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TRIF Signaling Is Essential for TLR4-Driven IgE Class Switching

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The TLR4 ligand LPS causes mouse B cells to undergo IgE and IgG1 isotype switching in the presence of IL-4. TLR4 activates two signaling pathways mediated by the adaptor molecules MyD88 and Toll/IL-IR domain-containing adapter-inducing IFN- β (TRIF)-related adaptor molecule (TRAM), which recruits TRIF. Following stimulation with LPS plus IL-4, *Tram*^{-/-} and *Trif*^{-/-} B cells completely failed to express C ϵ germline transcripts (GLT) and secrete IgE. In contrast, *Myd88*^{-/-} B cells had normal expression of C ϵ GLT but reduced IgE secretion in response to LPS plus IL-4. Following LPS plus IL-4 stimulation, C γ 1 GLT expression was modestly reduced in *Tram*^{-/-} and *Trif*^{-/-} B cells, whereas *Aicda* expression and IgG1 secretion were reduced in *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} B cells. B cells from all strains secreted normal amounts of IgE and IgG1 in response to anti-CD40 plus IL-4. Following stimulation with LPS plus IL-4, *Trif*^{-/-} B cells failed to sustain NF- κ B p65 nuclear translocation beyond 3 h and had reduced binding of p65 to the I ϵ promoter. Addition of the NF- κ B inhibitor, JSH-23, to wild-type B cells 15 h after LPS plus IL-4 stimulation selectively blocked C ϵ GLT expression and IgE secretion but had little effect on C γ 1 GLT expression and IgG secretion. These results indicate that sustained activation of NF- κ B driven by TRIF is essential for LPS plus IL-4-driven activation of the C ϵ locus and class switching to IgE. *The Journal of Immunology*, 2014, 192: 000–000.

Class switch recombination (CSR) allows a single B cell clone to produce Abs with the same fine specificity but different effector functions determined by the H chain isotype. The specificity of the H chain C region genes targeted for recombination depends on the cytokine signal received by the B cell. CSR involves activation of IgH germline transcripts (GLT) and the gene for activation-induced cytidine deaminase (AICDA), followed by deletional switch recombination, and expression of mature transcripts of the switched Ig isotype (1).

Stimulation of primary murine B cells with the TLR4 ligand LPS in the presence of IL-4 induces class switching to IgE and IgG1. Both signals synergize to induce optimal transcription of the ϵ GLT (2, 3), the γ 1 GLT (4), and *Aicda* loci (5), causing CSR to IgE and IgG1. TLR4 activates two signaling pathways mediated by the adaptor molecules, MyD88 (6) and the Toll/IL-IR domain-containing

adaptor-inducing IFN- β (TRIF)-related adaptor molecule (TRAM), which recruits TRIF (7, 8). Following ligand binding, TLR4, which is expressed on the cell membrane, recruits MyD88, resulting in rapid activation of NF- κ B (9, 10). After its ligand-driven internalization into endosomes, TLR4 recruits TRAM, which in turn recruits TRIF. TRIF-mediated activation of NF- κ B follows a late course, due to the time lag involved in the internalization of TLR4 into endosomes (7, 10).

Binding of IL-4 to its receptor recruits the Janus kinases, Jak1 and Jak3, which cause the phosphorylation and nuclear translocation of STAT6 (11, 12). STAT6 and NF- κ B bind to the C ϵ and C γ 1 promoters driving the expression of C ϵ and C γ 1 GLT (13, 14). STAT6 and NF- κ B also bind to the *Aicda* promoter (5). AICDA acts on switch regions in the C μ , C γ 1, and C ϵ genes to initiate deletional switch recombination and isotype switching to IgG1 and IgE (15).

The roles of the MyD88 and TRAM/TRIF pathways in TLR4-driven isotype switching have not been explored. In this study, we provide evidence that the TRAM/TRIF pathway is essential for IgE isotype switching of mouse B cells stimulated with LPS plus IL-4 due to its ability to cause the late phase of activation of NF- κ B, which is critical for the activation of the C ϵ locus in these cells.

Materials and Methods

Mice

Tram^{-/-} and *Myd88*^{-/-} mice were previously described (16). They were bred onto a BALB/c background and housed at the animal facility of Boston Children's Hospital. *Trif*^{-/-} and *Trif*^{-/-}/*Myd88*^{-/-} mice were previously described (16). These mice and genetically matched wild-type (WT) controls on Sv129/C57BL6 background were housed at the animal facility of the University of Massachusetts Medical School (Worcester, MA). All mice were bred and kept in specific pathogen-free conditions. All procedures performed on the animals were approved by the Animal Care and Use Committee of Boston Children's Hospital.

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Abbreviations used in this article: AICDA, activation-induced cytidine deaminase; ChIP, chromatin immunoprecipitation; CSR, class switch recombination; GLT, germline transcript; PARP, poly(ADP-ribose) polymerase; TRAM, Toll/IL-IR domain-containing adaptor-inducing IFN- β -related adaptor molecule; TRIF, Toll/IL-IR domain-containing adaptor-inducing IFN- β ; WT, wild-type.

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Ig production and proliferation by B cells

Naive B cells were negatively sorted from mouse splenocytes using biotin-conjugated mAbs to CD11b, CD43, Thy1.2, CD138, IgG1, IgG2a/b, IgG3, IgA, and IgE along with streptavidin magnetic beads (Dynabeads M-280; Invitrogen). Total B cells were purified by negative selection using microbeads conjugated to Abs against CD43 (Miltenyi Biotec). FACS analysis revealed that >98% of magnetically sorted cells were B220⁺ and CD3⁻. B cells were cultured at a concentration of 1×10^6 /ml in RPMI 1640 containing 10% FCS, L-glutamine, penicillin/streptomycin, and 50 mM 2-ME. B cells were stimulated with LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) at 10 µg/ml or anti-CD40 (BD Pharmingen) at 100 ng/ml in the presence of 50 ng/ml IL-4 (R&D Systems). The NF-κB inhibitor JSH-23 (Santa Cruz Biotechnology) was added at 20 µM to cultures at various time points. After 6 d in culture, supernatants were assayed for IgG1 and IgE by ELISA, as previously described (17). Baseline Ig secretion from unstimulated cultures (accounting for <1% of induced values) was subtracted, and the final results were calculated as a percentage of Ig secreted by WT B cells. For each individual experiment, B cells from three WT control mice and three mutant mice from the various strains were used. We divided each individual WT and mutant strain value by the mean of the three WT values (defined as 100%) and expressed it as a percentage of the mean. This allowed us to calculate a SD for both the WT and mutant strain samples. To assay proliferation, cultures were pulsed at 72 h with 1 µCi ³H-thymidine and then harvested after 16 h of additional culture. To examine the number of cell divisions, B cells were loaded with 1 µM Cell Tracker Violet (Life Technologies) prior to stimulation with LPS plus IL-4 or anti-CD40 plus IL-4, as described above. After 6 d in culture, cells were stained with anti-B220 FITC and 7-aminoactinomycin D (eBioscience). B220⁺7-aminoactinomycin D⁻ cells were gated on, and their Cell Tracker Violet profile was analyzed.

