased by day 14 (Liu et al., 1991). Antibody production in response to LPS is defective in *Tnfrsf13b*$^{-/-}$ mice (Ozcan et al., 2009). Therefore, we numerated NP-binding B cells 14 days after NP-LPS immunization. As suspected, we detected significantly more NP-binding B cells in NP-LPS-treated *Tnfrsf13b*$^{-/-}$ and *Fasl*$^{\Delta m/\Delta m}$ mice compared to NP-LPS-treated WT mice (Figures 3E and S3F; $p < 0.05$). *Fasl*$^{\Delta m/\Delta m}$ mice express TACI, thus allowing analysis of NP-specific IgM and IgG3 responses. Levels of NP-specific IgG3 and IgM remained high at day 14 after NP-LPS immunization in *Fasl*$^{\Delta m/\Delta m}$ mice but not in immunized WT mice in which these levels were substantially reduced (Figures 3F and 3G). Together, these results showed prolonged responses to LPS in *Tnfrsf13b*$^{-/-}$ and *Fasl*$^{\Delta m/\Delta m}$ mice, consistent with our observation of impaired FasL-mediated killing of MZ B cells in these mice. NP-KLH is a TD antigen used as a positive control for anti-NP antibody detection at day 14 (Figure 3F). Collectively, these results demonstrate that TLR4-activated MZ B cells require TACI and FasL to activate an innate activation-induced apoptosis program in vivo.

T Cells Are Not Required for LPS-Induced MZ B Cell Apoptosis

Considering that TLR4 stimulation and high TACI levels on MZ B cells cooperate to induce FasL and Fas on their surface (Figure 2), a possible scenario is that MZ B cells will undergo fratricide and/or suicide. Consistent with this notion, FasL^{+} MZ B cells from LPS-treated WT mice are cytolytic (Figure S3D; Hahne et al., 1996). However, it is also possible that upon LPS injection some MZ B cells migrate out of the MZ (Rubtsov et al., 2008) where they make contact with FasL^{+} activated T cells (Strasser et al., 2009). Indeed, CD4^{+} memory T cells from LPS-treated WT mice are FasL^{+} (Figure S2D). To test this, we injected LPS into WT and T-cell-deficient (*Tcrb*^{tm1Mom}-*Tcrd*^{tm1Mom}) mice (lacking αβ and γδ T cells). Upregulation of TACI, FasL, and Fas expression on MZ and T2-MZ B cells was normal in *Tcrb*^{tm1Mom}-*Tcrd*^{tm1Mom} mice as was LPS-induced MZ B cell apoptosis (Figure 4). This demonstrates that T cells are dispensable for this process and that MZ B cells may undergo fratricide or suicide within the MZ.

TACI Normally Represses the Expression of Caspase Inhibitors and FLIP (p43)

Fas-mediated apoptosis is negatively regulated by several anti-apoptotic factors including caspase inhibitors; this is relevant because the levels of activated caspases were significantly reduced in MZ B cells from LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figure 3B; $p < 0.01$). Therefore, we prepared B cell lysates from spleens of LPS- or PBS-treated WT or *Tnfrsf13b*$^{-/-}$ mice and analyzed the expression of regulators of the Fas signaling pathway by immunoblotting (Figures 5A and 5B). Interestingly, the basal levels of XIAP (a caspase inhibitor) and cIAP1/2, which are regulators of NF-κB signaling and necroptosis (reviewed in Han et al., 2011), were substantially greater in *Tnfrsf13b*$^{-/-}$ B cells compared to WT controls (Figures 5A and 5B). LPS treatment approximately doubled the levels of XIAP and cIAP1/2 in *Tnfrsf13b*$^{-/-}$ B cells compared to that of WT controls (Figures 5A and 5B). A similar result was obtained for cFLIP (Figure 5B). Bcl-2 and Bcl-x_L protein levels were unaffected by LPS in both WT and *Tnfrsf13b*$^{-/-}$ B cells (Figures 5B and S4A). Therefore, TACI signaling normally negatively regulates the expression of antiapoptotic factors cIAP1/2, XIAP, and cFLIP$_L$ in MZ B cells.

