B cell activation from actin rearrangements to cell survival

PhD thesis

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Summary

B cells are an essential component of the adaptive immune system. They recognize foreign antigens and mature to antibody secreting plasma cells to mediate humoral immunity. Recognition of the antigen is mediated by their surface antigen receptor the B cell receptor (BCR). Antigen encounter induces the aggregation of BCRs on the B cell surface resulting in the formation of microclusters. This process is a prerequisite of antigen processing and downstream signaling. Microcluster formation requires the reorganization of the actin cytoskeleton on a second timescale with an initial depolymerization and a subsequent repolymerization phase. Regulation of site-specific actin rearrangements is a poorly understood process.

We aimed to better understand how actin rearrangements can be mediated on such a rapid timescale. Calcium seemed to be an appropriate candidate. Calcium has a heterogeneous distribution in the cytoplasm, its concentration can vary on a large scale in only seconds and it regulates the activity of several actin binding proteins. Our goal was to test for a direct interdependence between the local concentration of calcium and the amount of filamentary actin. We have shown that large increases in the cytoplasmic calcium level lead to the loss of cell morphology, which is mediated by the collapse of the actin cytoskeleton. Induction of calcium oscillation results in fluctuations in the density of cortical actin, with maximum peaks of actin where calcium produces its minimums. We have found direct evidences that actin polymerization is favored in regions with lower calcium concentration, while actin decay dominates where calcium level is high, and that cells lower calcium concentration in subcellular regions where actin growth will occur.

B cells have an apoptosis sensitive phenotype, which can be lowered through the BCR signal. Following antigen encounter and microcluster formation a complex network of downstream signaling transmits a signal that can lower the apoptosis sensitiveness of B cells. A major route is the PI3K/Akt survival pathway.

We have shown that beside CD19 Gab2 adaptor molecule is a major way for PI3K recruitment and Akt phosphorylation. Gab2 upon antigen encounter becomes phosphorylated by both Lyn and Syk, and is recruited to the signalosome through its Pleckstrin homology domain, or through an interaction with the BCR. Phosphorylated Gab2 recruits PI3K and SHP2 to enhance Akt but not Erk phosphorylation and to support thereby BCR mediated cell survival.

Összefoglaló

A B limfociták nélkülözhetetlen elemei az adaptív immunrendszernek. Felismervén a kórokozókat ellenanyag szekretáló plazmasejtté érnek, és kialakítják a humorális immunválaszt. Az antigén felismerés a sejtfelszíni antigén receptorukon a B sejt receptoron (BCR) keresztül zajlik. Megkötve az antigént a BCR-ek aggregálódnak és ún. mikro-tömörüléseket hoznak létre. Ez a folyamat nélkülözhetetlen az antigén feldolgozásához és a jeltovábbításhoz. A mikro-tömörülések kialakításához az aktin sejtávz másodpercek alatt történő gyors átépülése szükséges egy kezdeti depolimerizációs és egy rákövetkező repolimerizációs lépésen át. A lokális aktin átépülések szabályozása egy mindezidáig kevéssé ismert folyamat.

Célunk az volt, hogy jobban megértsük miképpen szabályozhatóak az aktin átrendeződések helyfüggően másodpercek alatt. A kalcium egy megfelelő regulátornak jelöltnek tűnt. A kalcium ionok heterogén eloszlást mutatnak a citoplazmában, koncentrációjuk nagymértékben változtatható másodpercek alatt, és ismert, hogy számos aktin-kötő fehérje működését képesek szabályozni. Célul tűztük ki, hogy megvizsgáljuk létezik-e kapcsolat a kálcium és az aktin dinamikája között. Megmutattuk, hogy nagy kalcium felszabadulások a sejt morfológia vesztéséhez vezetnek az aktin citoszkeleton összeomlásán keresztül. Kalcium oszcillációk a kortikális aktin sűrűségének fluktuációját váltják ki maximum helyekkel ott, ahol a kalciumnak vannak a minimumai. Egyértelmű bizonyítékokat mutattunk arra, hogy az aktin épülés a citoplazma alacsonyabb kalcium koncentrációjú régióiban kedvező, míg ahol magasabb a kalcium koncentráció ott bomlás fog dominálni, valamint hogy a sejtek alacsonyabb kalciumot hoznak létre azokon a területeken, ahol aktin épülés fog bekövetkezni.

A B sejtek apoptózis érzékeny fenotípussal bírnak. Ezen tendeciájuk csökkenthető a BCR-en keresztül érkező szignálokon keresztül. Antigén kötést és a mikro-tömörülések létrejöttét követően egy komplex jelátviteli hálózat továbbítja a jelet a sejt belseje felé, mely végeredményben a sejt túlélését eredményezheti. Az egyik legfontosabb túlélési útvonal B sejtekben a PI3K/Akt útvonal.

Megmutattuk, hogy a CD19 mellett a Gab2 molekula biztosítja a PI3K szignaloszómába kerülésének és az Akt foszforilációnak egyik fő útvonalát B sejtekben. Antigén kötést követően a Gab2 Plecktrin homológ doméjén keresztül, vagy egy közvetlen kölcsönhatáson keresztül a B sejt receptorral a szignaloszómába kerül, ahol foszforilálja a Lyn és a Syk kináz, majd rekrutálja a PI3K-t és az SHP2-t, ezen keresztül fokozvav az Akt foszforilációt és a BCR mediált túlélést.

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1. Introduction

B cells are lymphocytes that mediate humoral immunity. Their principal functions are to make antibodies against antigens, perform the role of antigen-presenting cells (APCs) and eventually develop into memory B cells after activation by antigen interaction. B cells are an essential component of the adaptive immune system. The appropriate activation of B cells is critical in the development of the adaptive immune response. An accurate description of B cell activation requires investigation at both intercellular, intracellular and cell surface levels. This dissertation will follow and discuss the steps in B cell activation from antigen recognition to downstream signaling, and cell-fate decisions, with special focus on the author's contributions to the field.

1.1 What a B cell recognizes is determined by its B cell receptor

The effective humoral immune response requires recognition of foreign antigens and generation of antigen-specific effector cells. Antigen recognition in B cells is mediated by immunoglobulins (Ig). Each B cell clone produces a unique immunoglobulin, which recognizes a short sequence of an antigen called the epitope. Surface bound immunoglobulins serve as antigen receptors, and are known as the B cell receptor (BCR). The BCR is composed of an antigen-specific membrane bound immunoglobulin (mIg) paired with a signal transducing unit, a heterodimer of Igα and Igβ[1]. Multivalent or membrane-bound antigens trigger the oligomerization of the monomeric BCR complex. The signal of antigen encounter is transmitted by the intracellular domains of the Igα/Igβ heterodimer. Phosphorylation of the signal transducing unit and its subsequent interaction with signaling proteins starts a series of procedures which at the end result the reprogramming of the cells phenotype. This process turns resting B lymphocytes into activated cells and may subsequently induce proliferation and differentiation. Activated B cells may differentiate into antibody secreting plama cells or into memory B cells.

1.2 Selecting the right clone for the right antigen: clonal selection

After their generation in the bone marrow, naive B cells migrate to the secondary lymphoid tissues such as lymph nodes (LN) and the spleen to encounter antigen. Lymph nodes are strategically positioned in the body to provide the localization for the interaction between immune cells and pathogens. Small diffusible antigens such as toxins can directly enter from the lymph to the follicles in the LN interior[2], and gain access to the follicular B cells, while

larger antigens such as viruses or bacteria are actively transported on the surface of follicular dendritic cells (FDC)[3], dendritic cells (DC)[4], macrophages [5] or in the case of the spleen marginal-zone B-cells (MZB) [6]. The pool of naive mature B lymphocytes in the follicles of a lymph node represents a diverse cell population sensitive for various antigens with different affinities depending on the structure and sequence of the B cell receptor expressed on their surface. B cells that encounter antigen become activated. Some of these cells migrate to the primary follicle, forming germinal centers (GC) [7]. Here they undergo rapid rounds of proliferation and somatic hypermutation of their antibody genes, followed by a selection based on the affinity of their BCR for the antigen.

