VISA Is Required for B Cell Expression of TLR7

Liang-Guo Xu, Lei Jin, Bi-Cheng Zhang, Linda J. Akerlund, Hong-Bing Shu and John C. Cambier

*J Immunol* 2012; 188:248-258; Prepublished online 21 November 2011; doi: 10.4049/jimmunol.1100918

http://www.jimmunol.org/content/188/1/248

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/11/22/jimmunol.1100918.DC1

**References**

This article cites 42 articles, 18 of which you can access for free at:

http://www.jimmunol.org/content/188/1/248.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
VISA Is Required for B Cell Expression of TLR7

Liang-Guo Xu,*† Lei Jin,*† Bi-Cheng Zhang,*† Linda J. Akerlund,† Hong-Bing Shu,‡ and John C. Cambier*†

B cells play a critical role in the initialization and development of the systemic lupus erythematosus that is dependent on the expression of the endosomal ssRNA receptor TLR7. Previous studies have established that B cell expression of TLR7 is controlled by the type I IFN secreted by plasmacytoid dendritic cells. In this article, we report that VISA, also known as MAVS, IPS-1, and Cardif, essential for RIG-I/MDA5-mediated signaling following sensing of cytosolic RNA, regulate B cell expression of TLR7 and CD23. We found that B cells from a VISA−/− mouse express reduced TLR7 but normal basal levels of type I IFN. We also show that although IFN-β and TLR7 agonists synergize to promote TLR7 expression in VISA−/− B cells, they do not fully complement the defect seen in VISA−/− cells. Cell transfer experiments revealed that the observed effects of VISA−/− are B cell intrinsic. The reduced TLR7 expression in B cells is correlated with impaired TLR7 agonist-induced upregulation of activation markers CD69 and CD86, cell proliferation, production of IFN-α, TNF, and IL-12, and NF-κB activation. Finally, studies indicate that genetic background may influence the observed phenotype of our VISA−/− mice, because VISA−/− B cells differ in CD23 and TLR7 expression when on C57BL/6 versus 129Sv/C57BL/6 background. Thus, our findings suggest an unexpected link between VISA-mediated cytosolic RLR signaling and autoimmunity. 


Nucleic acid recognition by the innate immune system is mediated by two groups of pattern recognition receptors: the endosome-localized TLRs (TLR3, TLR7/8, and TLR9) and the cytosolic RNA (RIG-I and MDA5) and DNA sensors (1). A hallmark response to recognition of these ligands is the production of type I IFNs that activate the innate immune system, thereby modulating adaptive immune responses (1). Interplay between these innate sensory signaling pathways and various pathophysiologic conditions is only beginning to emerge.

Interesting in this context is the fact that many targets of autoantibodies produced in the autoimmune disease systemic lupus erythematosus (SLE) contain nucleic acids that act as endogenous ligands for nucleic acid-sensing pattern recognition receptors (2). A role for the endosomal TLRs in autoimmunity is revealed by studies of the Unc93b1 3d mutant mice, in which TLR3/7/9-mediated endosome nucleic acid sensing is abolished (3). These studies demonstrate that endosomal TLRs are required for production of IgG autoantibodies, IgM rheumatoid factors, and other clinical manifestations of the disease in B6-Fas−/−, as well as BXSB–Yaa-linked autoimmune acceleration (Yaa) lupus models (3). Furthermore, overexpression of TLR7 in BXSB-Yaa mice, which is due to TLR7 gene duplication, has been shown to be responsible for its development of lupus-like disease (4, 5). In contrast, the potential role of RIG-I–like cytosolic nucleic acid-sensing receptors (RLRs) in autoimmunity is unstudied.

B cells express the nucleic acid-sensing endosomal TLRs and cytosolic RLRs, and compelling evidence demonstrates a critical role for these cells in the pathogenesis of SLE, through a combination of Ab-mediated and Ab-independent actions (6). Multiple studies have demonstrated that DNA- and RNA-specific autoreactive B cells can become activated by virtue of their Ag specificity and expression of nucleic acid-sensing TLRs (TLR7 and TLR9) (7–11). Thus, by acting as targets of complex chromatin Ags carrying BCR ligands as well as TLR7 and/or TLR9 ligands, B cells may be initiators of SLE pathogenesis (6).

VISA, also known as IPS-1, MAVS, and Cardif, is a mitochondrial transmembrane protein essential for RLRs-mediated responses to cytosolic RNA (12–15). VISA−/− mice have impaired type I IFN production in response to cytosolic RNA stimulation and are susceptible to many RNA virus infections (12, 16). Despite its critical role in sensing cytosolic RNA, it is not clear whether RLR pathways mediated by VISA are involved in development of autoimmunity. Suggesting such a role are recent studies demonstrating that human VISA gene variants may be risk factors for SLE (17, 18). In this study, we generated a VISA−/− mouse and studied its phenotype on a mixed 129Sv/C57Bl6 background and show that in this context VISA is required for B cell expression of TLR7. It is suggested that TLR7 expression must be tightly regulated to prevent development of autoimmunity (19). Thus, the results provide the first evidence, to our knowledge, that, like the endosomal RNA sensing receptor TLR7, the VISA-mediated cytosolic RNA sensing RLRs may be involved in the development of lupus-like disease.