FLIP (p43) expression was substantially elevated in resting *Tnfrsf13b*$^{-/-}$ MZ B cells (Figures 5A and 5B). FLIP (p43) interacts directly or indirectly with other factors, such as TRAF-family adapters, RIP kinases, and possibly RAF-1 (Hyer et al., 2006). This pathway promotes cell survival and proliferation (Hyer et al., 2006) as well as the suppression of RIPK3-mediated necroptosis (Kaiser et al., 2011; Oberst et al., 2011). Consistent with this finding, proliferation (Ki-67 positivity) of MZ B cells from naive *Tnfrsf13b*$^{-/-}$ mice was significantly increased compared to WT MZ B cells (Figure 5C; $p < 0.01$).

TACI triggers the classical NF-κB pathway (He et al., 2010), which plays a role in regulating FasL and Fas expression (Kühnel et al., 2000). Electrophoretic mobility shift assays (EMSA)

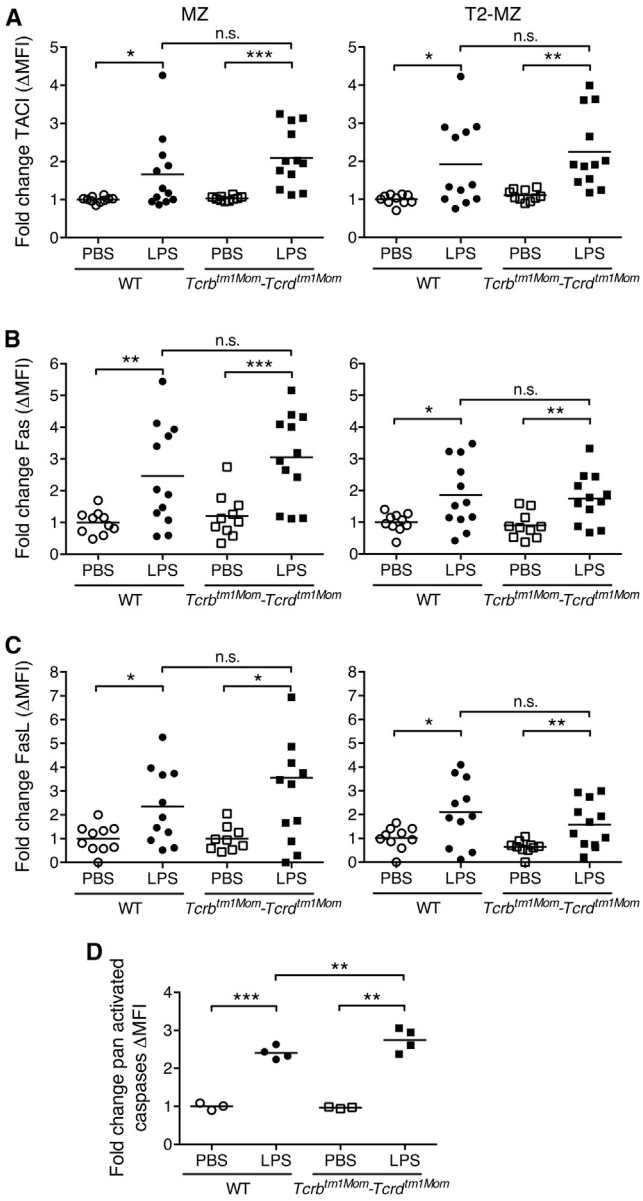

Figure 4. T Cells Are Dispensable for Upregulation of FasL, Fas, and TACI Expression and MZ B Cell Apoptosis upon LPS Activation In Vivo

(A–C) Surface expression of TACI (A), Fas (B), and FasL (C) on splenic MZ and T2-MZ B cells from WT or *Tcrb*^{tm1Mom}-*Tcrd*^{tm1Mom} mice 48 hr after injection with LPS or PBS.

