

Gold Standard Barcoded MHC Multimers for  
Antigen-Specific T Cell Single-Cell Multi-Omics

**dCODE Dextramer®**

**IMMUDEx®**  
PRECISION IMMUNE MONITORING

## The Journal of Immunology

RESEARCH ARTICLE | JANUARY 01 2014

### TRAF5 Negatively Regulates TLR Signaling in B Lymphocytes **FREE**

Claire M. Buchta; ... et. al

*J Immunol* (2014) 192 (1): 145–150.

<https://doi.org/10.4049/jimmunol.1301901>

#### Related Content

Roles of TRAF5 in TLR signaling (180.4)

*J Immunol* (May,2012)

TRAF5 negatively regulates Toll-like receptor signaling (P1226)

*J Immunol* (May,2013)

TRAF5 Deficiency Ameliorates the Severity of Dextran Sulfate Sodium Colitis by Decreasing TRAF2 Expression in Nonhematopoietic Cells

*Immunohorizons* (March,2020)

# TRAF5 Negatively Regulates TLR Signaling in B Lymphocytes

Claire M. Buchta\* and Gail A. Bishop\*,†,‡,§

**The cytoplasmic adaptor proteins TNFR-associated factor (TRAF)3 and TRAF6 are important mediators of TLR signaling. To our knowledge, we show in this study for the first time that another TRAF family member, TRAF5, is a negative regulator of TLR signaling. B lymphocytes from TRAF5<sup>-/-</sup> mice produced more IL-6, IL-12p40, IL-10, TNF- $\alpha$ , and IgM than did wild-type B cells after TLR stimulation. Consistent with these data, exogenous overexpression of TRAF5 in B cells inhibited TLR-mediated cytokine and Ab production. TLR stimulation of TRAF5-deficient B cells did not affect cell survival, proliferation, or NF- $\kappa$ B activation but resulted in markedly enhanced phosphorylation of the MAPKs ERK1/2 and JNK. TRAF5 negatively regulated TLR signaling in a cell-specific manner, because TRAF5<sup>-/-</sup> macrophages and dendritic cells showed less dramatic differences in TLR-mediated cytokine production than B cells. Following TLR stimulation, TRAF5 associated in a complex with the TLR adaptor protein MyD88 and the B cell-specific positive regulator of TLR signaling TAB2. Furthermore, TRAF5 negatively regulated the association of TAB2 with its signaling partner TRAF6 after TLR ligation in B cells. To our knowledge, these data provide the first evidence that TRAF5 acts as a negative regulator of TLR signaling. *The Journal of Immunology*, 2014, 192: 145–150.**

Toll-like receptors are pattern recognition receptors, providing a first-line defense against pathogens by recognizing pathogen-associated molecular patterns (1–3). The cytoplasmic adaptor proteins TNFR-associated factors (TRAFs) mediate signaling from the TNFR superfamily and the IL-1R/TLR superfamily of receptors (4). TRAF6 is recognized as an integral component of TLR signaling in multiple cell types (5). TRAF3 also mediates signaling after TLR ligation in myeloid cells while in contrast inhibiting TLR signaling in B lymphocytes (6–8).

Of the seven known TRAF family members, TRAF5 is relatively understudied. Although initially thought to be redundant with TRAF2, it is now appreciated that TRAF5 plays unique roles in CD8 T cell responses to infection, in limiting Th2 skewing, and in signaling to B cells through both CD40 and its viral mimic, latent membrane protein 1 (9–13). TRAF5 shares significant structural homology with TRAF3 and is composed of a C-terminal receptor binding domain, a coiled-coil, leucine-zipper domain, a zinc finger motif, and an N-terminal RING finger domain. TRAF5 forms heterotypic multimers with TRAF3 via coiled-coil, leucine-zipper domain interactions. This interaction is biologically important in TRAF5 recruitment to several types of membrane receptors (14–16).

TRAF5 has been implicated in the development of atherosclerosis in a mouse model (17). Because TLR dysregulation is known to contribute to atherogenesis (3), we hypothesized that like TRAFs 3 and 6, TRAF5 also plays an important regulatory role in

TLR signaling. To address this hypothesis, we used two complementary model systems. The first was a strain of genetically TRAF5-deficient mice. These mice breed and develop normally (12). Our laboratory previously backcrossed this strain onto the C57BL/6 genetic background and used the mice to examine roles of TRAF5 in T cell responses to infection (11) and in latent membrane protein 1-mediated B cell activation (13). The second model system inducibly overexpressed epitope-tagged TRAF5 in a well-studied B cell line to circumvent the poor quality and specificity of commercially available TRAF5-specific Abs and allowed examination of the contrasting effects of TRAF5 depletion versus excess.

Results from experiments in both models indicated that TRAF5 serves as an important negative regulator of TLR-mediated signaling, specifically in B lymphocytes. After TLR ligation, TRAF5-deficient B cells showed enhanced MAPK phosphorylation and produced more cytokines and Ab than control B cells. TRAF5 negatively regulated TLR signaling in a cell-specific manner because TRAF5-deficient dendritic cells and macrophages did not show dramatic differences in cytokine production in response to TLR agonists. Similarly, a recent study demonstrated that the TLR adaptor protein TAB2 acts in a cell-specific manner, positively regulating TLR signaling specifically in B lymphocytes. After TLR ligation, B lymphocytes from TAB2<sup>-/-</sup> mice show reduced phosphorylation of MAPKs and produce less IL-6 and Ab (18).

Thus, we hypothesized that TRAF5 negatively regulates TLR signaling in B lymphocytes by acting on the positive regulator TAB2. Our results showed association of TRAF5 with TAB2 after TLR ligation in B cells. In addition, TRAF5 negatively regulated the association of TAB2 with its known interacting partner TRAF6 after TLR ligation in B cells. These results demonstrate for the first time an important regulatory role for TRAF5 in TLR signaling.

## Materials and Methods

### Mice

TRAF5<sup>-/-</sup> mice on a C57BL/6 genetic background were described previously (13). Mice were maintained under pathogen-free conditions at the University of Iowa. Use of mice in this study was according to a protocol approved by the University of Iowa Animal Care and Use Committee.