Materials and Methods

Generation of VISA knockout mice

The VISA targeting vector was constructed by replacing a 4.3-kb genomic region of murine VISA gene, including exons 2, 3, 4, and 5, with a PGK-

*†Integrated Department of Immunology, University of Colorado School of Medicine and National Jewish Health, Denver, CO 80206; ‡College of Life Science, Wuhan University, Wuhan, 430072, People’s Republic of China

†For correspondence and reprint requests to Dr. John C. Cambier or Dr. Hong-Bing Shu, Integrated Department of Immunology, University of Colorado School of Medicine and National Jewish Health, 1400 Jackson Street, K303, Denver, CO 80206 (J.C.C.). J.C.C. is an Ida and Cecil Green Professor of Immunology.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100918

The online version of this article contains supplemental material.

Abbreviations used in this article: IRF, IFN regulatory factor; LOX, Loxoribine; PDC, plasmacytoid dendritic cell; qRT-PCR, quantitative RT-PCR; RLR, RIG-I–like cytosolic nucleic acid sensing receptor; SLE, systemic lupus erythematosus; WT, wild-type; Yaa, Y-linked autoimmune acceleration.
The Journal of Immunology 249

Neu selection cassette (Fig. 1). The targeting construct was transduced into TC1 ES line (derived from 129Sv strain). G418-resistant ES clones were screened by Southern blot analysis. Positive clone was microinjected into C57BL/6 blastocysts to produce chimera mice. Chimeric mice were bred to C57BL/6 mice to obtain germine transmission. The heterozygous F1 progenies were intercrossed to obtain VISA−/− mice. The VISA−/− mice were then backcrossed to C57BL/6 strain for four generations. The experiments were done on VISA−/− and their wild-type (WT) littermates. The genotypes of the mice were determined by genomic PCR. TLR7−/− mice were provided by Dr. P. Marrack (National Jewish Health). Animals were generated at the National Jewish Health Mouse Genetics Core Facility and used at 6–8 wk of age. Animal care and handling was performed as per Institutional Animal Care and Use Committee guidelines (protocol No. AS-2519-04-12).

Reagents
Recombinant mouse IFN-β protein (ab84998; Abcam), Loxoribine (LOX) (tirI-lox; InvivoGen), R848 (tirI-r848; InvivoGen), CpG-ODN 1668 (tirI-1668; InvivoGen), anti–IRAK1 Ab (No. 4504; Cell Signaling Technology), anti–IRAK4 Ab (No. 4363; Cell Signaling Technology), anti-MyD88 Ab (sc-11356; Santa Cruz Biotechnology), anti–TLR7 Ab (14-9079; eBioscience), anti–TLR9 Ab (14-9092; eBioscience), anti–TLR4 Ab (MAB2759; R&D Systems) and anti–TLR3 Ab (MAB3005; R&D Systems), anti–IκBα Ab (catalog No. 9242; Cell Signaling Technology), and anti–NF-κB p65 Ab (sc-203; Santa Cruz Biotechnology). Spleens from MAYS knockout mice were a gift from Dr. Z. Chen (UT Southwestern Medical School).

B cell purification and culture
Splenic B cells were purified from 6- to 8-wk-old mice using Miltenyi Biotec C11 selection kit as per the manufacturer’s instructions. Purified B cells were cultured at 5 × 10^5 cells/ml in IMDM (HyClone, Logan, UT) supplemented with 5% FBS (200-p500H; BioSource International), 1-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), sodium pyruvate (1 mM), and 2-ME (50 μg/ml). Cells were cultured at 37°C with 5% CO2. All culture reagents were from Life Technologies (Gaithersburg, MD).

Calcium mobilization ([Ca^{2+}]).
Intracellular calcium concentration was measured as described previously (20). Briefly, cells were loaded with indo-1AM (Molecular Probes, Eugene, OR) for 30 min at 37°C and then washed and suspended in IMDM supplemented with 2% FCS at 5 × 10^7 cells/ml. Cells were stimulated with goat anti-mouse IgM-F(ab)2 (115-006-020; Jackson ImmunoResearch Laboratories). Data were collected on an LSR II and analyzed by FlowJo (Tree Star, San Carlos, CA).

Mouse IFN-α, TNF, and IL-12 ELISA
IFN-α, TNF, and IL-12 were measured in culture supernatants using commercial ELISA kits (IFN-α kit, PBL Biomedical Laboratories; Vigi-Kine Mouse IFN-α ELISA Kit, 42100, TNF kit, 88-7324-22, eBioscience; and IL-12 p70 kit, M1270, R&D Systems).