(D) Apoptosis was determined by flow-cytometry-based CaspGLOW staining of MZ B cells from WT or *Tcrb*^{tm1Mom}-*Tcrd*^{tm1Mom} mice treated as in (A)–(C). A total of 10–12 mice per group were used; the data were consistent in two separate experiments with data from one experiment shown.

showed a delay in NF-κB activation in *Tnfrsf13b*$^{-/-}$ MZ B cells (Figure S4B), which may in part explain the delay and reduction in FasL and Fas upregulation on MZ B cells of LPS-treated *Tnfrsf13b*$^{-/-}$ mice (Figure S2C).

Collectively, these results show that TACI normally represses the expression of inhibitors of Fas-mediated apoptosis and promoters of MZ B cell proliferation.

DISCUSSION

MZ B cells have features of innate cells resulting from their ability to efficiently respond to innate activation, in particular TLR stimulation (reviewed in Martin and Kearney, 2002). MZ B cells offer a powerful first line of defense against pathogens entering the splenic MZ sinus. Their role as potent responders to microbial stimuli must be controlled to prevent adverse prolonged responses, which may cause collateral damage. In addition, the MZ B cell compartment contains self-reactive B cells (Chen et al., 1997), yet their activation by innate signals does not normally lead to autoimmunity, suggesting that MZ B cell activation is highly controlled.

Interestingly, MZ B cells express higher levels of TACI compared to Fo B cells and TACI expression is strongly augmented on MZ B cells after activation of various TLRs (Groom et al., 2007). Yet, such differential expression of TACI on MZ B cells has remained unexplained. Numbers of MZ B cells are abnormally increased in *Tnfrsf13b*$^{-/-}$ mice, and some *Tnfrsf13b*$^{-/-}$ mouse colonies (probably depending on genetic background and/or living conditions) eventually develop autoimmune disorders and lymphomas (Seshasayee et al., 2003; Yan et al., 2001). This suggests that TACI normally regulates B cell homeostasis. Moreover, TACI promotes the death of transformed B cells in vitro (Seshasayee et al., 2003), a result still lacking a molecular explanation. Pertinently, the cytoplasmic region of TACI does not contain a "death domain," which is required for apoptosis induction by classical "death receptors" from the TNF-R family (Strasser et al., 2009). Instead, TACI interacts with MyD88, which is critical for its role in supporting TI antibody responses, and TRAF6, which activates the classical NF-κB pathway (He et al., 2010). Therefore, nothing at the signaling level indicates a direct role for TACI in B cell apoptosis.

AICD driven by FasL and Fas is a mechanism involved in the termination of T and B cell responses and preservation of peripheral immunological tolerance (Strasser et al., 2009). Unlike TACI, the cytoplasmic region of Fas contains a death domain and activation of Fas triggers apoptosis in lymphocytes (Strasser et al., 2009). Fas expression on B cells is key for the maintenance of peripheral immune tolerance. Indeed, the specific loss of Fas expression in germinal center (GC) B cells was sufficient to trigger autoimmune disorders similar to that of *lpr* mice, which lack Fas in all tissues (Hao et al., 2008). However, what is unknown is whether expression of Fas on GC B cells by itself would be sufficient to restore immune tolerance in a Fas-deficient animal.

Little is known about the role of the FasL-Fas system in B cells activated by TI antigens. TLR4 is a known stimulator of Fas expression on B cells; what was unexpected was the role of the B cell survival factor BAFF (via TACI) in enhancing TLR4-induced Fas expression on B cells (Acosta-Rodríguez et al., 2007). Our study produced data that explain these findings, thereby uncovering a specific mechanism for innate activation-induced B cell death that has implications for peripheral B cell homeostasis and possibly prevention of autoimmunity. Our data show that TLR4 activation leads to two major outcomes: strong upregulation of TACI expression on MZ B cells and their precursors and strong FasL and Fas expression on these cells. Consistent with our findings, upregulation of FasL on