\*Graduate Program in Immunology, University of Iowa, Iowa City, IA 52242;

†Department of Microbiology, University of Iowa, Iowa City, IA 52242; ‡Department of Internal Medicine, University of Iowa, Iowa City, IA 52242; and §Iowa City Veterans Affairs Medical Center, Iowa City, IA 52242

Received for publication July 18, 2013. Accepted for publication October 22, 2013.

This work was supported by National Institutes of Health Grants R01AI28847, Department of Veterans Affairs Merit Review Grant 1101BX001702 (to G.A.B.), and the Roy J. and Lucille A. Carver Charitable Trust.

Address correspondence and reprint requests to Dr. Gail A. Bishop, Medical Education and Research Facility, 2193B, University of Iowa, 375 Newton Road, Iowa City, IA 52242. E-mail address: gail-bishop@uiowa.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; IPTG, isopropyl  $\beta$ -D-thiogalactoside; KO, knockout; TRAF, TNFR-associated factor; WT, wild-type.

### Cell lines

The mouse B cell line CH12.LX has been described previously (19). CH12.LX cells were stably transfected to inducibly express FLAG-tagged TRAF5 as described previously (20). Subclones expressing FLAG-tagged TRAF5 were maintained in B cell medium (BCM10) containing RPMI 1640 medium (Invitrogen, Grand Island, NY) with 10  $\mu$ M 2-ME (Invitrogen), 10% heat-inactivated FCS (Atlanta Biologicals, Atlanta, GA), antibiotics (Invitrogen), with added 400  $\mu$ g/ml G418 disulfate and 200  $\mu$ g/ml hygromycin (Invitrogen).

### Abs and reagents

Isopropyl  $\beta$ -D-thiogalactoside (IPTG) was purchased from Sigma-Aldrich (St. Louis, MO). The TLR7 agonist R848 was purchased from Enzo Life Sciences (Ann Arbor, MI). The synthetic oligonucleotide CpG B 2084, a TLR9 agonist, was purchased from Integrated DNA Technologies (Coralville, IA). LPS (*Escherichia coli* strain 0111:B4), alkaline phosphatase substrate tablets, and mouse anti-actin Ab were purchased from Sigma-Aldrich. ELISA Abs, PE-labeled mouse anti-IL-6 Ab, anti-CD21, and anti-IgM Abs were purchased from eBioscience (San Diego, CA). Rabbit anti-phospho-JNK Ab, rabbit anti-total JNK Ab, rabbit anti-phospho-p38 Ab, rabbit anti-phospho-ERK1/2 Ab, rabbit anti-total ERK1/2 Ab, rabbit anti-pI $\kappa$ B $\alpha$  Ab, rabbit anti-total I $\kappa$ B $\alpha$  Ab, rabbit anti-TAB2 Ab, and rabbit anti-MyD88 Ab were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-TRAF6 Ab for Western blotting was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-TRAF6 Ab for immunoprecipitation was purchased from Invitrogen. Goat anti-mouse IgG and goat anti-rabbit IgG secondary Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified mouse IgM polyclonal Ab for ELISA standards, goat anti-mouse IgM secondary polyclonal Ab, and alkaline phosphatase-conjugated goat anti-mouse IgM Ab were purchased from Southern Biotechnology Associates (Birmingham, AL).

### Detection of cytokine and Ab production in vitro

Primary splenic B cells were isolated using an anti-CD43 negative selection kit (Miltenyi Biotec, Auburn, CA) and stimulated with 100 nM CpG-B 2084, 1  $\mu$ g/ml R848, or 20  $\mu$ g/ml LPS for 48 h. Bone marrow-derived dendritic cells (BMDC) were differentiated using recombinant mouse GM-CSF and recombinant mouse IL-4 (R&D Systems, Minneapolis, MN). Bone marrow-derived macrophages (BMM) were differentiated using 30% L929 medium. Differentiated cells were stimulated with TLR agonists for 24 h. After stimulation, supernatants were collected and used in cytokine-specific ELISAs. For TNF- $\alpha$  detection, primary B cells were isolated as above, and in-plate ELISAs were performed as previously described, because B cells rapidly bind and internalize TNF (21). For detection of Ab production, primary cells were isolated as described above and cultured at  $1 \times 10^6$  cells/ml for 5 d in BCM10 with added TLR agonists. Ab production was determined by Ig-specific ELISA as described previously (22).

### Flow cytometry

Proliferation was measured by CFSE dilution using CellTrace (Invitrogen). Survival was measured with the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen). For intracellular IL-6 detection, primary B cells were isolated as above and stimulated with TLR agonists for 6 h in the presence of brefeldin A and monensin (BD Biosciences, San Jose, CA). The Cytofix/Cytoperm Plus kit (BD Biosciences) was used to detect intracellular IL-6 by flow cytometry. For B cell subset analysis, splenocytes were stained with anti-IgM and anti-CD21. Samples were analyzed on an LSR II flow cytometer (BD Biosciences), and results were analyzed using FlowJo software (Tree Star, Ashland, OR).

### Proximal signaling assays

Primary splenic B cells were isolated as for the in vitro cytokine production experiments above. Primary cells ( $4 \times 10^6$ ) were resuspended in 1 ml BCM10 in 1.5-ml Eppendorf tubes and stimulated with 1  $\mu$ g/ml R848 for 0, 2, 5, 10, 15, 30, and 60 min. After stimulation, cells were lysed in 100  $\mu$ l 2 $\times$  SDS-PAGE loading buffer (1% SDS, 2% 2-ME, and 62.5 mM Tris [pH 6.8]). Lysates were sonicated using a Branson Sonifier 250 (VWR International, Radnor, PA) with 10 pulses at 90% duty cycle, output 1.5. Samples were boiled for 10 min at 95°C.