Flow cytometric assay of immunofluorescence
Cells were stained with PerCP-anti-B220 (RA3.3A1; BD Pharmingen), FITC-anti-CD86 (GL1BD; Pharmingen) and allophycocyanin-anti-CD69 (H1.2F3; BioLegend), allophycocyanin- or PE-anti–CD23 (BD Pharmingen, clone B384), FITC-anti-CD21 (BD Pharmingen, 7G6), PE-anti–IgD (11-26; Santa Biotechnology Associates), and PerCP-anti–IgM (R6–60.2; BioLegend). FACs was performed using a FACScan flow cytometer (BD Biosciences) and analyzed by FlowJo (CellQuest).

Immunoprecipitation and SDS-PAGE and immunoblotting
Cells were lysed in cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM sodium pyrophosphate, 2 mM Na3VO4, 10 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) at 4°C for 1 h. Cell lysates were centrifuged at 12,000 × g at 4°C for 10 min. Immunoprecipitation was done in the lysates with indicated Abs conjugated Sepharose beads. The immunoprecipitates were run on an SDS-PAGE and probed with indicated Abs.

Quantitative real-time PCR
Quantitative real-time PCR (qPCR) were done as described previously (21). Briefly, RNA was isolated using RNeasy kit (74104; Qiagen), and cDNA was synthesized using the Superscript III First-Strand Synthesis kit (11752-050; Invitrogen). qPCRs were done in an ABI PRISM 5700 Sequence Detection System (Applied Biosystems). Data were normalized to GAPDH control and represented as fold change over WT control. Primers used in the qPCR are as follows: TLR7-F, 5′-CCACAGCTCCACC-ATACCTC-3′; TLR7-R, 5′-GGCATGTCCTAGGTGGTGACA-3′; TLR9-F, 5′-TGGGCCCATTTGATGAAC-3′; TLR9-R, 5′-TTGTTCTGCCACC-TCCAACAGT-3′; IFN-β-F, 5′-CCTCTGGAGAGAGCGGAAG-3′; IFN-β-R, 5′-TTCCACGGACGTCCAATGAC-3′; IFN-γ-F, 5′-AGAAGAGGAGGCTCCTCAGC-3′; GAPDH-F, 5′-ACCTGTGTTCGTAGCCGTATTCC-3′; and GAPDH-R: 5′-ACCTGTGTTCGTAGCCGTATTCC-3′. B cells were swollen in 500 μl buffer A (10 mM HEPES, 10 mM NaCl, 5 mM EDTA, 1.5 mM MgCl2, 10 μg/ml aprotinin and leupeptin, 2 mM DTT, and 1 mM PMSF [pH 7.8]) at 4°C for 10 min then lysed in equal volume of buffer B (buffer A plus 1.2% Nonidet P-40). Samples were vortexed for 10 s. The pellets were collected and washed with buffer A and then suspended in 4.5 μl buffer C (buffer A plus 10% glycerol). The suspension was mixed with 5 μl 4.1 M NaCl and left at 4°C for 30 min. The nuclear extract supernatant was collected and quantitated with Bio-Rad protein assay kit. The NF-κB targeting oligonucleotide 5′-GGGGACCTTCCC-3′ (Santa Cruz Biotechnology) labeled with [γ-32P]ATP by T4 DNA kinase was added into 20 μg nuclear extract and 0.8 μg poly(deoxyinosinic-deoxycytidylic) acid (20148E, Thermo Scientific) in binding buffer (20 mM Tris, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, and 5% glycerol [pH 7.5]) at room temperature for 15 min. The mixture was fractionated in 5% acrylamide gel prepared in 0.5× Tris borate–EDTA. The gel was dried and subjected to autoradiography.

[1H]Uptake assay
B cells (3 × 10^7 cells/well in 150 μl medium in 96-well plates) were stimulated with indicated TLR agonists for 24 h and then pulsed for 8 h with 1 μCi [1H]thymidine/well (Amersham Biosciences). Incorporation of [1H]thymidine was quantitated by a liquid scintillation beta counter (PerkinElmer).

Adoptive cell transfer
Purified splenic B cells were labeled with CFSE (2.5 μM) for 3 min in PBS buffer. Cells were then washed with PBS containing 5% FBS followed by three more washes with PBS. The labeled cells (1 × 10^7) were transferred (i.v.) to recipient mice (WT or VISA−/−).