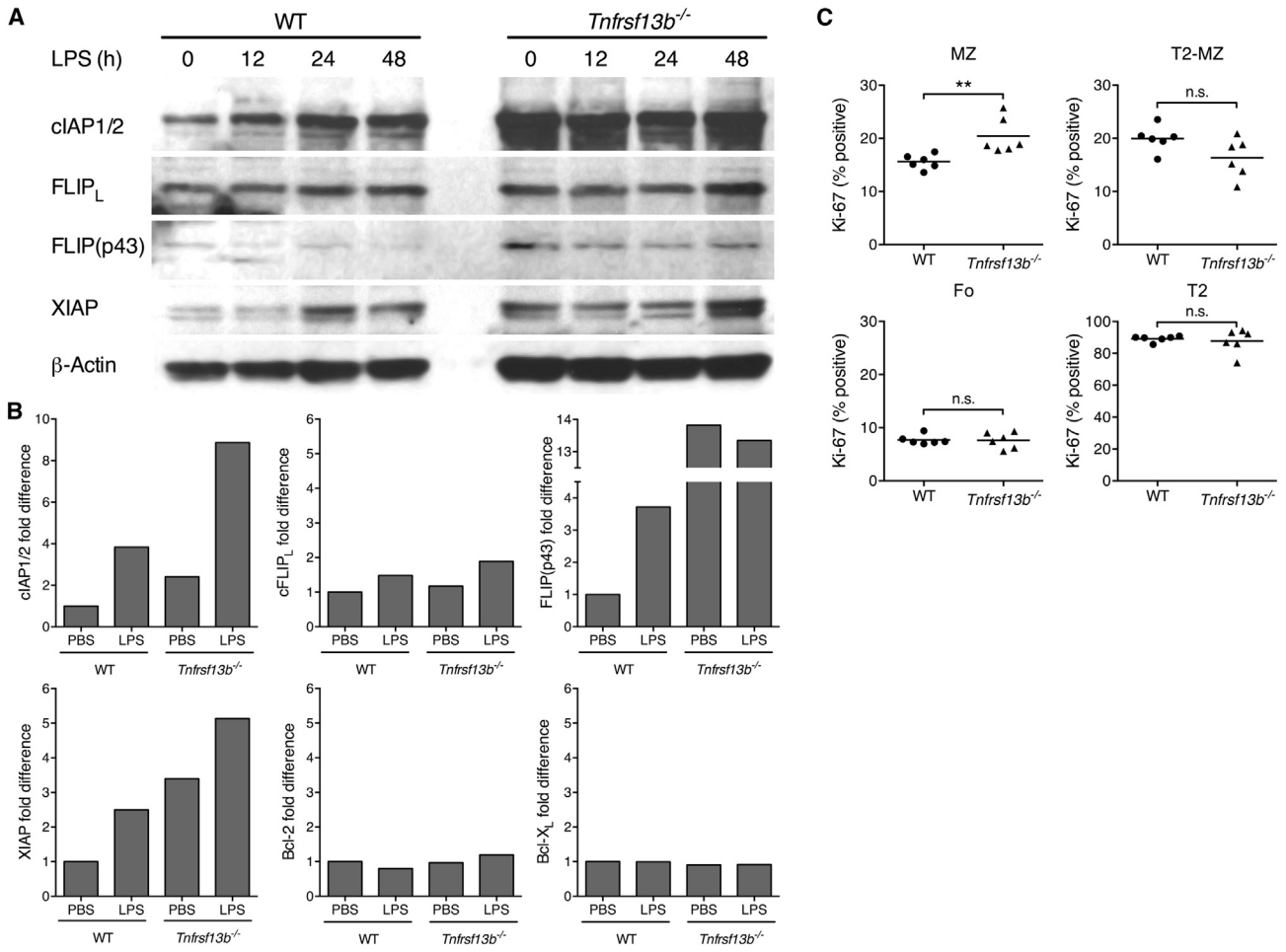

Figure 5. Overexpression of Antiapoptotic Factors in Splenic B Cells of LPS-Treated *Tnfrsf13b*$^{-/-}$ Mice

