

Understanding early morphological changes in B cell activation.

**A Thesis submitted towards partial fulfilment of
BS-MS Dual degree program.**



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CERTIFICATE

This is to certify that this dissertation entitled “**Understanding early morphological changes in B cell activation.**” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER) Pune represents original research carried out by **Mr. Anurag Mishra** at IISER Pune under the supervision of **Dr. Akanksha Chaturvedi**, Assistant Professor, Biology Division, IISER Pune during the academic year 2013-2014.

Dr. Akanksha Chaturvedi

Assistant Professor,

Biology Division, IISER Pune.

Declaration

I hereby declare that the matter embodied in the thesis entitled “**Understanding early morphological changes in B cell activation.**” are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of **Dr. Akanksha Chaturvedi**, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

B cells play a crucial role in the adaptive immune response by binding to an array of antigens and generating explicit responses against them. B cells sense the antigens through the surface expressed B cell receptors (BCRs). Antigen binding to the BCR initiates clustering of the BCRs that triggers a signaling cascade leading to the activation of MAP kinases and transcription of a variety of genes associated with B cell activation. In addition, antigen binding to the BCR also triggers the endocytosis of the antigen bound BCR and its trafficking to specialized intracellular compartments in which antigen processing occurs.

Recently it has become clear that B cells, in addition to clonally expressed BCRs also express various germ line encoded pattern recognition receptors, in particular Toll like receptors (TLRs). TLRs are innate immune receptors that recognize conserved molecular patterns present in microbes, referred to as pathogen associated molecular patterns (PAMPs). This dual expression allows B cells to not only sense antigen but also survey their environment for danger signals associated with the presence of pathogens. Although both BCR and TLRs initiate signal independently, in response to antigens and PAMPs, B cells are able to integrate both antigen-specific and danger signals into a qualitatively and quantitatively unique molecular response. Indeed, TLRs have been shown to significantly influence the outcome of antigen engagement by the BCR and contribute to the immune dysregulation observed in autoimmune disease and in some B cell tumors.

I have tried to study the early changes involved in B cell with response to TLR9 ligand. We have observed that some of these early events are manifested as morphological changes in B cells. Using field emission scanning electron microscopy and confocal microscopy, I have attempted to elucidate these preliminary changes. I have also planned an approach to look for the pathways involved in these morphological changes. Alongside, I have tried to check for some of the important molecules involved in the TLR9 ligand internalization and signaling.

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INTRODUCTION

B cells activation and signaling.

B cells are critical component of the adaptive immune system and central mediator of humoral immune response, providing both specific and long-lasting protection from a tremendously diverse range of potential pathogens. Deficiency of B cells can lead to the diseases like X-linked agammaglobulinemia, where complete lack of antibodies makes host susceptible to extracellular bacteria and viruses. On the contrary, abnormal and hyper-activated B cells are associated with many autoimmune diseases such as systemic lupus erythematosus (SLE) and certain B cell lymphomas(Shaffer et al., 2012; Yanaba et al., 2008).

B lymphocyte activation is initiated by the binding of Ags to the clonally expressed B cell receptors (BCRs). The BCR is composed of an Ag binding subunit, a membrane form of Ig (mIg) bound non-covalently to signaling subunits CD79a (Ig α) and CD79b (Ig β). The cytoplasmic domain of Ig α and Ig β contain immunoregulatory tyrosine activation motifs (ITAMs). Following Ag binding one of the first events is the phosphorylation of the BCR ITAMs by the Src-family kinase, Lyn(Kurosaki, 1999). In addition, antigen binding also initiates the clustering of the BCRs, leading to internalization of the antigen bound BCRs, to the specialized compartments where antigen is processed and presented to the Major Histocompatibility complex (MHC) class II molecule for the T cell help(Lanzavecchia, 1990). Following antigen contact and appropriate T cell help B cell proliferate in germinal center, and differentiate into antibody-secreting plasma cells and memory B cells.

BCR signaling is a multistep process, which is initiated by the sequential activation of three families of protein tyrosine kinases (PTKs), Src family PTKs, primarily Lyn, but also Blk, and Fyn, followed by the activation of Syk and the Tec family kinase, Btk. Following which signaling adaptor proteins, such as B cell linker protein (BLNK), Bam32 and BCAP are phosphorylated and recruited to the BCR. These adaptors facilitate the formation of BCR signalosome by providing multiple docking sites for downstream signaling mediators and bridging RTKs with their downstream effector proteins. Formation of the BCR signalosome ultimately triggers at least four major signaling cascade that include phospholipase C (PLC), the Rho family of GTPases, Ras, and phosphatidylinositol-3-kinase (PI3K) resulting in the generation of second

messengers and activation of the transcription of genes involved in B cell responses(Campbell, 1999; Kurosaki, 1999; Kurosaki et al., 2010).

Two second messengers in BCR signaling are calcium and DAG. Calcium responses are initiated following recruitment and activation of PLC γ 2, which hydrolyses PI(4,5)P₂ to generate - inositol-trisphosphate (IP₃) and diacyl glycerol (DAG)(Fu et al., 1998; Kurosaki and Tsukada, 2000). Binding of IP₃ to the IP₃ receptors on endoplasmic reticulum (ER) releases the stored calcium from the ER into the cytosol, leading to increase in cytosolic calcium and decrease in calcium concentration in the lumen of ER. The depletion of calcium in ER is sensed by the stromal interaction molecule 1 (STIM1) protein, which results in oligomerization of STIM1. STIM1 oligomers interacts with Orai, a calcium release activated channel (CRAC) at the junctions of the ER and plasma membrane and activates Orai to open calcium release activated channel (CRAC). Opening of CRAC channels initiate the influx of extracellular Calcium to the cytosol(Liou et al., 2005).

Increase in the cytosolic Calcium concentration, following BCR crosslinking is sensed by multiple downstream proteins such as calcium binding protein Calmodulin, which ultimately leads to the activation of transcription factor- NF-AT. Cytosolic calcium together with DAG, activates the protein kinase C - β pathway (PKC- β) initiating signaling cascade leading to activation of another important transcription factor, NF- κ B that plays essential role in B cell activation(Baba et al., 2013).

Activated PKC- β also facilitates the activation of Ras guanine nucleotide releasing protein 3(RasGRP3) which inturn activates Raf - MEK and ERK pathway(Chaturvedi, 2012; Kurosaki et al., 2010). In addition to ERK, other members of the MAP kinases such as JNK and p38 are also phosphorylated following BCR crosslinking. All three MAP kinases play important roles in the activation of transcription factors that regulate the expression of genes involved in B-cell proliferation, differentiation, and survival.

In addition to MAP kinases and transcription factor activation, another major event that takes place is the cytoskeletal rearrangement that facilitates internalization of the antigen bound BCR. Two crucial players of cytoskeletal rearrangement, Rac and cdc42 are recruited to the BCR signalosome and subsequently activated through the concerted action of PI3K, and adaptor Bam32. Both Rac and cdc42 have been shown to play crucial role in B cell activation by modulating the cytoskeleton machinery.

Once activated, B cells move to the secondary lymphoid organs to seed the germinal centers where they proliferate and differentiate into antibody producing plasma cells and memory B cells.

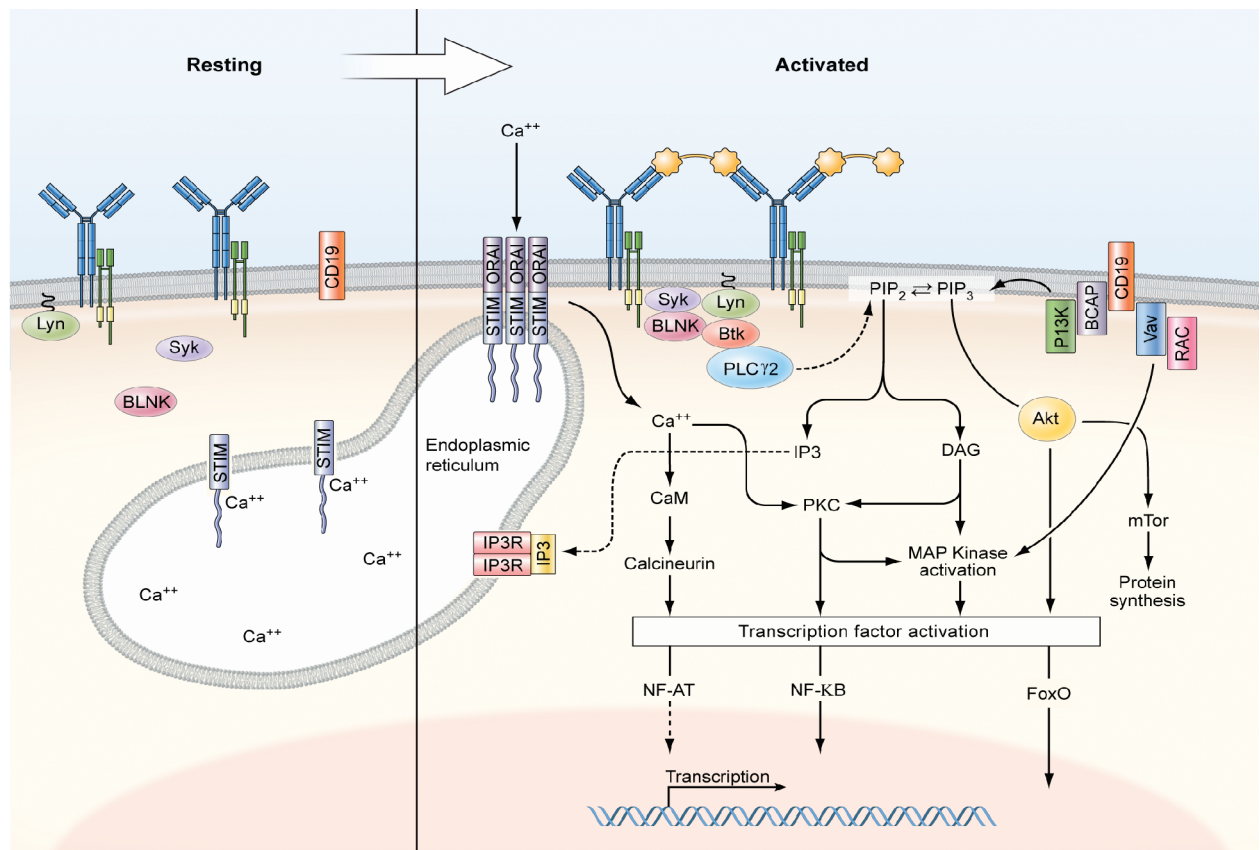


Figure1. Overview of B cell signaling: Antigen binding to the BCR leads to the formation of signalosome consisting of BCR, PTKs, adaptors and effector molecules. PLCγ2 hydrolyses the PIP₂ to IP₃ and DAG. IP₃ binds to the IP₃ receptor on the ER opening Ca²⁺ channels and releasing the stored Ca²⁺ from ER in the cytosol. Ca²⁺ depletion in the ER is sensed by STIM1 and STIM2, a Ca²⁺ binding protein, which results in its oligomerization and movement to regions of the ER membrane in close proximity to the plasma membrane where it interacts with ORA1 opening the CRAC channel, resulting in Ca²⁺ influx. Figure obtained from Fundamental immunology.

