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Differential STIM1 expression in T and B cell subsets suggests a role in determining antigen receptor signal amplitude

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Summary

Ca²⁺ acts ubiquitously as a second messenger in transmembrane signal transduction. In lymphocytes, calcium mobilization is triggered by antigen and chemokine receptors, among others, and controls cell functions ranging from proliferation to migration. The primary mechanism of extracellular Ca²⁺ entry in lymphocytes is the CRAC influx. STIM1 is a crucial component of the CRAC influx mechanism in lymphocytes, acting as a sensor of low Ca²⁺ concentration in the ER and an activator of the Ca²⁺ selective channel ORAI1 in the plasma membrane. While STIM1 function has been studied extensively, little is known regarding whether it is differentially expressed and thereby affects the magnitude of calcium mobilization responses. We report here that STIM1 expression differs in murine T and B lymphocytes, and in respective subsets. For example, mature T cells express ~4 times more STIM1 than mature B cells. Furthermore, we show that through the physiologic range of expression, STIM1 levels determine the magnitude of Ca²⁺ influx responses that follow BCR-induced intracellular store depletion. Considered in view of previous reports that differences in amplitude of lymphocyte Ca²⁺ mobilization determine alternate biological responses, these findings suggest that differential STIM1 expression may be important an determinant of biological responses.

Keywords

Adaptive immunity; lymphocytes; STIM1; Ca²⁺ influx

1. Introduction

Ca²⁺ is a crucial second messenger in the activation of a variety of cellular responses. CRAC channels are the primary mediators of receptor-activated Ca²⁺ entry in lymphocytes, and are functionally identified by their Ca²⁺ conductance in the cell plasma membrane following reduction in Ca²⁺ concentration in the ER. Antigen receptor signals mediate activation of this Ca²⁺ entry by triggering ER Ca²⁺ depletion (Conrad et al., 2007). The known molecular components of the CRAC mechanism include STIM1 (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005) and the Ca²⁺ selective channel-forming subunit,

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ORAI1 (Prakriya et al., 2006; Vig et al., 2006; Yeromin et al., 2006). STIM1 is an 84kD transmembrane glycoprotein oriented with its amino terminus in the ER lumen and carboxy terminus in the cytosol (Williams et al., 2001). The luminal domain contains a “sterile” α motif and an EF hand, which functions as a sensor of Ca^{2+} concentration in the ER lumen (Zhang et al., 2005). Reduction of ER Ca^{2+} triggers oligomerization and subsequent accumulation of STIM1 in an ER membrane compartment juxtaposed to the plasma membrane (Luik et al., 2006; Xu et al., 2006). The cytosolic domain of STIM1 contains a coiled-coil domain, a serine/proline rich region, and a lysine rich region. Recently, a cytosolic site, located in the STIM1 348-448 amino acid interval, was shown to interact with ORAI1 (Park et al., 2009). Upon store depletion, STIM1 oligomerizes and interacts with ORAI1, leading to its oligomerization. The complex accumulates in the adjacent plasma membrane, leading to channel formation, channel opening and Ca^{2+} influx.

The role of STIM1 in immunological processes became evident through studies using knock-out mice. Since STIM1 null ($\text{STIM1}^{-/-}$) mice are embryonic lethal (Baba et al., 2008), the role of STIM1 was studied in $\text{STIM1}^{-/-}$ mast cells grown in cultures from fetal liver progenitors. While mast cells developed normally from $\text{STIM1}^{-/-}$ fetal-liver, they displayed impaired Fc ϵ RI-mediated Ca^{2+} influx, as well as impaired activation of NF- κ B and NFAT transcription factors. Furthermore, Fc ϵ RI-mediated degranulation and cytokine production were impaired. In mice in which the Stim1 was ablated specifically in T cells, T cell development was normal but these cells displayed severely impaired store-operated Ca^{2+} influx, reduced cytokine production, and reduced translocation of NFAT transcription factor to the nucleus (Oh-Hora et al., 2008).