(A and B) Expression (A) and quantification (B) of the indicated antiapoptotic factors was examined by immunoblot analysis in lysates of splenic B cells isolated from PBS- or LPS-treated WT or *Tnfrsf13b*$^{-/-}$ mice and analyzed at intervals up to 48 hr as indicated in (A) or 48 hr in (B). Relative band intensities were adjusted to β-actin staining and displayed as the fold-change over the WT PBS-treated mean. Quantification in (B) was done on a repeated experiment as in (A).

(C) Percentages of Ki-67-positive cells within MZ, T2-MZ, Fo, and T2 splenic B cell subsets from WT and *Tnfrsf13b*$^{-/-}$ mice as determined by flow cytometry. Six mice per group were used. The data are representative of three independent experiments.

See also Figure S4.

LPS-activated B cells has been described (Hahne et al., 1996). Here, we reveal its specificity for MZ B cells and their precursors and demonstrate the essential role of TACI in this process.

The specificity of this mechanism for MZ B cells may relate to the fact that this population contains some self-reactive B cells (Chen et al., 1997) and/or that they express high levels of TACI in the resting state (Groom et al., 2007). High TACI expression suggests that this B cell subpopulation must be tightly controlled during nonspecific activation via TLR, presumably because this is a mode of activation unable to distinguish between safe and self-reactive B cells. Moreover, a significant proportion of MZ B cells undergo apoptosis after LPS injection into mice. This process required FasL expression but not T cells, suggesting that coexpression of FasL and Fas on TLR4-activated MZ B cells led to their fratricidal and/or suicidal death in situ, an idea supported by data showing that FasL on MZ B cells is a functional trigger of apoptosis. Some LPS-stimulated MZ B cells may migrate to different areas of the spleen (Cinamon et al., 2004) or differentiate into short-lived plasma cells (Genestier et al., 2007). Regardless, we demonstrated that once activated, these cells have initiated an apoptotic program. LPS can activate all MZ B cells regardless of the BCR specificity, but within the MZ B cell repertoire there may be some B cells with a BCR specific for LPS. Therefore, we cannot exclude the possibility that (tonic) BCR signaling in such B cells may protect them from apoptosis. The reality is that bacteria-derived LPS will activate TLR4 on a large number of B cells expressing a BCR specific for an antigen that will not be present at the time of LPS activation. The role of TACI in LPS-activated B cells is complex because TLR4 and TACI cooperate to drive B cell differentiation and Ig production, a process impaired in *Tnfrsf13b*$^{-/-}$ mice (Ozcan et al., 2009). Therefore, the impact of impaired apoptosis of *Tnfrsf13b*$^{-/-}$ MZ B cells after TLR4 activation is somewhat attenuated by the fact that these cells are also unable to differentiate into

plasma cells (Ozcan et al., 2009). However, prolonged survival of MZ B cells increases the risk of possible T cell help and T-cell-dependent antibody response, functional in *Tnfrsf13b*$^{-/-}$ mice. TACI is also an important regulator of Fo B cell homeostasis (Yan et al., 2001), yet unlike MZ B cells, Fo B cells express less TACI and TLR4-TACI signaling is less critical for Fas and FasL expression on these cells, suggesting a separate mechanism for regulating Fo B cell responses to LPS in vivo.

