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VISA is Required for B Cell Expression of TLR7

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Abstract

B cells play a critical role in the initialization and development of the Systemic Lupus Erythematosus (SLE) 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 (PDC). Here 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 VISA$^{-/-}$ mouse express reduced TLR7, but normal basal levels of Type I IFN. We also show that while IFNβ 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 up-regulation of activation markers CD69 and CD86, cell proliferation, production of IFNα, TNF, IL-12 and NF-κB activation. Finally, studies indicate that genetic background may influence the observed phenotype of our VISA$^{-/-}$ mice, since VISA$^{-/-}$ B cells differ in CD23 and TLR7 expression when on C57BL/6 vs 129Sv-C57BL/6 background. Thus, our findings suggest an unexpected link between VISA-mediated cytosolic RLR signaling and autoimmunity.

Introduction

Nucleic acid recognition by the innate immune system is mediated by two groups of innate pattern recognition receptors(PRRs): 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 PRRs (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*^{lpr}, as well as BXSB-Yaa (Y-

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linked autoimmune acceleration) 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 RIG-I-like receptors (RLRs), and compelling evidence demonstrates a critical role for these cells in the pathogenesis of SLE, through a combination of antibody-mediated and antibody-independent actions (6). Multiple studies have demonstrated that DNA- and RNA-specific autoreactive B cells can become activated by virtue of their antigen specificity and expression of nucleic acid-sensing TLRs (TLR7 and TLR9) (7–11). Thus, by acting as targets of complex chromatin antigens 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, 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 report, we generated 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, our results provide the first evidence 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 exon2, 3, 4 and 5, with a PGK-Neo selection cassette (Figure 1). The targeting construct was transfected 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 4 generations. The experiments were done on VISA$^{-/-}$ and their WT littermates. The genotypes of the mice were determined by genomic PCR. TLR7$^{-/-}$ mice were kindly provided by Dr. Philippa Marrack (National Jewish Health). Animals were generated at the National Jewish Health Mouse Genetics Core Facility and used at 6–8 wks of age. Animal care and handling was performed as per IACUC Guidelines (protocol # AS-2519-04-12).

Reagents

Recombinant mouse IFN-β protein (Abcam,ab84998), Loxoribine (Invivogen,tlrl-lox), R848 (Invivogen,tlrl-r848), CpG-ODN 1668 (Invivogen,tlrl-1668), anti-IRAK1 Ab (Cell Signaling, #4504), anti-IRAK4 Ab (Cell Signaling, # 4363), anti-MyD88 Ab (Santa Cruz, sc-11356), anti-TLR7 Ab (eBioscience,14-9079), anti-TLR9 Ab (eBioscience,14-9092), anti-TLR4 Ab (R&D system, MAB2759) and anti-TLR3 Ab (R&D system,MAB3005), anti-IκBα Ab (Cell Signaling, cat# 9242), anti-NF-κB 2 (p52) Ab (Santa Cruz, sc-298). Spleens

from MAVS knockout mice were a gift from Dr. Zhijian Chen at UT Southwestern Medical School.

B cell purification and culture

Splenic B cells were purified from 6~8 weeks-old mice using Miltenyi Biotec B cell isolation kit as per the manufacturer's instructions. Purified B cells were cultured at 5.0×10^6/ml in IMDM (HyClone, Logan, UT) supplemented with 5% FBS (Biosource, 200p-500HI), L-glutamine (2mM), penicillin (100 UI/ml), 100 μg/ml streptomycin, gentamycin (50 μg/ml), sodium pyruvate (1mM) and 2-ME (50 μM). Cells were cultured at 37°C with 7% CO_2. Allculture reagents were from Life Technologies (Gaithersburg, MD).

Calcium mobilization ($[Ca^{+2}]_i$)

Intracellular calcium concentration was measured as previous described (20). Briefly, cells were loaded with Indo-1AM (Molecular Probes, Eugene, OR) for 30min at 37°C, then washed and suspended in IMDM supplemented with 2% FCS at 10^6 cells/ml. Cells were stimulated with goat anti-mouse IgM-μF(ab′)$_2$ (Jackson ImmunoResearch, 115-006-020). Data were collected on LSR II and analyzed by Flow-Jo software (Tree Star, Inc., 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, *VeriKine*™ Mouse Interferon-Alpha ELISA Kit, 42100, TNF kit, eBioscience, 88-7324-22 and IL-12 p70 kit, R&D Systems, M1270)