Our understanding of the importance of STIM1 in a variety of cellular functions, and in a variety of cell types, is rapidly expanding. Available evidence indicates that at least two mechanisms regulate STIM1 function. STIM1 is regulated by phosphorylation (Pozo-Guisado et al., 2010; Smyth et al., 2009) as well as by the level of expression (Lioudyno et al., 2008; Oh-Hora et al., 2008). Super-antigen stimulation of primary human T cells was shown to up-regulate STIM mRNA expression consistent with increased protein expression. When these pre-treated cells were co-cultured with dendritic cells, a marked increase in Ca^{2+} influx was detected (Lioudyno et al., 2008). Furthermore, comparison of STIM1 levels in primary resting murine B220⁺ vs. CD4⁺ lymphocytes showed that CD4⁺ cells express more STIM1 than B220⁺ cells (Oh-Hora et al., 2008). However, in these studies, the difference in STIM1 expression was not quantified and additional subsets were not analyzed. Further, the effect of STIM1 expression level on antigen receptor mediated calcium signaling was not assessed.

While the importance of STIM1 in regulating cellular processes is clear, the potential role of differential STIM1 expression in these functions has not been established. Here, we show that STIM1 is differentially expressed in murine primary T and B cells and that STIM1 expression levels can affect the magnitude of calcium mobilization in lymphoid cells.

2. Materials and Methods

2.1. Animal, antibodies, and other reagents

C57BL/6 mice were purchased from Jackson (Bar Harbor, Maine) and housed in the National Jewish Health animal facility under an approved Institutional Animal Care and Use Committee (IACUC) protocol.

Anti-STIM1 (clone 44/GOK), anti-CD4 (clone L3T4)-PE, anti-CD19 (clone 1D3)-PE, anti-CD21 (clone 7G6)-FITC, anti-IgD (clone 11-26.2a)-PE, and anti-IgM (clone R6-60.2)-PerCP-Cy5.5 Ab were purchased from BD Pharmingen (Franklin Lakes, NJ). Anti-B220

(clone RA3-6B2)-APC-A750 Ab and Alexa-647 labeling kit were purchased from Invitrogen (Carlsbad, CA). Anti-CD3 ϵ (clone 145-2C11)-FITC was purchased from eBioscience (San Diego, CA). Anti-CD8-FITC was purchased from Zymed (San Francisco, CA). Anti-Actin (clone C-2) was purchased from Santa-Cruz bio-technology (Santa-Cruz, CA). Indo-AM was purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-LMP2A cytoplasmic tail Ab was produced and affinity purified in-house. The JA12.5 rat IgG1 anti-Mouse δ heavy chain Ab was produced in-house (a gift from Joseph Davie, Washington University, St. Louis, MO). The rat IgG1 anti-mouse μ heavy chain Ab b-7-6 Fab fragments (Leptin and Melchers, 1983) and anti-LMP2A Ab were prepared and Alexa 647 conjugated using standard methods. Goat anti-Mouse IRDye800 and Goat anti-Rabbit IRDye 680 secondary Ab were purchased from Li-COR (Lincoln, NE).

2.2. Generation of polyclonal anti-STIM1 Antibodies

The murine STIM1 cDNA sequence (NM 009287.4) encoding amino acid residues 483-620 was amplified using forward primer 5'-GAG ATT GTG TCG CCC TTG TCC ATG CAG-3' TCC and reverse primer 5'-ACT GGG GCT AAG AGA ATG GGA AGA ATC CAA AAG TGG-3'. The PCR product was cloned into the BamHI-EcoRI sites in pGEX-3x (27-4803-01, GE healthcare, Piscataway, NJ) to produce a glutathione S-transferase-STIM1 (483-620) fusion protein. This protein was adsorbed to glutathione Sepharose and cleaved using factor Xa. Released STIM1 polypeptide was used to immunize rabbits. The antibody was affinity purified using STIM1 (AA483-620) polypeptide-coupled Sepharose beads.