Importantly, we showed that the role of TACI is not solely limited to the control of FasL and Fas expression on TLR4-activated MZ B cells, a process that is dependent on the TLR4 signaling adaptor, TIR domain-containing adaptor protein (TIRAP, also known as Mal), and possibly reflected by the fact that greater proportions of MZ B cells were observed in *Tirap*$^{-/-}$ mice. Remarkably, in *Tnfrsf13b*$^{-/-}$ MZ B cells, expression of Fas signaling inhibitors and that of FLIP (p43), which has been implicated in a pathway promoting cell proliferation (Hyer et al., 2006; Kaiser et al., 2011; Oberst et al., 2011), are elevated. Accordingly, we observed abnormally increased proliferation of *Tnfrsf13b*$^{-/-}$ MZ B cells. High TACI expression on MZ B cells serves two purposes: it promotes the upregulation of both Fas and FasL and also represses the expression of proteins known to inhibit this apoptotic pathway. Together these processes promote the killing of innate-activated MZ B cells, regulate the response of these cells to innate activation, and possibly safeguard peripheral immune tolerance.

MZ B cells are located in the splenic MZ adjacent to the MZ sinus (Martin and Kearney, 2002), where the blood carries microbial agents. LPS on bacteria will activate (via TLR4) several MZ cell types, including MZ B cells, macrophages, and dendritic cells, which in response will produce BAFF (Mackay and Schneider, 2009). At the same time, LPS will trigger strong upregulation of TACI on MZ cells (Figure S5). Upon BAFF binding, two major functions of TACI are initiated. First, rapid and efficient TI antibody responses from MZ B cells (Oliver et al., 1997) will lead to the production of polyreactive antibodies (Chen et al., 1997) that constitute an efficient first line of defense by neutralizing bacteria (Martin and Kearney, 2002). The second process involves FasL-Fas-induced MZ B cell apoptosis preventing long-lasting activation, a potentially important safety feature, considering the autoreactive nature of some MZ B cells (Chen et al., 1997). This model is consistent with TLR4 being a trigger for autoimmune pathology in *lpr* mice, considering that *Tlr4*$^{-/-}$*Fas*$^{lpr/lpr}$ mice are protected from autoimmunity (Lartigue et al., 2009). We showed that responses to LPS in *Tnfrsf13b*$^{-/-}$ and *Fasl*$^{\Delta m/\Delta m}$ mice are prolonged and as such are dysregulated. Although in vitro work with isolated B cells suggested that the control of MZ B cells by TACI is B cell intrinsic, we cannot totally exclude that loss of TACI on other cell types such as MZ macrophages may have also contributed to help prolong responses to LPS in *Tnfrsf13b*$^{-/-}$ mice.

It is noteworthy that the autoreactive potential of MZ B cells stems from the largely polyreactive nature of their Ig (Li et al., 2002). This facilitates the highly desirable recognition of multiple conserved microbial epitopes during TI antibody responses. Nevertheless, although MZ B cells may be generated by design to produce an effective first line of defense against highly conserved microbial epitopes, their polyreactive nature requires that activation of these cells is tightly regulated.

We previously had little understanding of the regulation of B cells activated by nonspecific innate signals. Our work offers important clues and describes an effective new safety mechanism specific for the control of MZ B cell activation by TLR4, which we now define as innate activation-induced B cell death. Similar mechanisms might also exist for other B cell subsets and may involve other innate immune activation signals. The most exciting part of this work is the realization that innate signals from microbial agents, studied more often for their role in infectious diseases, also contribute to some important regulatory functions in the immune system, similar to the intimate relationship between the microbiota and immune tolerance (Ivanov and Littman, 2011; Maslowski and Mackay, 2011). The extent to which immune disorders arise from dysregulated T-cell-independent B cell responses remains to be explored but may offer promising new directions for understanding the basis of human immune diseases.

EXPERIMENTAL PROCEDURES

Mice

All mice are on a C57BL/6 background (12 generations of backcrossing) and have been described previously: *Tnfrsf13b*$^{-/-}$ mice (Seshasayee et al., 2003), BAFF Tg mice (Mackay et al., 1999), *Tirap*$^{-/-}$ mice (Greenhill et al., 2011), T-cell-deficient mice (homozygous *Tcrb*^{tm1Mom}-*Tcrd*^{tm1Mom} mice), *Fasl*$^{\Delta m/\Delta m}$ mice (lacking the membrane-bound form of FasL) (O' Reilly et al., 2009), and *Tlr4*$^{-/-}$ mice (Hoshino et al., 1999). C57BL/6 mice were used as WT controls in all experiments. Mice were housed under conventional barrier protection and handled with approval from institutional animal ethics committees, in compliance with the Australian code of practice for the care and use of animals for scientific purposes.