B cells with higher affinity for the antigen proliferate and differentiate into memory or plasma cells, while B cells with low receptor affinity undergo apoptosis. Thereby the BCR becomes the primary signal transducer to decide a B lymphocytes cell fate.

1.3 Antigen engagement

The fate of a B cell clone, whether it is going to have the fight against the pathogen or becomes the fatal loser of the competition called clonal selection, largely depends on the capability of the BCR to successfully grab the antigen and transmit the signal for survival.

One feature observed on successful B cell stimulation with antigen is the early formation of BCR microclusters[8]. These BCR microclusters comprise around 10-100 BCR molecules, containing IgM and IgD densly packed on the membrane surface. It was suggested that these microclusters are enriched in tyrosine kinases, positive regulators of the signal transduction, and exclude inhibitory phosphatasese[9] to allow a favorable environment for the BCR signal to reach the threshold of activation.

Microcluster formation of the BCR is a prerequisite of the activation process, and thereby fundamentally influences the fate of a B cell clone. The redistribution of the cell surface molecules is a cytoskeleton dependent procedure and requires the site-specific assembly and disassembly of actin filaments. Understanding the function of cytoskeleton brings us closer to understand B cell activation.

1.4 Actin is the “who” and “how” in cell shape, cell movement and plasma membrane organization

There are three main types of cytoskeletal polymers: actin filaments, microtubules and a group of polymers known collectively as intermediate filaments. Together, these three control

the shape and mechanics of eukaryotic cells[10]. All three are organized in networks that can resist deformation, but reorganize in response to external forces and stimuli. All of them have a major role in organizing intracellular compartments, but actin is the only one to control the plasma membrane compartmentalization and thereby BCR microcluster formation.

Actin filaments are much less rigid than microtubules, but the presence of high concentrations of crosslinkers that bind to actin filaments promotes the assembly of highly organized, stiff structures, including isotropic networks, bundled networks and branched networks.

The actin network under the inner layer of the plasma membrane is called cortical actin. The spatial distribution of cortical actin determines cell shape by site-specific polymerization and depolymerization of the filaments.

Outward actin growth under the plasma membrane leads to sheet-like membrane protrusions (such as lamellopodia and membrane ruffles) or finger-like membrane protrusions (such as microvilli and phillopodia)[11], while disassembly of the filaments results the tailing off of the cortical actin layer and thereby local rounding up of the cell. Cortical actin can regulate the lateral movement of receptors in the plasma membrane and thereby allow or prohibit microcluster formation in the plasma membrane of B cells.

1.4.1 Basic actin biochemistry

Actin is a globular, roughly 42 kDa protein. It is the monomer subunit, also known as globular actin (G-atin) of polymerized filamentous actin (F-atin).

The actin filament is a polarized helical polymer with a barbed (+) end (rapidly growing end of the filament) that has the higher affinity for G-actin subunits, and a pointed (-) end (the slowly-growing end of the filament) with the lower affinity for G-actin subunits. In the presence of ATP, the different rates of subunit dissociation and association at the two filament ends result in the ‘‘treadmilling’’ type of subunit turnover, characterized by a steady-state of permanent addition of ATP-G-actin at the barbed end and dissociation of ADP-G-actin from the pointed ends. The rate of filament growth is mainly limited by the G-actin concentration at two ends of the filaments.

1.4.2 Nucleation factors are the crystal nuclei of actin filaments

Spontaneous assembly of pure actin monomers is unfavorable owing to the instability of actin dimers and trimers. The initiation of the actin filament assembly requires a trimeric nucleus in

a process that is called nucleation. So far, three main classes of proteins have been identified that can promote the initiation of new filament assembly. These factors, commonly referred as nucleation factors or nucleators, are actin related protein-2/3 (Arp2/3) complex [12], spire [13] and formins [14]. Each promotes nucleation by a distinct mechanism.

Arp2/3 complex is thought to mimic an actin dimer or trimer and to function as a template for the initiation of a new actin filament that branch off of an existing filament, generating y-branched actin networks.

Spire recruits four ATP–actin monomers with its four tandem Wiskott-Aldrich syndrome protein homology domain-2 (WH2) domains to generate a novel single stranded actin tetramer that acts as a seed for new actin polymerization.

In the case of formins, a dimer of formin homology domain-2 (FH2) stabilizes two or three actin subunits to facilitate the nucleation.

Currently most of our knowledge is about Arp2/3, which seems to be the most abundant between the three classes of nucleators.

1.4.3 Nucleation promoting factors enable the crosstalk between extracellular stimuli and actin response

Eukaryotic cells are able to produce micron thick lamellopodias densely packed with actin filaments in only a few seconds. The enhancement of actin nucleation by Arp2/3 on its own is too weak to be alone responsible for this rapid actin growth. However, nucleation promoting factors can bind and activate the Arp2/3 complex and multiply its capacity.

There have been two protein subfamilies indentified to be responsible for the activation of the Arp2/3 complex, with WASP and neuronal N-WASP (neuronal Wiskott-Aldrich syndrome protein) in one subfamily and the Scar/WAVE (suppressor of cAMP receptor/ WASP-family verprolin homology protein) proteins in the other[15].

The Wasp/Scar/Wave family of proteins not just promotes actin growth and branching by activating the Arp2/3 complex, but also targets the actin growth to the plasma membrane. Namely, members of the family contain a pleckstrin homology (PH) domain like structure the Wiscott-Aldrich syndrome homology domain-1 (WH1) and a lysine-rich basic region to bind to Phosphatidylinositol 4,5-bisphosphate (PIP2)-enriched membrane surfaces[16]. Thereby the spatial distribution of PIP2 in the membrane positively affects the places of cortical actin growth.

Several stimuli including the B cell receptor signal induces PIP2 enrichment in the inner layer of the plasma membrane. PIP2 might be one of the major relays that enable the crosstalk between the extracellular signal and the cytoskeleton.

1.4.4 Elongation and shortening of actin filaments

There are five major regulatory processes to control the growing and shortening of actin filaments.

1.4.4.1 Cytoplasmic concentration of G-actin is the major limiting factor of F-actin growth

Actin filaments grow only when the concentration of cytoplasmic G-actin is above the critical concentration[17]. However the critical concentration at the pointed end (~0.6 μM), is considerably higher than at the barbed end (~0.1 μM). As a result, at a steady state condition, actin subunits are constantly depolymerized from the pointed end and added to the barbed end. This phenomenon is called treadmilling[18]. Since the rate of actin filament elongation is proportional to the concentration of subunits, filaments can only grow continuously if cells can maintain locally a pool of polymerization-ready actin subunits at high concentration[19].

1.4.4.2 Aging of filaments is determined by ATP hydrolysis

Hydrolysis of the ATP and dissociation of the γ-phosphate in the ATP-actin sets the inner timer that indicates the age of a filament, and triggers processes that disassemble actin filaments in the cell. Thereby an actin filament has an around 6 minutes long half-life[20] and can only exist longer with the turnover of the filament. Actin treadmiling is considered to be the major mechanism for actin turnover *in vivo*[19].

1.4.4.3 G-actin recycling enables continuous filament growth

To ensure the availability of G-actin subunits at the barbed end, and thereby continues growth of a filament a special recycling system is in run to recycle ADP-actin dissociated from the pointed end. ADF(actin depolymerizing factor)/cofilin is a ubiquitous actin-binding protein family[21]. It enhances treadmiling by, specifically binding to ADP-actin in the filaments and triggering their faster dissociation from the end of the filament, or by cutting the filament. By this mechanism ADF/cofilin increases the number of cytoplasmic ADP-actin subunits.

