Calcium signaling in B cells: Regulation of cytosolic Ca^{2+} increase and its sensor molecules, STIM1 and STIM2

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ABSTRACT

Calcium signals are crucial for diverse cellular functions including adhesion, differentiation, proliferation, effector functions and gene expression. After engagement of the B cell receptor, the intracellular calcium ion (Ca^{2+}) concentration is increased promoting the activation of various signaling cascades. While elevated Ca^{2+} in the cytosol initially comes from the endoplasmic reticulum (ER), a continuous influx of extracellular Ca^{2+} is required to maintain the increased level of cytosolic Ca^{2+} . Store-operated Ca^{2+} entry manages this process, which is regulated by an ER calcium sensor, stromal interaction molecule (STIM). STIM proteins sense changes in the levels of Ca^{2+} stored within the ER lumen and regulates the Ca^{2+} -release activated Ca^{2+} channel in the plasma membrane. This review focuses on the signaling pathways leading to Ca^{2+} influx and the role of Ca^{2+} signals in B cell functions.

Keywords: Ca^{2+} signal, store-operated Ca^{2+} entry (SOCE), stromal interaction molecule 1 (STIM1), IL-10, regulatory B cell

Abbreviations: BCR, B cell receptor; CRAC, Ca^{2+} -release activated Ca^{2+} ; PIP_2 , phosphatidylinositol-4,5-bisphosphate; IP_3 , inositol-1,4,5,-triphosphate; PIP_3 , phosphatidylinositol-3,4,5-trisphosphate; $\text{PLC-}\gamma 2$, phospholipase- $\gamma 2$; PI3K, phosphoinositide 3-kinase; EAE, experimental allergic encephalomyelitis

1. Store-operated calcium entry (SOCE)

There is no denying that calcium, an essential component of bone and teeth, is also of importance as a universal second messenger in various lineages of cells including immune cells. The intracellular calcium ion concentration ($[Ca^{2+}]$) controls a diverse range of cell functions and the elevation of intracellular calcium ions (Ca^{2+}) regulates various cellular events after cells are stimulated. Ligand binding to its specific receptor, such as the B cell receptor (BCR; Baba et al., 2006; Matsumoto et al., 2011), T cell receptor (TCR; Lewis, 2001, Hogan et al., 2010), Fc receptor (Beaven and Baumgartner, 1996; Melendez and Khaw, 2002; Bryceson et al., 2006; Baba et al., 2008; Braun et al., 2009), and chemokine receptors (Partida-Sánchez et al., 2004; Hsu et al., 2001; Barbet et al., 2008), or interactions between costimulatory molecules (Nicolaou et al., 2009; Tseng et al., 2008) all initiate the upregulation of intracellular $[Ca^{2+}]$, and there is a close correlation between increased Ca^{2+} levels and the regulation of cellular functions.

In the steady state, intracellular $[Ca^{2+}]$ is $\sim 10^{-7}$ M, approximately $1/10^4$ of extracellular $[Ca^{2+}]$ ($\sim 10^{-3}$ M). However, when cells are activated, intracellular $[Ca^{2+}]$ markedly increases ($\sim 10^{-6}$ M) because of the release of Ca^{2+} from intracellular storage, the endoplasmic reticulum (ER). While Ca^{2+} release from the ER generates primary Ca^{2+} signals, a continuous influx of extracellular Ca^{2+} is required to maintain the increased levels of Ca^{2+} inside cells because of the limitations of cellular Ca^{2+} storage. The mechanism for Ca^{2+} influx, where the entry of extracellular Ca^{2+} is initiated by the exhaustion of Ca^{2+} ER storage, is termed store-operated calcium entry (SOCE) through

the Ca^{2+} -release activated Ca^{2+} (CRAC) channel, a type of SOCE channel. Thus, SOCE is an essential process that ensures a continuous extracellular source of Ca^{2+} .