Results
Decreased CD23 expression in VISA−/− mice was observed by flow cytometry and immunofluorescence. Cells were stained with PerCP-anti-B220 (RA3.3A1; BD Pharmingen), FITC-anti-CD86 (GL1BD; Pharmingen) and allophycocyanin-anti-CD69 (H1.2F3; BioLegend), allophycocyanin- or PE-anti–CD23 (BD Pharmingen, clone B384), FITC-anti-CD21 (BD Pharmingen, 7G6), PE-anti–IgD (11-26; Santa Biotechnology Associates), and PerCP-anti–IgM (R6–60.2; BioLegend). FACs was performed using a FACScan flow cytometer (BD Biosciences) and analyzed by FlowJo (CellQuest).

Immunoprecipitation and SDS-PAGE and immunoblotting
Cells were lysed in cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM sodium pyrophosphate, 2 mM Na3VO4, 10 mM NaF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) at 4°C for 1 h. Cell lysates were centrifuged at 12,000 × g at 4°C for 10 min. Immunoprecipitation was done in the lysates with indicated Abs conjugated Sepharose beads. The immunoprecipitates were run on an SDS-PAGE and probed with indicated Abs.

Quantitative real-time PCR
Quantitative real-time PCR (qPCR) were done as described previously (21). Briefly, RNA was isolated using RNeasy kit (74104; Qiagen), and...
qPCR also confirmed that VISA/−/− B cells (CD43−) express low level of CD23 transcripts (Fig. 2D).

VISA/−/− B cells undergo slightly elevated calcium flux in response to low dose of anti-IgM stimulation

We also investigated BCR signaling in VISA/−/− mice. We found that VISA/−/− B cells have relative normal calcium mobilization in response to anti-IgM stimulation (Fig. 2E). However, at a low dose (0.26 μg/ml anti-IgM), VISA/−/− B cells elicit slightly great calcium response (Fig. 2E). VISA/−/− B cells also have normal up-regulation of CD69 costimulating factor in response to anti-IgM, anti-CD40, or IL-4 stimulation (Supplemental Fig. 1B). We concluded that the BCR signaling in VISA/−/− B cells is largely unaffected by the lack of VISA expression.

The low expression of CD23 in VISA/−/− mice is B cell intrinsic

Previous studies have shown that CD23 expression can be affected by matrix metalloproteases in the microenvironment of cells (21, 22). To begin to explore the role of microenvironmental factors in the observed loss of CD23 expression, we conducted adoptive transfer experiments in which B cells from WT or VISA/−/− mice were transferred to WT or VISA/−/− recipient mice in all permutations. Twenty-four hours later, recipients’ spleens were harvested, and B cell expression of CD23 was assessed. As shown in Fig. 2F, expression of CD23 was not affected by “parking” WT B cells in VISA/−/− mice. In addition, CD23 was not upregulated following parking B cells from VISA/−/− mice in WT mice. We conclude that the VISA requirement for normal expression of CD23 is B cell intrinsic.

Anti-CD40, LPS, and IL-4 stimulation upregulate CD23 expression in VISA/−/− B cells

B cell expression of CD23 can be upregulated by treatment of LPS (23), IL-4 (24), or anti-CD40 Abs (25) through the activation of transcriptional factors STAT6, NF-κB, or a combination of both (26). We determined whether CD23 upregulation is also impaired in VISA/−/− B cells. Purified splenic B cells were cultured with anti-CD40 mAb (FGK-45), LPS, or IL-4 for 24 h, and surface CD23 expressions were examined. Previous studies have found that CD23 is constitutively cleaved from the surface of human and mouse B cells by CD23-releasing enzyme ADAM10 (27, 28). Consistent with these reports, overnight culture of ex vivo B cells led to reduced surface CD23 expression regardless of stimulation (Fig. 3, mock). Also, in agreement with previous reports, anti-CD40 mAb, LPS, and IL-4 stimulation increased B cells expression of CD23 (Fig. 3A–C, solid thin lines). Importantly, CD23 expression was also increased in VISA+/− B cells (Fig. 3A–C, dotted thin lines). Comparing the relative increase in CD23 expression induced by these stimuli, we found that anti-CD40 mAb stimulates greater upregulation of CD23 expression by VISA+−/− (29-fold increase) than in WT B cells (~21-fold increase), whereas induced upregulation of CD23 by LPS and IL-4 was decreased in VISA+−/− B cells (Fig. 3E). There were no differences in the upregulation of CD69 between VISA+−/− and WT B cells by these stimuli (Fig. 3A–C, right panels). As a negative control, IFN-β did not affect CD23 expression in WT or VISA+−/− B cells although CD69 expression was upregulated in these cells (Fig. 3D). We concluded that VISA+−/− B cells can still upregulate CD23 expression in response to anti-CD40, LPS, or IL-4 stimulation but at a slightly different magnitude than WT B cells.