Innate Immune receptors:

Recent studies revealed that in addition to clonally expressed BCRs, B cells also express a wide repertoire of germ-line encoded innate immune receptors

commonly known as pattern recognition receptors (PRRs). Of particular interests are Toll-like receptors (TLRs), which are best-characterized and well-studied subfamily of PRRs. TLRs function as sentinels identifying the invading pathogen through the recognition of conserve pathogen associated molecular pattern (PAMPs). Recognition of PAMPs through the TLRs culminate in the up-regulation of variety of co-stimulatory molecules and the production of cytokines and interferons by the cells of innate immune system(Janeway and Medzhitov, 2002). For B cells, TLRs have been shown to directly regulate B cell antigen receptor signaling. TLRs belong to the family of interleukin-1 receptor family containing leucine-rich domain, which is responsible for the recognition of pathogen, transmembrane domain and cytoplasmic Toll/interleukin-1 receptor (TIR) domain that is involved in signal transduction(Takeda and Akira, 2004).

At least thirteen different TLRs have been identified in mice and human. Based on their subcellular location, TLRs can be broadly divided into surface expressing TLRs that includes TLR1,2,4,6 and 10 and intracellular TLRs such as TLR3,7,8, and 9. TLRs differ from each other in number of ways; they recognize different PAMPs, expressed in distinct cell types, and recruit different adaptor proteins for signaling(Takeda and Akira, 2004). Following binding to the PAMP, adaptors are recruited at the TIR domain of the TLRs, resulting in the activation of MAP family kinases, and NF- κ B.

One of the best-studied TLRs in this regard is TLR9, which recognizes unmethylated CpG motifs present in viral and bacterial DNA. How TLR9 signals in innate immune cells is known in great detail, however very little is known about TLR9 signaling in B cells. In B cells, ligation of TLR9 with unmethylated CpG ODN has been shown to initiate signaling cascades that ultimately converge on mitogen-activated protein (MAP) kinases and the transcription factor NF- κ B(Eckl-dorna and Batista, 2009). However, the details of the TLR9 signaling cascade leading to MAP kinase activation in B cells have not been mapped.

Interestingly, in B cells, the subcellular location of the TLR9 is dependent on the BCR. In naïve B cells where BCR is at the surface TLR9 resides in early endosomal compartments, however, the following the antigen binding to the BCR, which results in BCR internalization, TLR9 is recruited to the autophagosome like multivesicular compartments(Chaturvedi et al., 2008). The fact that in systemic lupus erythematosus,

activation of autoimmune B cells in response to DNA-containing antigens is dependent on both BCR and TLR9 clearly underscores the importance of the interaction between the BCR and TLR9(Christensen et al., 2005; Fields et al., 2006; Lartigue et al., 2006; Leadbetter et al., 2002; Viglianti et al., 2003). In innate immune cells, CD14 is one of the cofactor that modulates the function of TLR9(Baumann et al., 2010). However role for CD14 in B cells is not known, it is possible that CD14 also facilitates TLR9 signaling in B cells.

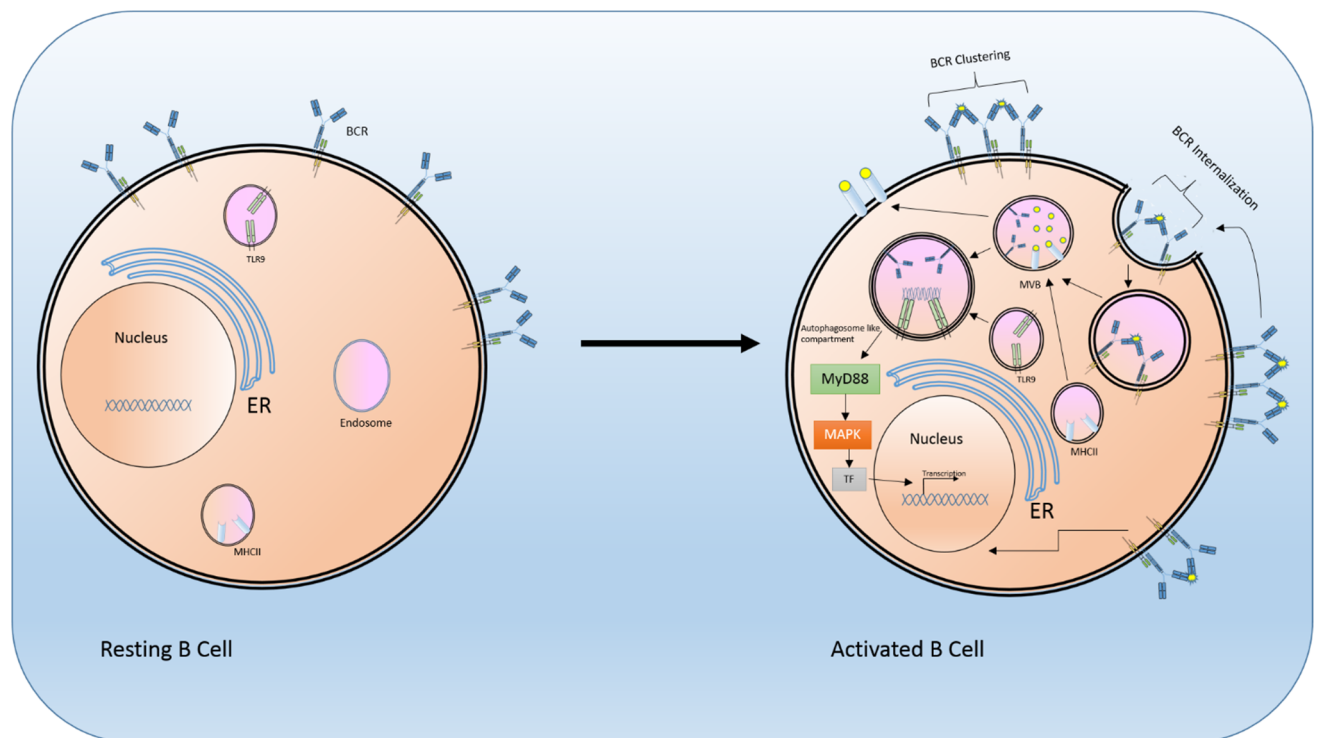


Figure2. Overview of TLR9 signaling in B cell: On antigen recognition through BCR, BCR are internalized and moved to the specialized compartment (multivesicular bodies; MVBs) where antigen is processed and presented onto the MHCII molecule. BCR crosslinking also induces relocalization of the TLR9 from early endosomal compartments to the autophagosome like compartments from where both BCR and TLR9 synergistically signal to MAP kinase activation and NF- κ B activation

Objective of this study

B cells respond differently to BCR and TLR9 ligands by undergoing antigen-specific and polyclonal activation respectively. Many of these differences are relatively late events occurring 15-60 min after ligand binding to the BCR and TLR9. Early responses to activating ligands that occur within several seconds are often manifested in morphological changes in cells. It is possible that cytoskeletal rearrangement and morphological changes in cells may differ in cells treated with BCR and TLR9 ligation and could potentially provide clue to their different mode of action.

Objectives of this work are -

1. To analyze the changes in the B cell surface morphology following BCR and TLR9 activation.
2. To clone CD14-GFP which in later studies will be used to map the trafficking of CD14 in B cells.

Materials and methods

Mice strains and Cells:

Both Balb/c and C57bl/6 mice strains were utilized. Mice were obtained from Reliance industries and maintained at INTOX as per institutional guidelines. Daudi and Ramos B cell lines were grown and maintained at 37°C in 5% CO₂ in Rosewell Park Memorial Institute medium (RPMI, obtained from Gibco) and 10 % (w/v) fetal bovine serum (Gibco, Cat. No.10082-147). CH27 B cell line was grown and maintained at 37°C in 5% CO₂ in Iscove's Modified Dulbecco Medium (IMDM, obtained from Gibco) and 15% (w/v) fetal bovine serum (Gibco). Splenic B cell were purified using negative selection on the same day of experiment.

B cell purification using negative selection:

Spleen was obtained from adult mice (6-8 weeks) and crushed with the syringe to make single cell suspension. RBC lysis buffer was added to remove the RBCs. Following RBC lysis, cells were plated in RPMI containing 20% FBS for panning at 37°C for 45 minutes to remove adherent cells. At the end of incubation, nonadherent cells were removed and incubated with anti-CD90.2 and anti-CD11b coated magnetic beads at 4°C for 30 minutes. Cells were passed through the magnet to remove non B cell population. Purified splenic B cells were suspended in RPMI containing 10% FBS and were used within 1-2 hours of purification.

Live cell imaging:

Lab-Tec chamber were coated with poly-L-lysine (Sigma) for 30 minutes prior to use. Cells were plated on to the chamber wells in RPMI containing 2% FBS and were imaged at 37°C images with or without stimulant. Both z-stacks and time series were taken on either Zeiss LSM 710 or 780 inverted microscopes (IISER Pune microscopy facility).

SEM sample preparation:

Silicon wafer were cut into 5mm X 7mm pieces and were coated with poly-L-lysine (0.1mg/ ml) and ~0.5 million cells were attached to them. Cells were stimulated with CpG ODN (3µM) and anti-IgM (10µg/ml) for the specified time and were fixed overnight with 2% glutaraldehyde + 4% paraformaldehyde. Cells were then post treated with 1%

OsO₄ for 30 minutes at room temperature. Dehydration the samples using series of 30%, 50%, 70% and 100% ethanol wash for 5 min each. Samples were gold sputtered (8 – 10 nm) in vacuum, then images were taken using Zeiss FE scanning electron microscope at physics microscope facility, IISER Pune.

Inhibitory experiments:

Silicon wafers were coated with poly-L-lysine and ~0.5 million cells were attached to the silicon wafer pretreated with the indicated inhibitors at 37°C for indicated time in 2% (w/v) FBS + RPMI. Indicated stimuli were added at 37°C for indicated time. Cells were then fixed with 4 % paraformaldehyde + 2% glutaraldehyde. Cells were post treated and dehydrated using 1% OsO₄ and alcohol respectively. Samples were gold splattered (8 – 10 nm) in vacuum, then images were taken using scanning electron microscope.

RNA isolation:

Total cellular RNA was isolated from the cells using trizol method. Cells were centrifuged to remove media and 500 µl of trizol was added to lyse the cells. Cell lysate was washed with chloroform to remove the phenol impurity. Subsequently cell lysate was washed with isopropanol to precipitate the RNAs. RNAs were washed with 70% (v/v) ethanol to remove the extra water content. RNAs were resuspended in nuclease free water and stored at -80°C.

cDNA synthesis:

Purified RNA was quantified using Thermo scientific Nanodrop spectrophotometer (2000c) with nanodrop2000 software at A230, A260 and A280. cDNA was synthesized using BIORAD iScript cDNA synthesis kit as per manufacturer's instructions. Following PCR cycle was used for cDNA synthesis: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min and hold it at 4°C for 5-10 minute.