Flow Cytometric assay of Immunofluorescence

Cells were stained with PerCp-anti-B220 (BD Pharmigen; RA3.3A1), FITC-anti-CD86 (BD Pharmigen, GL1) and APC-anit-CD69 (Biolegend, H1.2F3), APC or PE-anti- CD23(BD Pharmingen, clone B3B4), FITC-anti CD21(BD Pharmigen, 7G6), PE-anti-IgD(Southern Biotech, 11–26), PerCP-anti-IgM (Biolegend, R6-60.2). 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 (20mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton, 1mM PMSF, 10mM sodium pyrophosphate, 2mM Na_3VO_4, 10mM NaF, 1 μg/ml aprotinin, 1 μg/ml Leupetin) at 4°C for 1h. Cell lysates were centrifuged at 12,000g at 4°C for 10 min, Immunoprecipitation was done in the lysates with indicated Ab-conjugated sepharose beads. The immunoprecipitates were run on a SDS-PAGE and probed with indicated Abs.

Quantitative real-time PCR (qPCR)

qPCR were done as previously described (21). Briefly, RNA was isolated using RNeasy kit (Qiagen, 74104) and cDNA was synthesized using the Superscript III First-Strand Synthesis kit (Invitrogen, 11752-050). qPCR reactions 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

TLR7-F: 5′-CCACAGGCTCACCCATACTTC-3′;

TLR7-R: 5′-GGCATGTCCTAGGTGGTGACA-3′;

TLR9-F: 5′-TGGGCCCATTGTGATGAAC-3′;

TLR9-R: 5′-TTGGTCTGCACCTCCAACAGT-3′;

IFNβ-F: 5′-CCCTATGGAGATGACGGAGA-3′;

IFNβ-R: 5′-TCCCACGTCAATCTTTCCTC-3′;

IFNα4-F: 5′-TCCATCAGCAGCTCAATGAC-3′;

IFNα4-R: 5′-AGGAAGAGAGGGCTCTCCAG-3′;

GAPDH-F: 5′-TCAACAGCAACTCCCACTCTTCCA-3′;

GAPDH-R: 5′-ACCCTGTTGCTGTAGCCGTATTCA-3′.

EMSA assay

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, 2mM dithiothreitol, and 1mM phenylmethylsulfonyl fluoride, 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, then suspended in 4.5 μl of Buffer C (Buffer A plus 10% glycerol). The suspension was mixed with 5 μl of 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, GGGGACTTTCCC (Santa Cruz Biotechnology) labeled with [γ-32P]ATP by T4 DNA kinase, was added into 20 μg of nuclear extract and 0.8 μg of poly(dI-dC) in binding buffer (20 mM Tris, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 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.

^{3}[H] uptake assay

B cells (3×10^{5} cells/well in 150 μl medium in 96 well plates) were stimulated with indicated TLR agonists for 24 h, then pulsed for 8 h with (one microcurie of ^{3}[H]thymidine/per well (Amersham Bioscience). Incorporation of ^{3}[H] thymidine was quantified by a liquid scintillation beta counter (PerkinElmer).

Adoptive cell transfer

Purified splenic B cells were labeled with CFSE (2.5μM) for 3 min in PBS buffer. Cells were then washed with PBS containing 5% FBS followed by 3 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$^{-/-}$ B cells

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 (Figure 1A). Homologous recombination and the disruption of *VISA* were confirmed by Southern blot (Figure 1B) and Immunoblot (Figure 1C) analysis. Consistent with the role of VISA in antiviral response, Mouse Embryonic Fibroblasts (MEF) cells from our VISA$^{-/-}$ mice fail to activate transcriptional factors NFκB and IRF3 in response to Sendai virus infection (Figure 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 (Figure S1A) and similar numbers of B220^{+}CD93^{+} transitional and B220^{+}CD93^{-} mature B cells (Figure 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. While both compartments

were present and cells occurred in normal numbers based on IgM, B220, IgD and CD93 expression, CD23 expression was dramatically decreased on T2 cells from VISA$^{-/-}$ mice (Figure 2A, middle two panels). While mature B cells were also seen in normal frequency in VISA$^{-/-}$ mice, they too exhibited much reduced expression of CD23 (Figure 2B and 2C). Quantitative real-time PCR also confirmed that VISA$^{-/-}$ B cells (CD43-) express low level of CD23 transcripts (Figure 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 (Figure 2E). However at a low dose (0.26μg/ml anti-IgM), VISA$^{-/-}$ B cells elicit slightly great calcium response (Figure 2E). VISA$^{-/-}$ B cells also have normal up-regulation of CD69 co-stimulating factor in response to anti-IgM, anti-CD40 or IL-4 stimulation (Figure S1B). We concluded that the BCR signaling in VISA$^{-/-}$ B cells is largely un-affected by the lack of VISA expression.