VISA expression is required for TLR7-mediated upregulation of CD69 and CD86 in B cells

VISA is an antiviral protein that is required for RLR-initiated type I IFN production in immune cells as well as fibroblasts (12, 16). Although previous studies suggest that VISA is not involved in nucleic acid sensing and signaling by TLR3 and TLR9 (12, 16), it is not clear whether VISA is involved in signaling by TLR7, which senses endosomal ssRNA. To determine the effect of VISA expression on TLR7 signaling, we examined the upregulation of CD23 and CD69 in VISA+−/− B cells by TLR7 agonist LOX. Unlike the IL-4, anti-CD40, and LPS stimulation, LOX failed to induce increased CD23 and CD69 expression by VISA+−/− B cells (Fig. 3E, 3F).

We further examined the upregulation of CD69 in VISA+−/− B cells at different time points and with various doses of LOX or R848, another TLR7 agonist (Fig. 4A). Hyporesponsiveness was observed throughout the time course and stimulus dose range (Fig.
A. Splenocytes were stained with indicated Ab (left panel). Immature B cells (B220^+CD93^+) were further analyzed for CD23 expression (middle panel) and transitional B cells by IgM and IgD expression (right panel), T1 (IgM^{high}IgD^{low}), and T2 (IgM^{high}IgD^{high}). B. Mature B cells (B220^+CD93^-) were stained for CD21 and CD23 to separate marginal zone (MZ) and follicular (FO) populations. C. Splenocytes were analyzed for CD23 expression in B220^+ B cells and B220^- non-B cells. D. Total RNAs of B cells (CD43^-) or non-B cells (CD43^+) from WT and VISA^-/- mice were extracted, and qRT-PCR was performed as in Materials and Methods to analyze CD23 expression. Average ± SEM of three experiments. E. B220^+ B cells were loading with indo-1 AM and activated with indicated dose of goat anti-mouse IgM-F(ab')2 (Jackson ImmunoResearch Laboratories). F. Purified B cells from WT and VISA^-/- mice were labeled with CFSE and transferred i.v. to unirradiated recipients. After 24 h, CD23 expression in the spleen of recipient mice was analyzed. Data are representative of three or more independent experiments.

We also checked the upregulation of another costimulator factor CD86 in VISA^-/- B cells induced by LOX or R848. TLR7 agonist-induced CD86 upregulation was also dramatically inhibited in VISA^-/- B cells (Fig. 4B). As a control, we treated these B cells with TLR9 agonist CpG-ODN 1668. In agreement with a previous report, there was no difference in TLR9 agonist-induced upregulation of CD69 and CD86 expression (Fig. 4C, 4D) (16) by WT and VISA^-/- B cells. We conclude that VISA is required, selectively, for TLR7-mediated CD69 and CD86 upregulation in B cells.
A more dramatic effect was seen in IL-12 production, in which a less IFN-γ signaling and cytokine expression was observed in VISA−/− B cells (Fig. 5B). We found that although VISA−/− B cells respond similarly to CpG, their responses to TLR7 agonists LOX and R848 are impaired (Fig. 5A).

B cells also respond to TLR ligands by production of cytokines. To determine whether VISA deficiency affects cytokine production in B cells stimulated by TLR7 agonists, we cultured ex vivo B cells with TLR7 agonist LOX and measured subsequent production of IL-12 p70, TNF, and IFN-γ by ELISA. To eliminate the potential confounding effects of contaminating dendritic cells and macrophages in our B cell preparation, we isolated B cells that were >99.8% pure by CD43, CD11b, and CD11c depletion of splenocytes. We were able to detect ~65 pg/ml IFN-γ from LOX-stimulated WT B cells (Fig. 5B). However, VISA-deficient B cells produced ~50% less IFN-γ and TNF in response to LOX treatment (Fig. 5B, 5C).

A more dramatic effect was seen in IL-12 production, in which VISA−/− B cells made very little IL-12 relative to WT B cells (Fig. 5D). The production of IL-12 p70 by TLR agonists is known to depend on type I IFN signaling in dendritic cells (29, 30). Thus, it is likely that the difference in the production of IL-12 p70 is due to the low type I IFN production by LOX in VISA−/− B cells.

**VISA is required for TLR7-mediated NF-κB activation in B cells**

We then undertook studies of intermediary steps in the TLR signaling cascade in search of the site of action of VISA. Because TLR7 stimulation of activation marker and cytokine expression requires activation of NF-κB (31, 32), we examined whether the activation of NF-κB by TLR7 is impaired in VISA-deficient B cells. As shown in Fig. 6A, VISA−/− B cells displayed impaired LOX-induced NF-κB activation (Fig. 6A, left panel). The stimulation by LOX through TLR7 is specific, because LOX did not activate NF-κB in TLR7−/− B cells (Fig. 5A, right panel). Consistent with the previously seen specificity of VISA deficiency (Fig. 4), the TLR9 agonist CpG-ODN1668 induced equivalent NF-κB activation in WT and VISA−/− B cells (Fig. 6A, middle panel). Thus, VISA is required for TLR7, but not TLR9, mediated activation of NF-κB.