### Western blots

Samples (10  $\mu$ l) were resolved on 12% SDS-PAGE. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked in 4% milk or BSA for 1 h, washed in TBST (NaCl, Tris, Tween 20, and H<sub>2</sub>O), and incubated over-

night at 4°C in primary Ab. Blots were washed in TBST and incubated with secondary Ab overnight at 4°C and developed using Supersignal West Pico (Thermo Fisher Scientific, Rockford, IL). Western blot chemiluminescence was read with an LAS-4000 low-light camera and analyzed with Multi Gauge software (Fujifilm Life Science, Edison, NJ).

### Immunoprecipitation

FLAG-TRAF5 expression in transfected subclones of CH12.LX cells was induced by 20 h of culture in BCM10 with 100  $\mu$ M IPTG. A total of  $2 \times 10^7$  CH12.LX cells or  $5 \times 10^7$  primary splenic B cells were resuspended in 1 ml BCM10 in 1.5-ml Eppendorf tubes and stimulated with 1  $\mu$ g/ml R848 for the indicated time points. Cells were pelleted and lysed as described previously (23). A total of 2  $\mu$ g/ml mouse anti-FLAG Ab (Sigma-Aldrich), rabbit anti-MyD88 Ab (Cell Signaling Technology), rabbit anti-TRAF6 Ab (Invitrogen), or control mouse IgG1 Ab (MOPC21; BioLegend, San Diego, CA) were added, and samples were rotated at 4°C for 5 h. Dynal protein G magnetic beads (Invitrogen) were added for 1 h. Bead-bound proteins were resuspended in an equal volume of 2 $\times$  SDS-PAGE loading buffer and boiled for 10 min at 95°C.

### Statistical analysis

The *p* values were generated by using Student *t* test (unpaired, two-tailed, at 95% confidence interval).

## Results

### Effect of TRAF5 on TLR-mediated cytokine production

TLR ligation stimulates robust production of cytokines in both lymphoid and myeloid cells (1, 8). When stimulated through TLR4 (LPS), TLR7 (R848), or TLR9 (CpG DNA), TRAF5<sup>-/-</sup> (knock-out [KO]) B cells produced significantly more IL-6, IL-10, TNF- $\alpha$ , and IL-12p40 than wild-type (WT) TRAF5<sup>+/+</sup> B cells (Fig. 1A). Ligation of TLR2, TLR3, or TLR5 did not result in substantial B cell cytokine production. The enhanced cytokine production seen in TLR-stimulated TRAF5 KO B cells was not attributable to either enhanced cell survival or proliferation (Supplemental Fig. 1). Rather, TRAF5 KO B cells produced more cytokines on a per-cell basis (Supplemental Fig. 2). The enhanced cytokine production was seen in all splenic B cell subsets (Supplemental Fig. 2). These data indicate that TRAF5 inhibited TLR signals to B cells.

Consistent with results in TRAF5 KO B cells, CH12.LX B cells induced to overexpress transfected TRAF5 (CH12-TRAF5) produced significantly less IL-6, TNF- $\alpha$ , and IL-12p40 than parent CH12.LX clones expressing normal levels of endogenous TRAF5 (Fig. 1B). Parent CH12.LX cells constitutively produce IL-10, so differences in IL-10 production could not be quantified reliably in the cell lines.

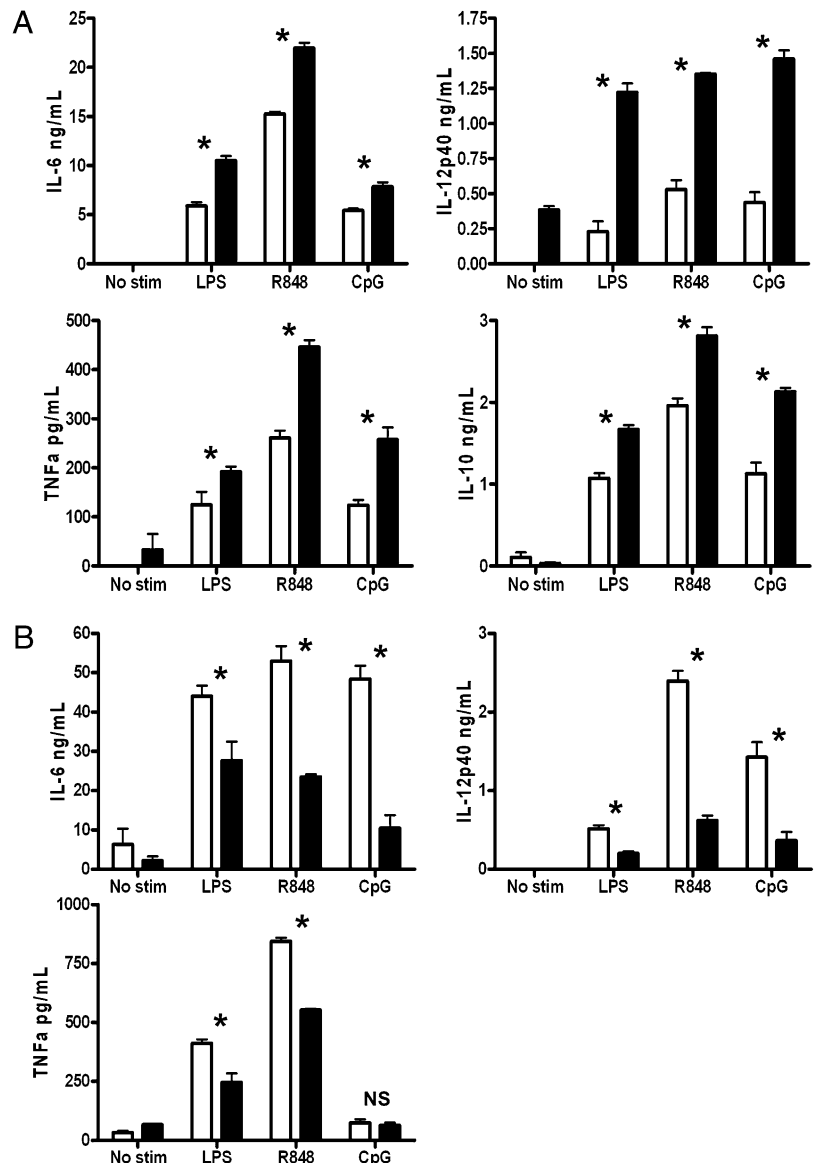
Most studies of TLRs focus on their important functions in myeloid cells. Although TRAF5-deficient B cells showed dramatic differences in cytokine production after TLR ligation, TRAF5<sup>-/-</sup> BMDC and BMM do not exhibit as striking a phenotype (Fig. 2). Similar to B cells, TRAF5<sup>-/-</sup> BMDC produced more IL-6 after TLR stimulation. However, TRAF5-deficient BMM showed enhanced IL-6 production only after stimulation through TLR7. With the exception of IL-12p40 production after TLR4 stimulation in BMDC and after TLR7 production in BMM, no significant differences in the production of IL-10 or IL-12p40 were observed (Fig. 2).