The low expression of CD23 in VISA$^{-/-}$ mice is B cells intrinsic

Previous studies have shown that CD23 expression can be affected by matrix metalloproteases in the microenvironment of cells (21, 22). To begin explore the role of micro-environmental 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 assessed. As shown in Figure 2F, expression of CD23 was not affected by "parking" WT B cells in VISA$^{-/-}$ mice. Nor was CD23 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 up-regulate CD23 expression in VISA$^{-/-}$ B cells

B cell expression of CD23 can be up-regulated by treatment of LPS(23), IL-4 (24) or anti-CD40 antibodies (25) through the activation of transcriptional factors STAT6, NFκB or a combination of both (26). We determined whether CD23 up-regulation is also impaired in VISA$^{-/-}$ B cells. Purified splenic B cells were cultured with anti-CD40 mAb (FGK-45), LPS or IL-4 for 24hrs 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 (Figure 3, mock). Also, in agreement with previous reports, anti-CD40 mAb, LPS and IL-4 stimulation increased B cells expression of CD23 (Figure 3A–3C, solid thin lines). Importantly, CD23 expression was also increased in VISA$^{-/-}$ B cells (Figure 3A–3C, 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) while induced up-regulation of CD23 by LPS and IL-4 was decreased in VISA$^{-/-}$ B cells (Figure 3E). There were no differences in the up-regulation of CD69 between VISA$^{-/-}$ and WT B cells by these stimuli (Figure 3A–3C, right panels). As a negative control, IFNβ did not affect CD23 expression in WT or VISA$^{-/-}$ B cells though CD69 expression was up-regulated in these cells (Figure 3D). We concluded that VISA$^{-/-}$ B cells can still up-regulate 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 up-regulation of CD69 and CD86 in B cells

VISA is an anti-viral protein that is required for RLRs initiated Type I IFN production in immune cells as well as fibroblasts (12, 16). Though 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, that senses endosomal ssRNA. To determine the effect of VISA expression on TLR7 signaling, we examined the up-regulation of CD23 and CD69 in VISA$^{-/-}$ B cells by TLR7 agonist Loxoribine (LOX). Unlike the IL-4, anti-CD40 and LPS stimulation, LOX failed to induce increased CD23 and CD69 expression by VISA$^{-/-}$B cells (Figure 3F, 3E).

We further examined the up-regulation of CD69 in VISA$^{-/-}$ B cells at different time-points and with various doses of LOX or R848, another TLR7 agoinst (Figure 4A). Hyporesponsiveness was observed throughout the time-course and stimulus dose range (Figure 4A). We also checked the up-regulation of another co-stimulator factor CD86 in VISA$^{-/-}$ B cells induced by LOX or R848. TLR7 agonist-induced CD86 upregulation was also dramatically inhibited in VISA$^{-/-}$ B cells (Figure 4B). As a control, we treated these B cells with TLR9 agonist CpG-ODN1668. In agreement with a previous report, there was no difference in TLR9 agonist-induced upregulation of CD69 and CD86 expression (16) (Figure 4C, 4D) by WT and VISA$^{-/-}$ B cells. We conclude that VISA is required, selectively, for TLR7-mediated CD69 and CD86 up-regulation in B cells.

VISA is required for TLR7 agonist-induced B-cell proliferation and cytokine production

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 while VISA$^{-/-}$ B cells respond similarly to CpG, their responses to TLR7 agonists LOX and R848 are impaired (Figure 5A).