2.3. Immunoprecipitation and immunoblotting

B cells were lysed in lysis buffer (1% NP-40, 150mM NaCl, 50mM Tris pH 7.4) supplemented with freshly added 10mM NaF, 0.4mM EDTA, 2mM Na Orthovanadate, 1mM phenylmethyl-sulfonylfluoride, 1 μ g/ml Aprotinin, 1 μ g/ml α -1-antitrypsin, and 1 μ g/ml Leupeptin, followed by centrifugation at 13,000rpm, for 15min in an Eppendorf centrifuge. The lysate was then used for blotting and immunoprecipitation. Immunoprecipitation was performed using anti-STIM1 antibody and protein G Sepharose. Samples were boiled for 10 min in 2x reducing SDS sample buffer (20% v/v Glycerol, 200mM Tris-base pH 6.8, 4% SDS, 10mM EDTA and freshly added 5% v/v beta Mercapto Ethanol) and fractionated using precast NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were transferred to PVDF membranes (Perkin Elmer, Belgium) and visualized using specific antibodies on an Odyssey imaging system (Li-Cor, Lincoln, NE).

2.4 Mass spectrometry

Bal-17.7.1 lymphoma cells (10^6) (a murine mIgM+, mIgD+, I-A^d B lymphoma provided by Dr. R. Asofsky, Bethesda, MD) were lysed in 1% NP-40 buffer, fractionated on SDS-PAGE gels and stained with SYPRO ruby protein gel stain (Molecular probes, Eugene, OR). A band of about 84kD was excised, destained using 50% (v/v) Acetonitrile and 25mM Ammonium Bicarbonate and dried using 100% Acetonitrile. Cysteines were reduced using 50mM dithiothreitol (D5545, Sigma, St. Louis, MO), and alkylated to prevent disulfide bonds formation using 100mM Iodoacetamide (I1149, Sigma, St. Louis, MO). The protein was then digested overnight at 37C using 25 ng/ μ l trypsin (V5280, Promega, Madison, WI). The digested protein was analyzed using nanospray liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) on an Agilent 6340 ion trap mass spectrometer. Results were analyzed using the Spectrum Mill (Agilent, Santa-Clara, CA) data search algorithm.

2.5. Intracellular staining

For intracellular staining, 'Fixation/Permeabilization' solution kit (cat. no. 554714, BD, Franklin Lakes, NJ) was used according to the manufacturer's instructions. Non-specific

immunoglobulin binding was blocked with 5% (v/v) normal rabbit serum for 15 min on ice before fixation. Cell surface staining was conducted before fixation.

2.6. Analysis of intracellular free Ca^{2+}

Cells ($10^7/\text{ml}$) in IMDM/2% FCS were loaded with $6.25\mu\text{M}$ Indo-AM (Molecular probes, Eugene, OR) for 45 min while rotating at 37°C (Buhl et al., 1997). Analysis was begun and baseline $[\text{Ca}^{+2}]_i$ established before cells were stimulated with $1\mu\text{M}$ Ionomycin (Calbiochem, San Diego, CA), $2.5\mu\text{g}/\text{ml}$ b-7-6 (anti- μ) or $1\mu\text{g}/\text{ml}$ JA12.5 (anti- δ) antibodies. Mean $[\text{Ca}^{+2}]_i$ was evaluated over time using a LSR II flow cytometer (BD, Franklin Lakes, NJ) and the ratio of bound to free Indo-1 was calculated using FlowJo data acquisition software (Tree star, San Carlos CA).

In some cases calcium mobilization was studied in 'untouched' B and T cells isolated from splenocytes by using CD43 depletion (cat no 130-09-801, Miltenyi Biotec, Germany) and pan T-cell isolation II (cat no 130-095-130, Miltenyi Biotec, Germany) kits, respectively.