The most striking effects of TRAF5 deficiency on TLR responses were seen in B cells, and the availability of a complementary model of TRAF5 overexpression in B cells suggested that this immune cell type was the best choice for further investigation of how TRAF5 regulates TLR responses.

### Effect of TRAF5 on TLR-induced Ab production

Production of Ab is the major unique immune function of the B lymphocyte, and TLR stimulation induces robust Ig production (8). The CH12.LX B cell line produces IgM specific for phosphatidyl choline, a major constituent of erythrocyte membranes (24). IgM-secreting CH12.LX cells were enumerated as lytic plaque-

**FIGURE 1.** Effect of TRAF5 status on TLR-mediated cytokine production by B lymphocytes. Splenic B cell isolation and detection of cytokine production were performed as described in *Materials and Methods*. **(A)** Splenic B cells were isolated from TRAF5 KO and TRAF5 WT mice and cultured with the indicated TLR ligands for 48 h. Cytokine production from TRAF5 KO B cells (■) was compared with TRAF5 WT B cells (□). **(B)** CH12.LX B cells were cultured for 20 h with IPTG to induce overexpression of TRAF5. Cytokine production after 48 h of TLR stimulation from these cells (■) was compared with CH12.LX cells that were not induced with IPTG (□). Data represent the mean values  $\pm$  SD of three experiments. Statistical analysis was conducted using Student *t* test. \**p* < 0.05.



forming cells on a lawn of sheep RBCs, as described previously (25). CH12-TRAF5 cells produced significantly less Ab following TLR4, TLR7, and TLR9 stimulation than the same cells that were not induced to overexpress TRAF5, demonstrating that TRAF5 negatively regulated Ab production after TLR ligation (Fig. 3A).

Consistent with these data, TRAF5 KO B cells produced more IgM when stimulated through TLR4 or TLR7 (Fig. 3B). Taken together with the cytokine data from Fig. 1, these results indicate that TRAF5 is a negative regulator of TLR effector functions in B cells.

#### TLR-induced early signaling events in TRAF5<sup>-/-</sup> B cells

Because B cell survival and proliferation were unaffected by TRAF5 deficiency (Supplemental Fig. 1), we hypothesized that the increases in cytokine and Ab production seen in TLR-stimulated TRAF5 KO B cells were directly attributable to enhanced signaling pathways after TLR ligation.

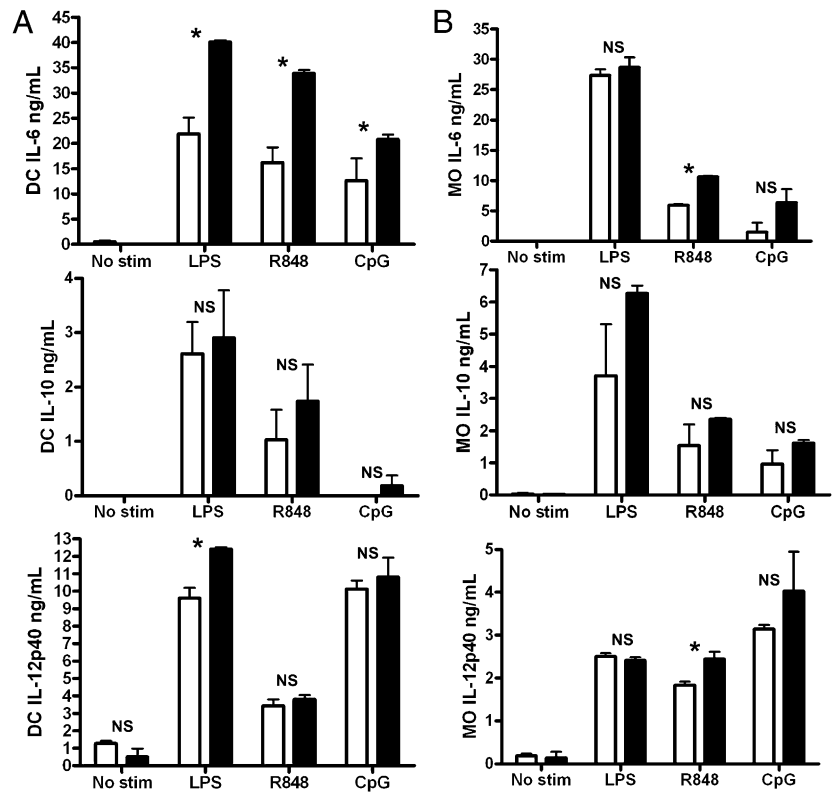
Fig. 1 results showed that ligation of TLR7 induced the most potent cytokine responses in both primary B cells and CH12-TRAF5 cells. The TLR7 agonist R848 was thus chosen as the optimal stimulus for characterizing the impact of TRAF5 on B cell TLR-mediated early signaling pathways. Following TLR7 ligation, TRAF5 KO B cells showed greater phosphorylation of the MAPK pathway proteins ERK1/2 and JNK compared with TRAF5

WT B cells, whereas phosphorylation of the MAPK p38 was unaffected (Fig. 4). TRAF5 status did not detectably impact the canonical NF- $\kappa$ B pathway as measured by the phosphorylation and degradation of I $\kappa$ B $\alpha$  (Fig. 5). Thus, TRAF5 regulates a distinct and specific subset of early TLR-mediated signaling pathways in B cells. Notably, this subset is distinct from that regulated by TRAF3 in B cell TLR responses (8).