PCR (Polymerase chain Reaction):

Above synthesized cDNA was stored at -20°C. Promega PCR master mix was used for the PCR reaction. Reaction mixture of 50µl was prepare using the 25µl of 2X PCR Master Mix, 2 µl of cDNA, 2.5µl of each forward and reverse primer (10µM) and nuclease free water to make up the volume. Following PCR cycle was used for all

PCR reaction with appropriate annealing temperature- 95°C for 5 min, 95°C for 40sec, annealing temperature for 40 sec, 72°C for 1min 30 sec, and 72°C for 5 min.

CD14 Primers (Annealing temperature- 68°C)

Forward primer: GCG AAG CTT CCA CCA TGG AGC GTG TGC

Reverse Primer: CGC GGA TCC TTA AAC AAA GAG GCG ATCT

RT- PCR

Total RNA was extracted from the Daudi cell line using standard Trizol method. cDNA was generated by using BIORAD iScript cDNA synthesis kit. PCR was conducted using the primer for TLR3, TLR7, TLR8 and TLR9 from Invivogen using Promega PCR master mix. All TLR3, TLR7, TLR8 and TLR9 were amplified using dsDNA (provided by Invivogen along with the primer) as control.

PCR Product analysis:

PCR product were analyzed on agarose gel (2% for up to 500 base pair and 0.8 % for more that 500 base pair). Standard marker and 6X loading dye was use for gel loading and gel was run at 100V for 1 hour. PCR images were taken on the SYNGENE G: BOX using Genesnap software from SynGene at COE facility IISER Pune.

Cloning:

Standard procedure was followed for the cloning. PCR product were purified using the Phenol-Chloroform-Isoamyl (PCI) procedure. These purified inserts and vector were digested with the appropriate restriction enzyme (NEB) and purified using gel extraction kit (Qiagen). 10µl ligation mixture was prepare in such a way that insert to vector ratio was 3:1. These purified insert and vector were ligated using T4 ligase from Promega for 16 hour at 16°C. After ligation, the mixture was transformed in DH5α E.Coli and plated with the appropriate antibiotic resistance. Colonies were screened for positive colony by colony PCR.

Vector Used for Cloning: EGFP-N1

Table 1: Magnetic beads used for B cell isolation.

Name	Company	Catalog	Concentration
CD90.2 Bead	BD Biosciences	551518	50 μ l / spleen
CD11b Bead	BD Biosciences	558013	30 μ l / spleen

Table 2: Stimulant used for the experiments.

Name	Company	Catalog	Stock Concentration
Type B CpG oligonucleotide - Human TLR9 ligand	Invivogen	tlrl-2006-1	0.5 mM
CpG oligonucleotide - Murine TLR9 ligand	Invivogen	tlrl-1826-1	0.5 mM
AffiniPure F (ab') ₂ Fragment Goat Anti-mouse IgM, μ Chain specific.	Jackson Immuno Research	115-006-020	1.2 mg / ml
AffiniPure F (ab') ₂ Fragment Goat Anti-Human IgM, F _c γ 2b fragment specific.	Jackson Immuno Research.	109-006-129	1.3 mg / ml

Table 3: Inhibitors used.

Sr No.	Inhibitor	Solvent	Concentration	Company
1	Latrunculin B	DMSO	500nM	CalBio Chem
2	Y-27632	Water	50 μ M	CalBio Chem
3	Blebbistatin	DMSO	100 μ M	CalBio Chem
4	ML-7	DMSO	600nM	CalBio Chem
5	PP2	DMSO	500nM	CalBio Chem
6	BTP2	DMSO	1180nM	CalBio Chem
7	TMB8	Water	1 μ M	CalBio Chem

Results

Dynamics of surface morphological changes by live cell imaging.

To understand the dynamics of BCR and TLR mediated surface morphological changes live cell bright-field imaging of purified and unlabeled murine splenic B cells from Balb/c mice was performed. To do so cells were coated on to the poly-l-lysine coated chamber plates and were directly imaged first without adding any stimuli (time 0 min), following which either unlabeled anti-IgM to crosslink the BCR (Figure 3A) or unlabeled CpG-ODN to ligate the TLR9 (Figure 3B) were added and cells were imaged for at least additional 40min. In both conditions, live cells showed extensive membrane ruffling which looked different when cells were treated with CpG compared to when treated with anti-IgM (Supplementary movie 1, 2). In addition, CpG treated cells appeared more polarized.

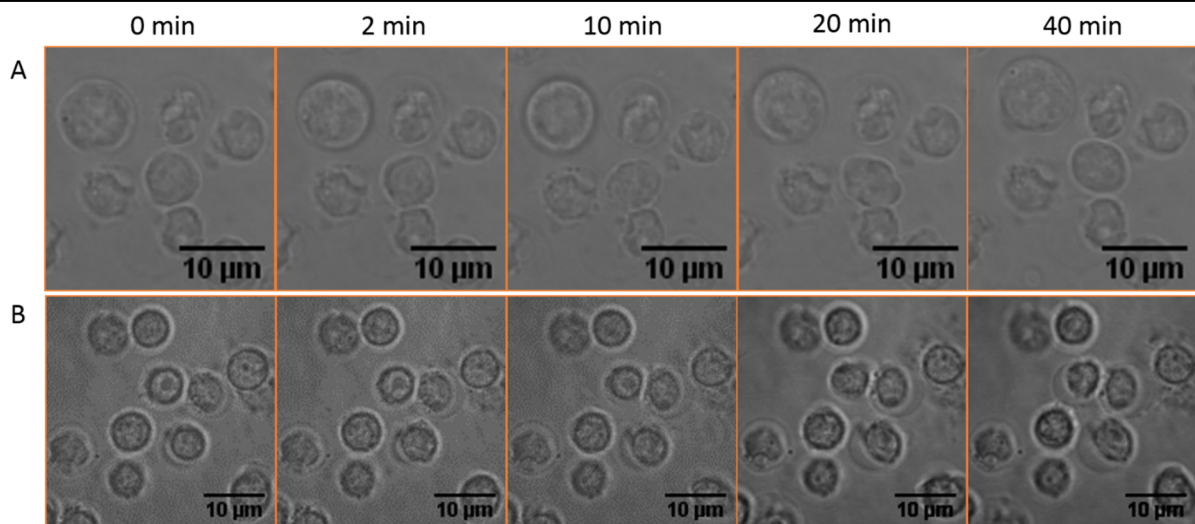


Figure 3. Treatment of anti-IgM and CpG to B cell leads to morphological changes: Murine splenic B cells from Balb/c mice were either left untreated (0 min) or treated with (a) F(ab)₂ fragment of anti-IgM to crosslink BCR or (b) CpG-ODN to ligate TLR9 for the indicated times. Cell were maintained in the RPMI plus 2% FBS as described in the material and methods. Live cell imaging was done using Zeiss confocal microscope.

Labelled ligands for BCR and TLR9 are internalized.

These results showed that BCR and TLR9 mediated morphological changes are very rapid which are observed within few seconds of addition of their respective ligands. To analyze whether B cells acquire ligands in this short period of time, the trafficking of the fluorescently labeled ligands was analyzed. Cells were plated onto the poly-L-lysine chamber plates and DyLight 647 labeled anti-IgM was used to crosslink the BCR (Figure 4A) and Cy3 labeled CpG-ODN to ligate the TLR9 (Figure 4B). Live cell imaging was performed immediately after addition of the ligands for at least 40min (Supplementary movie 3, 4). Both DyLight 647 labeled anti-IgM and Cy3 labeled CpG-ODN were taken up by B cells within a few seconds. Indeed, Cy3 labeled CpG ODN was seen clearly inside the cells right after addition, indicating that CpG internalization is a rapid event and that within few seconds TLR9 gets access to its ligand and precedes observed morphological changes.

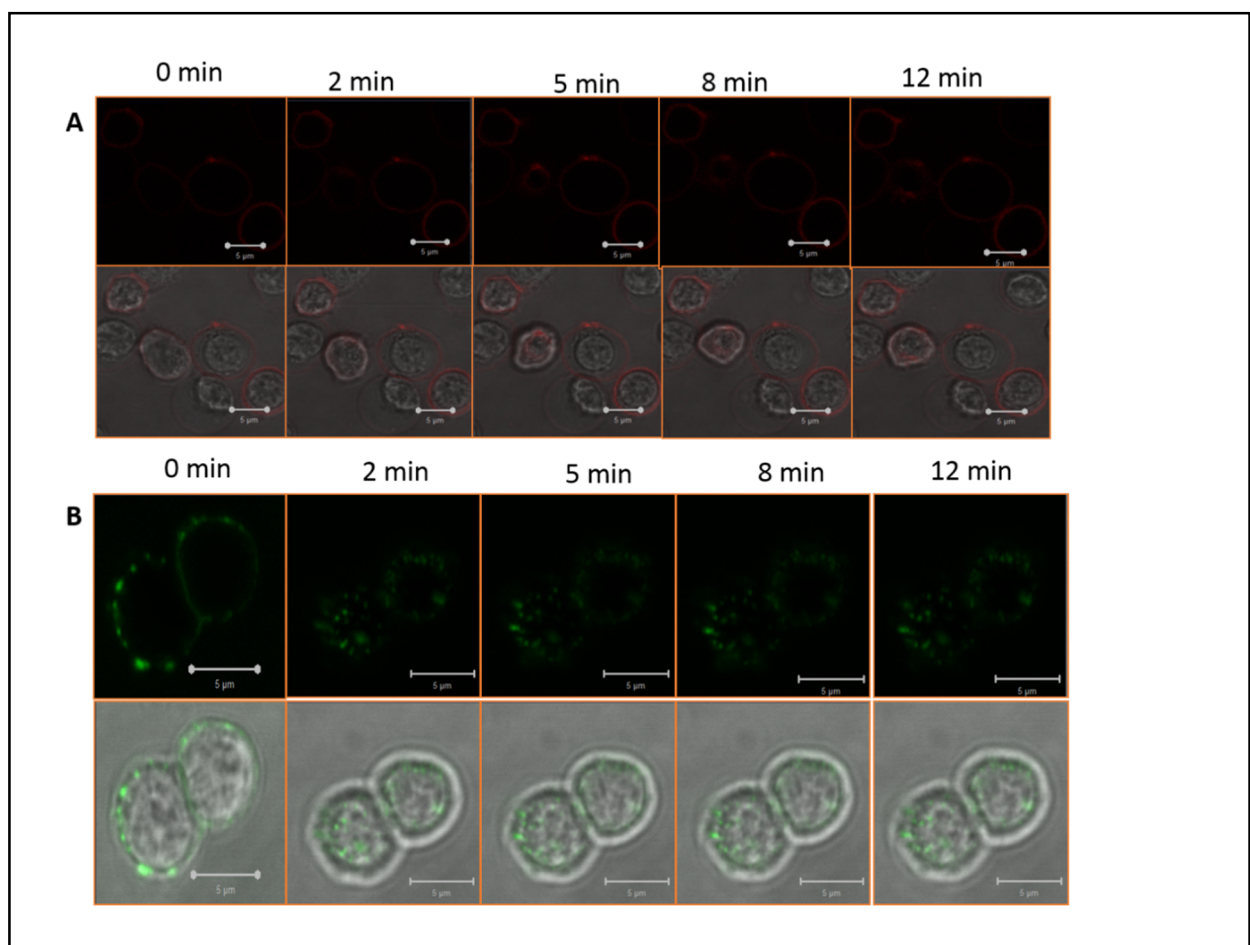


Figure 4. Internalization of anti-IgM and CpG: Murine splenic B cells from Balb/c mice were treated with labelled (a) DyLight 647 F (ab)₂ fragment of anti-IgM (to crosslink BCR) and (b) labelled Cy3-CpG (to ligate TLR9). Live cell imaging was done on confocal microscope to analyze the internalization of anti-IgM and CpG.