2. Ca^{2+} signaling in B cells

Following the activating stimulation of B cells via several functional molecules including BCR, $[\text{Ca}^{2+}]$ is increased and controls various cell functions. The binding of antigens to BCR initiates the activation of tyrosine kinase, and the phosphorylation of adaptor molecules, resulting in the activation of two main signal cascades: phospholipase- γ 2 (PLC- γ 2) and phosphoinositide 3-kinase (PI3K) pathways (Kurosaki, 2009) (Fig. 1).

B-cell linker (BLNK), identified as a protein tyrosine-phosphorylated after BCR stimulation and as a substrate of Syk (Goitsuka et al., 1998), is a pivotal adaptor molecule in the BCR to PLC- γ 2 activation signaling cascade. After BCR stimulation, Syk is recruited to phosphorylated ITAM (immunoreceptor tyrosine-based activation motif) in $\text{Ig}\alpha/\text{Ig}\beta$, which then phosphorylates BLNK allowing phosphorylated BLNK to bind to PLC- γ 2 and Btk through their SH2 domains. Eventually, PLC- γ 2 becomes tyrosine phosphorylated and activated by Syk and Btk. Activated PLC- γ 2 hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5,-triphosphate (IP_3) and diacylglycerol (DAG), and the resultant IP_3 binds to the receptor for IP_3 (IP_3R), an IP_3 -gated intracellular Ca^{2+} channel itself, on the ER membrane. This leads to the release of Ca^{2+} stored in the lumen of the ER. Our previous study on the mutated N-terminal Src homology 2 (SH2) domain of PLC- γ 2 demonstrated a central role for the SH2 domain

in directing PLC- γ 2 to close proximity to the BCR signaling complex by its association with BLNK (Ishiai et al., 1999). Thus, BLNK functions as a bridging molecule in the PLC- γ 2 pathway to recruit Syk and Btk to PLC- γ 2. It should be noted that Ca^{2+} mobilization is significantly reduced when examined in DT40 B cells deficient in Syk, Btk, BLNK, or PLC- γ 2, indicating the importance of the concerted action of this molecular complex (Kurosaki and Tsukada, 2000). After the IP_3 -mediated transient release of Ca^{2+} from the ER, extracellular Ca^{2+} entry is controlled by SOCE and CRAC channels.

The PI3K pathway positively regulates BCR-elicited Ca^{2+} flux (Scharenberg and Kinet, 1998; Okkenhaug and Vanhaesebroeck, 2003). The activation of PI3K is mainly mediated by CD19 or BCAP (B cell adaptor molecule for PI3K) that contains a binding site for the SH2 domain in the p85 α subunit of PI3K. After stimulation of BCR, CD19 is phosphorylated by Lyn, and BCAP is phosphorylated by Syk and Btk. Phosphorylated CD19 or BCAP (by binding to the p85 subunit of PI3K) activates PI3K to produce phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from PIP_2 , and PIP_3 transmits signals downstream (Okada et al., 2000). The accumulation of PIP_3 allows Btk to interact with its PH domains, thereby promoting their membrane targeting and activation. Subsequently, Btk effectively activates PLC- γ 2 to induce Ca^{2+} release and influx. In particular, p110 δ , a catalytic subunit of PI3K, is thought to be critical for BCR-mediated Ca^{2+} mobilization (Okkenhaug et al., 2002). Interestingly, in BCAP-deficient B cells, reduced Ca^{2+} mobilization (60-70% of the normal level) is observed suggesting insufficient PLC- γ 2 activation in BCAP-deficient B cells contributes to the

decreased Ca^{2+} flux. The normal tyrosine phosphorylation status of PLC- γ 2 was observed in BCAP-deficient B cells, suggesting BCAP participates in PLC- γ 2 activation in a phosphorylation-independent manner (Yamazaki et al., 2002).

In addition to BCR signaling, the signaling pathway through CD20 also induces cytosolic Ca^{2+} influx. Using inhibitor analyses, fluorescence resonance energy transfer and variant Ramos human B cell lines lacking BCR, Ca^{2+} mobilization via CD20 was shown to be highly correlated with BCR expression, suggesting the direct association of CD20 with BCR "hijacks" the signaling potential downstream of BCR (Walshe et al., 2008).