Profilin on the other hand, binds to ADP-actin subunits, and catalyzes the exchange of ADP for ATP, subsequently it returns the ATP-actin subunits to the pool of ready to associate G-actins at the barbed end[22].

1.4.4.4 Barbed end elongation can be controlled by capping and un-capping filaments

Capping proteins such as CapZ and gelsolin bind the barbed end of a filament and interfere subunit addition to limit the length of the filament[23]. In cell motility where the driving force is the push forward of the plasma membrane, capping proteins have a substantial role[19]. They enable the building of short filaments that are stiffer than long ones, and therefore more effective at pushing on the membrane. Capping proteins also control where actin filaments "push". Since only barbed ends in contact with the lamellopodial membrane are effective in generating protrusive force, global capping of barbed ends avoid non-productive actin-subunit consumption elsewhere in the cell.

1.4.4.5 Actin severing proteins enable site-specific rapid collapse of the actin cytoskeleton

The actin cytoskeleton can collapse in only a few minutes upon certain extracellular stimuli. Spontaneous dissociation of G-actin from the pointed ends of the filaments does not allow this fast disassembly of the actin network. To accelerate the process, the dissociation of actin from the pointed ends need to be enhanced, or the number of filament endings needs to be increased by filament severing.

Actin severing proteins such as gelsolin[24] have both capping and severing capability. Upon certain stimuli, they are able to cap the barbed end of the filament prohibiting thereby the addition of new actin subunits, and cutting the filaments into shorter sections to increase the number of disassembling pointed ends.

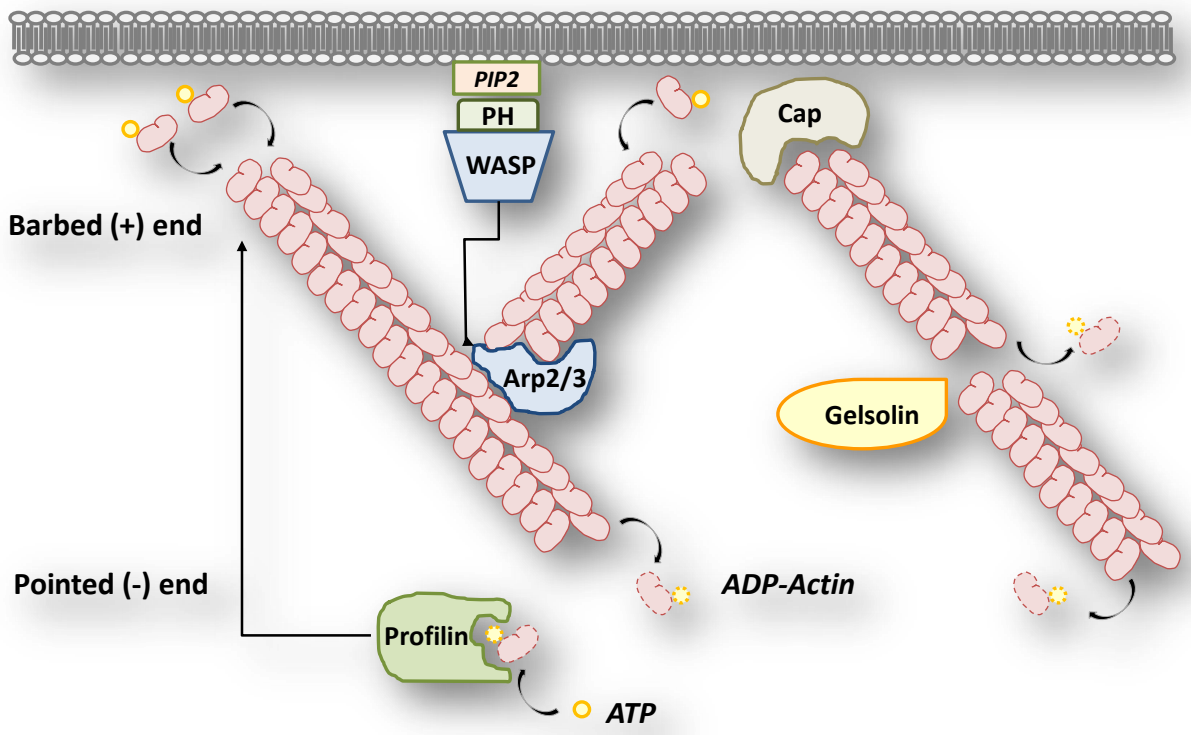

Figure 1. A schematic model of the regulation of actin growth and decay. *Actin filaments are polarized structures with a growing barbed end and a disintegrating pointed end. New actin filaments grow with the help of nucleation factors such as Arp2/3. Nucleation factors are activated by nucleation promoting such as WASP. Actin filament growth can be terminated with the attachment of capping proteins to the barbed end. Actin severing proteins such as gelsolin can cut actin filaments into multiple smaller filaments. Profilin recycles actin monomers at the pointed end to enable G-actin supply at the barbed end.*

1.4.5 High-scale actin structures are formed with the help of F-actin cross-linking and bundling proteins

Cells have the ability to organize their filaments into high-scale actin structures with the cross-linking and bundling of filaments. These higher order structures have a profound influence on cell shape, division, plasma membrane organization and cellular signaling. The organization of filamentous actin in actin networks is coordinated by various F-actin crosslinking proteins.

Around 60 actin binding proteins have been identified in mammalian cells until now, among which 7 are F-actin cross-linking/bundling proteins[25]. The function of actin cross-linking/bundling proteins is believed to mediate interactions between actin filaments to form

both orthogonal networks and ordered bundles. The type of the actin cross-linking/bundling protein used to form higher scale-structures largely depends on the compartment where the actin network is formed[26]. Stress fibers in adherent epithelial cells and fibroblasts contain actinin, fascin, and tensin. Focal adhesions (FA), which mechanically couple the actin cytoskeleton to the extracellular matrix via cell surface receptors integrins, contain the actin cross-linking proteins actinin, talin, plectin, fimbrin, and vinculin. Actin-filament bundles in stable microvilli and isotropic meshworks in the lamellipodia of motile cells also contain several actin cross-linking proteins, including fascin and actinin.

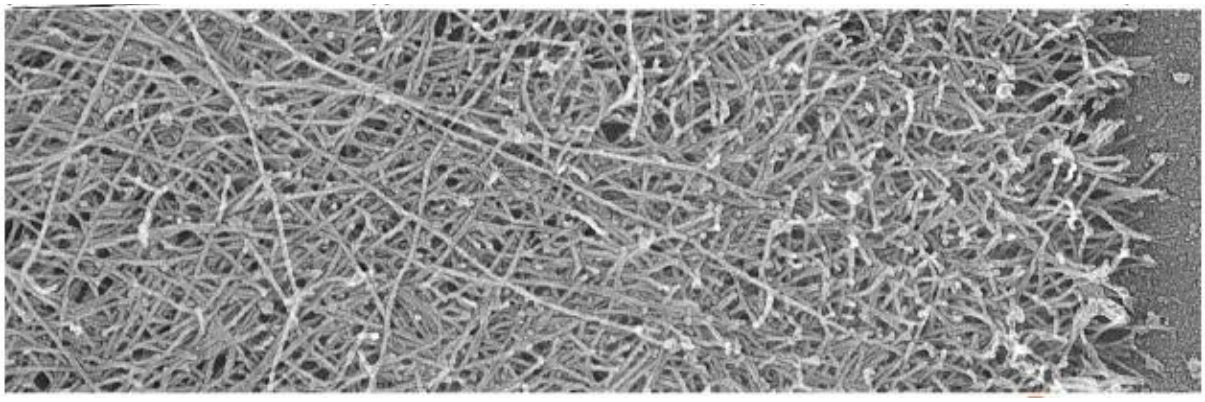

Figure 2. Electron microscopic image of an actin meshwork. *(From Pollard and Borisy)*

1.5 Linking the actin cytoskeleton to the plasma membrane

The connection between the plasma membrane and the underlying actin cytoskeleton allows the bidirectional interaction of the two structures. Proteins at the boundary between the cytoskeleton and the plasma membrane control cell shape, delimit specialized membrane domains, and stabilize attachments to other cells and to the substrate. These proteins also control cell locomotion and cytoplasmic responses to external stimuli.