B cell survival assay

B cells were stained on day 3 after stimulation with annexin V-FITC and propidium iodide (Bio Vision) and analyzed by FACS.

RT-PCR analysis

RNA was extracted from 4-d cultures using TRIzol (Invitrogen) and reverse transcribed by Superscript II RT (Invitrogen). PCR primers and conditions used to detect *Iε-Cε*, *Iμ-Cε*, *Iγ1-Cγ1*, *Iμ-Cγ1*, *AICDA*, and β_2 microglobulin were previously described (17). To ensure adequate detection, the cycle number for the *Iε-Cε* and *Iμ-Cε* transcripts was increased to 42, whereas 30 cycles were used to amplify *Iγ1-Cγ1*, *Iμ-Cγ1*, *AICDA*, and β_2 microglobulin. PCR conditions were tested with three dilutions of cDNA to ensure that the products measured were in the linear range (Supplemental Fig. 1A). Amplified products were separated on agarose gels and stained with ethidium bromide.

To examine expression of *Bcl2*, *Id2*, and *PU.1*, B cells were stimulated with LPS plus IL-4. RNA was extracted from cultures at various time points and reverse transcribed. TaqMan primers for *Bcl2*, *Id2*, and *PU.1* were obtained from Applied Biosystems, and real-time PCR reactions were run on cDNA using the ABI Prism 7300 (Applied Biosystems). Relative expression was based on β_2 microglobulin expression for each sample.

Nuclear translocation of the NF-κB p65 subunit and STAT6

Nuclear extracts were prepared using a nuclear extract kit (Active Motif). Protein content was measured using Bradford reagent (Pierce, Rockford, IL). Immunoblotting was performed using Abs to p65, STAT6, and poly (ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology). Nuclear translocation of p65 and STAT6 was quantified using the ImageJ program from the National Institutes of Health and normalized to the nucleus-specific protein PARP.

Chromatin immunoprecipitation assay

Total B cells were purified from splenocytes of *Trif*^{-/-} mice and genetically matched WT controls, stimulated with LPS plus IL-4 or anti-CD40 plus IL-4, washed, and then treated with 1% formaldehyde. The B cells were processed further using the provided protocol from the chromatin immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnologies) with the following modification: 125 nM glycine was added for 5 min to terminate cross-linking, and then the cells were washed with PBS and resuspended in the ChIP Assay Kit lysis buffer. Cells were sonicated (Misonix Sonicators) to shear DNA and obtain chromatin with lengths between 400 and 1500 bp. Sonicated cell supernatants were diluted 10-fold in ChIP dilution buffer. A

total of 5 µg p65 (rabbit polyclonal Ab; Abcam) or rabbit IgG isotype control (Imgenex) Abs was added to the chromatin solution and incubated overnight at 4°C. Magnetic protein G-coated beads were then added. Following incubation for 1 h, the beads were washed and DNA was eluted. The eluted DNA was purified by phenol/chloroform extraction and ethanol precipitation. Real-time PCR of the *IgE* promoter was performed on 10-fold DNA dilutions of immunoprecipitates using the following primers: forward, 5'-AAGGGAACCTTCCAAGGCTGCTAAG-3'; reverse, 5'-CCATTAAATTCATGCCAGATGGTAG-3'; and FAM-probe, 5'-TTTAGCTGAGGGCACTGAGGCAGA-3'. RT-PCR was also performed using mouse β -globin primers forward, 5'-GACGAGTCACAGAGGACTTCCTGG-3'; reverse, 5'-CTGTGAGGAGCAGAGAGGGCAAG-3'; and FAM-probe, 5'-ATGGATTGCAGAGCCCAGAGGGAGCCAT-3' to calculate the non-specific background DNA binding. The primers were obtained from Integrated DNA Technologies. The $\Delta\Delta C_q$ method was used to generate relative expression values (18).

Statistics

The *p* values were calculated using the paired *t* test with PRISM software (Prism Software).

Results

The TRAM/TRIF pathway is essential for LPS- and IL-4-driven IgE production

We compared the response of negatively sorted naive B cells (95% IgM⁺ IgD⁺ cells) from *Tram*^{-/-}, *Myd88*^{-/-}, and WT BALB/c controls stimulated with LPS plus IL-4. *Tram*^{-/-} B cells failed to secrete detectable amounts of IgE in response to stimulation with LPS plus IL-4 (Fig. 1A). Similar data were obtained when *Trif*^{-/-} B cells were compared with B cells from genetically matched Sv129/C57BL6 WT controls (Fig. 1A). The failure of *Tram*^{-/-} and *Trif*^{-/-} B cells to undergo IgE isotype switching in response to LPS plus IL-4 did not represent a global defect in IgE isotype switching or in responsiveness to IL-4, because the same B cells produced normal amounts of IgE when stimulated with anti-CD40 plus IL-4 (Fig. 1B). *Myd88*^{-/-} B cells secreted reduced amounts of IgE compared with WT B cells in response to LPS plus IL-4, but normal amounts in response to anti-CD40 plus IL-4 (Fig. 1A, 1B).

In contrast to the absence of detectable IgE secretion after LPS plus IL-4 stimulation, IgG1 secretion by B cells from *Tram*^{-/-} and *Trif*^{-/-} mice was only partially reduced compared with B cells from WT controls (Fig. 1C). IgG1 secretion by LPS plus IL-4-stimulated B cells from *Myd88*^{-/-} mice was also partially reduced (Fig. 1C). B cells from all three knockout strains secreted comparable amounts of IgG1 in response to anti-CD40 plus IL-4 (Fig. 1D). Because IgG1 production following LPS plus IL-4 stimulation was partially preserved in *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} B cells, we investigated whether pathways other than TRIF and MyD88 were involved in LPS plus IL-4-driven isotype switching. *Myd88*^{-/-}/*Trif*^{-/-} B cells completely failed to secrete IgE and IgG1 in response to LPS plus IL-4 (Supplemental Fig. 2A). These results indicate that the TRAM/TRIF pathway plays an essential and nonredundant role in TLR4-driven IgE switching, and the TRAM/TRIF and MyD88 pathways play important but partially redundant roles in TLR4-driven IgG1 switching.