B cells also respond to TLR ligands by production of cytokines. To determine if 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 IL12 p70, TNF and IFNα by ELISA. To eliminate the potential confounding effects of contaminating DC 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 about 65 pg/ml of IFNα from loxoribine-stimulated WT B cells (Figure 5B). However, VISA deficient B cells produced ~50% less IFNα and TNF in response to loxoribine treatment (Figure 5B, 5C). A more dramatic effect was seen in IL-12 production, where VISA$^{-/-}$ B cells made very little IL-12 relative to WT B cells (Figure 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. Since 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 Figure 6A, VISA$^{-/-}$ B cells displayed impaired LOX-induced NF-κB activation (left panel). The stimulation by LOX through TLR7 is specific, as LOX did not activate NF-κB in TLR7$^{-/-}$ B cells (Figure 5A, right panel). Consistent with the previously seen specificity of VISA deficiency (Figure 4), the TLR9 agonist CpG-ODN1668 induced equivalent NF-κB activation in WT and VISA$^{-/-}$ B cells (Figure 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 (IKKβ)(33). Consistent with our EMSA result, we found that degradation of IκBα is inhibited in LOX-activated VISA$^{-/-}$ B cells (Figure 6B).

The non-canonical NF-κB pathway is mediated by IκB kinase α (IKKα) 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 (Figure 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 loxoribine-induced MyD88- IRAK1 association is dramatically decreased in VISA$^{-/-}$ B cells (Figure 6C). We also found that MyD88 is constitutively associated with IRAK4 in B cells, and this is independent of VISA expression (Figure 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 receptor 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 (Figure 7A, CD43^{-} splenocytes). This VISA requirement is TLR7 specific since VISA deficiency does not affect TLR3, TLR4 and TLR9 expression (Figure 7A). Finally, RT-PCR also confirmed the decrease of TLR7, but not TLR9, transcripts in VISA$^{-/-}$ B cells (Figure 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 (35, 36, 38). Furthermore, type I IFN treatment can increase B cell expression of TLR7 (36). We confirmed that treatment of B cells with IFNβ increases TLR7 expression (Figure 7C). However, we found that treating B cells with both the TLR7 agonist LOX and IFNβ can lead to much greater induction of TLR7 expression than either treatment alone (Figure 7C). This increase is best seen after 24hrs (Figure 7D). TLR7 stimulation of purified B cells leads to additional Type I IFN production (Figure 5B), which is far less than the IFNβ we added here (400U/ml). We concluded that it is TLR7 signaling, and not the additional Type I IFN generated by TLR7 stimulation, that augments IFNβ induced TLR7 expression.

We also confirmed that Type I IFN signaling is absolutely required for the up-regulation of TLR7 expression in B cells because the combination of LOX and IFNβ stimulation does not increased TLR7 expression in IFNRA$^{-/-}$ B cells (Figure 7E). Thus, in the presence of IFNβ, TLR7 signaling acts to further up-regulate its own expression. The combined treatment also

leads to much greater upregulation of CD69 than loxoribine or IFNβ treatment alone (Figure 7F).

We then tested the ability of INFβ and TLR7 agonists to restore TLR7 expression in B cells from VISA$^{-/-}$ mice. Although LOX and IFNβ treatment increased TLR7 expression (Figure 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 IFNβ 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 IFNα or IFNβ in spleen measured by qRT-PCR (Figure 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 24hr did not result in restoration of TLR7 mRNA levels (Figure 7G). Transfer of WT B cells to VISA$^{-/-}$ mice also did not lead to changes in TLR7 mRNA levels (Figure 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-KO. MAVS is another name for VISA. The MAVS-KO 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 (Figure 8A). Unlike the VISA$^{-/-}$ B cells, the MAVS$^{-/-}$ B cells also undergo normal up-regulation of CD69 (Figure 8B, upper two panels) and proliferation in response to TLR7 activation (Figure 8C). Finally, WT and MAVS$^{-/-}$ B cells have similar levels of TLR7 expression (Figure 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 report, 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, as 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 IFNβ, 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 antigens (7–11). Furthermore over-expression of TLR7 due to 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 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 IFNAR1$^{-/-}$, IFNAR2$^{-/-}$, 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 (Figure 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 (Figure 9). We hypothesis that a steady state of VISA signal through RIG-I/MDA, either by tonic or non-microbial RNA ligands, directly drives the expression of TLR7, possibly by VISA mediated activation of NF-κB (Figure 9).