1.5.1 Spectrin/ankyrin network organizes the fluid membrane bilayer

Spectrins are flexible rods 0.2 micron in length and with binding sites for F-actin at each end. They are formed from α and β subunits assembled into heterodimers, which in turn form tetramers[27]. Spectrin is coupled to the inner surface of the membrane primarily through association with ankyrin. Ankyrin interacts with spectrin through its spectrin binding domain, and with most of its membrane partners through ankyrin (ANK) repeats. Membrane partners of ankyrin are mostly ion-channels such as the inositol 1,4,5-triphosphate receptor (IP_3R) and

ionic exchangers as the sodium/calcium exchanger (NCX). Spectrin and ankyrin together build a triangular network beneath the plasma membrane and link short actin filaments to themselves.

The spectrin/ankyrin network is required for the organization of transmembrane proteins in the plasma membrane by forming a barrier for lateral diffusion[28], and by promoting the assembly of specialized membrane microdomains.

1.5.2 PIP2 regulates key players in actin-membrane linkage

PIP2 transmits signals originating at the plasma membrane to the underlying actin cytoskeleton. It regulates actin dynamics in several ways: by activating WASP/N-WASp thereby Arp2/3-mediated actin branching and actin growth, by binding and impairing the activity of actin-severing proteins, such as gelsolin and cofilin/ADF, and by uncapping actin filaments for the addition on new actin monomers[29]. Besides these functions PIP2 binds to, and influences the activity of actin/plasma membrane crosslinking proteins.

Actin networks attach to the plasma membrane via a number of proteins including vinculin, talin, and Ezrin/Radixin/Moezin (ERM). Most of these proteins crosslink actin and transmembrane receptors in a PIP2 dependent manner[16].

1.5.3 The Vinculin/Talin duo targets integrins to the cytoskeleton

Focal adhesions (FA) are interconnections of the extracellular matrix and the cell through which mechanical force can be transmitted from the actin cytoskeleton to integrins. To transmit this force integrins need first to become attached to the cytoskeleton. Talins are high-molecular-weight proteins concentrated in these focal adhesions. They have an N-terminal head structure and a C-terminal rod. Talins bind phosphorylated integrins via their FERM-domain (4.1, ezrin, radixin, moezin) in the N-terminal head, and direct them to vinculin[30]. Vinculin is a ubiquitous cytoskeletal protein. In resting cells it has a closed conformation and needs to bind PIP2 to become phosphorylated upon external stimuli and turn into an active conformation. Active vinculin binds talin and links the talin integrin complex to the actin cytoskeleton[31].

1.5.4 Ezrin/Radixin/Moezin: turning on and off the receptor actin cytoskeleton interactions

Ezrin, radixin and moezin are closely related proteins. All of them link actin filaments to the plasma membrane either directly via binding the cytoplasmic tails of transmembrane proteins

or indirectly via scaffolding proteins attached to transmembrane proteins[32]. ERM proteins resemble a combination of vinculin and talin. They contain the globular head with the FERM domain of talin, and have a C-terminal helical F-actin binding site like vinculin. Their regulation is very much like that of vinculins. In resting cells they have a closed conformation phosphorylation and PIP2 binding turns them into active conformation. Substrates of ERM family include a large variety of proteins.

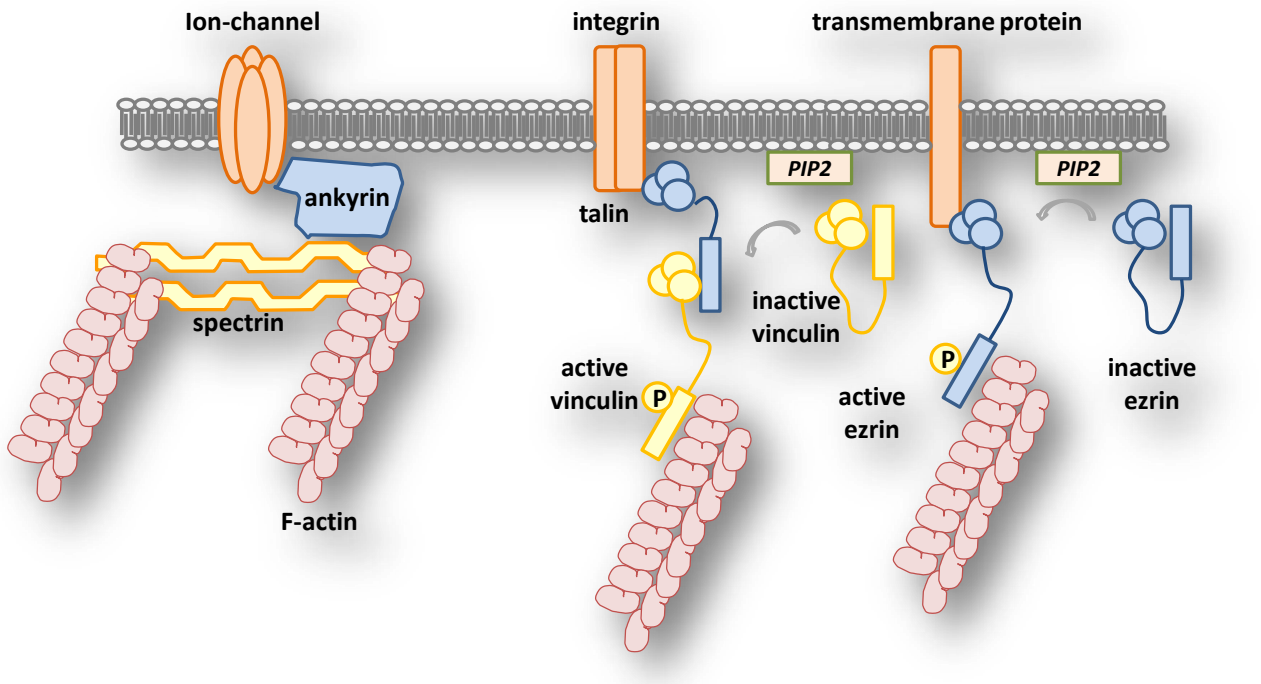

Figure 3. Plasma membrane can be dynamically linked to the actin cytoskeleton.

1.6 Early steps in B cell activation

Engagement of the BCR with specific antigen induces the redistribution of cell surface receptors in the plasma membrane in an actin-dependent manner. This rearrangement of the BCR is a prerequisite of intracellular signaling.

Signaling is achieved through the Igα/Igβ subunits that contain the cytoplasmic domain immunoreceptor tyrosin-based activation motifs (ITAMs). Cross-linking or oligomerization of the B cell receptor after ligand binding results in phosphorylation of the ITAM tyrosines by Src family kinase Lyn. However, the BCR and Lyn reside in distinct compartments of the

plasma membrane in resting cells. A clue as to how the BCR associates with Lyn kinases after ligation has come from observations of membrane microdomains called lipid rafts.

1.6.1 BCR is excluded from lipid rafts in resting cells

Lipid rafts are liquid-ordered microdomains in the plasma membrane, enriched in sphingolipids and cholesterol that allow their tight packing and separation from more loosely organized regions of the membrane[33]. They appear to function as platforms for both signaling and membrane trafficking[34]. A central feature of rafts is their ability to selectively include or exclude membrane proteins.