The TRAM/TRIF and MyD88 pathways both contribute to B cell proliferation and survival in response to LPS plus IL-4

Isotype switching is linked to proliferation (19). *Tram*^{-/-} and *Myd88*^{-/-} B cells showed a significant reduction in proliferation and underwent fewer cell divisions in response to LPS plus IL-4 relative to WT B cells. In contrast, they proliferated and divided normally in response to anti-CD40 plus IL-4 (Fig. 2A, 2B). Similar results were obtained with *Trif*^{-/-} B cells (Supplemental

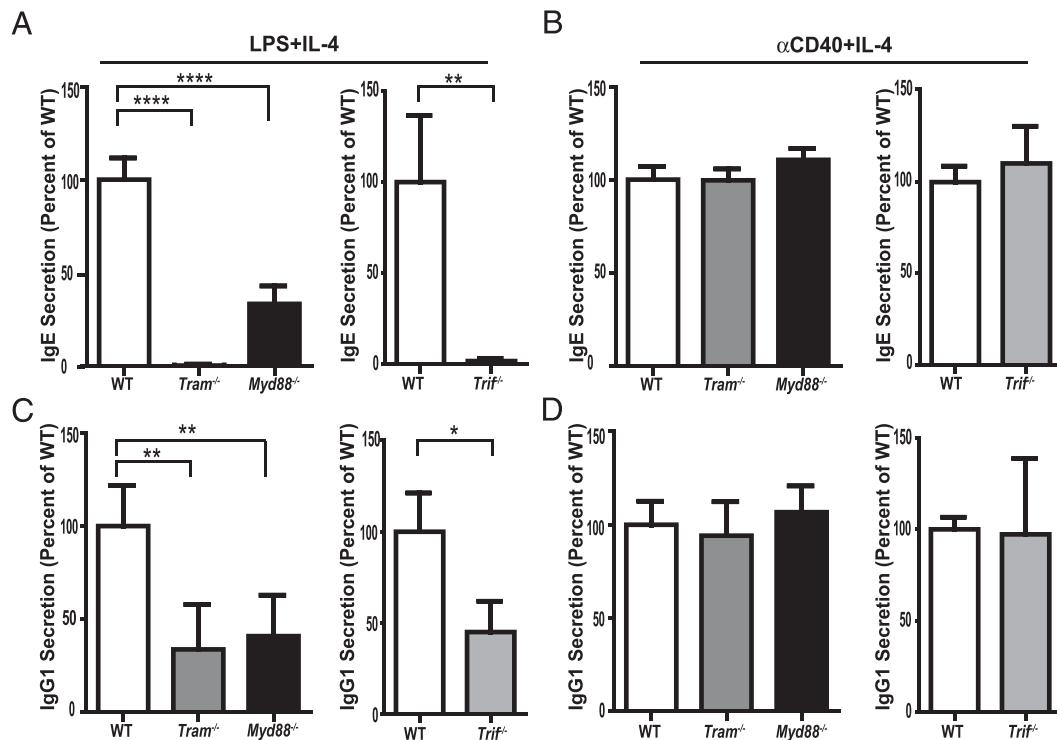


FIGURE 1. IgE and IgG1 secretion by *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} B cells in response to LPS plus IL-4 and anti-CD40 plus IL-4. (**A** and **B**) IgE secretion by naive B cells from *Tram*^{-/-}, *Myd88*^{-/-}, and WT mice (left panels) and from *Trif*^{-/-} and WT controls (right panels) after 6 d in culture. (**C** and **D**) IgG1 secretion by naive B cells from *Tram*^{-/-}, *Myd88*^{-/-}, and WT mice (left panels) and *Trif*^{-/-} mice (right panels) along with their WT controls after 6 d in culture. Results represent the mean $\pm$ SD of secreted Ig normalized to WT control from five independent experiments. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

Fig. 3). *Myd88*^{-/-}/*Trif*^{-/-} B cells completely failed to proliferate in response to LPS plus IL-4 (Supplemental Fig. 2B). Whereas *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} B cells had a comparable partial defect in proliferation in response to LPS plus IL-4, *Tram*^{-/-} and *Trif*^{-/-} B cells have a complete inability to produce IgE, whereas *Myd88*^{-/-} B cells had only a partial reduction in their capacity to produce IgE. This suggests that impaired proliferation is not the sole reason for the failure of *Tram*^{-/-} and *Trif*^{-/-} B cells to produce IgE in response to LPS plus IL-4.

When assessed by annexin V and propidium iodide staining, *Tram*^{-/-} and *Myd88*^{-/-} B cells had reduced survival by day 4 of culture with LPS plus IL-4. In contrast, after 4 d of anti-CD40 plus IL-4 stimulation, B cell survival was comparable among *Tram*^{-/-}, *Myd88*^{-/-}, and WT B cells (Fig. 2C). Similar results were observed with *Trif*^{-/-} B cells (data not shown). These results indicate that the TRAM/TRIF and MyD88 pathways play essential but partially redundant roles in TLR4-driven B cell survival and proliferation.

The TRAM/TRIF pathway is essential for LPS plus IL-4 induction of C ϵ GLT expression