In contrast to these "positive-signal" delivering molecules, CD22 is an inhibitory coreceptor of BCR exclusively expressed on B cells (Nitschke and Tsubata, 2004). Engagement of CD22 initiates its phosphorylation, subsequently activating SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1). This leads to the attenuation of BCR signaling via dephosphorylation of BCR intracellular signaling pathway substrates, including $\text{Ig}\alpha$, $\text{Ig}\beta$, Syk, and BLNK (Tamir et al., 2000). Accordingly, reduced BCR-induced Ca^{2+} mobilization was observed through the ligation of CD22 (using anti-CD22 F(ab)_2 mAb); a long-term decrease in intracellular $[\text{Ca}^{2+}]$ was observed in anti-CD22 mAb pretreated B cells though an initially rapid increase in intracellular $[\text{Ca}^{2+}]$ was detected after BCR activation. This suggests Ca^{2+} mobilization regulation by CD22 is focused on the influx of extracellular Ca^{2+} . Interestingly, adding Ca^{2+} to EGTA-treated cells induced an immediate influx of Ca^{2+} , although a significantly faster reduction in intracellular Ca^{2+} was observed in B cells

pretreated with anti-CD22 F(ab)₂ mAb. This indicates that the ligation of CD22 not only inhibits kinase phosphorylation events downstream of BCR, but also modulates Ca²⁺ efflux pump activity, and thus regulates Ca²⁺ homeostasis (Sieger et al., 2013).

3. Key molecule for SOCE: STIM

The key molecule for the operation of SOCE was recently identified as stromal interaction molecule 1 (STIM1), a ubiquitously expressed single-transmembrane protein localized in the ER that is conserved from *Drosophila* to mammals. It was reported to possess dual functions: as a sensor for [Ca²⁺] levels in the ER, and an activator for Ca²⁺ channels in the plasma membrane (Lewis, 2007; Putney, 2007). In addition, a novel protein for the CRAC channel, Orai1 (also known as CRACM1), was identified (Feske et al., 2006; Vig et al., 2006), and the functional and intracellular spatial linkage of STIM1 and Orai1 has been determined. Upon Ca²⁺ store depletion after stimulation, STIM1 rapidly is redistributed into discrete puncta located underneath the plasma membrane; STIM1 and Orai1 are then redistributed to ER-plasma membrane junctions (Wu et al., 2006). The CRAC activation domain (CAD, a 107-amino acid region, STIM1₃₄₂₋₄₄₈) of STIM1 directly binds to the N and C termini of Orai1 to open the CRAC channel, and the direct binding of STIM1 to Orai1 drives the accumulation and activation of CRAC channels at ER-plasma membrane junctions (Park et al., 2009). Furthermore, it was reported that Orai channels are fully activated by the conserved STIM1 amino acid fragment 344-442, SOAR (the STIM1 Orai activating region), and that SOAR can regulate the strength of interactions with Orai1 (Yuan et al., 2009). Thus,

upon depletion of the Ca^{2+} store, STIM1 migrates from a diffuse ER localization into puncta at the cell periphery close to the plasma membrane, whereby it directly activates Orai1 channels on the plasma membrane by protein–protein interactions (Hogan et al., 2010). In the case of B cells, STIM1 is also a key component in the SOCE/CRAC axis. Our studies using DT40 B cells clearly showed the physiological importance of STIM1 in B cell activation where a significant suppression of BCR-mediated Ca^{2+} influx was observed in STIM1-deficient cells (Baba et al., 2006).