Standardizing the SEM.

To obtain a high resolution view of the BCR and TLR9 induced morphological changes, field emission scanning electron microscopy (FESEM) was performed, which required extensive standardization. Purified splenic B cells were resuspended in RPMI containing 2% FCS, and were attached to poly-L-Lysine coated silicon wafers. Cells were left untreated or treated with anti-IgM or CpG, fixed, and washed in phosphate buffer saline (PBS), air dried and gold plating was done and cells were imaged with FESEM. As shown in figure 5, the surface was not resolved properly and in addition multiple salt crystals were seen. To remove the salt content was replaced with filtered sterilized phosphate buffer (PB). To get better contrast many conditions were tried, such as cells were imaged after washing directly with 100% ethanol with or without gold sputtering. Alternatively, cells were post fixed with osmium tetroxide followed by gradient ethanol wash, and gold sputtering. Representative images are shown in figure 6. Ethanol wash was useful in removing the extra water content, which ultimately reduced the charging effect obtained in cells where post fixation treatment was not performed.

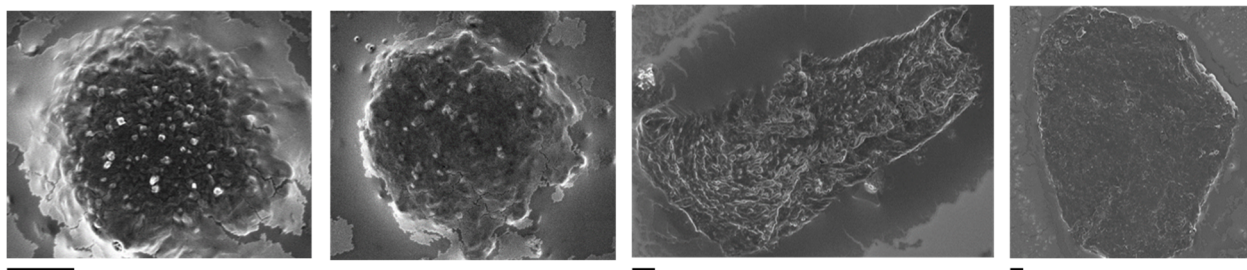


Figure 5. SEM images of B cells. B cells were attached to the Poly L Lysine coated silicon wafer. Attached cells were fixed using paraformaldehyde and SEM images were acquired using FESEM. (Scale Bar: 2µm)

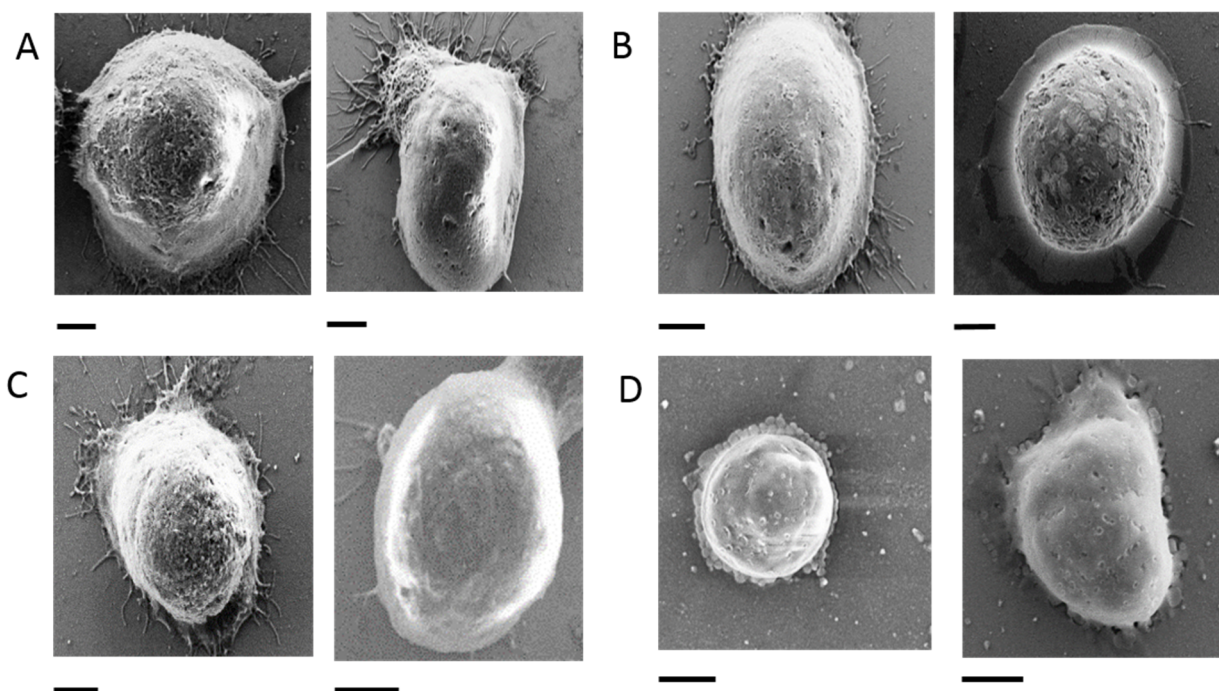


Figure 6. Standardization of the fixation and post fixation conditions for SEM, B cell were attached to the Poly L Lysine coated silicon wafer. Attached cells were fixed using paraformaldehyde and various post treatments were done (A) Cells were simply washed with 100 % ethanol (B) Cells were washed with ethanol and gold coated. (C) Cells treated with OsO₄ and ethanol. (D) Cells were treated with ethanol, OsO₄ and gold coated. Images were acquired after post treatment using FESEM. (Scale Bar: 2µm)

Changes in B cell surface morphological changes following BCR and TLR9 ligand.

To understand whether BCR crosslinking and TLR9 ligation induce any changes on the surface morphology of the B cells, murine splenic B cells from C57bl/6 mice were analyzed by scanning electron microscopy. To do so cells were treated with either unlabeled F(ab')₂ fragment of anti-mouse IgM (anti- IgM) to crosslink the BCR (Figure 7A) or unlabeled CpG ODN to ligate the TLR9 (Figure 7B) for the indicated times. At the end of incubation cells were fixed and post processed as described in material and methods. Within 30 seconds of BCR crosslinking, cells showed extensive membrane expansions. By 120 seconds membrane expansions in majority of the cells were retracted. On the contrary, ligation of TLR9 with CpG ODN did not show membrane expansions, rather multiple bleb like protrusions were observed on the surface of the

B cells in as early as 30 seconds following TLR9 ligation. These results clearly indicate that BCR crosslinking and TLR9 ligation induce distinct surface morphological changes on B cells. T-test was performed to test the statistically differences, data were collected from three different experiment (Figure 8).

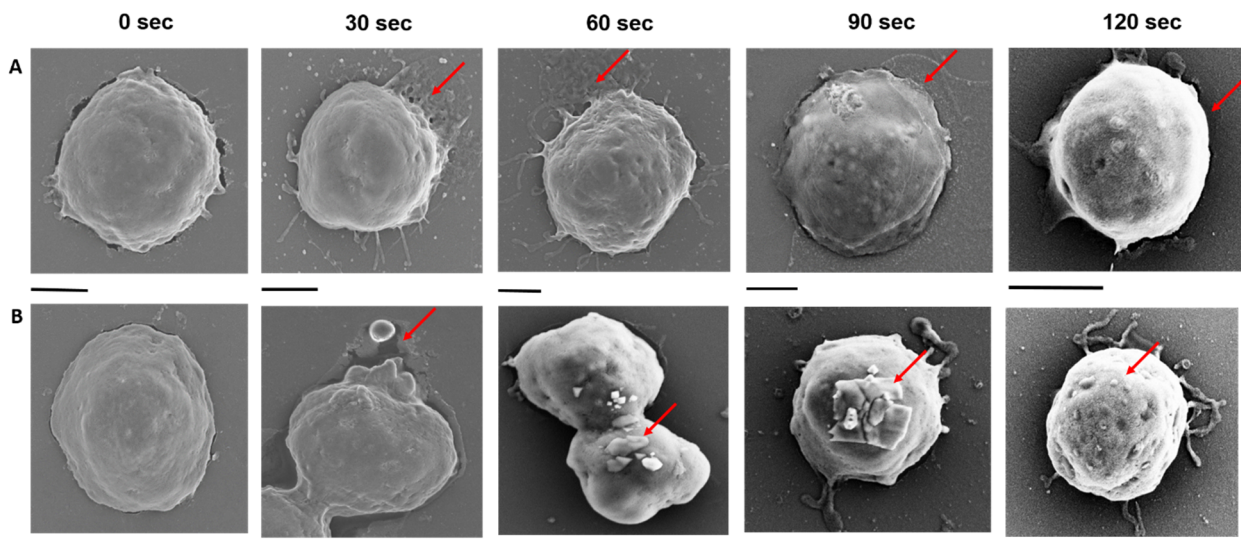


Figure 7. Changes in B cell surface morphology following BCR and TLR9 ligation: Murine splenic B cells from C57Bl/6 mice were either left untreated (0sec), or treated with F(ab)₂ fragment of anti-IgM (A) or CpG ODN (B) for the indicated times. Cells were fixed and post processed. Images were acquired on Zeiss FESEM. (Scale Bar: 2μm)

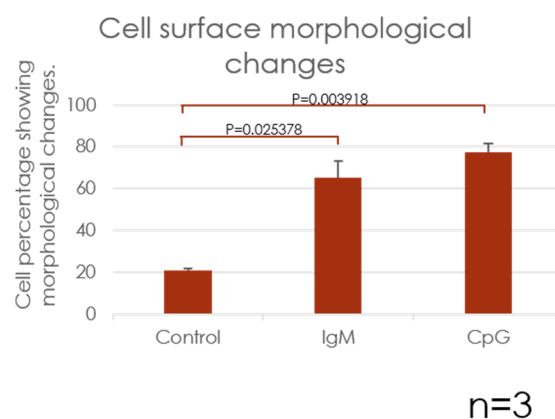


Figure 8. Quantification of changes in cell surface morphology: The positive change in cell surface morphology is quantified using T test for three independent experiments.