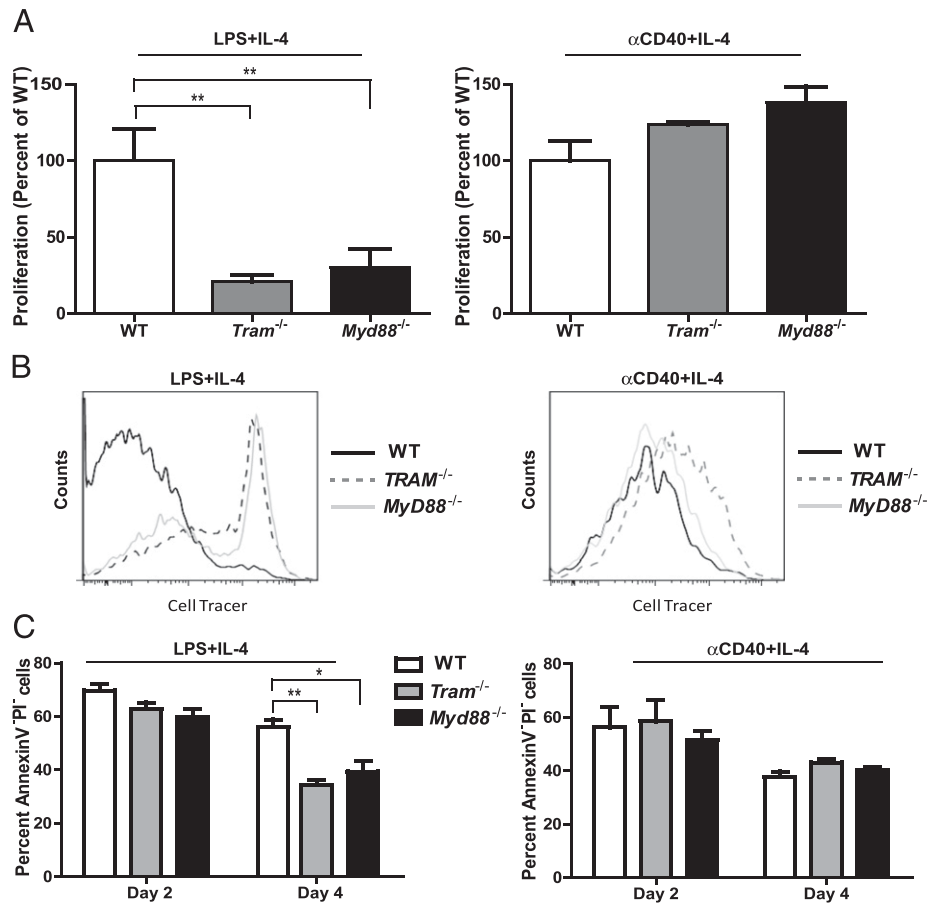
CSR requires expression of GLT and *Aicda*, followed by deletional switch recombination and expression of postswitch mature I μ -C μ transcripts (1). In response to LPS plus IL-4, WT B cells begin transcribing the C ϵ GLT and the C γ 1 GLT by 24 h (2, 3). C ϵ GLT transcription by WT B cells was weakly detected on days 1–3, and it becomes robust on day 4 following LPS plus IL-4 stimulation. In contrast, C γ 1 GLT transcription was readily detectable by day 1 and became robust and sustained by day 2 (Supplemental Fig. 1B). LPS plus IL-4 failed to drive expression of C ϵ GLT and I μ -C ϵ mature transcripts in *Tram*^{-/-} and *Trif*^{-/-} B cells (Fig. 3A, Supplemental Fig. 1). In contrast, following stimulation with anti-CD40 plus IL-4, *Tram*^{-/-} B cells expressed C ϵ GLT and I μ -C ϵ

mature transcripts in amounts comparable to WT B cells (Fig. 3B). Because expression of C ϵ GLT is critical for CSR to IgE (20), the inability to activate transcription at the C ϵ locus underlies the failure of *Tram*^{-/-} and *Trif*^{-/-} B cells to undergo IgE isotype switching in response to stimulation with LPS plus IL-4. We were unable to detect C ϵ GLT expression or IgE secretion in WT B cells stimulated with the TLR2 ligand Pam₃CSK₄, which signals exclusively via MyD88 (data not shown), further supporting the conclusion that the TRAM/TRIF pathway is strictly necessary to drive C ϵ GLT expression in B cells stimulated with LPS plus IL-4.

LPS plus IL-4-stimulated *Tram*^{-/-} and *Trif*^{-/-} B cells expressed a modest decrease in the amount of C γ 1 GLTs on day 4 compared with WT B cells (Fig. 3, Supplemental Fig. 1). The levels of *Aicda* mRNA and of I μ -C γ 1 mature transcripts were reduced in LPS plus IL-4-stimulated *Tram*^{-/-} and *Trif*^{-/-} B cells (Fig. 3A, Supplemental Fig. 1). Following anti-CD40 plus IL-4 stimulation, *Tram*^{-/-} B cells had normal to slightly decreased expression of C γ 1 GLT and normal expression of *Aicda* and I μ -C γ 1 mature transcripts (Fig. 3B). These results suggest that the TRAM/TRIF pathway is important for optimal *Aicda* expression following LPS plus IL-4 stimulation and that the reduced ability to express *Aicda* underlies the lower expression of I μ -C γ 1 mature transcripts and decreased production of IgG1 by *Tram*^{-/-} and *Trif*^{-/-} B cells stimulated with LPS plus IL-4. The observation that LPS plus IL-4-stimulated *Tram*^{-/-} and *Trif*^{-/-} B cells had only a modest reduction in C γ 1 GLT expression, but no detectable C ϵ GLT, following LPS plus IL-4 stimulation, demonstrates the specific requirement of the TRIF/TRAM pathway in the activation of C ϵ transcription by LPS plus IL-4.

Myd88^{-/-} B cells expressed normal amounts of C ϵ and C γ 1 GLT following LPS plus IL-4 stimulation (Fig. 3A), indicating that MyD88 is not essential for the activation of the C ϵ and C γ 1

FIGURE 2. Proliferation and viability of *Tram*^{-/-}, *Trif*^{-/-}, *Myd88*^{-/-}, and WT B cells stimulated with LPS plus IL-4 and anti-CD40 plus IL-4. (**A** and **B**) Proliferation was assessed by ³H-thymidine incorporation into DNA after 3 d in culture (A). Proliferation was also measured using dilution of a cell-labeling dye (Cell Trace Violet) after 6 d in culture (B). (**C**) Survival was measured by flow cytometric analysis of annexin V⁻PI⁻ *Tram*^{-/-}, *Myd88*^{-/-}, and WT B cells. Columns and bars represent mean ± SD normalized to WT of three independent experiments. **p* < 0.05, ***p* < 0.01.



loci by LPS plus IL-4. However, following LPS plus IL-4 stimulation, *Myd88*^{-/-} B cells transcribed reduced amounts of *Aicda* and had decreased expression of mature Iμ-Cε and Iμ-Cγ1 transcripts consistent with their reduced ability to secrete IgE and IgG1 (Figs. 1A, 1C, 3A). *Myd88*^{-/-} B cells had normal expression of *Aicda* following anti-CD40 plus IL-4 stimulation. Given that Cε and Cγ1 GLT levels were normal in LPS plus IL-4-stimulated *Myd88*^{-/-} B cells, the decreased expression of mature Iμ-Cε and Iμ-Cγ1 transcripts and IgE and IgG secretion by these cells is most likely a result of decreased *Aicda* expression. The decreased *Aicda* expression in *Myd88*^{-/-} as well as *Tram*^{-/-} and *Trif*^{-/-} B cells may reflect their decreased viability and proliferation following stimulation with LPS plus IL-4. Following LPS plus IL-4 stimulation, expression of Cε and Cγ1 GLT, *Aicda*, and mature Iμ-Cε and Iμ-Cγ1 transcripts was abolished in *Myd88*^{-/-}/*Trif*^{-/-} B cells, but they expressed all of these transcripts normally with anti-CD40 plus IL-4 stimulation (data not shown).