Reagents

Reagents for B cell activation in vitro, unless otherwise specified, included ultrapure grade LPS from *E. coli* K12 (20 μg/ml), CpG ODN1826 (0.5 μg/ml), non-CpG ODN1826 control (0.5 μg/ml), recombinant mouse APRIL (50 ng/ml) (Peprotech), BAFF (50 ng/ml) (Alexis Biochemicals), and CD40L (750 ng/ml) (R&D Systems). High-grade LPS was used, purified with enzymatic hydrolysis and phenol-TEA-DOC extraction to eliminate lipopeptide contaminants (TLR2 ligands). BAFF and APRIL were denatured at 95°C for 5 min. For LPS injections, a single, sublethal LPS dose of 1 mg/kg of body weight was administered intraperitoneally to mice. Unless otherwise specified, mice were sacrificed 48 hr after injection.

Flow Cytometry

Mouse splenocyte suspensions were treated with red blood cell lysis buffer (eBioscience) and washed with FACS buffer (PBS, 1% BSA, 2 mM EDTA). Lymphocytes were stained with fluorochrome-conjugated rat mAbs to mouse CD1d, CD21/35, CD23, B220, and IgM (BD Biosciences) and analyzed with LSR Fortessa, LSR II, FACS Canto II, or FACSCalibur flow cytometers (BD Biosciences) and FlowJo software (TreeStar). Transitional type 1 (T1), T2, T2-MZ, T2-Fo, T3, Fo, and MZ B cell subset were gated as previously detailed (Allman and Pillai, 2008). Phycoerythrin (R-PE)-conjugated mAbs to mouse Fas, FasL, TLR4 (eBioscience), Ki-67 (BD Biosciences), and TACI (R&D Systems and eBioscience) were used. Delta mean of fluorescence intensity (ΔMFI) values for Fas, FasL, TLR4, or TACI staining were calculated by subtracting the MFI of samples stained with fluorophore-matched Ig isotype-matched control mAbs (BD Biosciences) or fluorescence-minus-one controls.

B Cell Preparation and Culture

Pan-B cell purification was performed with Dynabeads Mouse CD43 Untouched B cell negative isolation kits (Invitrogen) according to the instructions. Purity of >95% was routinely achieved. Mouse B cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere, at 5 × 10^5 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum,

50 μM 2-merceptoethanol, L-glutamine (2 mM), 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco).

Analysis of B Cell Apoptosis

Apoptosis of B cells was measured with a flow-cytometry-based TUNEL assay with an ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon International) or FlowTACS Flow Cytometry Apoptosis Detection Kit (R&D Systems), which quantify DNA fragmentation. The ΔMFI was calculated by subtracting the MFI of TdT enzyme nontreated controls from the sample MFI (Figure S3A). Total activated caspases were measured with a flow-cytometry-based CaspGLOW Fluorescein Caspase Detection kit (BioVision, Life Research) according to the instructions. The ΔMFI was determined by subtracting the MFI of control cells, not stained with FITC-VAD-FMK.