Recent studies showed that SOCE activity was regulated by binding partners of STIM1 and Orai1. CRACR2A (CRAC regulator 2A) physically associates with Orai1 and STIM1 in the cytosol to form a ternary complex, which dissociates following Ca^{2+} binding with EF-hand motif of CRACR2A (Srikanth et al., 2010). The siRNA-mediated depletion of CRACR2A showed a significant reduction of SOCE. When EF-hand was mutated to prevent Ca^{2+} binding, STIM1 formed clustering independent of store depletion, suggesting Ca^{2+} -bound CRACR2A may be involved in a negative feedback loop to elicit disassembly of the SOCE complex. The Ca^{2+} -binding integral ER membrane protein Junctate was also identified as a binding protein of Orai1/STIM1 (Srikanth et al., 2012). Junctate contains an EF-hand motif in the ER luminal side and mutation of this motif to prevent Ca^{2+} binding resulted in clustering of STIM1 and subsequent SOCE induction independent of store depletion. Surf4 (Surfeit locus protein 4) was identified as an binding partner of STIM1 (Fujii et al., 2012) and interacted with the ER-luminal region of STIM1. Surf4 deficiency promoted STIM1 puncta formation at ER-PM junctions and SOCE, suggesting it functions as a negative modulator of

STIM1. Another negative regulator of SOCE, SARAF (SOCE-associated regulatory Factor), an ER transmembrane protein, interacts with STIM in the cytosol, which negatively regulates basal ER and cytosolic Ca^{2+} levels and SOCE activity (Palty et al., 2012). The reduction of SARAF by siRNA resulted in constitutive clustering of STIM1 and increased SOCE. SARAF may promote slow CARC inactivation after Ca^{2+} entry and ER refilling to prevent the risk of Ca^{2+} overload.

Although the mechanism of puncta formation of STIM1 and Orai1 during SOCE activation is unclear, a recent finding demonstrated the importance of septin filaments (Sharma et al., 2013). It was shown that septin rearrangement is essential for PIP_2 reorganization and recruitment of Orai1 to ER-plasma membrane junctions, but only partially affects STIM1 translocation. After store depletion, septin colocalized with PIP_2 , and accumulated preferentially in the circumference of the Orai1 cluster. Furthermore, knock down of septin resulted in a marked reduction of SOCE. These data suggest septins function as scaffold proteins to organize PIP_2 microdomains for the SOCE signalosome. Both PLC- γ 2 and PI3K signals may affect Ca^{2+} release from the ER and SOCE directly because phosphoinositides are involved in STIM1-Orai1 complex formation. STIM1 has a polybasic region at the C-terminal, which facilitates membrane targeting of STIM1 through interactions with PIP_2 or PIP_3 (Ercan et al., 2009; Walsh et al., 2010). In addition, PI3K/PI4K inhibitors reduce SOCE and STIM1 relocation. As described above, given that septin/ PIP_2 redistribution is essential for STIM1/Orai1 coupling, phosphoinositide dynamics induced by activation of PLC- γ 2 and PI3K may influence communication of STIM1 and Orai1 at ER-plasma membrane junctions.

STIM2, a closely related homolog of STIM1, also functions as an ER Ca^{2+} sensor to activate CRAC channels in the plasma membrane. Though STIM1 triggers Ca^{2+} influx in response to receptor-mediated depletion of ER Ca^{2+} stores, STIM2 activates Ca^{2+} influx upon smaller decreases in ER Ca^{2+} and causes Ca^{2+} influx via activation of Orai1. Thus, STIM2 might be a feedback regulator that stabilizes basal cytosolic and ER Ca^{2+} levels (Brandman et al., 2007). In accordance with this, Thiel et al. recently reported that molecular silencing of STIM2 by siRNA suppressed SOCE and agonist-mediated Ca^{2+} oscillations at low levels of store depletion, without interfering with STIM1-mediated signals induced by full store depletion in mast cells (RBL), T cells (Jurkat) and human embryonic kidney cells (HEK293) (Thiel et al., 2013). This indicated that STIM2 is preferentially activated by low-level physiological agonist concentrations that cause mild reductions in ER Ca^{2+} levels. Therefore, it can be speculated that SOCE is mediated initially by endogenous STIM2 and incrementally by STIM1, enabling the differential modulation of Ca^{2+} entry depending upon store depletion.