2.6. STIM1 knock-down and over-expression

For STIM1 knock-down experiments, an shRNA (referred to as 2606) targeting STIM1 mRNA was used. STIM1 forward shRNA 5'-TGCAGGAGTGATCTCTTAATTCAAGAGATTAAGAGATACACTCCTGCTTTTTTC-3' and reverse shRNA 5'-TCGAGAAAAAGCAGGAGTGATCTCTTAATCTCTTGAATTAAGAGATACACTCCTGCA-3' were annealed and inserted into XhoI and EcoRI sites in MSCV-IRES-eGFP vector. The vector, together with the packaging vector pCL-Ecotrophic, was used to transfect HEK-293 (Graham et al., 1977) Phoenix cells. The virus-containing supernatant was then used to transduce murine Bal-17 B cell lymphoma (Chiles and Rothstein, 1992). Retrovirally transduced Bal-17 cells were sorted based on GFP expression prior to experiments. To modulate expression of STIM1 *in vivo*, hematopoietic stem cells were enriched in bone marrow by *i.v* injection of 3.75mg 5-Fluorouracil per mouse (ICN, Costa Mesa, CA) 5 days before harvesting of bone marrow. Cells were incubated overnight in DMEM containing 10% FCS, stem cell factor, IL-3, and IL-6. Cells were spin-infected as described above, then injected *intravenously* into lethally irradiated mice (600rad followed by 400rad 3Hr later). Splenocytes were isolated 4–5 weeks after reconstitution. This time point was chosen because following longer periods of reconstitution, i.e. >7 weeks, STIM1 knocked-down cells were no longer detectable.

For STIM1 over-expression, *stim1* cDNA was amplified from murine B cell mRNA using forward primer 5'-ATGGATGTGTGCGCCCGTCTTGCCCTGT-3' and reverse primer 5'-CTACTCTTAAGAGGCTTCTTAAAAATTTTGAGAGGAACTTCTTCCTGCCTGGA CTGG-3'. The resultant fragment was inserted into BglII and XhoI sites in MSCV-IRES-eGFP vector. pCL-Ecotrophic, together with MSCV IRES-eGFP, either with or without *stim1* cDNA, were packaged in HEK-293 Phoenix cells. Bal-17 cells were transduced using the packaged virus, propagated, and then sorted based on GFP expression.

2.7. Statistical analysis

ANOVA was used to calculate p values. Results shown are representative of four replicate experiments.

3. Results

3.1. Quantification of STIM 1 expression

Assessment of differential STIM1 expression in lymphoid cells required anti-STIM1 Ab effective for immunofluorescence staining. Since appropriate antibodies were not commercially available, we produced polyclonal Abs in rabbits by immunization with a bacterially expressed polypeptide composed of STIM 1 residues 483-620 (Fig. 1A). Resultant Abs were affinity purified from immune serum using immobilized STIM 1 residues 483-620 and tested for their ability to immunoprecipitate STIM1 from whole cell lysates of the Bal-17 murine B lymphoma cell that either over-expressed STIM1 or STIM1 knocked-down. Immunoprecipitates were fractionated by SDS-PAGE and transferred to PVDF membrane. Blotting of transfers revealed a distinct band of about 84kD corresponding to the relative mass of STIM1 (Fig. 1B). An additional band was detected and determined to be rabbit IgG heavy chain based on the fact that it was also seen in the control lane in which the immune adsorption was conducted without added cell lysate. The membrane was then stripped and re-blotted with a commercial anti-STIM1 Ab, and the results confirmed that the purified polyclonal anti-STIM1 Ab recognized STIM1 specifically (Fig. 1B, bottom). This analysis confirmed that the affinity purified anti-STIM1 antibody reacts with native and denatured STIM1.

The specificity of the anti-STIM1 Ab was further verified using Bal-17 lymphoma cells in which endogenous STIM1 expression was knocked-down using shRNA, or STIM1 was over-expressed by *Stim1* cDNA transduction. Whole cell lysates were produced from parental Bal-17, empty vector-transduced, STIM1 knock-down, or STIM1 over-expressing cells. Immunoblotting of these lysates showed an 84kD band in the parental, empty vector, and STIM1 over-expressing transduced cells but not in the knock-down cells. This genetic approach verified the specific reactivity of the Ab to STIM1 as well as the effectiveness of knock-down strategy (Fig. 1C).

Final confirmation of anti-STIM1 Ab reactivity was undertaken using mass spectrometry. Presumptive STIM1 was immunoprecipitated from Bal-17 lysates and the eluates were fractionated using SDS-PAGE. A SYPRO ruby stained band with a molecular weight of about 84kD was excised, digested in-gel using trypsin, and resulting peptides were analyzed using LC/MS/MS on an Agilent 6340 ion trap mass spectrometer. The sequence of the peptides identified the immunoprecipitated protein as STIM1 (Fig. 1D).