The low expression of CD23 may also be explained by the lack of VISA-mediated activation of NF-κB since 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 since TLR7 activation fails to up-regulate CD23 expression in these cells (Figure 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 BXSB strain of mice spontaneously develops an SLE-like phenotypes due to the presence of the *Yaa* (Y-linked autoimmune acceleration) mutation that carries an extra copy of TLR7 gene (4, 5). However, the *Yaa* mutation by itself is unable to induce significant autoimmune responses in C57BL/6 background (42). Two recent reports have suggested that certain human VISA variants, including one loss-of-function human VISA variant, are associated with SLE in some human populations (17, 18). Thus, our finding that VISA deficiency 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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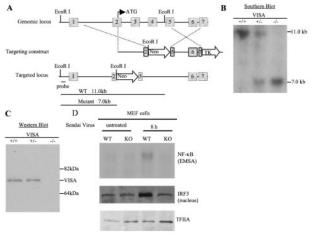

Figure 1. Generation of VISA$^{-/-}$ mice

A. The genomic structure of murine *VISA* gene, the targeting vector, and the predicated 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 7.0kb band for homozygote mutant(−/−), and both bands for heterozygote(+/−). **C.** 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 before (13). EMSA was performed as described in *Material and Methods*.

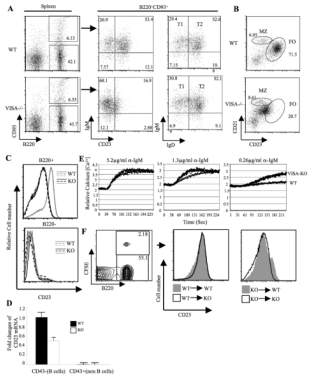

Figure 2. VISA-deficient B cells have dramatically decreased CD23 expression

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 ($IgM^{high}IgD^{low}$), T2 ($IgM^{high}IgD^{high}$). **B.** Mature B cells ($B220^{+}CD93^{-}$) were stained for CD21 and CD23 to separate MZ and 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 $VISA^{-/-}$spleen were extracted, and qRT-PCR was performed as in *Materials and Methods* to analyzed 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-μF$(ab')_2$ (Jackson ImmunoResearch). **F**. Purified B cells from WT and $VISA^{-/-}$ mice were labeled with CFSE, and transferred i.v. to unirradiated recipients. After 24 hrs, CD23 expression in the spleen of recipient mice was analyzed. Data are representative of three or more independent experiments.

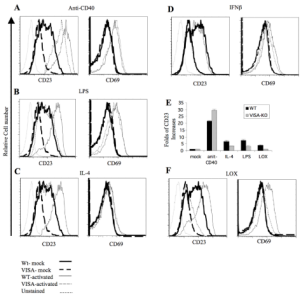

Figure 3. Anti-CD40, LPS, IL-4, but not TLR7, stimulation up-regulate CD23 expression in VISA$^{-/-}$ B cells

A–D, 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 (20ng/ml), IFNβ (250U/ml) and loxoribine (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 MFI of CD23 from activated cells divided by MFI of mock treated cells.

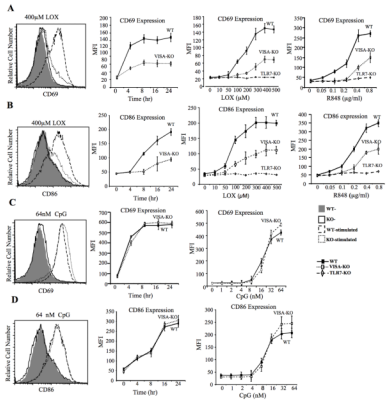

Figure 4. VISA is required for TLR7 but not TLR9-mediated up-regulation of CD69 and CD86 in B cells

A–B. Splenic B220^{+} B cells from WT and VISA$^{-/-}$ mice were cultured with loxoribine (400 μM) or mock for 20 h. Cells were then stained for CD69 **(A)** or CD86 (B) (*left panel*), ■ WT-, □KO-, ⬚ WT-activated, ⬚ KO-activated. The middle two panels examined CD69 **(A)** or CD86 **(B)** expression at indicated time (400 μM of loxoribine) and doses of loxoribine. The right panel examined CD69 **(A)** or CD86 **(B)** expression at the indicated dose of R848. Average ± SEM of three experiments. **C–D.**, CD69 **(C)** or CD86 **(D)** expression were examined in B cells from WT or VISA−/− in response to CpG-ODN 1668 (64nM) or indicated doses as **A** and **B**, Average ± SEM of three experiments.