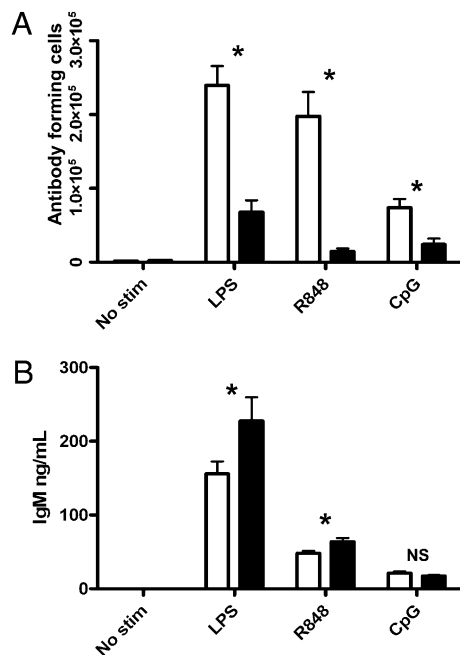
#### TLR-induced association of TRAF5 with TLR signaling proteins

Figs. 1–5 show that TRAF5 negatively regulated MAPK signaling after TLR ligation and also restrained cytokine and Ab production in B cells while having little effect in TLR-stimulated myeloid cells. In addition, its manner of action is independent of cell survival, proliferation, and NF- $\kappa$ B activation. A recent publication identified TAB2 as a positive regulator of TLR signaling, specifically in B lymphocytes. After TLR ligation, B lymphocytes from TAB2<sup>-/-</sup> mice show reduced phosphorylation of MAPKs and produce less IL-6 and Ab. However, TAB2 deficiency has no effect on cell survival, proliferation, or NF- $\kappa$ B activation and does not affect TLR signaling in macrophages. In addition, TAB2<sup>-/-</sup> mice have decreased marginal zone B cells in the spleen in direct opposition to the increased marginal zone B cell population observed in TRAF5<sup>-/-</sup> mice (Supplemental Fig. 3) (18).

**FIGURE 2.** Effect of TRAF5 deficiency on TLR-mediated cytokine production by BMDC. BMDC (A) and BMM (B) differentiation was performed as described in *Materials and Methods*. BMDC and BMM derived from TRAF5 WT and TRAF5 KO mice were cultured with the indicated TLR ligands for 24 h. Cytokine production from TRAF5 KO BMDC or BMM (■) was compared with TRAF5 WT BMDC or BMM (□). Data represent the mean values  $\pm$  SD of three experiments. Statistical analysis was conducted using Student *t* test. \**p* < 0.05.



Because the phenotypes of these mice are directly opposite, we examined the potential interaction of TRAF5 with TAB2 as well as with the TLR adaptor protein MyD88. A carefully orchestrated



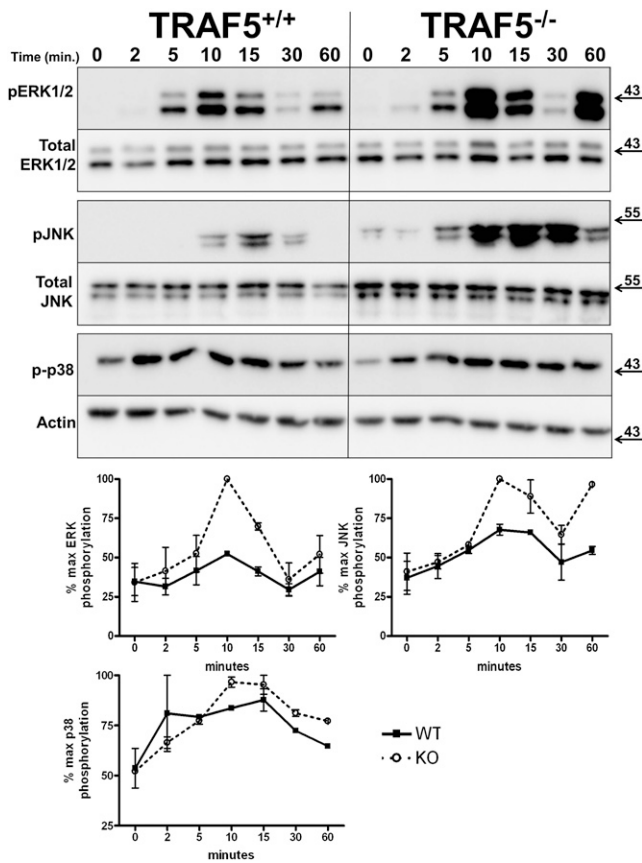
**FIGURE 3.** Effect of TRAF5 status on TLR-induced Ab production. Ab production was measured as described in *Materials and Methods*. (A) CH12.LX B cells were cultured for 20 h with IPTG to induce over-expression of TRAF5 (CH12-TRAF5). IgM production after TLR ligation from these cells (■) was compared with CH12.LX cells that were not induced with IPTG (□). (B) Splenic B cells were isolated from TRAF5 KO and TRAF5 WT mice and cultured with the indicated TLR ligands for 5 d. IgM production from TRAF5 KO B cells (■) was compared with TRAF5 WT B cells (□). Data represent the mean values  $\pm$  SD of three experiments. Statistical analysis was conducted using Student *t* test. \**p* < 0.05.

series of protein–protein interactions mediates TLR signaling, and prior reports have established that TRAFs 6 and 3 participate in these interactions. Results presented above indicate that TRAF5 is also likely to interact with signaling proteins in TLR pathways. To address this prediction, expression of FLAG-tagged TRAF5 was induced in CH12.LX cells prior to stimulation through TLR7. Cell lysates were then subjected to immunoprecipitation using anti-FLAG Ab.