In contrast to these studies, STIM2 has been reported to be an inhibitor of STIM1-mediated SOCE when examined in STIM2 (and also STIM1) overexpressing cell lines (HEK293, PC12, A7r5, and Jurkat T cells) (Soboloff et al., 2006). In this report, STIM1 was confirmed as a required mediator of SOCE, while STIM2 was a powerful inhibitor of this process, interfering with STIM1-mediated SOCE at a point downstream of puncta formation. Therefore, STIM1 and STIM2 might play a coordinated role in controlling SOCE by their opposing functions.

As described above, ligation of BCR induces the mobilization of Ca^{2+} , and emptying the ER Ca^{2+} store is sensed by STIM1 and STIM2 that mediate the CRAC channel in the plasma membrane (Baba and Kurosaki, 2009). Interestingly, these biphasic Ca^{2+} influxes were recently recorded spatiotemporally by real-time imaging of a single B cell (Ramos and DT40 B cells) using confocal laser scanning microscopy. After the ligation of BCR with anti-IgM, translocation of BLNK from the cytosol to the plasma membrane preceded the release of Ca^{2+} from the ER and membrane association of the BLNK-assembled complex continued after Ca^{2+} influx. Furthermore, recruiting very small amounts of the cellular BLNK pool to the plasma membrane is sufficient to pass the release Ca^{2+} threshold (Junek et al., 2012).

4. STIM1 and STIM2 function in B cell regulation

To examine directly the physiological function of STIM1 and STIM2 in B cells, we prepared mice with B cell-specific deletion of STIM1 and STIM2 using the cre recombinase-*loxP* system (Matsumoto et al., 2011). These mice (*Stim1^{fl/fl}Stim2^{fl/fl}Mb1^{cre/+}*) showed the following B cell physiological properties. 1) Development of B cells in the spleen, bone marrow and peritoneal cavity was equivalent to that of control *Mb1^{cre/+}* mice, indicating STIM1 and STIM2 are dispensable for B cell development. 2) Deficiency of both STIM1 and STIM2 caused almost complete suppression of SOCE induced by stimulation with thapsigargin or anti-IgM Ab. 3) BCR-mediated proliferation was completely inhibited in *Stim1^{fl/fl}Stim2^{fl/fl}Mb1^{cre/+}* B cells, whereas anti-CD40 Ab- or LPS-mediated proliferation was normal, and thus BCR-mediated B cell

proliferation is dependent on SOCE. 4) *Stim1^{ff}Stim2^{ff}Mb1^{cre/+}* mice showed normal antibody responses against T-dependent and T-independent antigens, indicating STIM-mediated SOCE was dispensable for the induction of antibody responses. Thus, although B cell-specific deletion of STIM1 and STIM2 caused profound defects in BCR-induced SOCE and resultant proliferation, B cell development and antibody responses were not affected.

B cells mediate multiple functions that overlap between immune and inflammatory responses, and B cells with negative regulatory functions have recently gained much attention (Mauri et al., 2011). It was reported that B cell-derived IL-10 plays a key role in controlling autoimmunity where Th1-driven experimental autoimmune encephalomyelitis persists in the absence of IL-10 production by B cells (Fillatreau et al., 2002). A unique B cell subset with an immunophenotype of CD1d^{hi}CD5⁺ was observed in mice as regulatory B cells that preferentially produced IL-10 (Yanaba et al., 2008). Furthermore, these immunosuppressive functions of regulatory B cells were observed in several murine models of chronic inflammation, including collagen-induced arthritis and inflammatory bowel disease. In humans, CD19⁺CD24^{hi}CD38^{hi} B cells were reported to suppress the differentiation of Th1 cells partially through the production of IL-10 (Blair et al., 2010).