The monospecificity of the affinity purified anti-STIM1 Ab in immunofluorescence staining was tested by conjugating the antibody with Alexa-647 and staining the same set of cells as in Fig. 1C. The staining was detected in cells over-expressing STIM1 while lower levels were detected in the empty vector-transduced cells. The lowest level was detected in STIM1 knocked-down cells (Fig 1E). Staining in STIM1 knock-down cells was similar in intensity to that of cells stained with an equal amount of affinity purified rabbit anti-LMP2A Ab. Since murine B cells do not express Epstein-Barr LMP2A protein, this staining served as a negative control. These results were consistent with those observed with immunoblotting, demonstrating that among native (non-denatured) cellular proteins the anti-STIM1 Ab reacts significantly only with STIM1 and thus can be used to determine the expression levels of STIM1 by intracellular staining and flow cytometry.

3.2. Differential STIM1 expression and store-activated Ca^{2+} influx in B and T lymphocytes

In order to compare STIM1 levels in B and T lymphocytes, splenocytes were isolated from C57BL/6 mice, stained with CD3 ϵ and CD19 (Fig 2A), and a portion was sorted to isolate B or T cell populations. Whole cell lysates were prepared from these populations and subjected to anti-STIM1 immunoblotting. A ~4x higher expression of STIM1 in T cells was

detected compared to B cells after normalizing to actin (Fig. 2B) (see supplemental figure 2 for statistical analysis). This result was verified by intracellular STIM1 staining in figure 2C. Unsorted splenocytes were fixed, permeabilized and stained. Consistent with the immunoblotting result, T cells stained ~3x fold (MFI 1607.75 vs 625.5, background measured 57.7, $p < 0.0001$) more brightly than B cells.

The experimental results above are consistent with the possibility that differences in STIM1 expression in mature T and B cells may dictate differential response to intracellular Ca^{2+} store depletion between these cells. To test this possibility directly, untouched isolated B and T splenocytes were Indo-1 loaded. Splenocytes were suspended in Ca^{2+} -depleted medium ($[\text{Ca}^{2+}] \sim 100\text{nM}$) immediately before intracellular Ca^{2+} analysis ($[\text{Ca}^{2+}]_i$). After baseline $[\text{Ca}^{2+}]_i$ was established (~30sec), cells were stimulated with increasing concentrations of ionomycin to achieve intracellular store release. The intracellular release response was monitored and at 240 seconds, Ca^{2+} in the medium was restored to 2mM and Ca^{2+} influx was recorded. As shown in figure 2D, B and T cells showed similar Ca^{2+} release from ER stores. However, consistent with their reduced STIM1 expression, B cells responded to Ca^{2+} repletion with a slower and more gradual Ca^{2+} influx that reached lower maximal amplitude compared to T cells (Fig. 2D). Taken together, these data indicate that STIM1 levels correlate with the differential capacity of T and B cells to mobilize Ca^{2+} . While this experiment does not prove that these differences are the direct result of differential STIM1 expression, they are consistent with this possibility.

3.3. Differential expression of STIM1 in lymphocyte subsets

Intracellular staining was utilized to further assess STIM1 expression in subpopulations of B and T cells. Thymocytes were isolated from C57BL/6 mice, stained with anti-CD4 and anti-CD8 Ab, fixed, permeabilized, and stained for intracellular STIM1. DN, DP, as well as CD4^+ and CD8^+ single positive subsets were gated and their STIM1 expression was compared. We found that STIM1 expression was 1.5x fold greater in DN cells than DP cells (MFI 1350.25 vs 926.75, $p = 0.0002$). During differentiation to the single positive stage, STIM1 expression increased compared to DP. Interestingly, a 1.6x fold higher level was observed in the CD8^+ compared to CD4^+ thymocytes, (MFI 2046.75 vs 1294.25, $p < 0.0001$) (Fig. 3A) (see supplemental figure 3 for statistical analysis). Similar (1.3x) differential expression of STIM1 by CD8^+ and CD4^+ cells was seen in splenic compartment (MFI 1837.75 vs 1418, $p < 0.0001$) (Fig. 3B).