An association between TRAF5 and MyD88 was markedly induced early after TLR ligation (Fig. 6A), demonstrating that TRAF5 was recruited to the TLR signaling complex. The reciprocal immunoprecipitation experiment using anti-MyD88 Ab confirmed the interaction between TRAF5 and MyD88 (Fig. 6B). TRAF5 also associates with TAB2 after TLR ligation (Fig. 6A). To further elucidate this interaction, TRAF6 was immunoprecipitated from both TRAF5-sufficient and TRAF5-deficient primary mouse B cells as well as parent CH12.LX and CH12-TRAF5 cells before and after TLR7 stimulation. TRAF6 is a known binding partner of TAB2. After normalization to amount of TRAF6 in the immunoprecipitates, more TAB2 associated with TRAF6 in TRAF5<sup>-/-</sup> B cells 30 min after TLR7 ligation, whereas samples from CH12-TRAF5 cells showed reduced association between TAB2 and TRAF6 after TLR ligation, suggesting that TRAF5 acts on TAB2 in TLR signaling in B cells (Fig. 6C). These data demonstrate that TRAF5 was recruited to TLR signaling complexes after TLR ligation where it associated with the B cell-specific positive regulator of TLR signaling, TAB2, decreasing its association with TRAF6.

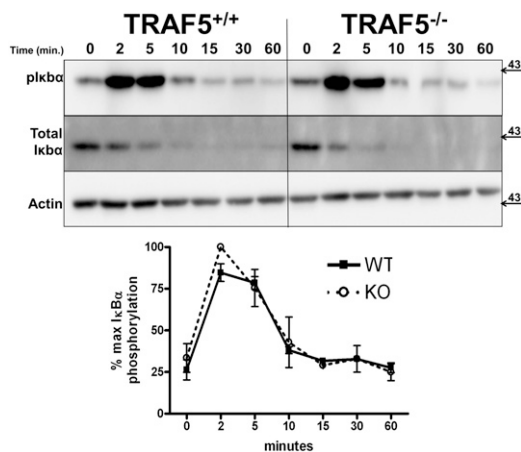
## Discussion

Although TLRs are expressed by a variety of cell types, the majority of published reports on this important receptor family have examined their functions in myeloid cells, including the roles played by TRAF molecules in these functions (6, 7, 26–28). However, TLR functions also have major effects on B lymphocytes (29), and it has been recently demonstrated that TRAF3 plays very distinct roles in TLR signaling to B versus myeloid cells (8). In the current study, the

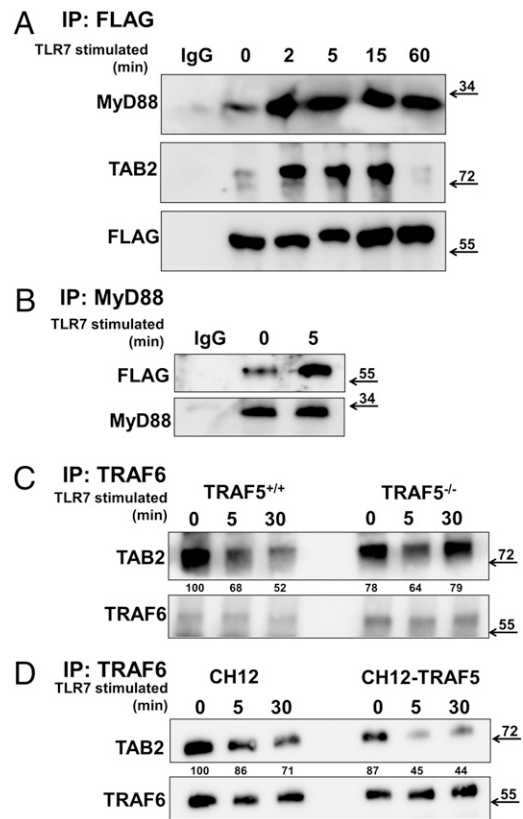


**FIGURE 4.** TLR-induced MAPK activation in TRAF5-deficient B lymphocytes. Splenic B cells were isolated from TRAF5 KO and WT mice as described in *Materials and Methods* and stimulated with TLR7 agonist R848 for the indicated times. Preparation of whole-cell lysates and Western blotting were as described in *Materials and Methods*. Western blots shown are representative of three experiments, whereas the quantitation provided combines three separate experiments.

adaptor protein TRAF5 was revealed to inhibit TLR function in B lymphocytes, and to a lesser extent in myeloid cells. The regulatory impact was most marked in B cells, further supporting the



**FIGURE 5.** TLR-induced NF- $\kappa$ B activation in TRAF5-deficient B lymphocytes. Splenic B cells were isolated from TRAF5 KO and WT mice as described in *Materials and Methods* and stimulated with TLR7 agonist R848 for the indicated times. Preparation of whole-cell lysates and Western blotting were as described in *Materials and Methods*. Western blots shown are representative of three experiments, whereas the quantitation provided combines three separate experiments.



**FIGURE 6.** TLR-induced association of TRAF5 with TLR signaling proteins. CH12.LX B cells (**A**, **B**, **D**) were cultured for 20 h with IPTG to induce expression of FLAG-tagged TRAF5. Splenic B cell isolation was performed as described in *Materials and Methods* (**C**). Cells were stimulated with TLR7 agonist R848 for the indicated times. Immunoprecipitation of FLAG (**A**), MyD88 (**B**), and TRAF6 (**C**, **D**) and Western blotting were performed as described in *Materials and Methods*. Quantitation in (**C**) and (**D**) is shown below the blots as percent of maximum association.

concept that important TLR functions and regulatory mechanisms can be distinct in B lymphocytes versus myeloid cells.