By using *Stim1^{ff}Stim2^{ff}Mb1^{cre/+}* mice, we then determined whether B cell regulatory functions were affected by the loss of both STIM1 and STIM2. We demonstrated that B cells lacking both STIM1 and STIM2 failed to produce IL-10 (Matsumoto et al., 2011). It is well known that IL-10 production is regulated by MAP

kinases and various Ca^{2+} -activated transcription factors including CREB, NF- κ B and NFAT (Saraiva and O'Garra, 2010). Although activation of Jnk, p38, Erk, CREB, or I κ B α was unaffected when phosphorylation was examined, BCR-induced dephosphorylation of NFAT was markedly impaired in *Stim1^{flf}Stim2^{flf}Mb1^{cre/+}* B cells. Serine/threonine phosphatase activity of calcineurin is usually upregulated after binding to calmodulin in a high $[\text{Ca}^{2+}]$ environment, and NFAT is dephosphorylated by activated calcineurin, causing the nuclear translocation of dephosphorylated NFAT. The loss of SOCE in *Stim1^{flf}Stim2^{flf}Mb1^{cre/+}* B cells impairs the calmodulin-calcineurin pathway resulting in the inhibition of nuclear translocation of NFAT. Therefore, the loss of IL-10 production in these B cells was attributable to the defective activation of NFAT.

As described above, IL-10-producing B cells have a protective role in T cell-mediated autoimmune diseases, and this was confirmed in our studies where *Stim1^{flf}Stim2^{flf}Mb1^{cre/+}* mice exhibited an intense exacerbation of the development and progression of experimental allergic encephalomyelitis (EAE) after immunization with myelin oligodendrocyte glycoprotein (MOG) peptide. Furthermore, severe EAE observed in *Stim1^{flf}Stim2^{flf}Mb1^{cre/+}* mice was ameliorated by the adoptive transfer of B cells transduced with an IL-10 gene. Thus, STIM-mediated IL-10 production contributes to B cell regulatory functions *in vivo*, and controls the development of EAE. In summary, STIM1 and STIM2 are critical in SOCE in B cells, and are indispensable mediators of B cell regulatory functions that might limit autoimmune diseases by supporting IL-10 production.

5. Conclusion

In this review, we have attempted to highlight the currently understood signaling cascade leading to Ca^{2+} influx and its physiological impact in B cells. A long-standing hypothesis is that Ca^{2+} influx is important for a variety of B cell functions such as cell development and humoral responses. The recent discovery of the essential components for SOCE, STIM1 and STIM2, have uncovered a different role whereby SOCE in B cells participates in IL-10 production to limit autoimmunity. However, how cytosolic Ca^{2+} signals in B cells is achieved by a variety of inputs and decoded to specific and appropriate physiological outputs remains unclear. Determining the functional difference between Ca^{2+} release from ER and SOCE is also a challenging issue. Finally, understanding the biology of Ca^{2+} signals and the molecular mechanisms in immune cells might be useful for the development of clinical strategies to modulate immune responses to treat autoimmune, inflammatory and allergic diseases.

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Figure Legend

Figure 1. Ca^{2+} signaling cascade. After BCR stimulation, Syk is recruited to phosphorylated ITAMs in $\text{Ig}\alpha/\text{Ig}\beta$, and then phosphorylates BLNK. Phosphorylated BLNK provides binding sites for Btk and $\text{PLC-}\gamma 2$. Subsequently, $\text{PLC-}\gamma 2$ is fully activated by Syk and Btk through phosphorylation. BCR stimulation induces phosphorylation of CD19 by Lyn and BCAP by Syk, which activates PI3K to produce phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_3 is important for the membrane recruitment of Btk through its PH domain. PIP_2 serves as a substrate of $\text{PLC-}\gamma 2$ to produce diacylglycerol (DAG) and inositol-1,4,5,-triphosphate (IP_3). The resultant IP_3 binds to its receptor IP_3R , which leads to Ca^{2+} release from the ER. The reduction of Ca^{2+} in the ER lumen causes STIM relocation and association of STIM and Orai into puncta at the ER-plasma membrane junction. Subsequently, the Orai channel is activated and induces a Ca^{2+} influx (store-operated Ca^{2+} entry: SOCE). Sustained Ca^{2+} increase by SOCE activates the calmodulin/calcineurin pathway. Phosphatase calcineurin dephosphorylates NFAT, which leads to its nuclear localization to regulate gene expression.

Figure 1