STIM1 expression in B cell subpopulations was also measured using splenocytes isolated from C57BL/6 mice. B220 and CD21 surface markers were used to distinguish FO from $\text{CD21}^{\text{high}}$ B cells. mIgM and mIgD staining was used to further distinguish T2 and MZ B cells within the $\text{CD21}^{\text{high}}$ population. Both T2 and MZ B cells expressed STIM1 at higher levels than FO B cells (MFI 1822.25, 1336.25, and 652.25, respectively, $p < 0.0001$) (Fig. 3C). The data presented above demonstrate that STIM1 is expressed at different levels in B and T cell subsets and these differences are statistically significant.

3.4. Reduction of STIM1 expression using shRNAs leads to reduced Ca^{2+} influx following cell stimulation

In order to further address whether STIM1 levels determine the magnitude of store depletion-induced calcium influx, we analyzed calcium mobilization in Bal-17 expressing different levels of STIM1 (Fig. 4A). The parental Bal-17, the empty vector-transduced, the STIM1 knockdown, or the STIM1 over-expressing cells were stimulated as in figure 2D and the store depletion-induced influx was measured. Ca^{2+} released from the intracellular stores was similar in the parental Bal-17, the empty vector-transduced, and the STIM1 knockdown. In contrast, the STIM1 over-expressing cells showed elevated baseline $[\text{Ca}^{2+}]_i$ and increase

Ca²⁺ mobilization responses. Increased basal [Ca²⁺]_i may reflect a previously unrecognized role for STIM1 in resting Ca²⁺ homeostasis. STIM1 over-expression may cause increased ER storage of calcium or alternatively, expression of more STIM1 may lead to more efficient release of calcium from stores and more efficient activation of ORAI1. When extracellular [Ca²⁺] was restored to physiological levels, the STIM1 over-expressing cells showed higher amplitude of Ca²⁺ influx than the parental cells. Conversely, STIM1 knock-down had no effect on ER store release but resulted in greatly muted Ca²⁺ influx compared to the parental cells. The duration of Ca²⁺ changes was also altered, with the STIM1 over-expressing cells exhibiting the most rapid changes in both the ER release and the extracellular influx. While the rapidity of the increase was probably due to the high expression of STIM1, the more rapid decrease may result from enhanced [Ca²⁺]_i activation of Ca²⁺ re-sequestration. In all, the data presented in this figure demonstrate that STIM1 levels can modulate the amplitude of store-depletion-activated Ca²⁺ influx.

We next examined whether this is also the case in *ex vivo* lymphoid populations. Bone marrow cells from 5-Fluorouracil treated mice were transduced with LMP retroviruses encoding STIM1 shRNA and eGFP, and these cells were used to reconstitute lethally irradiated recipients. In a subsequent flow analysis of splenocytes from reconstituted animals, transduced splenic B cells were gated based on anti-CD19 staining and GFP expression (derived from transducing vector) (figure 4B). Analysis of STIM1 expression in these cells revealed that, as expected, GFP levels were inversely correlated with STIM1 levels (see figure 4C). Analysis of Ca²⁺ mobilization revealed that cells expressing progressively less STIM1 underwent corresponding decreased Ca²⁺ influx upon store depletion using ionomycin (Fig. 4D). To determine whether STIM1 levels also limit calcium mobilization responses mediated by mIgM and mIgD, B cells were stimulated using anti-μ (Fig. 4E) or anti-δ (Fig. 4F). The magnitude of responses to BCR stimulations was also affected by STIM1 level. These results show that through the physiologic range of expression levels, STIM1 determines the magnitude of the calcium influx response to both internal store depletion and BCR stimulation.

4. Discussion

It was previously shown that differences in Ca²⁺ influx amplitude and duration determine the magnitude of activation of NFAT and NF-κB pathways and this, in turn, leads to different cell responses (Dolmetsch et al., 1997). Here, we show that STIM1 expression level is a direct determinant of amplitude of antigen receptor-mediated Ca²⁺ influx responses in *ex vivo* lymphocyte populations. Furthermore, we demonstrate that STIM1 is differently expressed in primary murine T cells, B cells, and their subsets. Taken together, these observations indicate that the level of STIM1 expression in various lymphoid compartments may play an important role in determining the biologic responses of cells to variety of stimuli. In essence, STIM1 expression levels may determine cell sensitivity to ligand.