NF-κB signaling consists of two distinct pathways: the canonical NF-κB pathway that is indicated by the degradation of the NF-κB inhibitor IκBα that allows the transcriptional factors NF-κB1 (RelA) and p50 to enter into nucleus (33). The degradation of IκBα, thus the activation of the canonical NF-κB, is mediated by IκB kinase β (33). Consistent with our EMSA result, we found that degradation of IκBα is inhibited in LOX-activated VISA−/− B cells (Fig. 6B).

The noncanonical NF-κB pathway is mediated by IκB kinase α via processing of NF-κB2 (p100) to transcriptional factor p52 (33). There was no significant processing of p100 to p52 in LOX-stimulated B cells (Fig. 6B). We concluded that LOX mainly activates canonical NF-κB pathway in B cells and VISA is required for this activation.

**VISA is required for TLR7 agonist induced MyD88–IRAK1 association**

Moving closer to the level of TLR7 signal initiation, we examined the effect of VISA deficiency on TLR7-mediated association of

---

**FIGURE 3.** Anti-CD40, LPS, and IL-4, but not TLR7, stimulation up-regulate CD23 expression in VISA−/− B cells. A–D, and F, CD43-depleted splenic cells (B cells) from WT and VISA−/− mice were cultured with anti-CD40 mAb (FGK-45) (10 μg/ml), LPS (10 μg/ml), IL-4 (20 ng/ml), IFN-β (250 U/ml), and LOX (400 μM) or mock for 20 h. Cells were then stained for CD23 (left panel) or CD69 (right panel). E, Relative increase of CD23 expression was calculated from the mean fluorescence intensity of CD23 from activated cells divided by the mean fluorescence intensity of mock-treated cells.

A–D, Anti-CD40, LPS, and IFN-β. A, Relative cell number.

E, Relative cell number.

F, Relative cell number.

VISA REGULATES B CELL EXPRESSION OF TLR7 AND CD23
MyD88 with IRAK1. MyD88 is a key adaptor protein that mediates TLR7 signaling by recruiting the ser/thr kinase IRAK1 leading to NF-κB activation (31). Indeed, we found that LOX-induced MyD88–IRAK1 association is dramatically decreased in VISA−/− B cells (Fig. 6C). We also found that MyD88 is constitutively associated with IRAK4 in B cells, and this is independent of VISA expression (Fig. 6C, second panel).

VISA-deficient B cells exhibit reduced expression of TLR7

VISA is a potent type I IFN stimulator (13, 34). Two recent studies have shown that type I IFN signaling in B cells (35, 36). Furthermore, B cells from these mice have very low TLR7 expression (35, 36). The findings discussed above are consistent with the possibility that VISA is required for expression of TLR7. We then tested TLR7 expression in VISA−/− B cells. Using Abs against various TLRs, we found that the expression of TLR7 is dramatically decreased in VISA−/− B cells (Fig. 7A, CD43+ splenocytes). This VISA requirement is TLR7 specific because VISA deficiency does not affect TLR3, TLR4, and TLR9 expression (Fig. 7A). Finally, RT-PCR also confirmed the decrease of TLR7, but not TLR9, transcripts in VISA−/− B cells (Fig. 7B). These findings further indicate that the VISA signaling pathway specifically regulates TLR7 expression, and this regulation is exercised at the level of gene transcription.

TLR7 agonist synergizes with IFNβ to increase B cell expression of TLR7

We then began to explore potential mechanisms by which VISA deficiency affects TLR7 expression. It is established that B cell expression of TLR7 is controlled in part by type I IFN signaling (35–37). B cells from IFNAR1−/−, IFNAR2−/−, STAT1−/−, or IRF9−/− mice all have dramatically decreased TLR7 expression...
Furthermore, type I IFN treatment can increase B cell expression of TLR7 (36). We confirmed that treatment of B cells with IFN-β increases TLR7 expression (Fig. 7C). However, we found that treating B cells with both the TLR7 agonist LOX and IFN-β can lead to much greater induction of TLR7 expression than either treatment alone (Fig. 7C). This increase is best seen after 24 h (Fig. 7D). TLR7 stimulation of purified B cells leads to additional type I IFN production (Fig. 5B), which is far less than the IFN-β we added in this study (400 U/ml). We concluded that it is TLR7 signaling and not the additional type I IFN generated by FIGURE 5. VISA-deficient B cells proliferate poorly and have decreased cytokine productions in response to TLR7 agonists. A, Splenic B cells from WT and VISA−/− mice were stimulated with indicated concentration of LOX, R848, or CpG-ODN 1668, and proliferation was measured by [3H]thymidine uptake. Average ± SEM of three experiments. B–D, Splenic B cells from WT and VISA−/− mice were stimulated with indicated concentration of LOX for 20 h. Levels of IFN-α, TNF, and IL-12 in culture supernatants were measured by ELISA. Average ± SEM of three experiments.