To ascertain whether strain specificity has any role to play in TLR9 and BCR dependent morphological changes in B cells we used murine splenic B cells from Balb/c mouse and similar analysis was done as mentioned above and images were taken in FESEM (Figure 9). We observed the similar morphological changes were induced in B cells derived from Balb/c mice clearly indicating that these morphological changes are not restricted to a particular strain.

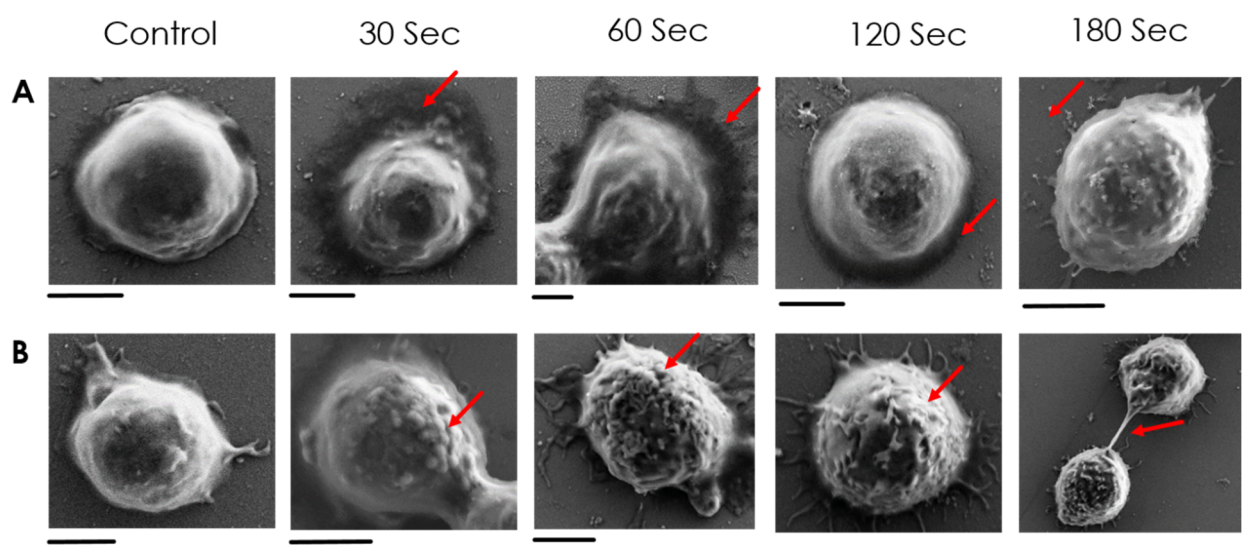


Figure 9. Changes in B cell surface morphology following BCR and TLR9 ligation: Murine splenic B cells from Balb/c mice were either left untreated (Control), or treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9 (B) for indicated times. Cells were fixed and post processed. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Both surface attached and in suspension B cells show similar morphological changes.

To identify if the results obtained are not because of attaching cells to the silicon wafers and to understand whether similar morphological changes are observed if B cells are treated in suspension, splenic B cells were treated with anti-IgM or CpG, fixed and

post treated in solution. Following which, cells were attached to silicon wafers and imaged under FESEM. BCR mediated membrane spreading and TLR9 mediated morphological changes are independent of whether cells are attached or are in suspension (Figure 10).

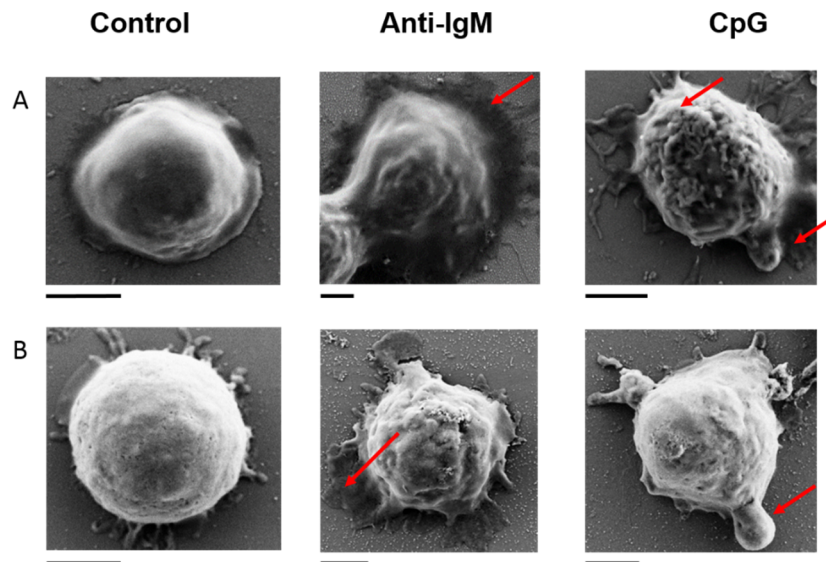


Figure 10. Comparison of changes in Splenic B cell surface morphology following BCR and TLR9 ligation on attached B cells and in solution. Murine splenic B cells from Balb/c mice were either (A) first attached to Poly L Lysine coated silicon wafer and then stimulated, fixed and post processed or (B) they were stimulated in solution, post processed and attached to Poly L Lysine coated silicon wafer. The cells were either left untreated (Control), or treated with F(ab)₂ fragment of anti-IgM or CpG ODN for 60 sec before fixing. Images were acquired using FESEM. (Scale Bar: 2µm)

Study of surface morphological in human B cell lines.

TLR9 has been shown to play a crucial role in human B cells also. Indeed TLR9 ligands have been shown to promote survival of memory B cells. To analyze whether human B cells also show similar morphological changes, two human B cell lines: Daudi and Ramos were utilized. Both Daudi and Ramos express IgM chain at the surface similar to the naïve B cells. However, to analyze whether TLRs expression in these cell lines is similar to naïve B cells, expression of all intracellular TLRs was analyzed by RT-PCR.

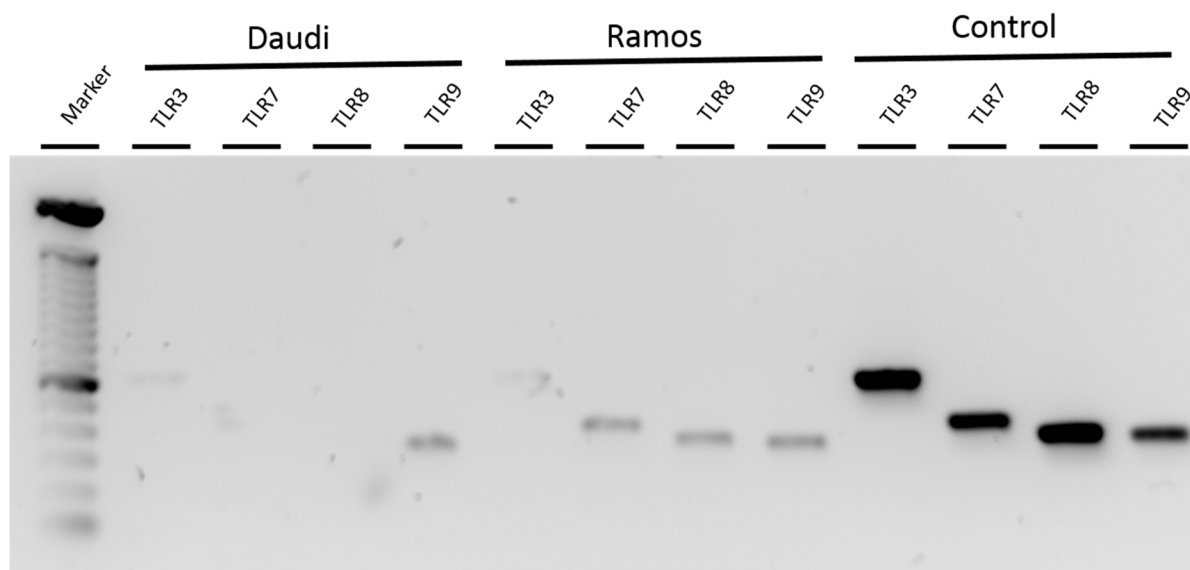


Figure 11. Intracellular TLR expression in Daudi and Ramos cell lines: TLR3, TLR4, TLR8 and TLR9 mRNA expression is analyzed by PCR with the cDNA from Daudi and Ramos. Human dsDNA from Invivogen was used as control.

Transcript for TLR9 was clearly observed in Daudi cells, low amount of TLR3 was also observed, and however the other two intracellular TLRs, TLR7 and TLR8 were completely absent. However, in Ramos cells transcripts for TLR7, TLR8 and TLR9 was clearly seen. In these cells too, TLR3 expression was very low (Figure 11).

After ascertaining the presence of TLR9 in the B cell lines, morphological changes following BCR and TLR9 ligation were analyzed in Daudi. For which, Daudi B cells were remained untreated or treated with either unlabeled F(ab')₂ fragment of anti-human IgM (anti-IgM) to crosslink the BCR (Figure 12A) or unlabeled CpG ODN to ligate the TLR9 (Figure 12B) for 90s. At the end of incubation cells were fixed and post processed as described in material and methods. Similar experiments were also performed for Ramos B cells (Figure 13 A, B)

In both Daudi and Ramos B cells BCR cross linking and TLR9 ligation triggered extensive surface morphological changes which looked very similar to what was observed in murine primary cell lines. BCR crosslinking triggered massive membrane expansions in both Daudi and Ramos B cell. Effect of ligation of TLR9 with CpG were more dramatic in both Daudi and Ramos compared to the murine primary B cells. Numerous bleb like structures were observed in both Daudi and Ramos B cell surface.

Interestingly, CpG ODN treated Daudi cells were extremely polarized and it appeared that they were trying to move away from the surface. These results clearly indicate that CpG mediated signaling triggers morphological changes in both human and murine B cells that are distinct from BCR crosslinking induced morphological changes.