TRIF is important for sustained NF-κB activation in LPS plus IL-4-stimulated B cells

Cε GLT is induced by NF-κB and STAT6, which are activated, respectively, by LPS and IL-4. Both of these transcription factors reside in the cytoplasm of resting cells and translocate to the nucleus following stimulation. LPS causes a MyD88-dependent early phase and a TRAM/TRIF-dependent late phase of NF-κB activation in fibroblasts and macrophages (7, 21). We performed a kinetic analysis of nuclear translocation in primary B cells over the 15-h period following LPS plus IL-4 stimulation. Nuclear translocation of p65 in WT B cells was noted at 30 min, peaked at 3 h, and persisted at a lower level through 15 h (Fig. 4A, 4B). *Trif*^{-/-} B cells had a well-preserved early phase of p65 nuclear translocation compared with WT B cells with a similar signal at 30 min and 1 h. In contrast, the late phase of p65 nuclear translocation (3, 6, 15 h) was decreased in comparison with WT B cells (Fig. 4A, 4B). ChIP assays performed 15 h poststimulation with LPS plus IL-4 demonstrated reduced binding of p65 to the Iε

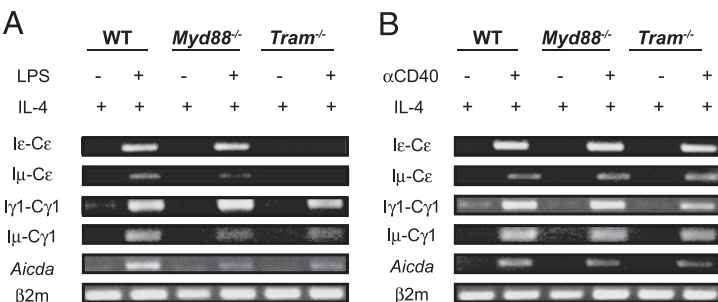


FIGURE 3. Molecular events involved in IgE and IgG1 CSR in *Tram*^{-/-}, *Myd88*^{-/-}, and WT B cells stimulated with LPS plus IL-4 and anti-CD40 plus IL-4. Expression of Cε GLT (Iε-Cε), mature Cε transcripts (Iμ-Cε), Cγ1 GLT (Iγ1-Cγ1), mature Cγ1 transcripts (Iμ-Cγ1), and *Aicda* mRNA from *Tram*^{-/-}, *Myd88*^{-/-}, and WT B cells in response to 4 d of stimulation with LPS plus IL-4 (**A**) and anti-CD40 plus IL-4 (**B**). Similar results were obtained in three independent experiments.

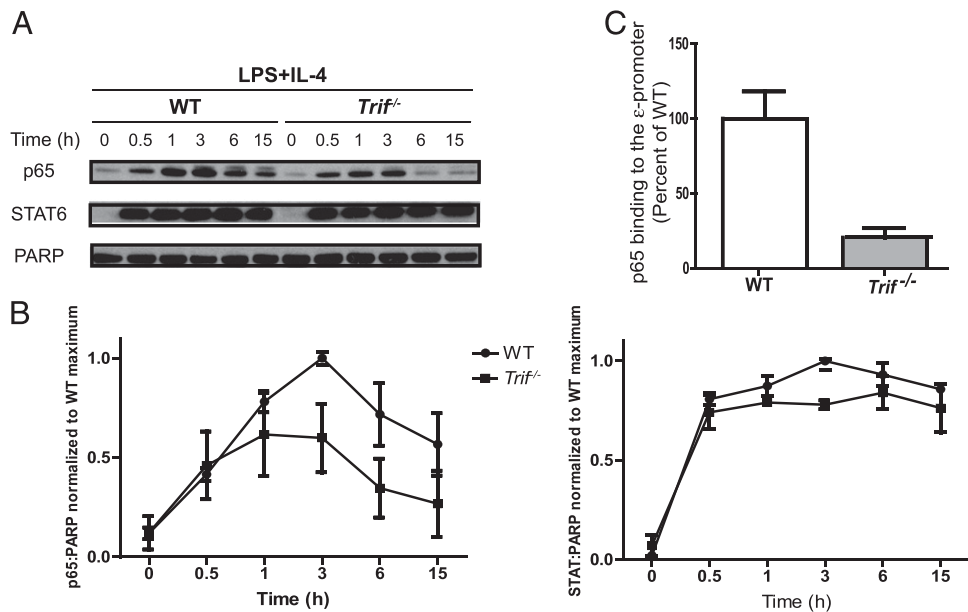


FIGURE 4. Nuclear translocation of p65 and STAT6 in *Trif*^{-/-}, *Myd88*^{-/-}, and WT B cells stimulated with LPS plus IL-4 and anti-CD40 plus IL-4. **(A)** Immunoblotting of nuclear extracts from *Trif*^{-/-} and WT naive B cells for the p65 subunit of NF-κB and STAT6 following stimulation with LPS plus IL-4 for the indicated times. PARP was used as a loading control. Results are representative of three independent experiments. **(B)** Quantitative analysis of the nuclear translocation of the p65 and STAT6 in *Trif*^{-/-} and WT B cells following stimulation with LPS plus IL-4. Values represent the mean ratio of the intensity of the p65:PARP and STAT6:PARP bands normalized to the WT maximum as determined by densitometry scanning. **(C)** ChIP assay for binding of p65 to the Iε promoter. Results are expressed as the ratio p65 bound DNA in *Trif*^{-/-} B cells compared with WT B cells. Values represent mean ± SD of three independent experiments in (B) and two independent experiments in (C).

promoter in *Trif*^{-/-} B cells compared with WT B cells (Fig. 4C). The nuclear translocation defect in LPS plus IL-4-stimulated *Trif*^{-/-} B cells was selective for p65 following stimulation with LPS plus IL-4, because STAT6 nuclear translocation was preserved over 15 h in *Trif*^{-/-} B cells (Fig. 4A, 4B).

The finding that both NF-κB and STAT6 activation are sustained in normal B cells undergoing CSR to IgE is in agreement with the observation that robust transcription at the Cε occurs relatively late after stimulation with LPS plus IL-4 (Supplemental Fig. 1B) (22). It suggests that a lag period poststimulation is required for the Cε locus to become susceptible to activation by these two transcription factors. This lag time could be necessary to down-regulate the expression of transcription factors that repress Cε germline transcription, such as Bcl6, Id2, and PU.1 (23, 24). *Trif*^{-/-} and *Myd88*^{-/-} B cells normally downregulated the expression of these transcription factors following LPS plus IL-4 stimulation (Supplemental Fig. 4), excluding a role for these factors in the failure of LPS plus IL-4 to activate the Cε locus in *Trif*^{-/-} B cells.