Rafts have been shown to concentrate Src family kinases and exclude the BCR in resting cells[35]. The affinity of the monomeric BCR for rafts is presumably low, giving the appearance of exclusion. When oligomerized the BCRs affinity for rafts is increased, shifting equilibrium towards raft association and raising the possibility to translocate into the same compartments where Lyn kinases reside[36].

1.6.2 Actin cytoskeleton limits the rate of BCR diffusion in the plasma membrane

Translocation into the lipid rafts, oligomerization and microcluster formation of the BCR, all of this process require the unattached diffusion of the B cell receptor in the plasma membrane. The rate of diffusion is determined by the density of ezrin defined actin networks beneath the membrane. In unstimulated cells, ezrin is phosphorylated and links the actin cytoskeleton to the plasma membrane and prohibits BCR translocation to lipid rafts[37].

1.6.3 Uncoupling the cytoskeleton from the plasma membrane is a critical step towards BCR translocation into lipid rafts and microcluster formation

A critical step in BCR oligomerization is the decoupling of the cytoskeleton from the plasma membrane. Following antigen engagement, ezrin becomes transiently dephosphorylated and concomitantly detaches actin[38], thereby possibly allowing the free diffusion, translocation to the rafts and microcluster formation of BCRs for a short time window.

After microcluster formation, the actin network is reorganized around the BCR microclusters forming corrals, that restrict the diffusion of the BCR from the microclusters, maintaining microcluster integrity[37].

These two phases are in accordance with the finding that upon BCR engagement actin cytoskeleton partially depolymerizes in a signal strength dependent manner. This early event of actin decay allows the translocation of the BCR to rafts, and subsequent coalescence of raft

microdomains, also called capping. The depolymerization is followed by a polarized re-polymerization phase of the actin cytoskeleton[39].

1.6.4 BCR-raft microclusters transmit the signals

Rafts were shown to be enriched in proteins that fall into two main categories: proteins involved in signal transduction[40] and components of the cytoskeleton[41]. Thus, rafts appear to function as platforms for signaling where the signaling complexes are assembled and receptors become attached to the plasma membrane, also called signalosomes.

1.7 BCR signaling versus internalization

Encountering the antigen triggers two distinct events in B cells. One is the initiation of a complex cascade of signaling events that ultimately leads to the activation of B lymphocytes, which can then initiate its maturation to extrafollicular plasma cells. The other one is the internalization and processing of the BCR-antigen complex, in order to present the antigen on MHC class II (Major histocompatibility complex class II) molecules to primed CD4 T cells – a process referred as T-cell B-cell cooperation[42] -, which is a prerequisite of successful GC formation.

1.7.1 BCR signaling and internalization are mutually exclusive events

BCR translocation into the raft compartment enables src-family kinase Lyn to phosphorylate the ITAM motif of the Igα/Igβ subunits, leading to the recruitment and activation of two other major protein tyrosine kinases, the spleen tyrosine kinase (Syk) and the Burton's agammaglobulinemia tyrosine kinase (Btk). Syk and Btk activity are required for downstream signaling, and for the regulation of cell fate and differentiation[43].

However, it was shown recently that only a small fraction of Igα/Igβ is phosphorylated upon Ag stimulation, and that phosphorylation prevents the internalization of the corresponding BCR-Ag complexes[44]. BCR phosphorylation and internalization are therefore mutually exclusive events. While the phosphorylated B-cell receptor transmits the antigen signal, unphosphorylated BCR-Ag complexes become internalized for the processing and presentation of the antigen.

1.8 B cells in the germinal center are ticking time bombs

In case of an infection a large pool of naive B lymphocytes stand by in the lymph node. They possess a great variety of B cell receptors with the capability to recognize almost any antigen getting across the natural barriers of the body. B cells able to recognize the antigen enter the germinal center to undergo an improvement in their receptor affinity – called affinity maturation. Affinity maturation is a process whereby the mutation of antibody variable-region genes occurs. The site-directed mutation of variable-regions is followed by the selection of higher-affinity clones in the germinal center. The process leads to an increase in average antibody affinity for an antigen as an immune response progresses. The selection part is thought to be a competitive process in which B cells compete with each other to capture decreasing amounts of antigen[45].

Only B cells with the highest affinity receptors survive and differentiate into plasma cells or memory B cells, the rest of the pool undergoes apoptosis. Thereby B cells not only compete for antigen, but for survival.

B cells in the GC are programmed to die, and show a typical apoptosis-sensitive phenotype. They express low levels of the anti-apoptotic protein Bcl-2 and high levels of the pro-apoptotic proteins Fas, Bax and c-Myc[46]. The tissue environment with the expression of death receptors further enhances their tendency. In order to survive, B cells need to gather survival signals that turn on anti-apoptotic signaling pathways in the cell. The major source for survival signals is the B cell receptor/antigen interaction itself.

1.9 In the decision between “live or die” Bcl-2 family members bring the verdict

Apoptosis is a naturally occurring process by which a cell is directed to programmed death. There are two major pathways how apoptosis can run its course. One referred to as extrinsic pathways, is initiated at cell-surface death receptors such as Fas and directly activates effector caspases[47]. The other pathway, referred as the cell intrinsic pathway, disrupts mitochondrial integrity and leads to cytochrome c release and caspase activation[48]. The point of no return in this pathway is the permeabilization of the mitochondrial outer membrane. It seems that a single family of proteins, the Bcl-2 family set up the signal threshold for the point of no return, and thereby the commitment of the cell to apoptosis.

Proteins of the Bcl-2 family have either pro or anti-apoptotic activities, and regulate the mitochondrial pathway of apoptosis. Once the pro-apoptotic family members Bcl-2-associated

X protein (Bax) or Bcl-2 antagonist/killer-1 (Bak) become active, they homo-oligomerize and participate in the formation of pores in the outer mitochondrial membrane through which pro-apoptotic molecules escape, including second mitochondria-derived activator of caspase (Smac) (also known as Diablo) and cytochrome *c*. Release of these molecules leads to the activation of caspases, which are proteases that cleave key cellular proteins.

Expression of Bcl-2 anti-apoptotic proteins, including myeloid cell leukemia-1 (Mcl-1), Bcl-2-like 1 (Bcl-XL), Bcl-2-like 2 (Bcl-w) and Bcl-2-related protein A1 (Bfl-1), block cell death by preventing both the activation and homo-oligomerization of Bax and Bak[49].

1.10 Turning off the death

A cells sensitivity to undergo apoptosis largely depends on its Bcl-2 protein expression profile. Expression of anti-apoptotic Bcl-2 members can be induced by the BCR signal[50], thereby lowering a B cells sensitivity to die by apoptosis.

1.10.1 NF-κB pathway: the ultimate way to counteract apoptosis

The activation of the inducible transcription factor NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1) was found to have a key role in desensitizing cells regarding apoptosis. NF-κB[51] protects against both forms of apoptosis by activating the transcription of genes that encode anti-apoptotic proteins[52, 53]. NF-κB in the inactive form consists of a heterodimer, which is the actual transcription factor, sequestered in the cytoplasm by the inhibitory molecules IκB. The activation of NF-κB involves the phosphorylation of IκB by IκB-kinases, leading to its degradation, and the release of DNA-binding NF-κB, which translocates to the nucleus to activate gene transcription. NF-κB can be the downstream effector molecule of several signaling pathways.

1.10.2 The phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway suppresses apoptosis and cell cycle arrest

PI3Ks are a class of enzymes that mediate the phosphorylation of phosphoinositides (PI) and its derivates PI(4)P and $PI(4,5)P_2$ in the 3-position to give PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$. These lipids promote the assembly of signaling complexes at the plasma membrane by recruiting proteins with domains that selectively bind 3-phosphoinositides[54].

The best known 3-phosphoinositides binding domain is the PH-domain. Many of the signaling molecules posses a PH-domain that selectively bind PI3K products and are downstream effectors of PI3K function.