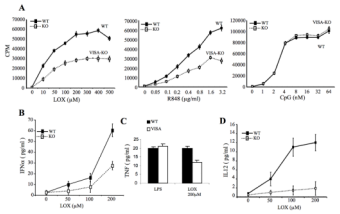

Figure 5. VISA-deficient B cells proliferate poorly and have decreased cytokine productions in response to TLR7 agonists

A. Splenic B cells from WT and VISA$^{-/-}$mice were stimulated with indicated concentration of loxoribine, R848 or CpG-ODN 1668, and proliferation was measured by ^{3}[H]thymidine uptake. Average ± SEM of three experiments. **B–D.** Splenic B cells from WT and VISA$^{-/-}$ mice were stimulated with indicated concentration of loxoribine for 20 h. Levels of IFNα, TNF and IL-12 in culture supernatants were measured by ELISA, Average ± SEM of three experiments.

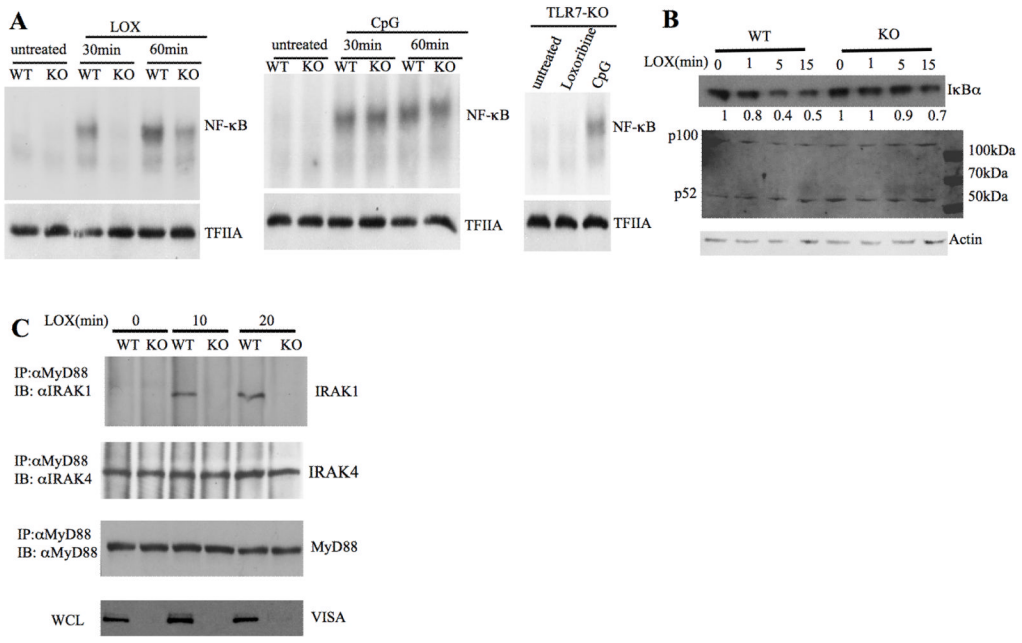

Figure 6. B cells from VISA$^{-/-}$ mice have impaired TLR7 signaling

A. Purified B cells from WT, VISA$^{-/-}$ mice were treated with 400 μM Loxoribine (*left panel*) or 64 nM of 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 Loxoribine 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 loxoribine (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 loxoribine (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.

