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VISA Is Required for B Cell Expression of TLR7

Liang-Guo Xu,*1 Lei Jin,*1 Bi-Cheng Zhang,*1 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−/− B cells are cell intrinsic. The reduced TLR7 expression in B cells is correlated with impaired TLR7 agonist-induced upregulation of activation markers CD69 and CD23, 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. The Journal of Immunology, 2012, 188: 248–258.

Nucleic acid recognition by the innate immune system is mediated by two groups of innate 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 pathophysiological 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 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 understudied.

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/C57B6L 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 studies 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-

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Abbreviations used in this article: IRF, IFN regulatory factor; LOX, Loxoribine; NOD, NOD2/CARD15; 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.

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Neo selection cassette (Fig. 1). The targeting construct was transfections 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 chimeric mice. Chimeric mice were bred to C57BL/6 mice to obtain germline 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. TL7−/− 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), and IL-12 p70 kit, M1270, R&D Systems). Knockout mice were a gift from Dr. Z. Chen (UT Southwestern Medical Laboratories). Data were collected on an LSR II and analyzed by Flow-Jo software (TreeStar, San Carlos, CA).

B cell purification and culture
Splenic B cells were purified from 6- to 8-wk-old mice using Miltenyi Biotec B cell isolation kit as per the manufacturer’s instructions. Purified B cells were cultured at 5 × 10^6 in IMDM (HyClone, Logan, UT) supplemented with 5% FBS (200p-500FH; 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 µM). Cells were cultured at 37°C with 5% CO². All culture reagents were from Life Technologies (Gaithersburg, MD).

Calcium mobilization ([Ca²⁺]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 10^6 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 Flow-Jo software (TreeStar, 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; Véri-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 Pharamingen), FITC-anti-CD86 (GL183; Pharmingen) and allophycocyanin-anti-CD69 (H1.2F3; BioLegend), allophycocyanin- or PE-anti-CD23(BD Pharamingen, clone B384), FITC-anti-CD21(BD Pharamingen, 706), PE-anti-IgD (11-26; Southern Biological Technology Associates), and PerCP-anti-IgM (R6-60.2; BioLegend). FACS was performed using a FACSscan flow cytometer (BD Biosciences) and analyzed by FlowJo software (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 NaVO₄, 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). Results were normalized to GAPDH control and represented as fold change over WT control. Primers used in the qPCR are as follows: TL7R-F, 5′-CCACAGCTCACC-ATACCTC-3′; TL7R-5′-GGCATGTCCTAGGTGGTGACA-3′; TL9R-F, 5′-GGGCCATTTGATGAAAC-3′; TL9R-5′-TTGCTGCCACC-TCCAACAGT-3′; IFN-βF, 5′-CCCTAGGAGATCCGGAAG-3′; IFNβ-R, 5′-CCCTAGTCATCTTCTCC-3′; IFNα-4-F, 5′-TCCAATGCG-AGCTCTAGAC-3′; IFNα-R, 5′-AGGAGAGGGCGCTCTCC-3′; GAPDH-F, 5′-ACCCTGGTCTGATGCGGATT-3′; and GAPDH-R: 5′-ACCCTGGTCTGATGCGGATT-3′. EMSA
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 B 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 quantified with Bio-Rad protein assay kit. The NF-κB targeting oligonucleotide 5′-GGGACCTTCCC-3′ (Santa Cruz Biotechnology) labeled with [³²P]ATP by T4 DNA kinase was added into 20 µg nuclear extract and 0.8 µg poly(deoxyinosinic-deoxy-cytidylic) 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.

['H] uptake assay
B cells (3 × 10⁵ 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 ['H]thymidine/well (Amersham Biosciences). Incorporation of ['H]thymidine was quantified 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 × 10⁷) were transferred (i.v.) to recipient mice (WT or VISA−/−).

Results
Decreased CD23 expression in VISA−/− mice
To investigate the in vivo functions of VISA in B cells, we generated VISA−/− mice by homologous recombination. The VISA gene was disrupted by replacing its exons 2, 3, and 4 with a neo gene (Fig. 1A). Homologous recombination and the disruption of VISA were confirmed by Southern blot (Fig. 1B) and immunoblot (Fig. 1C) analyses. Consistent with the role of VISA in antiviral response, mouse embryonic fibroblast cells from our VISA−/− mice fail to activate transcriptional factors NF-κB and IFN regulatory factor (IRF3) in response to Sendai virus infection (Fig. 1D). VISA−/− mice are born at the expected Mendelian ratio and bred normally. B cells were isolated by erythrocyte lysis and CD43 depletion of splenocytes, yielding a cell population that was 99.5% B cells. B cells from VISA−/− were found to have normal surface IgM and IgD expression (Supplemental Fig. 1A) and similar numbers of B220⁺CD93⁺ transitional and B220⁺CD93⁺ mature B cells (Fig. 2A, left two panels). The B220⁺CD93⁺ splenic transitional B cells can be further separated into T1 and T2 compartments based on their IgM, IgD, and CD23 expression. Although both compartments were present and cells occurred in normal numbers based on IgM, B220, IgD, and CD39 expression, CD23 expression was dramatically decreased on T2 cells from VISA−/− mice (Fig. 2A, middle two panels). Although mature B cells were also seen in normal frequency in VISA−/− mice, they too exhibited much reduced expression of CD23 (Fig. 2B, 2C).
Ab. analysis was performed by using anti-VISA (+/+)
(+/−) and heterozygote mutant (+/−). Genomic DNA was
targeting vector, and the predicted mutated
mutant (−/−), and both bands for heterozygote
probes indicated. The
blotting gave a single 11.0-kb band for WT
and hybridized with a probe indicated. The
(+/+), a single 7.0-kb band for homozygote
mutant (−/−), and both bands for heterozygote
(+/−). Total splenic cells were harvested from
WT (+/+), heterozygote (+/−), and homozygote mutant (−/−), and Western blotting
analysis was performed by using anti-VISA
Ab. D. MEF cells from WT and VISA−/− mice were
stimulated with Sendai virus, and nuclear translocation of IRF3 was done as described
previously (13). EMSA was performed as
described in Materials and Methods.