In this regard, it was shown previously that TRAF5 cooperates with TRAF3 to activate NF- $\kappa$ B and the transcription factor IRF3 after stimulation of the RNA helicases retinoic acid-inducible gene I (RIG-I) and MDA-5 (30). In contrast, TRAF5 and TRAF3 affect TLR signaling pathways by distinct and nonredundant, complementary mechanisms in B cells. B lymphocytes deficient in either TRAF3 or TRAF5 show enhanced cytokine and Ab secretion after TLR ligation. However, the signaling pathways affected by these specific TRAFs show distinct differences. TRAF5<sup>-/-</sup> B cells displayed elevated ERK1/2 and JNK activation, whereas the canonical NF- $\kappa$ B pathway was unaffected (Figs. 4, 5). In contrast, TRAF3<sup>-/-</sup> B cells show enhanced canonical NF- $\kappa$ B activation after TLR ligation but do not exhibit changes in MAPK activation (8).

TRAF5 is less well examined and understood compared with other TRAF proteins, and most reports to date have focused upon its function in lymphocytes. It was shown previously that unlike TRAF5-deficient B cells, TRAF5<sup>-/-</sup> BMDC do not show defects in surface molecule upregulation after CD40 ligation (11, 12). Taken together with data shown in this study, this suggests that TRAF5 may play a lesser role in myeloid versus lymphoid cells. This emphasizes how TRAF proteins function in both a cell-type and receptor-specific fashion (29).

Fig. 6 demonstrates the association of TRAF5 with the adaptor protein MyD88, indicating that like TRAFs 3 and 6, TRAF5 is present within the TLR signaling complex. In addition, TRAF5

associates with TAB2 after TLR ligation. A recent publication identified TAB2 as a positive regulator of TLR signaling, specifically in B lymphocytes. After TLR ligation, B lymphocytes from TAB2-deficient mice show reduced phosphorylation of MAPKs and produce less IL-6 and Ab. However, TAB2 deficiency has no effect on cell survival, proliferation, or NF- $\kappa$ B activation and does not affect TLR signaling in macrophages. In addition, TAB2<sup>-/-</sup> mice have decreased marginal zone B cells in the spleen, which contrasts with the increased marginal zone B cell population that we observed in TRAF5<sup>-/-</sup> mice (Supplemental Fig. 3) (18). Figs. 1–5 show that TRAF5 negatively regulated MAPK signaling after TLR ligation, resulting in restrained cytokine and Ab production in B cells, while having little effect in TLR-stimulated myeloid cells. In addition, its manner of action was independent of cell survival, proliferation, and NF- $\kappa$ B activation.

Because TAB2<sup>-/-</sup> and TRAF5<sup>-/-</sup> mice exhibit opposite phenotypes and TRAF5 associates with TAB2 after TLR stimulation in B cells, it is reasonable to hypothesize that TRAF5 negatively regulates TLR signaling in B lymphocytes by acting on the positive regulator TAB2. After TLR ligation, TAB2 links TRAF6 to TGF- $\beta$ -activated kinase 1 (TAK1), facilitating downstream activation of MAPKs (31). In the absence of TRAF5, we observed greater association of TAB2 with TRAF6 after TLR7 ligation in B cells (Fig. 6C). The reciprocal experiment showed a reduced association of TAB2 with TRAF6 when TRAF5 was overexpressed in B cells (Fig. 6D). This could result from competition between TAB2 and TRAF5 for association with TRAF6. Because TAB2 positively regulates MAPK pathways in B cells, its increased association with TRAF6 in the absence of TRAF5 could account for the increased MAPK phosphorylation and resulting enhancement of cytokine production seen in TLR-stimulated TRAF5-deficient B cells (Figs. 1, 4). This agrees with a recent report in which TRAF5 negatively regulates the ERK1/2 pathway in a model of cardiac hypertrophy (32).

TLR signaling to B cells is biologically important in both normal and pathogenic immunity. Aberrant TLR signaling in B cells has been linked to autoimmune diseases, making identification of novel TLR signaling targets clinically relevant (3, 33). To our knowledge, the present study shows for the first time that TRAF5 negatively regulates TLR signaling and downstream functions in B lymphocytes, associating with TLR signaling proteins and altering the association of TAB2 with TRAF6. Future investigations will seek to characterize in detail the mechanism(s) by which TRAF5 exerts its regulatory activities.