The serine/threonine kinase Akt, also known as protein kinase B is one of these molecules. Activation of Akt requires the generation of $PI(3,4,5)P_3$ in the plasma membrane, subsequent recruitment to the plasma membrane through its PH-domain, and phosphorylation by two kinases the phosphoinositide-dependent protein kinase 1 and 2 (PDPK1, PDPK2).

Akt activation promotes various cell responses that are associated with cell survival, suppression of apoptosis and induction of cell division[55]. The level of activated Akt in lymphocytes correlates with augmented NF-κB function, and the upregulation of anti-apoptotic Bcl-2 proteins[56]. Activated Akt also induces the sequestration, and degradation of the Forkhead Box, Subgroup O family (FOXO). FOXO members are transcription factors that positively regulate the transcription of genes involved in cell-cycle arrest and apoptosis, such as apoptosis inducing ligands as TRAIL (tumor necrosis factor-related apoptosis inducing ligand) and the FasL (Fas ligand).

1.10.3 The mitogen-activated protein kinase (MAPK) cascade promotes cell cycle progression and differentiation

The MAPK cascade comprises a series of kinases, generally referred to as MAP3K-MAP2K-MAPK, amplifying the incoming signal by consecutively phosphorylating each other, with a terminal effector kinase, the MAPK[57]. Activation of these cellular pathways can give rise to cell proliferation, differentiation and survival.

In B cells, the initial step of the cascade is the activation of the 21 kDa, small GTP-binding Ras protein. Ras in resting cells is inactive in a GDP-bound form. Upon BCR ligation the guanine nucleotide exchange factor (GEF) son of sevenless homolog (Sos) causes the inactive Ras to release GDP and bind GTP instead. This converts Ras into an active conformation and forms a binding-site for the MAP3K, Raf. Once Raf serine/threonine kinase is fully activated it phosphorylates the downstream MAP2K kinases, MEK1/2 on serine residues. MEK1/2 kinases are dual specificity kinases that phosphorylate the effector MAPK kinases Erk1/2 on both threonine and tyrosine residues. Phosphorylated Erks translocate into the nucleus and regulate transcription factors such as Ets, Elk, Jun and Myc resulting in the expression of genes required for proliferation, survival and differentiation.

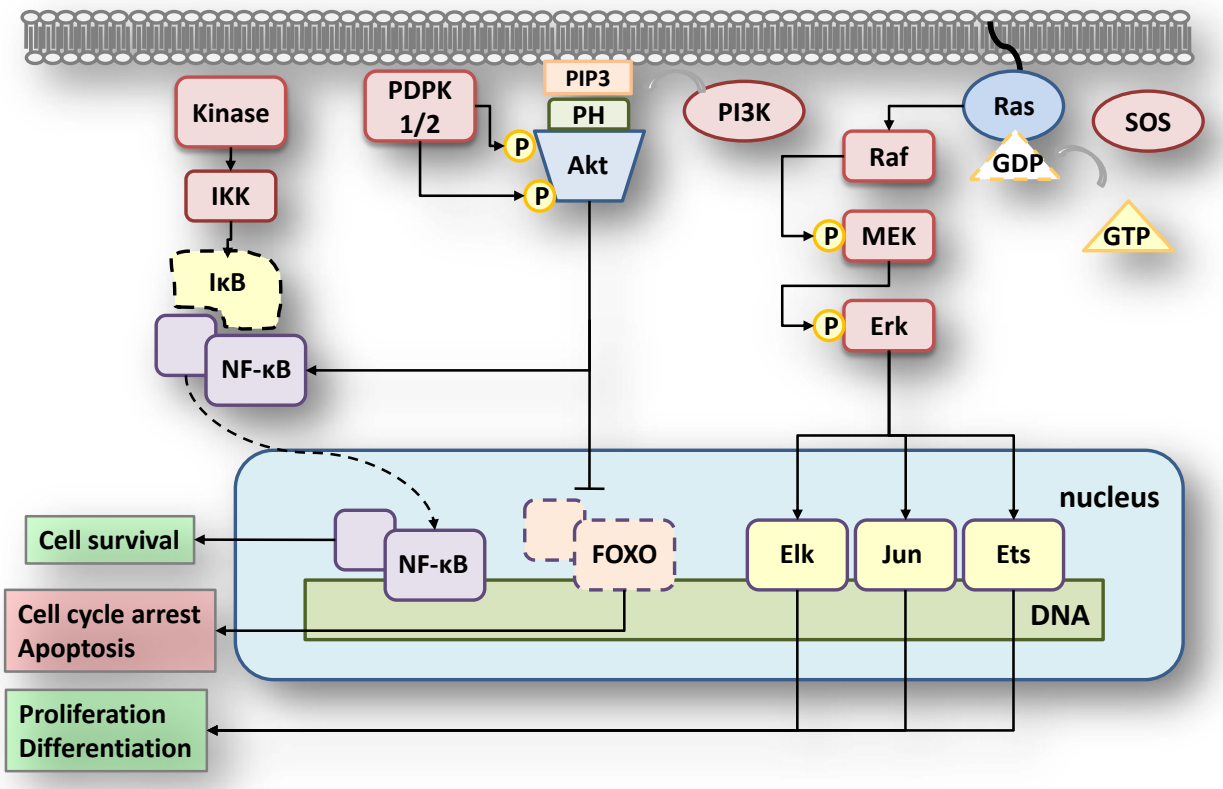

Figure 4. Survival pathways in B cells. *(1) The NF-κB pathway is initiated by the phosphorylation and degradation of the regulatory molecule IκB. Subsequently NF- κB is released and transported to the nucleas. (2) Akt requires synthesis of PIP3 by PI3K in the plasma membrane to become recruited to the signalosome and phosphorylated by kinases PDPK1 and PDPK2. (3) The MAPK cascade is initiated by the GDP/GTP exchange of Ras by guanine nucleotide exchange factor Sos. The terminal MAPK Erk translocates to the nucleus and regulates transcription factors such as Elk, Jun and Ets.*

1.11 Calcium in the BCR signaling

B cells exhibit a rise in their cytoplasmic calcium concentration upon BCR antigen encounter. Ca^{2+} signals affect a variety of cell functions that are central to cell-fate decisions in B cells. Changes in the intracellular level of calcium regulate intracellular processes including activation of protein kinases, apoptosis signaling, gene transcription and proliferation. Each cell type expresses a unique set of components from the Ca^{2+}-signaling toolkit to set up a system with different spatial and temporal properties. In B cells phospholipase C gamma (PLCγ) seems to have the central role in the regulation of calcium signaling[58]. PLCγ mediates the initial signal by cleaving phosphatidyl-inositol-4,5-biphosphate (PIP2) to produce the lipid

second messenger diacylglycerol (DAG)[59] and inositol-1,4,5-trisphosphate (IP_3)[60]. IP3 subsequently opens the endoplasmatic reticulum (ER) IP_3R calcium channels.

1.11.1 IP_3R mediated calcium transients initiate store-operated calcium entry

PLCγ catalyzed IP_3 binds to IP3R calcium channels in the ER to induce their opening and to allow the calcium efflux from ER stores to the cytosol. ER stores however are limited in Ca^{2+} supply and can only support a transient increase in the intracellular calcium level. For a sustained increase in the cytoplasmic Ca^{2+} concentration another store, the unlimited extracellular calcium pool needs to be opened. A process known as store-operated calcium entry (SOCE)[61] is responsible for this event. The Ca^{2+} efflux from the ER activates stromal interaction molecule (STIM) which is a type II transmembrane protein located in the ER[62]. STIM senses the reduced calcium concentration in the ER, which leads to its direct interaction with plasma membrane calcium-release activated calcium (CRAC) channel, ORAI[63]. This interaction activates ORAI and allows the influx of extracellular calcium into the cytoplasm.
SOCE is an 'all or nothing' mechanism, where the level of PLCγ activity sets threshold. This means that either there is enough IP3 available to empty the ER stores and activate SOCE to induce sustained calcium or PLCγ activity is below threshold and a transient calcium signal will arise.