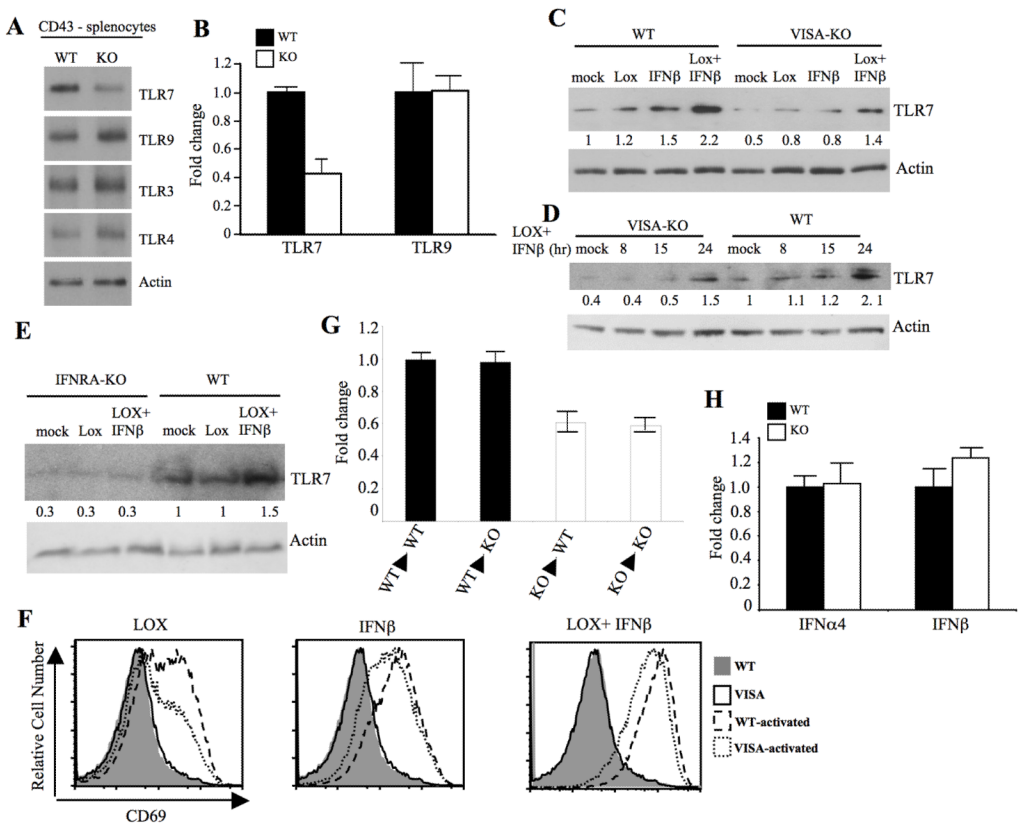

Figure 7. Decreased TLR7 expression in VISA$^{-/-}$ B cells

A. CD43^{-} (B cells) splenic cells from WT and VISA$^{-/-}$ mice were lysed, run on a SDS-PAGE and probed for indicated Abs. **B.** Total RNAs of B cells from WT and VISA$^{-/-}$ spleen were extracted, and qRT-PCR was performed as in *Materials and Methods* to analyzed TLR7 and TLR9 expression. Average ± SEM of three experiments. **C–E.** Purified B cells from WT, VISA$^{-/-}$ or IFNRA$^{-/-}$mice were stimulated with loxoribine (400 μM), IFNβ (400U/ml), or both Loxoribine and IFNβ for 20h (C, E) or at indicated time (D). The WCL were analyzed for TLR7 expression by western blot. F. Purified B cells from WT, VISA$^{-/-}$ mice were stimulated with loxoribine (400 μM), IFNβ (400U/ml), or both Loxoribine and IFNβ for 20h. B cell expression of CD69 was measured as before. **G**. CFSE labeled B cells (1×10^7) were transferred (i.v.) to unirradiated recipients (WT or VISA$^{-/-}$). After 24 hrs, the CFSE labeled B cells were sorted out from the receipts and RNA was extracted. qRT-PCR was performed to analyze the expression of TLR7. Average ± SEM of three experiments. H. qRT-PCR was performed in total RNAs from WT and VISA$^{-/-}$ spleen to analyze the IFNα4 and IFNβ expression.