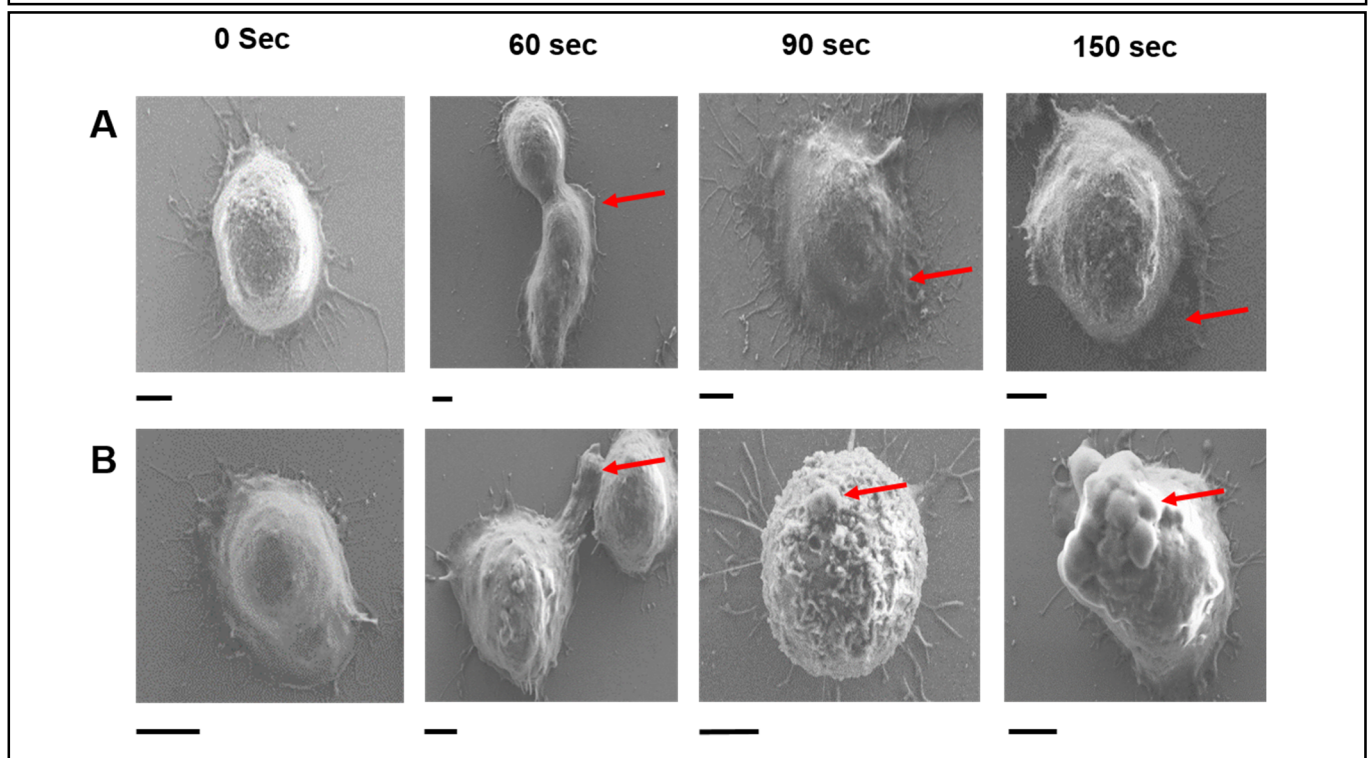
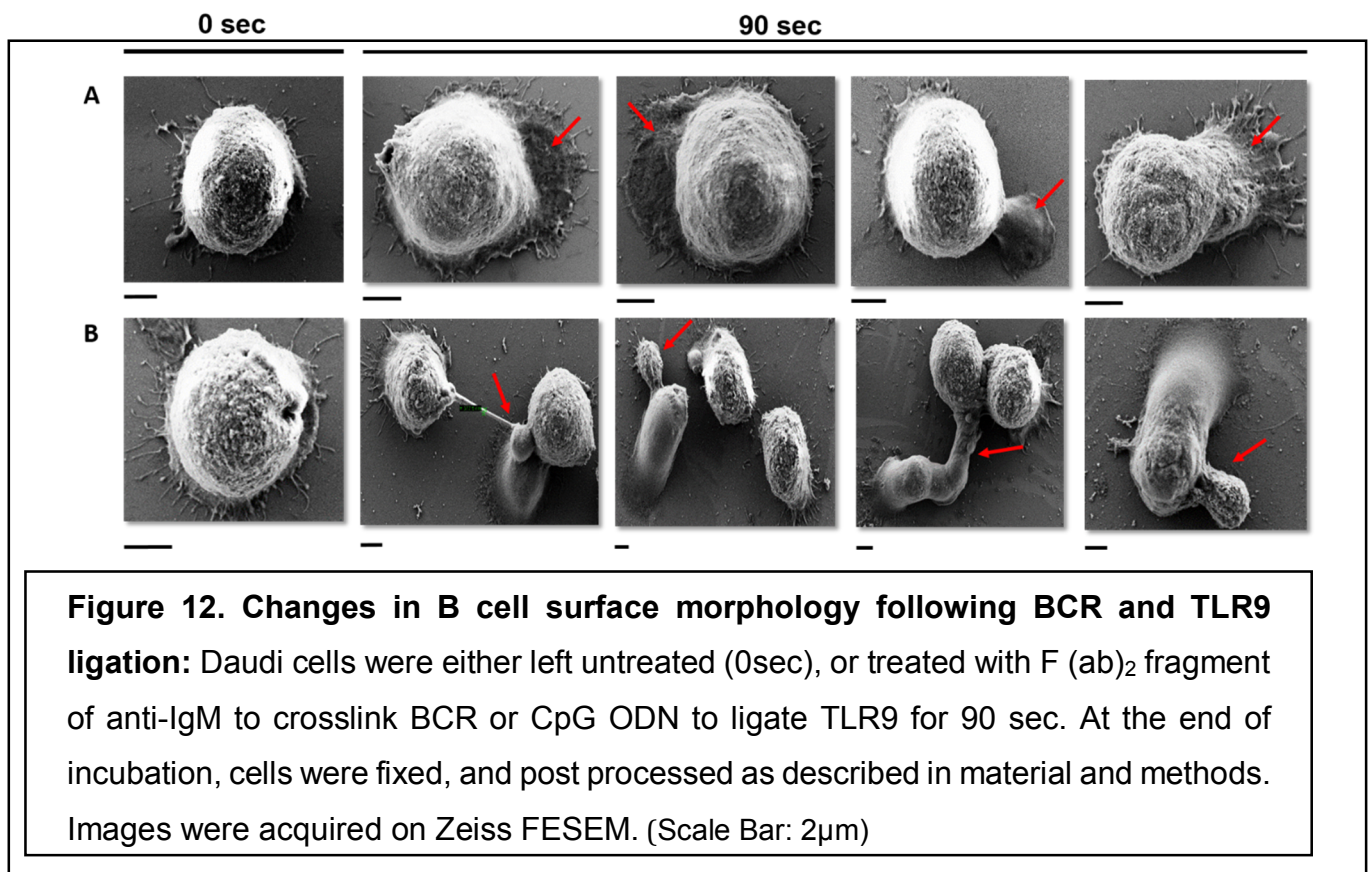


Figure 13. Changes in B cell surface morphology following BCR and TLR9 ligation: Ramos cells were either left untreated (0sec), or treated with F(ab)₂ fragment of anti-IgM to crosslink BCR or CpG ODN to ligate TLR9 for the indicated times. At the end of incubation, cells were fixed, and post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Role of actin in BCR and TLR9 mediated morphological changes.

Actin cytoskeleton and myosin proteins play essential role in maintaining the cellular shape. To determine the role of actin in the BCR and TLR mediated morphological changes, the murine splenic B cell were preincubated with Latrunculin B, an actin polymerization inhibitor, followed by treatment with anti-IgM and CpG. The Latrunculin B completely blocked the BCR mediated membrane expansion whereas TLR9 mediated blebbing was slightly reduced but was not completely inhibited, suggesting distinct roles for actin in BCR and TLR9 mediated morphological changes (Figure 14).

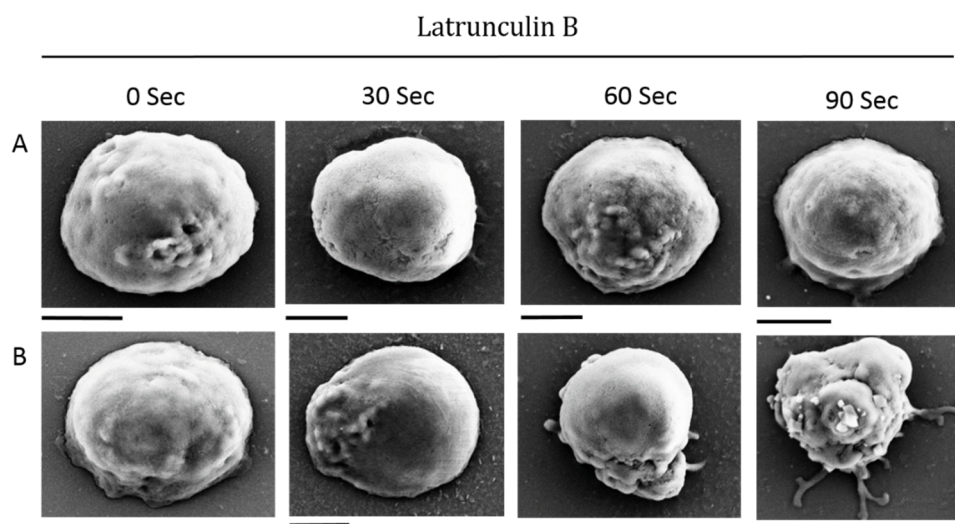


Figure 14. Role of actin polymerization in BCR and TLR mediated morphological changes. Murine splenic B cells from Balb/c mice were incubated with Latrunculin B and treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9(B) and fixed at the indicated times. Following fixation, cells were post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

To determine the role for myosin in the BCR and TLR9 mediated morphological changes, cells were pretreated with blebbistatin, which blocks the ATPase activity of Myosin followed by treatment with anti-IgM and CpG. In addition cells were also pretreated with myosin light chain kinase inhibitor, ML-7 and ROCK inhibitor, Y-27632. While blebbistatin and Y-27632 has completely blocked the BCR and TLR9 mediated morphological changes, partial effects were observed with ML-7 (Figure 15 A, B).

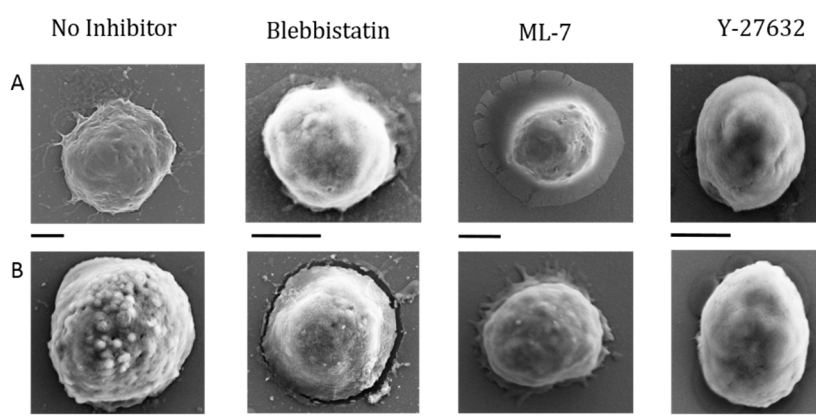


Figure 15. Role of Myosin in BCR and TLR mediated morphological changes.

Murine splenic B cells from Balb/c mice were incubated with blebbistatin, ML-7 and Y-27632, and treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9(B) and fixed at the 60 sec. Following fixation, cells were post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Role for Src family kinase and Calcium pathway in BCR and TLR9 mediated morphological changes.