FIGURE 1. Generation of VISA−/− mice. A. The
genomic structure of murine VISA gene, the
targeting construct, and the predicted mutated
allele are shown. B. Southern blot analysis of
WT (+/+), heterozygote (+/−), and homozygote mutant (−/−). Genomic DNA was
extracted and digested with EcoRI, electrophoresed,
and hybridized with a probe indicated. The
blotting gave a single 11.0-kb band for WT
(+/+), a single 7.0-kb band for homozygote
mutant (−/−), and both bands for heterozygote
(+/−). C. Total splenic cells were harvested from
WT (+/+), heterozygote (+/−), and homozygote mutant (−/−), and Western blotting
expression was also increased in VISA−/− mice
(right panels). 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−/− mice (−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.

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 greater calcium response (Fig. 2E). VISA−/− B cells also have normal upregulation 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−/− mice (−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

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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.
We also checked the upregulation of another costimulator factor CD86 in VISA<sup>−/−</sup> B cells induced by LOX or R848. TLR7 agonist-induced CD86 upregulation was also dramatically inhibited in VISA<sup>−/−</sup> 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<sup>−/−</sup> 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 cascade in search of the site of action of VISA. Because TLR7 stimulation of activation marker and cytokine expression

To further explore the basis of the requirement for VISA in TLR7 signaling, we compared the ability of WT and VISA−/− B cells respond to TLR7 agonists by proliferation. 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-α 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-α from LOX-stimulated WT B cells (Fig. 5B). However, VISA-deficient B cells produced ~50% less IFN-α 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
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−/− 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 knockout mice have abnormal TLR7 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-\(\beta\) increases TLR7 expression (Fig. 7C). However, we found that treating B cells with both the TLR7 agonist LOX and IFN-\(\beta\) 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-\(\beta\) 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 \(^{3}H\)thyidine 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-\(\alpha\), 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 \(\mu\)M 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-\(\kappa\)B activation was indicated. At the right panel, B cells from TLR7\(^{-/-}\) mice were treated with 400 \(\mu\)M LOX or 64 nM CpG for 60 min, and NF-\(\kappa\)B activation was measured by EMSA as the other panels. B. B cells from WT and VISA\(^{-/-}\) mice were treated with LOX (400 \(\mu\)M) 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 \(\mu\)M) 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 IFNRA-/- 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 VISA-/- 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 VISA-/- B cells may be B cell intrinsic. Otherwise, LOX and IFN-β would be expected to completely restore the TLR7 in VISA-/- 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 VISA-/- is B cell intrinsic

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

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

Both the CD23 and TLR7 defect in our VISA-/- mouse are B cell intrinsic. Considering the fact that our VISA-/- 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. 8, 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-β 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](http://www.jimmunol.org/)

**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 μM) 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 μM). Cell proliferation was measured by [³H]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](http://www.jimmunol.org/)

**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-κB activation, thus affect signal 2 and TLR7 expression in B cells. In contrast, VISA-mediated NF-κB activation is also required for CD23 expression in B cells (signal 3). This NF-κB 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−/−, IFNAR−/−, STAT1−/−, or IRF9−/− 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−/− 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 hypothesis 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-κB (Fig. 9).

The low expression of CD23 may also be explained by the lack of VISA-mediated activation of NF-κB because it has been shown that B cell expression of CD23 depends on the activation of NF-κB 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−/− mouse, because the VISA−/− mouse in C57BL/6 background (MAVS−/−) 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 BXXSB strain of mice spontaneously develops an SLE-like phenotype as a result of the presence of the Yaa mutation that carries an extra copy of TLR7 gene (4, 5). However, the phenotypes as a result of the presence of the Yaa mutation affects B cell expression of TLR7 in the certain genetic background will shed an important light on the role of VISA-mediated intracellular RNA sensing signaling in the development of autoimmune diseases. Furthermore, this VISA−/− mouse could be a valuable tool to understand the genetic requirement for the development of SLE in certain human population.

In summary, we demonstrate that the adaptor protein VISA, essential for RLR-mediated signaling following intracellular recognition of RNA, is also important for TLR7 signaling in B cells. Future studies should be focused on the role of VISA in the manifestation of autoimmune disease SLE.

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