1.11.2 Calcium and DAG activate the NF-κB pathway

The activation of the NF-κB pathway and thereby cell survival strongly depends on the peak amplitude of the Ca^{2+} signal[64]. Protein kinase C β (PKCβ) functions downstream of the BCR and promotes the activation of NF-κB-dependent gene transcription. The full activation of PKCβ requires the binding of second messenger DAG and Ca^{2+}. Active PKCβ is essential for the phosphorylation of the IκB-kinase (IKK) and subsequent phosphorylation and degradation of the inhibitory molecule IκB[65].

1.11.3 Terminating the calcium signal: calcium pumps and exchangers

Four different pumping mechanisms are responsible for the lowering of the cytoplasmic calcium concentration – the plasma membrane calcium ATPase (PMCA), the Na^+/Ca^{2+} exchanger, the sarcoplasmic/endoplasmic reticulum ATPase (SERCA) and the Mitochondrial uniporter. These pumping mechanisms have different threshold for activity and different subcellular localization. PMCA and SERCA pumps have lower transport rates but high affinity, which means that they can respond to modest elevation and set basal Ca^{2+} levels. The

NCX and mitochondrial uniporter have much greater transport rates, thus can limit transients over a wider dynamic range. SERCA resides in the ER and refills the store in the endoplasmatic reticulum. PMCA pumps sequester the calcium in the vicinity of the plasma membrane. Mitochondria are motile, and can translocate to regions in the cells where calcium sequestration is urgent. They rapidly lower the local calcium concentration with their high transport uniporters and after stimulus tunnel back calcium ions in the ER[66].

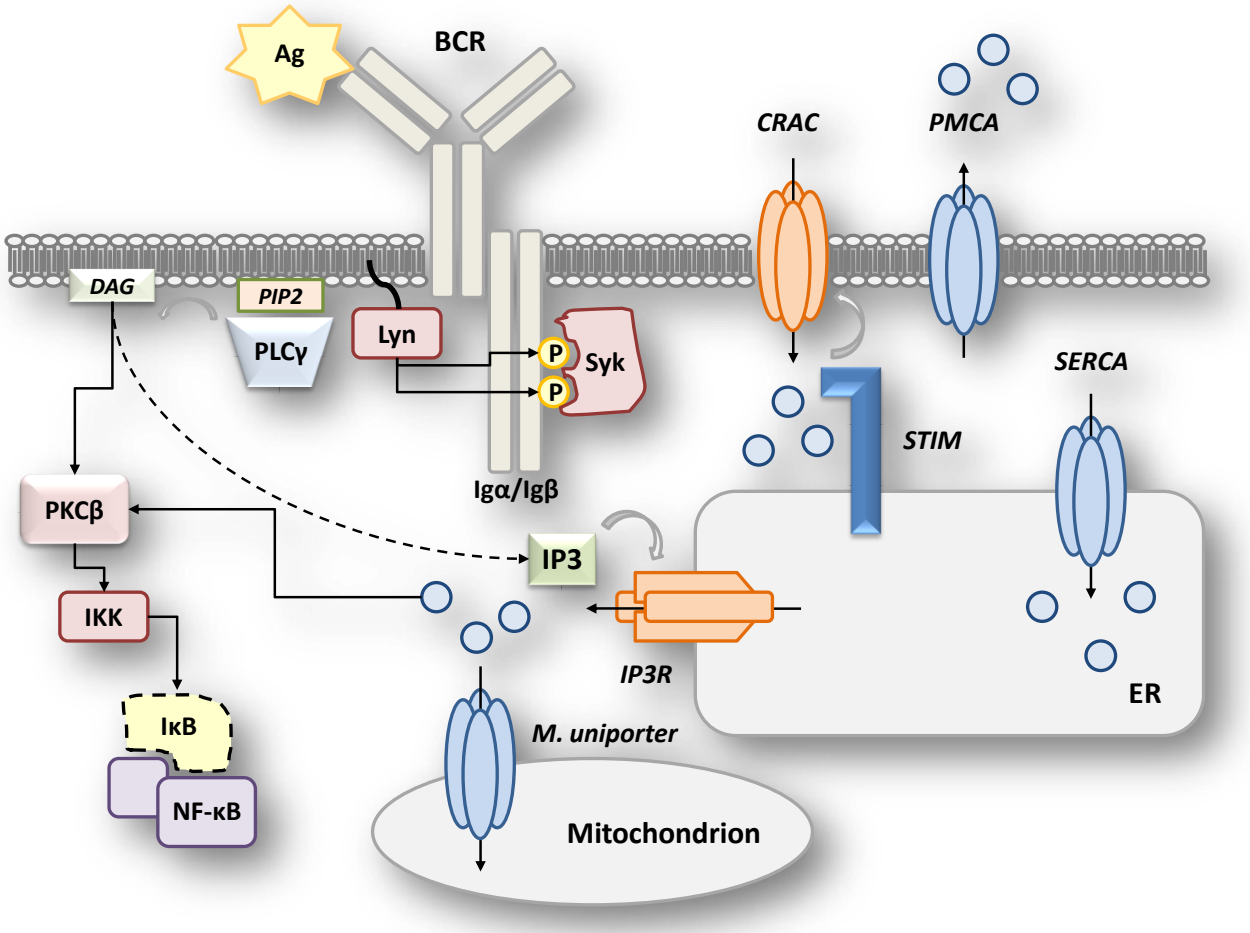

Figure 5. Calcium release and sequestering. *(1) Calcium release is initiated by IP3 release mediated by PLCγ. (2) IP3 opens the ER calcium channel IP3R. (3) Lowering the ER calcium concentration activates the calcium release activated calcium entry (CRAC) a process that is regulated by the STIM/Orai interplay. (4) Cytoplasmic calcium regulates the function of several proteins in between PKCβ. PKCβ requires binding of DAG and* Ca^{2+} *to become active and initiate the NF-B pathway. (5) Mitochondrial uniporters, SERCA pumps and plasma membrane calcium ATPases restore cytoplasmic calcium concentration.*

1.12 Linking the BCR to the downstream signaling pathways: a task for adaptor molecules

We have seen that a variety of signaling pathways are available to counteract a B cells natural course to die by apoptosis. However, still the question remained how the BCR engagement is linked to these downstream pathways. Adaptor proteins are able to bridge this gap. Adaptor proteins are commonly defined as proteins that possess multiple tyrosine residues that are potential targets for tyrosine kinases, and protein-protein or protein-lipid interaction domains such as the phosphotyrosine binding PTB domain and src homology domain 2 (SH2), the proline-rich sequence binding SH3 domain and PIP3-binding PH domains, but do not exhibit enzymatic activity. They are able to bring enzymes and substrates into close proximity and to determine the subcellular localization of binding partners[67].

1.12.1 Control of Lyn activation by adaptor molecule Pag

As discussed previously a very initial step in B cell receptor signaling is the translocation of the BCR into lipid raft microdomains and subsequent phosphorylation of Igα/Igβ ITAM motifs by Lyn. Lyn however in resting cells is in an inactive state. Phosphorylation of a carboxy terminal tyrosine residue (Tyr527) mediates an intramolecular association with the SH2 domain of the same kinase, which leads to repressed kinase activity[68]. Phosphorylation of this residue is mediated by the c-SRC tyrosine kinase (Csk)[69], while dephosphorylation by transmembrane phosphatase CD45[70]. Subcellular localization of Csk is dependent on the adaptor protein phosphoprotein associated with GEMs (Pag). Pag is a constitutively raft associated protein as Lyn. In unstimulated cells, Pag is tyrosine phosphorylated and associates with the SH2 domain of Csk and brings thereby Csk and Lyn into the same compartment. Regulatory phosphorylation of Lyn by Csk keeps Lyn in the inactive state. Upon BCR engagement Pag becomes dephosphorylated and releases Csk[71], thereby allowing Lyn to become dephosphorylated by CD45 and to take up the active conformation.