To examine which of the signaling pathway are involved in these morphological changes in B cell, we used a number of inhibitor to interrupt the specific signaling pathway. Src family kinase is known to be the first kinase which gets activates on BCR crosslinking. To ascertain the role of Src-family kinase in BCR and TLR9 mediated morphological changes, cells were preincubated with PP2 and followed by incubation with anti-IgM and CpG. As expected, PP2 completely blocked the BCR mediated

membrane expansion (Figure 16A). However, PP2 also completely blocked TLR9 mediated morphological changes, suggesting a role for Src kinase in TLR9 mediated signaling in B cell (Figure 16B).

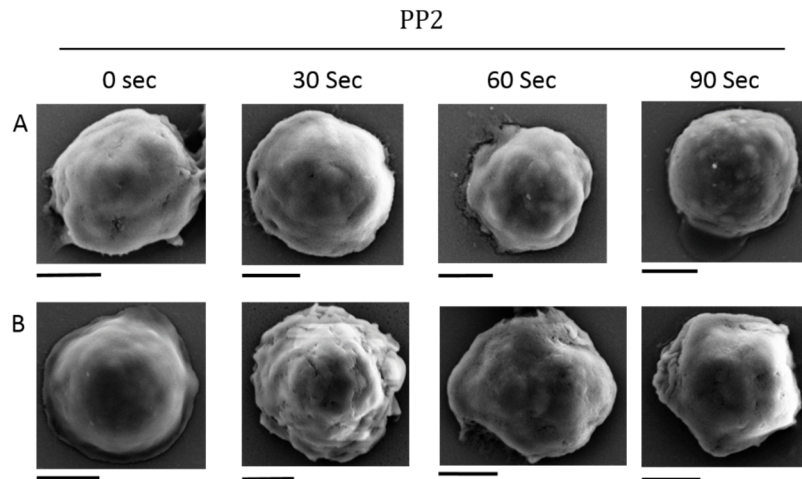


Figure 16. Src family kinase inhibitor blocks BCR and TLR9 mediated morphological changes. Murine splenic B cells from Balb/c mice were incubated with PP2 and treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9(B) and fixed at the indicated times. Following fixation, cells were post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Crosslinking of BCR induces calcium release first from the intracellular stores followed by calcium mobilization from the extracellular environment. To understand whether BCR mediated membrane expansion require calcium, cells were pretreated with two calcium channel inhibitors, TMB8, which inhibits binding of IP₃ to IP3R on the endoplasmic reticulum membrane and a CRAC channel inhibitor BTP2. Both TMB8 and BTP2, completely blocked the BCR mediated morphological changes, as expected (Figure 17A, 18A). However, TLR9 mediated morphological changes were also partially blocked by TMB8 and completely by BTP2. (Figure 17B, 18B) suggesting a role for calcium signaling in TLR9 mediated morphological changes.

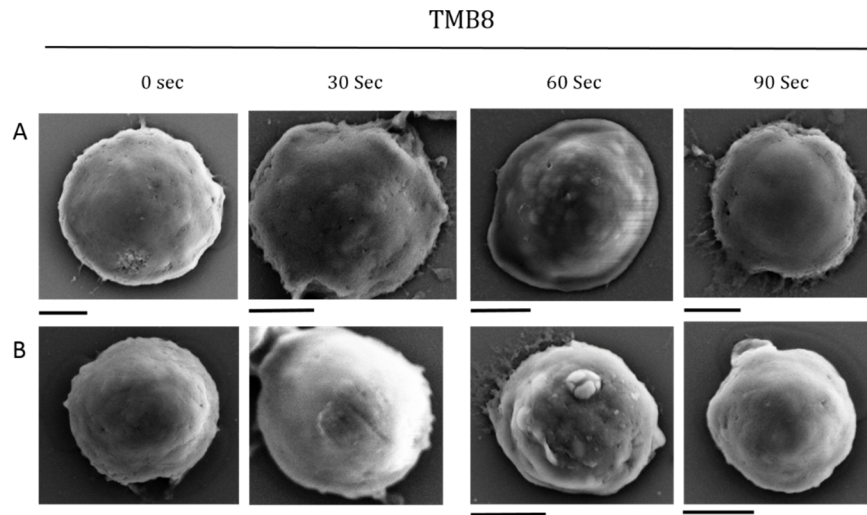


Figure 17. Role of intracellular stored calcium signaling. Murine splenic B cells from Balb/c mice were incubated with TMB8 and treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9(B) and fixed at the indicated times. Following fixation, cells were post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

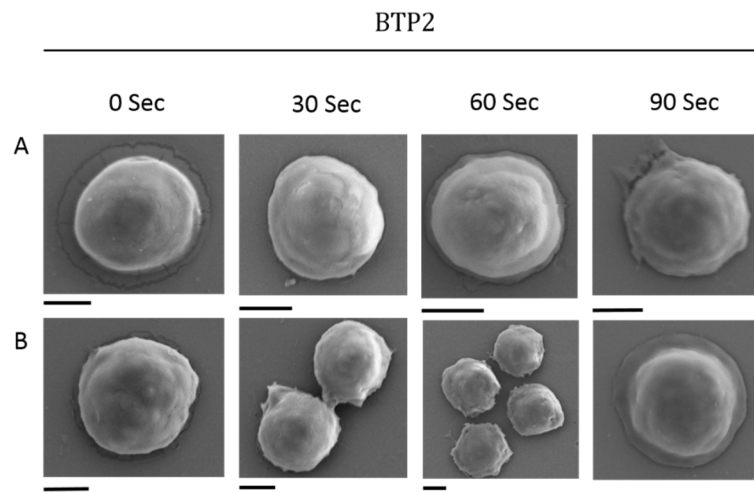


Figure 18. Role of CRAC channel inhibitor on BCR and TLR9 mediated morphological changes. Murine splenic B cells from Balb/c mice were incubated with BTP2 and treated with F(ab)₂ fragment of anti-IgM to crosslink BCR (A) or CpG ODN to ligate TLR9(B) and fixed at the indicated times. Following fixation, cells were post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Role of CD14 in TLR9 and TLR7 Signaling.

How the ligand for TLR9 is internalized and how its signaling is initiated in the B cell is completely unknown. It has been recently shown that CD14 is a co receptor for the TLR7 and TLR9 (Baumann et al., 2010). It is possible that CD14 might facilitate CpG internalization or trafficking, we plan to express CD14 in B cell lines and analyze if it co-localizes with TLR9 or it facilitates entry to CpG. To get the GFP tagged CD14, CD14 was cloned in EGFP N1 vector. CD14 was PCR amplified using the cDNA from splenic B cells (Figure 19A). To standardize the PCR condition I have used gradient PCR. pEGFP N1 and CD14 were both double digested with the BamH1 and HindIII restriction enzyme as described in material and methods. Digested products were run on the gel and were extracted using gel extraction kit (Figure 19B). Eluted product were ligated and transformed in bacteria. Positive clones were confirmed using colony PCR where we have used the CMV forward primer from vector and CD14 reverse primer (Figure 19C).

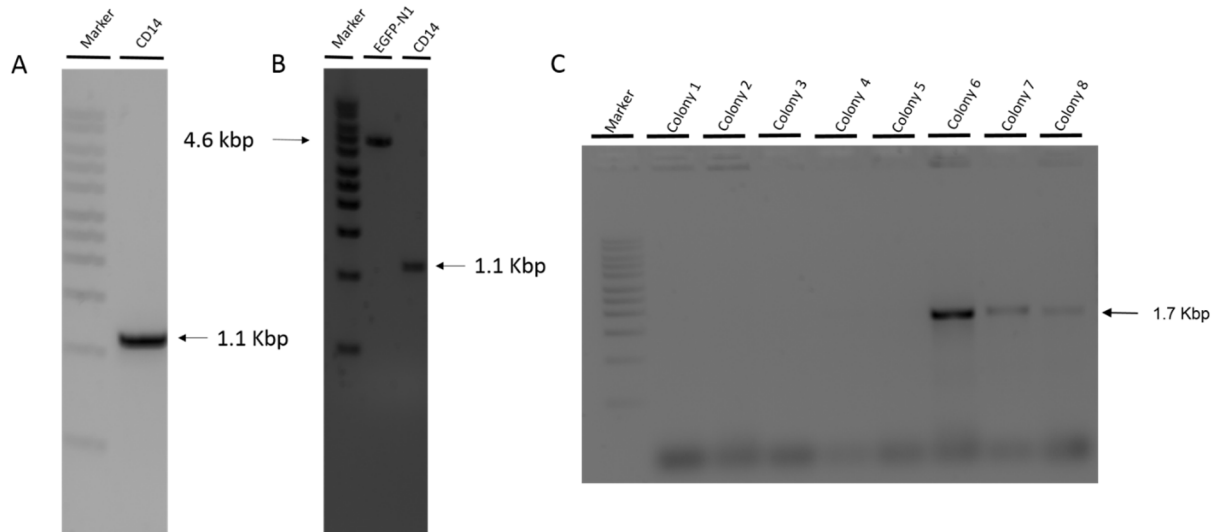


Figure 19. Cloning murine CD14 into pEGFP-N1: A) PCR amplification of CD14 using Balb/c murine cDNA. B) BamH1 and HindIII digested products of PCR amplified CD14 (1.1kb) and pEGFP-N1 (4.6 kb).

To understand what signaling pathways are involved in BCR and TLR9 induced morphological changes, we plan to knock down specific proteins to analyze their individual role. However, it is difficult to transfect the murine primary B cells, where change in morphology was observed. We utilized IgM expressing murine B cell line, CH27 and performed the similar experiments (Figure 20). Similar results were observed in the CH27 cell lines. BCR crosslinking induced extensive membrane spreading whereas TLR9 ligation induced bleb like structures. Therefore, it is possible to do future knockdown experiments in CH27 cells to identify the molecular mechanisms of morphological changes.