Fluorescence Microscopy

Spleen cryosections (7 μm) were cut onto SuperFrost Plus slides (Menzel-Glaser), acetone fixed for 5 min, and air-dried at RT. Sections were rehydrated in PBS and nuclear membranes mildly permeabilized with 1% Triton X-100, 1% (w/v) sodium acetate in PBS for 2 min at RT. Sections were washed in PBS and TUNEL staining was carried out with a TdT reaction mixture (6 μM fluorescein-12-dUTP [Roche], 1 mM $CoCl_2$ [Sigma], 60 μM dATP, 25 U TdT enzyme, and reaction buffer [Promega] in 50 μl) for 1 hr at 37°C. Slides were washed and blocked with normal rat serum and stained with polyclonal rat anti-mouse IgM-Cy5 antibodies (Southern Biotech) and rat anti-mouse IgD-PE mAbs (eBioscience). After washing, slides were mounted in Fluoromount aqueous mounting medium (Sigma) and viewed under a BX61 fluorescence microscope (Olympus). An array of all images was uniformly analyzed by ImageJ software (v.1.43u, National Institutes of Health) with the investigators blind to the treatment groups. MZ B cells were identified as $IgM^{hi}IgD^{lo}$ cells surrounding the $IgM^{hi}IgD^{lo}$ B cell follicles. Numbers of TUNEL-positive B cells per mm^2 of MZ were calculated.

Real-Time PCR

RNA from cells was extracted with an RNeasy Mini Kit (QIAGEN) and 1.5 μg of RNA was used for reverse transcription to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Predesigned TaqMan assays for mouse *Rn18S* (Mm03928990_g1), *Fas* (Mm00433237_m1), *Fasl* (Mm00438864_m1), *Tnfsf13* (APRIL) (Mm03809849_s1), and *Tnfsf13b* (BAFF) (Mm00446347_m1) mRNA were used. RT-PCR was performed with an ABI 7900 HT sequence Detection System (Applied Biosystems). Each sample was assayed in triplicate. Target mRNA was normalized to 18S rRNA as the endogenous control and the relative fold difference in expression was calculated via the $2^{-\Delta\Delta Ct}$ method.

Immunoblot Analysis

B cell lysates were prepared with RIPA buffer with Halt Protease Inhibitor Cocktail (Pierce) and protein concentration determined by BCA Protein Assay (Pierce). Proteins were resolved on Criterion precast 10%, 12.5%, or 15% SDS-PAGE gels (Bio-Rad) and then transferred to PVDF membranes. These were incubated first in blocking buffer (0.2% nonfat milk powder in TBS), then with polyclonal rabbit antibodies against mouse β-Actin, cFLIP, XIAP (Cell Signaling), Bcl-2 (Pro-Sci), Bcl-X_L (Alexis Biochemicals), or cIAP-1/2 (R&D Systems), followed by goat anti-rabbit Ig antibodies coupled to AP (Invitrogen) and developed with the Immun-Star AP chemiluminescent protein detection system (Bio-Rad) according to the instructions.

Immunizations

Mice were injected once i.p. with LPS coupled to (4-hydroxy-3-nitrophenyl) acetyl (NP), i.e., $NP_{0.5}$-LPS (Biosearch Technologies), 1 mg/kg. Sera were collected from the tail vein 2 days prior (naive sera) and 7 and 14 days after NP-LPS injection. As a positive control for an anti-NP T-cell-dependent humoral response detectable at day 14 after immunization, mice were injected i.p. with 50 μg of NP-KLH (Biosearch Technologies) precipitated in alum.

ELISA

384-well high-binding assay plates (Corning) were coated with NP_{16}-BSA (Biosearch Technologies) at 2 μg/ml in 50 mM sodium carbonate buffer overnight at 4°C. Plates were blocked with 4% BSA in PBS and incubated with 2-fold serial dilutions of serum samples for 1 hr at 37°C. NP-binding antibodies were detected with anti-mouse isotype-specific alkaline phosphatase-labeled antibodies (Southern Biotechnology) and revealed by *P*-NP phosphate substrate (Sigma). Titer was defined as the serum dilution giving an OD_{405nm} four times higher than background (where 1 = 1/200).

Statistical Analysis

Student's t tests (normally distributed data) or Mann-Whitney tests (abnormal distribution) were used where appropriate. Error bars indicate standard error of the mean (SEM). Statistically significant differences are shown for *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s. indicates not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.05.019.

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