The amplitude of calcium influx has been clearly shown to influence biological responses in lymphocytes (Dolmetsch et al., 1997). While the transcriptional factor NFAT is activated by low and sustained [Ca²⁺]_i plateau, NF-κB is activated by large more transient [Ca²⁺]_i responses. Differences in the Ca²⁺ influx amplitude, therefore, translate to differences in gene transcription.

The data presented in figure 2D clearly shows differences in Ca²⁺ mobilization kinetics between B and T cells. In Ca²⁺ depleted media, T cells more rapidly reincorporated Ca²⁺ into the ER stores than B cells. After extracellular Ca²⁺ repletion, T cell internalized Ca²⁺ more quickly than B cells. While this difference can be explained by differential STIM1

expression (figure 2B), it can also result from numerous molecular differences between B and T cells. However, it is interesting that B cells that were modified only by overexpression of STIM1 (figure 4A) resembled T cells in their Ca^{2+} influx kinetics. This suggests that the level of STIM1 expression determines, at least in part, the pattern of Ca^{2+} mobilization in lymphocytes. These results underscore the importance of STIM1 expression levels in regulating Ca^{2+} influx.

It was previously suggested that over-expression of both STIM1 and ORAI1 is needed to influence Ca^{2+} influx in HEK cells, indicating that both are limiting components of the calcium influx pathway (Peinelt et al., 2006). Here, we have shown that in B lymphocytes over-expression of STIM1 alone is sufficient to increase Ca^{2+} influx responses in B lymphocytes, as seen in Fig. 4. This discrepancy probably results from the differences in cellular models employed, e.g. HEK cells may simply express less ORAI1 than BAL17. These results highlight the importance of the relative abundance of STIM1 and ORAI1 in determining the magnitude Ca^{2+} influx responses.

In our experiments, differences in STIM1 expression were correlated with amplitude of store-depletion-activated calcium influx, suggesting that the magnitude of calcium mobilization responses in lymphoid cells is determined, in part, by STIM1 expression. Based on this relationship, one might expect that lymphoid populations that express higher levels of STIM1 might be more sensitive to stimulation by low avidity antigens. Indeed, it has been shown that BCR stimulation leads to greater calcium influx responses in immature and transitional B cells than in mature B cells (Benschop et al., 2001; Benschop et al., 1999). This could be because, as shown in figure 3, they express more STIM1 than mature B cells. The potential biologic importance of this difference may lie in the fact that immature and transitional B cells are more sensitive to antigen as measured by immune tolerance (Klinman, 1996; Monroe, 1996) as well as receptor editing (Ramsey et al., 2011) and CD86 upregulation (Benschop et al., 1999). This heightened sensitivity may provide an added measure of assurance that potentially dangerous autoreactive cells are purged from the repertoire.

The data presented in this work clearly shows that STIM1 is differentially expressed in lymphocytes and their subsets, and indicates the immune system may utilize STIM1 expression to control the repertoire.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CRAC	Ca ²⁺ release activated Ca ²⁺
STIM1	stromal interacting molecule 1
ER	endoplasmic reticulum
Ab	antibody
PVDF	Polyvinylidene difluoride
ANOVA	analysis of variance
DN	Double negative T cells
DP	Double positive T cells
FO	follicular
T2	transitional 2
MZ	marginal zone
TCR	T cell receptor

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Figure 1. Characterization of a specific anti-STIM 1 antibody effective for cell staining and immunoblotting