FIGURE 6. B cells from VISA−/− mice have impaired TLR7 signaling. A, Purified B cells from WT and VISA−/− mice were treated with 400 μM LOX (left panel) or 64 nM CpG-ODN 1668 (middle panel) for the indicated time course. EMSA was done as described in Materials and Methods. NF-κB activation was indicated. At the right panel, B cells from TLR7−/− mice were treated with 400 μM LOX or 64 nM CpG for 60 min, and NF-κB activation was measured by EMSA as the other panels. B, B cells from WT and VISA−/− mice were treated with LOX (400 μM) for the indicated time. The cells were lysed, and the whole-cell lysates (WCL) were run on a SDS-PAGE and probed with indicated Abs. C, B cells from WT and VISA−/− mice were treated with LOX (400 μM) for the indicated time. Cells were lysed and precipitated by anti-MyD88 Ab. The immunoprecipitates were run on a SDS-PAGE and probed with indicated Abs.
TLR7 stimulation, which augments IFN-β–induced TLR7 expression.

We also confirmed that type I IFN signaling is absolutely required for the upregulation of TLR7 expression in B cells, because the combination of LOX and IFN-β stimulation does not increase TLR7 expression in IFNRA2/2 B cells (Fig. 7E). Thus, in the presence of IFN-β, TLR7 signaling acts to further upregulate its own expression. The combined treatment also leads to much greater upregulation of CD69 than LOX or IFN-β treatment alone (Fig. 7F).

We then tested the ability of IFN-β and TLR7 agonists to restore TLR7 expression in B cells from VISA2/2 mice. Although LOX and IFN-β treatment increased TLR7 expression (Fig. 7C, 7D), levels did not approach that seen in WT B cells. This suggests that low expression of TLR7 in VISA2/2 B cells may be B cell intrinsic. Otherwise, LOX and IFN-β would be expected to completely restore the TLR7 in VISA2/2 B cells to levels seen in similarly stimulated WT B cells. This hypothesis is supported by our observation that VISA deficiency does not affect the autonomous production of either IFN-α or IFN-β in spleen measured by quantitative RT-PCR (qRT-PCR) (Fig. 7H).

The TLR7 expression defect in VISA2/2 is B cell intrinsic

To explore the possibility that the defect in TLR7 expression in VISA2/2 is, like the CD23 expression defect, B cell intrinsic, we conducted reciprocal adoptive transfer experiments. B cells from WT or VISA2/2 mice were purified, labeled with CFSE, and transferred into either VISA2/2 or WT mice. Parking of VISA2/2 B cells in WT mice for 24 h did not result in restoration of TLR7 mRNA levels (Fig. 7G). Transfer of WT B cells to VISA2/2 mice also did not lead to changes in TLR7 mRNA levels (Fig. 7G). We conclude that the abnormal expression of TLR7 in VISA2/2 mice is, like the CD23 defect, B cell intrinsic.

The decreased expressions of CD23 and TLR7 in VISA2/2 B cell are likely due to genetic background of the VISA2/2 mouse

Both the CD23 and TLR7 defect in our VISA2/2 mouse are B cell intrinsic. Considering the fact that our VISA2/2 mouse is on a 129Sv/C57BL6 mixed background, it is possible that genetic background may play a role in the development of these defects. To test this hypothesis, we obtained B cells from MAVS knockout. MAVS is another name for VISA. The MAVS knockout mouse has the same deletion in VISA gene but is in C57BL/6 background.
(12). Surprisingly, we found MAVS\(^{−/−}\) B cells have normal CD23 expression (Fig. 8A). Unlike the VISA\(^{−/−}\) B cells, the MAVS\(^{−/−}\) B cells also undergo normal upregulation of CD69 (Fig. 8B, upper two panels) and proliferation in response to TLR7 activation (Fig. 8C). Finally, WT and MAVS\(^{−/−}\) B cells have similar levels of TLR7 expression (Fig. 8D). We conclude that defects in CD23 and TLR7 expression seen in VISA\(^{−/−}\) B cells are likely due in part to genetic background.

**Discussion**

VISA mediates signaling following RIG-I/MDA5 sensing of cytosolic RNA and thereby plays a critical role in host defense against RNA virus infection. In this paper, we describe two previously unrecognized functions of VISA. We found that VISA regulates the expression of CD23 and TLR7. The observed effect on TLRs is TLR7 specific, because no reduction was seen in TLR3, TLR4, or TLR9. Studies further show that VISA promotion of TLR7 expression is B cell intrinsic and cannot be completely compensated by exogenous IFN-\(\beta\) but rather must involve an as-yet unidentified VISA function that acts in a cell-intrinsic manner. VISA regulation of CD23 expression was also found to be cell intrinsic.