Late inhibition of NF-κB partially inhibits Cε germline transcription and IgE isotype switching in WT LPS plus IL-4-stimulated B cells

We investigated whether IgE isotype switching was selectively dependent on sustained NF-κB activation. Addition of the NF-κB inhibitor JSH-23 (25) to WT B cells at 0, 7.5, and 15 h poststim-

ulation with LPS plus IL-4 caused a drastic reduction in IgE secretion. Similarly, early addition of JSH-23 at 0 h led to a dramatic decrease in IgG1 secretion (Fig. 5A). However, IgG1 secretion rebounded and was normal when JSH-23 was added later at 7.5 or 15 h. Likewise, JSH-23 affected B cell proliferation when added at earlier time points, but much less so when added at 15 h (Fig. 5B). The effects on IgG1 secretion correlate with the decreased proliferation and *Aicda* mRNA expression seen when JSH-23 is added at 0 h (Fig. 5C). However, by the time JSH-23 is added at 15 h, the effects are primarily seen on the Cε locus with decreased Cε GLT and mature Iμ-Cε transcripts (Fig. 5). These findings support the notion that the importance of late-phase activation of NF-κB is selective for IgE.

Discussion

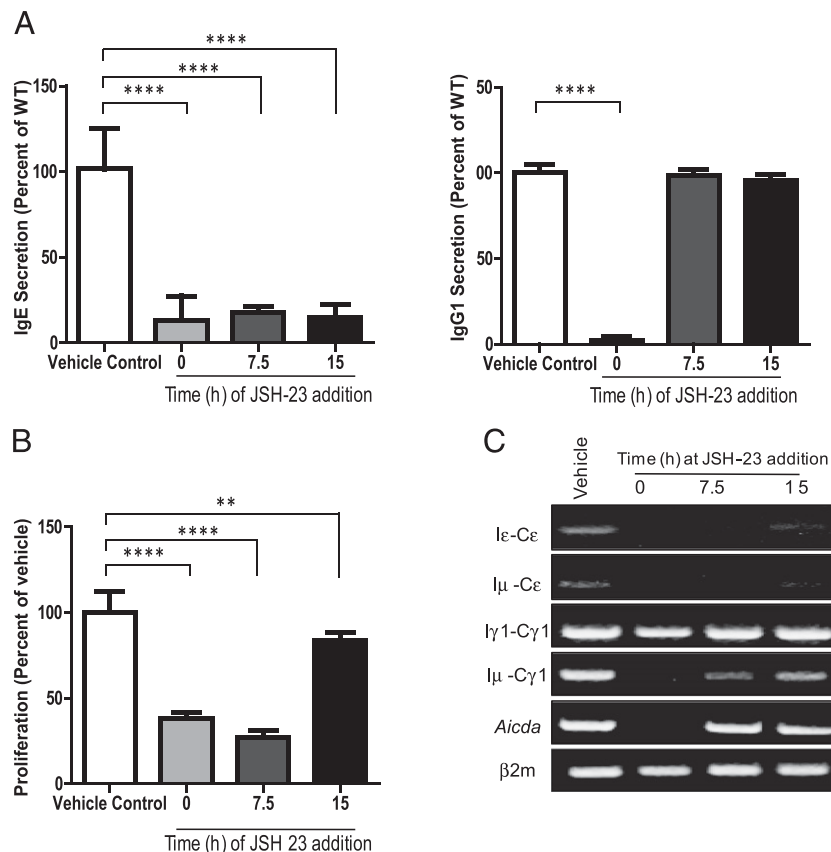
Our results that are summarized in Table I demonstrate that TRIF is critical for TLR4-driven activation of the Cε locus and IgE isotype switching in IL-4-stimulated mouse B cells. B cells from *Tram*^{-/-} and *Trif*^{-/-} mice failed to express Cε GLT and mature Cε transcripts and to secrete IgE in response to stimulation with LPS plus IL-4. In contrast, B cells from *Myd88*^{-/-} mice expressed comparable amounts of Cε GLT as WT B cells in response to stimulation with LPS plus IL-4 but had diminished amounts of Iμ-Cε mature transcripts and secreted reduced quantities of IgE that

Table I. Summary of results after stimulation of B cells from *Trif*^{-/-}, *Tram*^{-/-}, and *Myd88*^{-/-} mice with LPS + IL-4

Genotype	Proliferation ^a	<i>AICDA</i> ^b	IgG1 Switching			IgE Switching		
			Iγ1-Cγ1 ^b	Iμ-Cγ1 ^b	IgG1 ^c	Iε-Cε ^b	Iμ-Cε ^b	IgE ^c
<i>Tram</i> ^{-/-} or <i>Trif</i> ^{-/-}	↓	↓	Normal to ↓	↓	↓	Absent	Absent	Absent
<i>MyD88</i> ^{-/-}	↓	↓	Normal	↓	↓	Normal	↓	↓

^aProliferation was evaluated after 4–6 d of stimulation with LPS + IL-4.
^bTranscript production was measured by RT-PCR after 4 d of stimulation with LPS + IL-4.
^cIg secretion was measured after 6 d of stimulation with LPS + IL-4.

FIGURE 5. Late inactivation of NF- κ B inhibits IgE CSR in WT B cells. **(A and B)** Effect of the addition of the NF- κ B inhibitor JSH-23 0, 7.5, and 15 h poststimulation with LPS plus IL-4 on IgE and IgG1 secretion (A) and proliferation (B) by WT total B cells. Values are expressed as percentage of vehicle (DMSO) control. Columns and bars represent mean $\pm$ SD of three independent experiments. $**p < 0.01$, $****p < 0.0001$. **(C)** Effect of addition of JSH-23 0, 7.5, and 15 h poststimulation with LPS plus IL-4 on C γ 1 GLT, mature C ϵ transcripts, C γ 1 GLT, mature C γ 1 transcripts, and *Aicda* expression. Results are representative of three independent experiments.



correlated with their reduced expression of *Aicda*. These findings strongly suggest that the reduction in IgE secretion by these B cells is a consequence of their reduced *Aicda* expression, because AICDA is essential for CSR (26).

B cells from *Tram*^{-/-} and *Trif*^{-/-} mice expressed a modest reduction in the levels of C γ 1 GLT following LPS plus IL-4 stimulation. The decreased expression of mature C γ 1 transcripts and reduced secretion of IgG1 by *Tram*^{-/-} and *Trif*^{-/-} B cells were most likely due to both the modest decrease in C γ 1 GLT and the decreased expression of *Aicda* in these B cells. B cells from *Myd88*^{-/-} mice expressed normal levels of C γ 1 GLT following LPS plus IL-4 stimulation but had reduced expression of mature I μ -C γ 1 transcripts and reduced secretion of IgG1 by these B cells, both likely secondary to decreased *Aicda* expression by these cells. The regulation of C γ 1 GLT expression following stimulation with LPS is complex (4, 27, 28). A difference in the composition of the residual NF- κ B complexes might explain the reduction in the levels of C γ 1 GLT observed in LPS-stimulated in TRAM/TRIF-deficient B cells but not in LPS-stimulated MyD88-deficient B cells. Expression of *Aicda* is regulated by NF- κ B (5). Decreased activation of NF- κ B in B cells from *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} mice most likely contributed to the decreased *Aicda* expression in these cells following stimulation with LPS and IL-4.