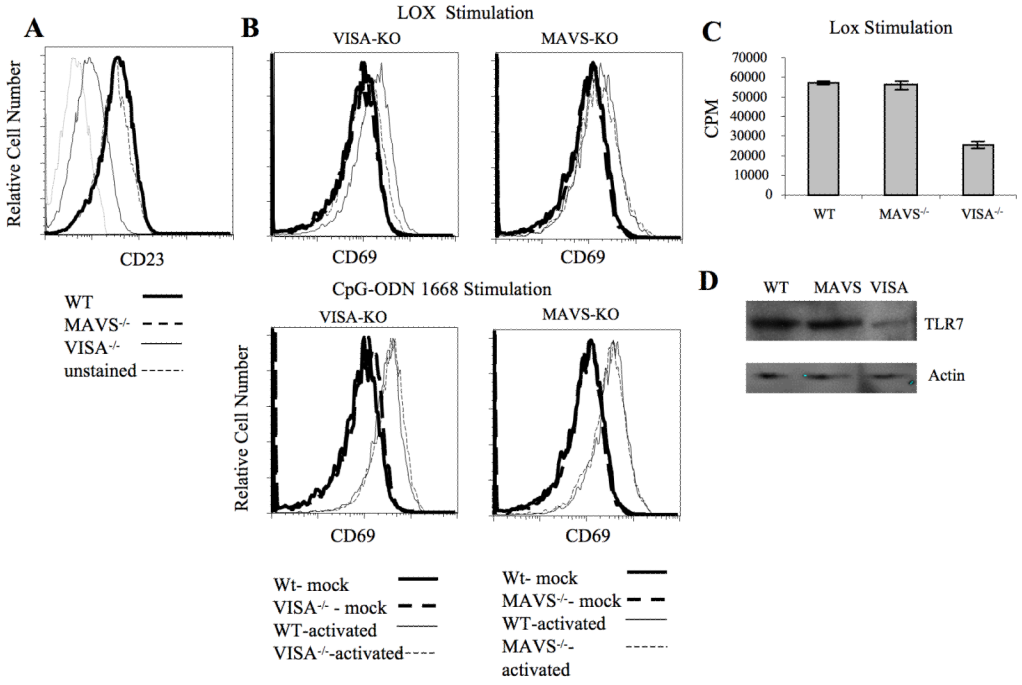

Figure 8. B cells from MAVS−/− mouse have normal CD23 and TLR7 expression

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$^{-/-}$, VISA$^{-/-}$ and their WT littermate control mice were stimulated with Loxoribine (400 μM) or CpG-ODN 1668 (50nM) for 20hrs. CD69 expression was measured as before. **C**. Purified splenic B cells from MAVS$^{-/-}$, VISA$^{-/-}$ and their WT littermate control mice were stimulated with Loxoribine (400 μM). 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.

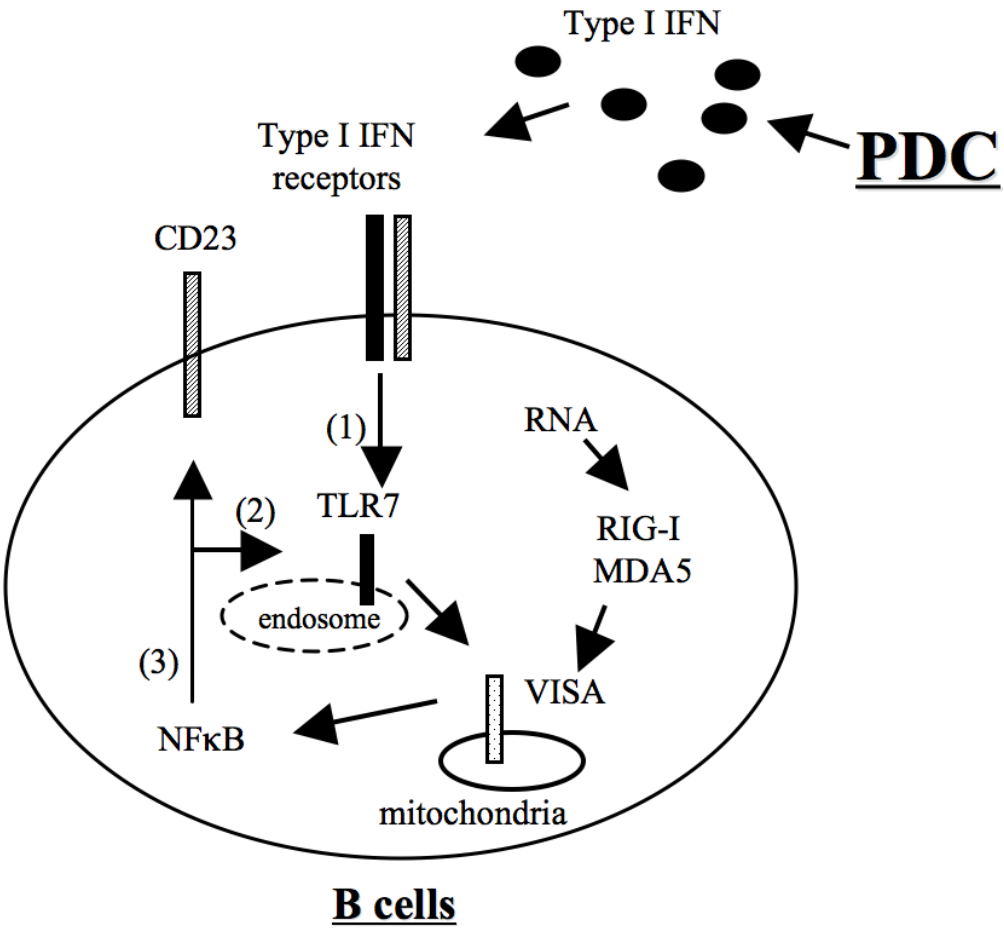

Figure 9. Model: VISA regulates B cell expression of CD23 and TLR7

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-κB activation, thus affect signal 2 and TLR7 expression in B cells. On the other hand, 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.