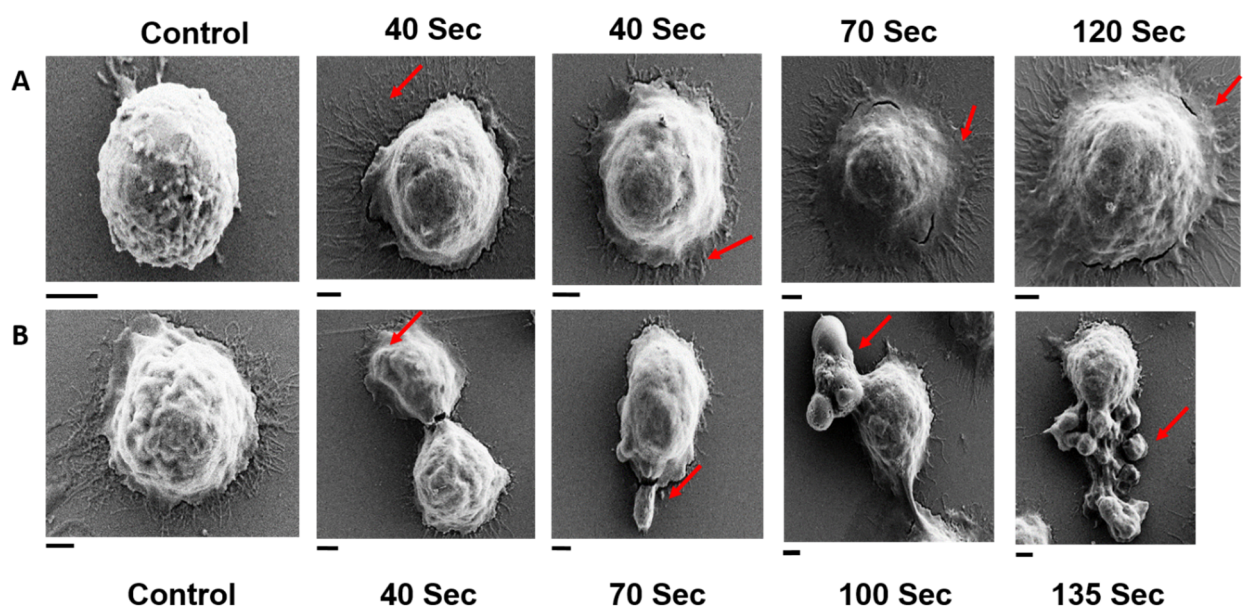


Figure 20. Changes in B cell surface morphology following BCR and TLR9 ligation: CH27 cells were either left untreated (0sec), or treated with F(ab)₂ fragment of anti-IgM to crosslink BCR or CpG ODN to ligate TLR9 for the indicated times. At the end of incubation, cells were fixed, and post processed as described in material and methods. Images were acquired on Zeiss FESEM. (Scale Bar: 2µm)

Discussion

These results clearly indicate that murine and human B cells undergo discrete morphological changes following BCR crosslinking and TLR9 ligation. BCR crosslinking by soluble antigens induced extensive membrane expansion. B cells have been reported to show significant characteristic morphological change in response to surface immobilized antigen. These morphological changes include the spreading of B cells (Fleire et al., 2006), necessary for the maximum accumulation of antigen on B cell surface. However, B cells acquire their ligands in solution, we here show that in response to soluble ligands, B cells undergo similar morphological changes.

However, TLR9 ligand induced extensive membrane blebbing that was initiated as early as 30 seconds following TLR9 ligation and were retracted in later time points. Initially thought to be involved in apoptosis, blebs have now been shown to play a crucial role in a variety of cellular mechanisms. In general, blebs are the result of separation of plasma membrane with cell cortex (Charras and Paluch, 2008).

In both murine and human cells polarization of B cells following TLR9 ligation was observed, which was clearly more evident in Daudi B cells, suggesting that CpG induced blebs in B cells might facilitate migration of B cells or make cells more responsive to chemokines. Blebs have previously been associated with primordial germ cells (PGCs) migration (Blaser et al., 2006). Indeed, metastatic cancer cells have also been shown to utilize blebs for their migration (Charras and Paluch, 2008; Fackler and Grosse, 2008; Laser-Azogui et al., 2013; Paluch and Raz, 2013; Sahai and Marshall, 2003). Plasma membrane blebbing has also been shown to be important in membrane repair. (Babiychuk et al., 2011). In addition, blebs were also observed during the cell division from the anaphase to the cytokinesis.

One possibility is that CpG induced blebbing might facilitate further uptake of ligand itself. Blebs indeed have been shown to be utilized by Vaccinia virus to enter the host cell and it has been reported that the cell with decreased number of blebbing has low infection rate (Mercer and Helenius, 2008). In addition, we observed that following TLR9 ligation multiple bleb-like structures were dissociated from the membrane and multiple vesicles were released. It is possible that TLR9 induced blebs might be released from the cells, which then play a role in intercellular communication. Secreted

vesicles and exosomes have been shown to play various physiological roles. (Regev-Rudzki et al., 2013)

Here we have also looked at the signaling pathway involve in the TLR9 mediated morphological changes. We have looked at the

Taken together, present study have shown that B cells undergo extensive and different morphological changes following binding of BCR and TLR9 ligands. TLR9 induced blebbing is a novel phenomenon, which has not been described earlier.

Future Work:

Future studies will focus on identifying the underlying molecular mechanisms by using pharmacological inhibitors in murine B cells and siRNA mediated knockdown in B cell lines. We will also try to identify the physiological significance of the BCR and TLR9 induced morphological changes. Another important question is to understand if TLR9 induced membrane blebs are unique to this receptor or other intracellular TLRs such TLR3, TLR7 and TLR8 also exhibit similar response. Additionally, we will identify whether CD14 plays any role in TLR9 ligand internalization or trafficking.

B cells response against pathogen is largely dependent on both developmental and environmental contexts. Most of what we learned about the responses of B cells to both BCR and TLR9 ligands came from biochemical studies. CpG has extensively been studied as potential vaccine adjuvant. Understanding very early events of how B cells recognize and respond to TLR and BCR ligands will aid in our efforts to develop effective vaccines and to target therapies to block hyper B cell responses as in systemic autoimmune diseases.

References:

1. Baba, Y., Matsumoto, M., and Kurosaki, T. (2013). Calcium signaling in B cells: Regulation of cytosolic Ca²⁺ increase and its sensor molecules, STIM1 and STIM2. *Mol. Immunol.* 2, 2–6.
2. Babiychuk, E.B., Monastyrskaya, K., Potez, S., and Draeger, A. (2011). Blebbing confers resistance against cell lysis. *Cell Death Differ.* 18, 80–89.
3. Baumann, C.L., Aspalter, I.M., Sharif, O., Pichlmair, A., Blüml, S., Grebien, F., Bruckner, M., Pasierbek, P., Aumayr, K., Planyavsky, M., et al. (2010). CD14 is a coreceptor of Toll-like receptors 7 and 9. *J. Exp. Med.* 207, 2689–2701.
4. Blaser, H., Reichman-Fried, M., Castanon, I., Dumstrei, K., Marlow, F.L., Kawakami, K., Solnica-Krezel, L., Heisenberg, C.-P., and Raz, E. (2006). Migration of zebrafish primordial germ cells: a role for myosin contraction and cytoplasmic flow. *Dev. Cell* 11, 613–627.
5. Campbell, K.S. (1999). Signal transduction from the B cell antigen-receptor. *Curr. Opin. Immunol.* 11, 256–264.
6. Charras, G., and Paluch, E. (2008). Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.* 9, 730–736.
7. Chaturvedi, A. (2012). *Fundamental Immunology* (Lippincott Williams & Wilkins).
8. Chaturvedi, A., Dorward, D., and Pierce, S.K. (2008). The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponse to DNA-containing antigens. *Immunity* 28, 799–809.
9. Christensen, S.R., Kashgarian, M., Alexopoulou, L., Flavell, R. a, Akira, S., and Shlomchik, M.J. (2005). Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J. Exp. Med.* 202, 321–331.
10. Eckl-dorna, J., and Batista, F.D. (2009). BCR-mediated uptake of antigen linked to TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. *Blood* 113, 3969–3977.
11. Fackler, O.T., and Grosse, R. (2008). Cell motility through plasma membrane blebbing. *J. Cell Biol.* 181, 879–884.
12. Fields, M.L., Metzgar, M.H., Hondowicz, B.D., Kang, S.-A., Alexander, S.T., Hazard, K.D., Hsu, A.C., Du, Y.-Z., Prak, E.L., Monestier, M., et al. (2006).

- Exogenous and endogenous TLR ligands activate anti-chromatin and polyreactive B cells. *J. Immunol.* 176, 6491–6502.
13. Fleire, S.J., Goldman, J.P., Carrasco, Y.R., Weber, M., Bray, D., and Batista, F.D. (2006). B cell ligand discrimination through a spreading and contraction response. *Science* 312, 738–741.
 14. Fu, C., Turck, C.W., Kurosaki, T., and Chan, a C. (1998). BLNK: a central linker protein in B cell activation. *Immunity* 9, 93–103.
 15. Janeway, C. a, and Medzhitov, R. (2002). Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216.
 16. Kurosaki, T. (1999). Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* 17, 555–592.
 17. Kurosaki, T., and Tsukada, S. (2000). BLNK: connecting Syk and Btk to calcium signals. *Immunity* 12, 1–5.
 18. Kurosaki, T., Shinohara, H., and Baba, Y. (2010). B cell signaling and fate decision. *Annu. Rev. Immunol.* 28, 21–55.
 19. Lanzavecchia, A. (1990). Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 8, 773–793.
 20. Lartigue, A., Courville, P., Auquit, I., François, A., Arnoult, C., Tron, F., Gilbert, D., and Musette, P. (2006). Role of TLR9 in Anti-Nucleosome and Anti-DNA Antibody Production in Ipr Mutation-Induced Murine Lupus. *J. Immunol.* 177 , 1349–1354.
 21. Laser-Azogui, A., Diamant-Levi, T., Israeli, S., Roytman, Y., and Tsarfaty, I. (2013). Met-induced membrane blebbing leads to amoeboid cell motility and invasion. *Oncogene* 231, 1–11.
 22. Leadbetter, E. a, Rifkin, I.R., Hohlbaum, A.M., Beaudette, B.C., Shlomchik, M.J., and Marshak-Rothstein, A. (2002). Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416, 603–607.
 23. Liou, J., Kim, M.L., Heo, W. Do, Jones, J.T., Myers, J.W., Ferrell, J.E., and Meyer, T. (2005). STIM is a Ca²⁺ sensor essential for Ca²⁺-store-depletion-triggered Ca²⁺ influx. *Curr. Biol.* 15, 1235–1241.
 24. Mercer, J., and Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320, 531–535.

25. Paluch, E.K., and Raz, E. (2013). The role and regulation of blebs in cell migration. *Curr. Opin. Cell Biol.* 25, 582–590.
26. Regev-Rudzki, N., Wilson, D.W., Carvalho, T.G., Siqueira, X., Coleman, B.M., Rug, M., Bursac, D., Angrisano, F., Gee, M., Hill, A.F., et al. (2013). Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* 153, 1120–1133.
27. Sahai, E., and Marshall, C.J. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat. Cell Biol.* 5, 711–719.
28. Shaffer, A.L., Young, R.M., and Staudt, L.M. (2012). Pathogenesis of human B cell lymphomas. *Annu. Rev. Immunol.* 30, 565–610.
29. Takeda, K., and Akira, S. (2004). TLR signaling pathways. *Semin. Immunol.* 16, 3–9.
30. Viglianti, G. a, Lau, C.M., Hanley, T.M., Miko, B. a, Shlomchik, M.J., and Marshak-Rothstein, A. (2003). Activation of autoreactive B cells by CpG dsDNA. *Immunity* 19, 837–847.
31. Yanaba, K., Bouaziz, J.-D., Matsushita, T., Magro, C.M., St Clair, E.W., and Tedder, T.F. (2008). B-lymphocyte contributions to human autoimmune disease. *Immunol. Rev.* 223, 284–299.