Both TRIF and MyD88 were important for optimal proliferation and survival of LPS plus IL-4-stimulated B cells. This is not surprising as NF- κ B plays an important role in B cell proliferation (29, 30). The decreased proliferation and survival of B cells from *Tram*^{-/-}, *Trif*^{-/-}, and *Myd88*^{-/-} mice most likely contribute to their reduced *Aicda* expression following stimulation with LPS plus IL-4. In the absence of both TRIF and MyD88, B cells completely failed to proliferate and secrete IgE and IgG1 following stimulation with LPS plus IL-4, again demonstrating the

critical role NF- κ B plays in B cell proliferation. These findings indicate that the TRAM/TRIF and MyD88 pathways play important but partially redundant roles in TLR4-driven B cell activation.

It has been shown that B cells must undergo five or more divisions to class switch to IgE (19). However, the failure of *Trif*^{-/-} B cells to undergo IgE isotype switching in response to LPS plus IL-4 cannot be solely due to their defective cell division. A comparable defect in proliferation was found in *Myd88*^{-/-} B cells, as measured both by thymidine incorporation and Cell Tracer Violet dye dilution. In contrast to *Trif*^{-/-} B cells, which secreted no detectable IgE, *Myd88*^{-/-} B cells secreted ~50% of the IgE secreted by WT B cells. Late addition of the NF- κ B inhibitor JSH-23 to WT B cells, to mimic the reduction in the late-phase activation of NF- κ B found in *Trif*^{-/-} B cells, strongly inhibited IgE production but had only a modest effect on proliferation. This supports the conclusion that the failure of *Trif*^{-/-} B cells to secrete IgE in response to stimulation with LPS plus IL-4 is not solely due to their defective proliferation.

LPS activates NF- κ B, whereas IL-4 activates STAT6 (11, 12). The two transcription factors synergize to drive C ϵ GLT expression in both human and mouse B cells (14, 31). In LPS-stimulated mouse B cells, a NF- κ B complex containing p50 interacts with a c/EBP family member to bind to a NF- κ B site in the C ϵ promoter (32). LPS stimulation of mouse B cells has been shown to result in the formation of nuclear NF- κ B complexes that consist of p50:p50 dimers and p50:p65 dimers with negligible amounts of p50:RelB (33). Studies with knockout mice indicate that p50 is essential for IgE isotype switching, whereas RelB is not required (29, 30). Because p50:p50 dimers are inactive, it is likely that p50:p65 complexes induced by LPS are important for driving C ϵ GLT transcription in mouse B cells, as has been demonstrated for LPS-driven C γ 1 germline transcription in these cells (28). *Trif*^{-/-}

B cells had a preserved early phase of NF- κ B p65 nuclear translocation compared with WT B cells. However, in contrast to WT B cells, they failed to sustain NF- κ B p65 nuclear translocation beyond 3 h. CHIP assays, performed 15 h poststimulation with LPS plus IL-4, demonstrated reduced binding of p65 to the I ϵ promoter in *Trif*^{-/-} B cells compared with WT B cells. IL-4-driven STAT6 nuclear translocation was normal in *Trif*^{-/-} B cells, consistent with their intact IgE production in response to anti-CD40 plus IL-4 stimulation. These findings strongly suggest that the failure of *Trif*^{-/-} B cells to activate the C ϵ locus in response to LPS plus IL-4 stimulation is due to their inability to sustain NF- κ B activation.

Further support for the above hypothesis was provided by experiments using timed addition of the NF- κ B inhibitor JSH-23. Addition of JSH-23, 15 h after LPS plus IL-4 stimulation, selectively blocked C ϵ GLT expression and IgE secretion in WT B cells, with minimal effect on C γ 1 GLT expression. Thus, taken together, our results suggest that TRIF-dependent sustained activation of NF- κ B is essential for the transcriptional activation of the C ϵ locus in LPS plus IL-4-stimulated B cells. It is of note that IgE isotype switching requires sustained exposure of the B cells to IL-4 for at least 72 h, suggesting that sustained activation of STAT6 may also be necessary for transcription at the C ϵ locus (34). Sustained activation of both NF- κ B and STAT6 may be required because a lag period may be needed for the C ϵ locus to become susceptible for activation. This lag period could be explained by the need to downregulate the expression of the transcription factors Bcl6, Id2, and PU.1 that inhibit transcription of the C ϵ locus (23, 24).

TLR3 has been reported to mediate isotype switching to IgG1 by tonsillar B cells. Although a dominant-negative TRIF inhibited TLR3-driven NF- κ B activation in these cells, the role of TRIF in isotype switching was not formally demonstrated (35). TRIF has been shown recently to be important for TLR4-dependent IgG production in response to immunization with the autoantigen histidyl-tRNA synthetase (36) and for the IgG Ab response to vaccination with a peptide presented with monophosphoryl lipid A on liposomes (37). The role we demonstrated for TRIF in LPS-driven IgE isotype switching is novel and has potential implications for IgE-mediated allergic responses elicited by mechanisms that may involve TRIF activation.

Disclosures

The authors have no financial conflicts of interest.