These findings are interesting in the context of the role of TLR7 expression in lupus-like autoimmune disease. Manipulation of TLR7 expression in various lupus-prone mouse strains has demonstrated that TLR7 is critically involved in the development of lupus nephritis and production of autoantibodies against RNA-associated nuclear Ags (7–11). Furthermore, overexpression of TLR7 because of duplication of the TLR7 gene has been shown to underlie lupus-like disease in the BXSB-Yaa mouse (4, 5). It has been proposed that TLR7 expression levels set a threshold for the development of autoimmunity (19). Thus, by virtue of its requirement for establishing TLR7 expression levels in B cells, we speculate that VISA may play a role in the development of autoimmune disease. Future studies, such as introduction of VISA deficient into lupus-prone mice, will be done to determine the role of VISA in the initiation and development of lupus-like diseases.

The question remains how intrinsic VISA functions could regulate TLR7 expression in B cells. Previous study showed that

**FIGURE 8.** B cells from MAVS\(^{−/−}\) mouse have normal CD23 and TLR7 expression. A, Splenic cells from MAVS\(^{−/−}\), VISA\(^{−/−}\), and the WT control mice were gated on B220 and CD23 expression was measured as before. B, Purified splenic B cells from MAVS\(^{−/−}\) and VISA\(^{−/−}\) and their WT littermate control mice were stimulated with LOX (400 \(\mu\)M) or CpG-ODN 1668 (50 nM) for 20 h. CD69 expression was measured as before. C, Purified splenic B cells from MAVS\(^{−/−}\) and VISA\(^{−/−}\) and their WT littermate control mice were stimulated with LOX (400 \(\mu\)M). Cell proliferation was measured by \[^{3}H\]thymidine uptake as in Fig 5A. Average ± SEM of three experiments. D, TLR7 expression in splenic B cells from indicated mice was detect as in Fig 7.

**FIGURE 9.** Model: VISA regulates B cell expression of CD23 and TLR7. B cell expression of TLR7 is regulated by type I IFN secreted by PDC (signal 1) and TLR7 signaling itself (signal 2). VISA mediates TLR7-induced NF-\(\kappa\)B activation, thus affect signal 2 and TLR7 expression in B cells. In contrast, VISA-mediated NF-\(\kappa\)B activation is also required for CD23 expression in B cells (signal 3). This NF-\(\kappa\)B activation could come from the autonomous VISA-mediated TLR7 signaling, RIG-I/MDA5 signaling, or both that control CD23 expression.
B cell expression of TLR7 is controlled by type I IFN secreted by plasmacytoid dendritic cells (PDC) (37). Later studies also found that B cells from IFNAR<sup>−/−</sup>, IFNAR2<sup>−/−</sup>, STAT1<sup>−/−</sup>, or IRF9<sup>−/−</sup> mice have low TLR7 expression (35, 36, 38), which confirms the notion that B cell expression of TLR7 is controlled by type I IFN provided by PDC. However, VISA is not required for the type I IFN productions in PDC (12). Consistent with that report, we showed that the basal type I IFN levels are normal in spleen from VISA<sup>−/−</sup> mice (Fig. 7H). This suggests that the type I IFN signaling is required but not sufficient for B cell expression of TLR7. A second B cell-intrinsic signal from VISA is also required for its expression (Fig. 9). We hypothesize that a steady state of VISA signal through RIG-I/MDA, either by tonic or nonmicrobial RNA ligands, directly drives the expression of TLR7, possibly by VISA-mediated activation of NF-kB (Fig. 9).

The low expression of CD23 may also be explained by the lack of VISA-mediated activation of NF-kB because it has been shown that B cell expression of CD23 depends on the activation of NF-kB pathway (39). Alternatively, decreased CD23 expression may be due to the impaired TLR7 signaling in these B cells, because TLR7 activation fails to upregulate CD23 expression in these cells (Fig. 2).

We also found that defects in expression of both CD23 and TLR7 are B cell intrinsic and due to the genetic background of our VISA<sup>−/−</sup> mouse, because the VISA<sup>−/−</sup> mouse in C57BL/6 background (MAVS<sup>−/−</sup>) does not have these phenotypes. Thus, the low TLR7 expression as well as the decreased CD23 by VISA deficiency only manifests on a permissive genetic background.

It has been recognized that genetic background of mouse strain plays a big role in the development of autoimmune disease (40, 41). The BXSB strain of mice spontaneously develops an SLN-like phenotypes as a result of the presence of the Yaa mutation that carries an extra copy of TLR7 gene (4, 5). However, the Yaa mutation affects B cell expression of TLR7 in the certain genetic background human populations (17, 18). Thus, our finding that VISA deficiency of-function human VISA variant, are associated with a subset of systemic lupus patients. Possible association of VISA gene polymorphisms with susceptibility to systemic lupus erythematosus in Chinese population. Mol. Biol. Rep. 38: 4583–4588.