A. Diagram of STIM-1 illustrates the peptide (aa483-620) used for immunization. B. Affinity purified rabbit anti-STIM-1 aa483-620 antibody was used to immunoprecipitate antigen from whole cell lysates of Bal-17 lymphoma cells. Immunoprecipitates were fractionated by SDS-PAGE, transferred to PVDF membrane, and blotted with the same antibody, with appropriate secondary antibody. The upper band is STIM-1 and the lower band is Ig heavy chain detected by the secondary Ab. Lower panel shows blotting after stripping and re-probing with a commercial anti-STIM-1 antibody. C. Whole cell lysates were prepared from parental Bal-17 cells, Bal-17 transduced with STIM-1, STIM-1 targeted hairpin, or empty vector. Lysates were fractionated as above and blotted with anti-STIM-1 Ab. Anti-Actin was used as a control for equal loading. D. Whole cell lysate from Bal-17 was fractionated by SDS-PAGE, the gel was SYPRO ruby stained and a band at about 84kD

excised. The gel slice was destained, Cysteines were reduced and alkylated to prevent disulfide bonding, and the protein was digested with trypsin. The analyzed peptides are shown in bold in the complete amino acid sequence of STIM1. E. The indicated cells were fixed, permeabilized, and stained using Alexa-647 conjugated anti-STIM-1 Ab or rabbit polyclonal affinity purified anti-LMP2A-Alexa 647 Ab.

Figure 2. T cells express more STIM-1 than B cells

A. Splenocytes were isolated from C57BL/6 mice and stained for cell surface markers, CD3ε (T) and CD19 (B), for gating and sorting. These cells were then analyzed and data presented in Fig. 2B-2D. B. Sorted T and B cell populations were lysed and fractionated by SDS-PAGE, transferred to PVDF membrane, and blotted with the anti-STIM-1 as in figure 1. Anti-Actin was used as a control for equal loading. STIM-1 values were normalized to the corresponding Actin band values. C. Stained splenocytes (A) were fixed, permeabilized, and stained with anti-STIM-1 (as in Fig. 1) or anti-LMP2A as a negative control. Gated T cell and B cell populations were analyzed for STIM-1 expression using flow cytometry. Four mice were analyzed per group. D. B cells were isolated from splenocytes by negative selection using CD43 magnetic beads depletion. T cells were isolated using a cocktail of magnetic beads against non-T splenocytes beads. Both untouched B and T were loaded with Indo-AM. Immediately (< 5 s) before recording was begun, EGTA was added to the sample to reduce $[Ca^{2+}]$ to ~ 100 nM. Splenocytes were stimulated using ionomycin at the indicated concentration. $[Ca^{2+}]$ was restored to physiologic levels at the indicated time point (dashed arrow) and the analysis was continued.

Figure 3. STIM-1 expression levels differ among T and B subpopulations

A. Thymocytes were stained for cell surface CD4, CD8, and intracellular STIM-1. STIM-1 expression (right panel) is compared in DN, DP, CD4 and CD8 T cells. B. Splenocytes were stained as in (A) and intracellular STIM-1 level was compared between CD4 and CD8 cells. C. Splenocytes were stained for cell surface B220, CD21, IgM, IgD, and intracellular STIM-1. STIM-1 levels were compared among B cell subsets including T2, MZ, and FO cells. Four mice were analyzed per group.

Figure 4. Differences in STIM-1 expression level lead to correlated differences in Ca^{2+} influx following store depletion

A. Indo-AM loaded Bal-17 cells were suspended in IMDM. Immediately (< 5s) before recording was begun, EGTA was added to the sample to reduce $[Ca^{2+}]$ to ~100nm. Cells were stimulated with ionomycin (1 μ M) at the time indicated by the solid arrow. $[Ca^{2+}]$ was restored at the indicated time point (dashed arrow) and the analysis was continued. B. Splenocytes from animals reconstituted with STIM-1-targeted shRNA/GFP-transduced HSC were loaded with Indo-AM and stained with anti-CD19. B cells (CD19⁺) were gated according to GFP expression. C. Splenocytes were stained intracellularly for STIM1, MFI of STIM1-A647 and GFP measured, and plotted on a log₂ scale for each of the GFP⁺ groups shown in (B). D. Cells were stimulated with Ionomycin (1 μ M) at the time indicated by the arrow. Analysis of Ca^{2+} mobilization assay was performed as in (A). E. Cells were stimulated with b-7-6 anti- μ antibody (2.5 μ g/ml) at the time indicated by the solid arrow and analyzed as in (A). F. Cells were stimulated with JA12.5 anti-IgD antibody (1 μ g/ml) at the time indicated by the solid arrow and analyzed as in (A).