References

- Xu, Z., H. Zan, E. J. Pone, T. Mai, and P. Casali. 2012. Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. *Nat. Rev. Immunol.* 12: 517–531.
- Rothman, P., Y. Y. Chen, S. Lutzker, S. C. Li, V. Stewart, R. Coffman, and F. W. Alt. 1990. Structure and expression of germ line immunoglobulin heavy-chain epsilon transcripts: interleukin-4 plus lipopolysaccharide-directed switching to C epsilon. *Mol. Cell. Biol.* 10: 1672–1679.
- Gerondakis, S. 1990. Structure and expression of murine germ-line immunoglobulin epsilon heavy chain transcripts induced by interleukin 4. *Proc. Natl. Acad. Sci. USA* 87: 1581–1585.
- Kepron, M. R., Y. W. Chen, J. W. Uhr, and E. S. Vitetta. 1989. IL-4 induces the specific rearrangement of gamma 1 genes on the expressed and unexpressed chromosomes of lipopolysaccharide-activated normal murine B cells. *J. Immunol.* 143: 334–339.
- Dedeoglu, F., B. Horwitz, J. Chaudhuri, F. W. Alt, and R. S. Geha. 2004. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NF-kappaB. *Int. Immunol.* 16: 395–404.
- Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
- Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 301: 640–643.
- Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the Toll adapters TRAM and TRIF. *J. Exp. Med.* 198: 1043–1055.
- Latz, E., A. Visintin, E. Lien, K. A. Fitzgerald, B. G. Monks, E. A. Kurt-Jones, D. T. Golenbock, and T. Espevik. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the Toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J. Biol. Chem.* 277: 47834–47843.
- McGettrick, A. F., and L. A. O'Neill. 2010. Localisation and trafficking of Toll-like receptors: an important mode of regulation. *Curr. Opin. Immunol.* 22: 20–27.
- Rolling, C., D. Treton, S. Pellegrini, P. Galanaud, and Y. Richard. 1996. IL4 and IL13 receptors share the gamma c chain and activate STAT6, STAT3 and STAT5 proteins in normal human B cells. *FEBS Lett.* 393: 53–56.
- Jiang, H., M. B. Harris, and P. Rothman. 2000. IL-4/IL-13 signaling beyond JAK/STAT. *J. Allergy Clin. Immunol.* 105: 1063–1070.
- Linehan, L. A., W. D. Warren, P. A. Thompson, M. J. Grusby, and M. T. Berton. 1998. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J. Immunol.* 161: 302–310.
- Mao, C. S., and J. Stavnezer. 2001. Differential regulation of mouse germline Ig gamma 1 and epsilon promoters by IL-4 and CD40. *J. Immunol.* 167: 1522–1534.
- Luo, Z., D. Ronai, and M. D. Scharff. 2004. The role of activation-induced cytidine deaminase in antibody diversification, immunodeficiency, and B-cell malignancies. *J. Allergy Clin. Immunol.* 114: 726–735, quiz 736.
- Hise, A. G., K. Dachner, I. Gillette-Ferguson, E. Cho, H. F. McGarry, M. J. Taylor, D. T. Golenbock, K. A. Fitzgerald, J. W. Kazura, and E. Pearlman. 2007. Innate immune responses to endosymbiotic *Wolbachia* bacteria in *Brugia malayi* and *Onchocerca volvulus* are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM. *J. Immunol.* 178: 1068–1076.
- Jabara, H., D. Laouini, E. Tsitsikov, E. Mizoguchi, A. Bhan, E. Castigli, F. Dedeoglu, V. Pivniouk, S. Brodeur, and R. Geha. 2002. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity* 17: 265–276.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402–408.
- Hasbold, J., A. B. Lyons, M. R. Kehry, and P. D. Hodgkin. 1998. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur. J. Immunol.* 28: 1040–1051.
- Thyphronitis, G., I. M. Katona, W. C. Gause, and F. D. Finkelman. 1993. Germline and productive C epsilon gene expression during in vivo IgE responses. *J. Immunol.* 151: 4128–4136.
- Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4: 1144–1150.
- Stavnezer, J., G. Radcliffe, Y. C. Lin, J. Nietupski, L. Berggren, R. Sitia, and E. Severinson. 1988. Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. *Proc. Natl. Acad. Sci. USA* 85: 7704–7708.
- Wei, F., K. Zaprazna, J. Wang, and M. L. Atchison. 2009. PU.1 can recruit BCL6 to DNA to repress gene expression in germinal center B cells. *Mol. Cell. Biol.* 29: 4612–4622.
- Sugai, M., H. Gonda, T. Kusunoki, T. Kataki, Y. Yokota, and A. Shimizu. 2003. Essential role of Id2 in negative regulation of IgE class switching. *Nat. Immunol.* 4: 25–30.
- Shin, H. M., M. H. Kim, B. H. Kim, S. H. Jung, Y. S. Kim, H. J. Park, J. T. Hong, K. R. Min, and Y. Kim. 2004. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Lett.* 571: 50–54.
- Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102: 553–563.
- Zelazowski, P., D. Carrasco, F. R. Rosas, M. A. Moorman, R. Bravo, and C. M. Snapper. 1997. B cells genetically deficient in the c-Rel transactivation domain have selective defects in germline CH transcription and Ig class switching. *J. Immunol.* 159: 3133–3139.
- Lin, S. C., H. H. Wortis, and J. Stavnezer. 1998. The ability of CD40L, but not lipopolysaccharide, to initiate immunoglobulin switching to immunoglobulin G1 is explained by differential induction of NF-kappaB/Rel proteins. *Mol. Cell. Biol.* 18: 5523–5532.
- Snapper, C. M., P. Zelazowski, F. R. Rosas, M. R. Kehry, M. Tian, D. Baltimore, and W. C. Sha. 1996. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J. Immunol.* 156: 183–191.
- Snapper, C. M., F. R. Rosas, P. Zelazowski, M. A. Moorman, M. R. Kehry, R. Bravo, and F. Weih. 1996. B cells lacking RelB are defective in proliferative responses, but undergo normal B cell maturation to Ig secretion and Ig class switching. *J. Exp. Med.* 184: 1537–1541.
- Stütz, A. M., and M. Woisetschlager. 1999. Functional synergism of STAT6 with either NF-kappa B or PU.1 to mediate IL-4-induced activation of IgE germline gene transcription. *J. Immunol.* 163: 4383–4391.
- Delphin, S., and J. Stavnezer. 1995. Characterization of an interleukin 4 (IL-4) responsive region in the immunoglobulin heavy chain germline epsilon pro-

- moter: regulation by NF-IL-4, a C/EBP family member and NF-kappa B/p50. *J. Exp. Med.* 181: 181–192.
33. Doyle, S. L., and L. A. O'Neill. 2006. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem. Pharmacol.* 72: 1102–1113.
 34. Moon, H. B., E. Severinson, C. Heusser, S. G. Johansson, G. Möller, and U. Persson. 1989. Regulation of IgG1 and IgE synthesis by interleukin 4 in mouse B cells. *Scand. J. Immunol.* 30: 355–361.
 35. Xu, W., P. A. Santini, A. J. Matthews, A. Chiu, A. Plebani, B. He, K. Chen, and A. Cerutti. 2008. Viral double-stranded RNA triggers Ig class switching by activating upper respiratory mucosa B cells through an innate TLR3 pathway involving BAFF. *J. Immunol.* 181: 276–287.
 36. Harlow, L., I. Fernandez, M. Soejima, W. M. Ridgway, and D. P. Ascherman. 2012. Characterization of TLR4-mediated auto-antibody production in a mouse model of histidyl-tRNA synthetase-induced myositis. *Innate Immun.* 18: 876–885.
 37. Pihlgren, M., A. B. Silva, R. Madani, V. Giriens, Y. Waeckerle-Men, A. Fettelschoss, D. T. Hickman, M. P. López-Deber, D. M. Ndao, M. Vukicevic, et al. 2013. TLR4- and TRIF-dependent stimulation of B lymphocytes by peptide liposomes enables T cell-independent isotype switch in mice. *Blood* 121: 85–94.