## Acknowledgments

We thank Timothy Rosean for assistance with flow cytometry. This material is based on work supported in part by the use of facilities and resources of the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406: 782–787.
- Akira, S. 2003. Toll-like receptor signaling. *J. Biol. Chem.* 278: 38105–38108.
- Huang, Q., and R. M. Pope. 2010. Toll-like receptor signaling: a potential link among rheumatoid arthritis, systemic lupus, and atherosclerosis. *J. Leukoc. Biol.* 88: 253–262.
- Ha, H., D. Han, and Y. Choi. 2009. TRAF-mediated TNFR-family signaling. In *Current Protocols in Immunology*. J. E. Coligan, B. E. Bierer, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Chapter 11: Unit11 19D.
- Kirschning, C. J., H. Wesche, T. Merrill Ayres, and M. Rothe. 1998. Human TLR2 confers responsiveness to bacterial LPS. *J. Exp. Med.* 188: 2091–2097.
- Häcker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Häcker, et al. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439: 204–207.
- Oganesyan, G., S. K. Saha, B. Guo, J. Q. He, A. Shahangian, B. Zarnegar, A. Perry, and G. Cheng. 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 439: 208–211.
- Xie, P., J. Poovassery, L. L. Stunz, S. M. Smith, M. L. Schultz, L. E. Carlin, and G. A. Bishop. 2011. Enhanced TLR responses of TRAF3-deficient B lymphocytes. *J. Leukoc. Biol.* 90: 1149–1157.
- Akiba, H., H. Nakano, S. Nishinaka, M. Shindo, T. Kobata, M. Atsuta, C. Morimoto, C. F. Ware, N. L. Malinin, D. Wallach, et al. 1998. CD27, a member of the TNFR superfamily, activates NF- $\kappa$ B and stress-activated protein kinase/JNK via TRAF2, TRAF5, and NF- $\kappa$ B-inducing kinase. *J. Biol. Chem.* 273: 13353–13358.
- So, T., S. Salek-Ardakani, H. Nakano, C. F. Ware, and M. Croft. 2004. TRAF5 limits the induction of Th2 immune responses. *J. Immunol.* 172: 4292–4297.
- Kraus, Z. J., J. S. Haring, and G. A. Bishop. 2008. TRAF5 is required for optimal T cell expansion and survival in response to infection. *J. Immunol.* 181: 7800–7809.
- Nakano, H., S. Sakon, H. Koseki, T. Takemori, K. Tada, M. Matsumoto, E. Munechika, T. Sakai, T. Shirasawa, H. Akiba, et al. 1999. Targeted disruption of *Traf5* gene causes defects in CD40- and CD27-mediated lymphocyte activation. *Proc. Natl. Acad. Sci. USA* 96: 9803–9808.
- Kraus, Z. J., H. Nakano, and G. A. Bishop. 2009. TRAF5 is a critical mediator of in vitro signals and in vivo functions of LMP1, the viral oncogenic mimic of CD40. *Proc. Natl. Acad. Sci. USA* 106: 17140–17145.
- Pullen, S. S., H. G. Miller, D. S. Everdeen, T. T. Dang, J. J. Crute, and M. R. Kehry. 1998. CD40-TRAF interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* 37: 11836–11845.
- Arcipowski, K. M., L. L. Stunz, J. P. Graham, Z. J. Kraus, T. J. Vanden Bush, and G. A. Bishop. 2011. Molecular mechanisms of TRAF6 utilization by the oncogenic viral mimic of CD40, LMP1. *J. Biol. Chem.* 286: 9948–9955.
- He, L., A. C. Grammer, X. Wu, and P. E. Lipsky. 2004. TRAF3 forms heterotrimers with TRAF2 and modulates its ability to mediate NF- $\kappa$ B activation. *J. Biol. Chem.* 279: 55855–55865.
- Missiou, A., P. Rudolf, P. Stachon, D. Wolf, N. Varo, P. Aichele, C. Colberg, N. Hoppe, S. Ernst, C. Müntel, et al. 2010. TRAF5 deficiency accelerates atherosclerosis in mice by increasing inflammatory cell recruitment and foam cell formation. *Circ. Res.* 107: 757–766.
- Ori, D., H. Kato, H. Sanjo, S. Tarte, T. Mino, S. Akira, and O. Takeuchi. 2013. Essential roles of K63-linked polyubiquitin-binding proteins TAB2 and TAB3 in B cell activation via MAPKs. *J. Immunol.* 190: 4037–4045.
- Bishop, G. A., and G. Haughton. 1986. Induced differentiation of a transformed clone of Ly-1<sup>+</sup> B cells by clonal T cells and antigen. *Proc. Natl. Acad. Sci. USA* 83: 7410–7414.
- Busch, L. K., and G. A. Bishop. 1999. The EBV transforming protein, LMP1, mimics and cooperates with CD40 signaling in B lymphocytes. *J. Immunol.* 162: 2555–2561.
- Hostager, B. S., and G. A. Bishop. 2002. Role of TRAF2 in the activation of IgM secretion by CD40 and CD120b. *J. Immunol.* 168: 3318–3322.
- Arcipowski, K. M., and G. A. Bishop. 2012. TRAF binding is required for a distinct subset of *in vivo* B cell functions of the oncoprotein LMP1. *J. Immunol.* 189: 5165–5170.
- Rowland, S. L., M. M. Tremblay, J. M. Ellison, L. L. Stunz, G. A. Bishop, and B. S. Hostager. 2007. A novel mechanism for TRAF6-dependent CD40 signaling. *J. Immunol.* 179: 4645–4653.
- Mercolino, T. J., L. W. Arnold, and G. Haughton. 1986. Phosphatidyl choline is recognized by a series of Ly-1<sup>+</sup> murine B cell lymphomas specific for erythrocyte membranes. *J. Exp. Med.* 163: 155–165.
- Bishop, G. A. 1991. Requirements of class II-mediated B cell differentiation for class II cross-linking and cyclic AMP. *J. Immunol.* 147: 1107–1114.
- Butt, A. Q., S. Ahmed, A. Maratha, and S. M. Miggin. 2012. 14-3-3 $\epsilon$  and 14-3-3 $\sigma$  inhibit TLR-mediated proinflammatory cytokine induction. *J. Biol. Chem.* 287: 38665–38679.
- Schneider, M., A. G. Zimmermann, R. A. Roberts, L. Zhang, K. V. Swanson, H. Wen, B. K. Davis, I. C. Allen, E. K. Holl, Z. Ye, et al. 2012. The innate immune sensor NLR3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF- $\kappa$ B. *Nat. Immunol.* 13: 823–831.
- Kobayashi, T., P. T. Walsh, M. C. Walsh, K. M. Speirs, E. Chiffolleau, C. G. King, W. W. Hancock, J. H. Caamano, C. A. Hunter, P. Scott, et al. 2003. TRAF6 is a critical factor for dendritic cell maturation and development. *Immunity* 19: 353–363.
- Bishop, G. A. 2004. The multifaceted roles of TRAFs in the regulation of B-cell function. *Nat. Rev. Immunol.* 4: 775–786.
- Tang, E. D., and C. Y. Wang. 2010. TRAF5 is a downstream target of MAVS in antiviral innate immune signaling. *PLoS One* 5: e9172.
- Takaesu, G., S. Kishida, A. Hiyaama, K. Yamaguchi, H. Shibuya, K. Irie, J. Ninomiya-Tsuji, and K. Matsumoto. 2000. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol. Cell* 5: 649–658.
- Bian, Z., J. Dai, N. Hiroyasu, H. Guan, Y. Yuan, L. Gan, H. Zhou, J. Zong, Y. Zhang, F. Li, et al. Disruption of TRAF5 exacerbates pressure overload cardiac hypertrophy and fibrosis. *J. Cell. Biochem.* In press.
- Sabroe, I., R. C. Read, M. K. Whyte, D. H. Dockrell, S. N. Vogel, and S. K. Dower. 2003. Toll-like receptors in health and disease: complex questions remain. *J. Immunol.* 171: 1630–1635.