1.12.2 Control of PLCγ activation by adaptor molecule BLNK

The importance of PLCγ activation upon BCR engagement was indicated previously. However, the question remained open how the translocation of PLCγ to the lipid rafts and its subsequent activation is carried out. An adaptor protein, the B-cell linker (BLNK) was shown to be the key docking protein of PLCγ[72]. Following recruitment of Syk to the Igα/Igβ, Syk phosphorylates BLNK to provide binding sites for the SH2 domains of PLCγ and for Btk.

BLNK thus brings Btk and PLCγ into close proximity, to allow the subsequent phosphorylation and activation of PLCγ by Btk[73].

1.12.3 Regulation of PI3K recruitment by adaptor molecules

Recruitment of PI3K to the signalosome is crucial in the activation of several downstream pathways. CD19, a transmembrane adaptor molecule was suggested to be the main adaptor of PI3K. On BCR ligation, the cytoplasmic tail of CD19 is phosphorylated by Lyn, and provides binding sites for the SH2 domains of PI3K subunit p85[74].

Although involvement of CD19 in PI3K recruitment is clear, this mechanism does not seem to fully account for the activation of PI3K. BCR-mediated activation of Akt, a readout of PI3K activation is inhibited, but still occurs in B cells from CD19$^{-/-}$ mice[75]. This suggests that B cells use more adaptor proteins to target PI3K.

The B-cell adaptor for PI3K (Bcap) is another candidate. However Bcap$^{-/-}$ mice have seemingly normal Akt activation[76].

Grb2-associated binding proteins (Gab) were also shown to function as PI3K adaptors in other cell types[77]. Gab family proteins could be promising candidates in B cells as well[78].

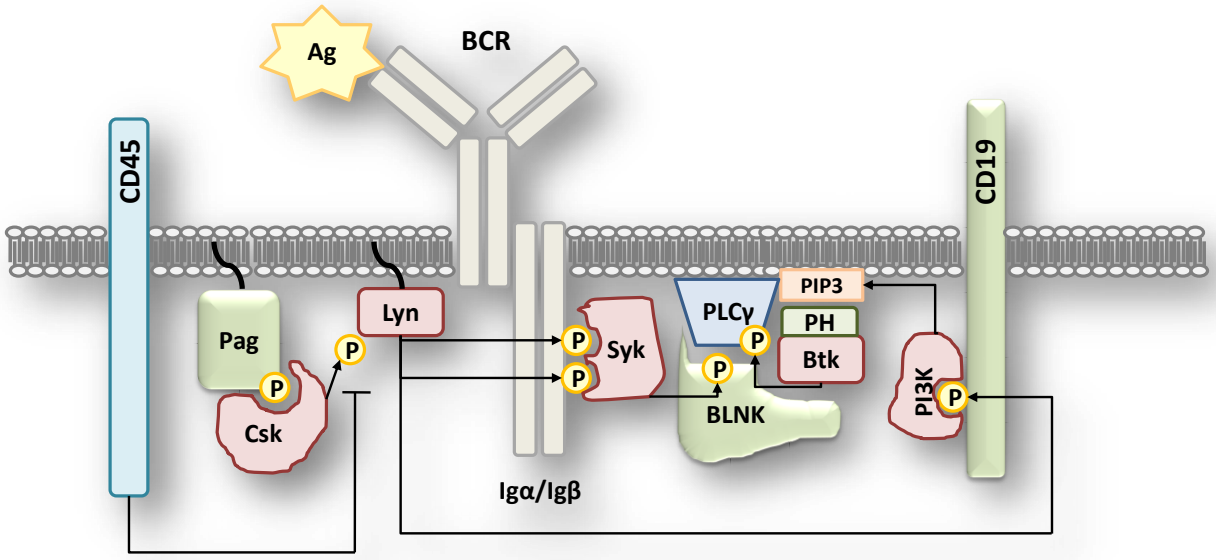

Figure 6. Adaptor proteins regulate several signaling pathways in B cells. *(1) Dephosphorylation of the adaptor molecule Pag is required for the release of Csk a negative regulator of Lyn. Release of Csk allows CD45 to dephosphorylate regulatory tyrosine of Lyn and thereby initiate BCR mediated signal transduction. (2) Active Lyn phosphorylates*

Igα/Igβ, ITAM motifs, docking sites for Syk. (3) Adaptor protein BLNK links PLCγ to Btk and enables thereby initiation of calcium signaling. (4) CD19 is the major adaptor of PI3K a protein that catalyzes synthesis of PIP3. PIP3 allows recruitment of PH domain containing proteins such as Btk.

1.13 Gab adaptor protein family

Since Gab proteins might be the missing gap between the PI3K/Akt survival pathway and BCR engagement, we will discuss their supposed role in more detail.

In mammals three members of the family have been described, Gab1, Gab2 and Gab3, their expression varies between different tissues[79, 80]. Gab proteins comprise a distinct family of adaptor proteins characterized by similar overall structural organization and ~40-50% sequence similarity[81].

1.13.1 Recruitment of Gab proteins to receptors and membranes

Gab proteins can be recruited to activated receptors through direct or indirect mechanisms.

Direct recruitment has been demonstrated only for the interaction between Gab1 and the hepatocyte growth factor receptor[82].

Most Gab protein-receptor interactions are mediated indirectly via Grb2. Gab proteins contain proline-rich motifs that can mediate their binding to Grb2[83]. Grb2 on the other hand contains an SH2 domain to target the constitutive Gab-Grb2 complex to a receptor.

In cytokine signaling, where receptors lack the direct Grb2 binding site, Grb2-Gab complexes are recruited via an additional molecule the src-homology domain 2 containing transforming protein (SHC)[84].

All of the Gab family members contain an N-terminal PH domain that prefers the binding to lipid products of the PI3K, especially PIP3. The PH domain might enable Gab proteins to be recruited to the signalosome independently from a receptor.

1.13.2 Kinases that phosphorylate Gab proteins

Gab proteins undergo tyrosine and serine/threonine phosphorylation in response to diverse stimuli. The particular kinases to be involved in this process vary between cell types. In mast cell src-family kinase Fyn was suggested to be the primary kinase of Gab2[85]. In T cells the zeta-chain associated protein kinase 70 kDa (ZAP-70), T cell homologue of the kinase Syk was suggested to be responsible for the tyrosil phosphorylation of Gab2[86]. The precise identity of serine/threonine kinases involved in the phosphorylation of Gab proteins remains

to be elucidated; however there is experimental evidence that they are downstream of PI3K and the MAPK cascade[87].

1.13.3 Signaling from Gab proteins

Gab proteins contain multiple tyrosine phosphorylation sites, which upon phosphorylation recruit and activate key SH2-domain-containing signal relay molecules, most prominently the SH2-domain containing phosphatase (SHP2) and PI3K.

1.13.4 The role of SHP2/Gab interaction

All Gab proteins contain at least one conserved tyrosine motif capable to recruit SHP2. Gab/SHP2 interaction was shown to be required for full activation of the MAPK cascade in many signaling pathways. Fibroblasts from Gab1-/- mice are defective in Erk activation in response to multiple growth factors[88]. Overexpression of a Gab2 mutant unable to bind SHP2 impairs Erk activation in response to colony stimulating factors[89]. The mechanism by which Gab/SHP2 interaction promotes Erk activation is currently unknown.

1.13.5 The role of PI3K/Gab interaction

All Gab proteins have at least one potential binding site for the p85 regulatory subunit of PI3K. By binding p85, Gab proteins may provide a route to the PI3K/Akt pathway and thereby to cell survival. And indeed, it was shown in mast cells and fibroblasts as well, that Gab family members have a key role in the sufficient activation of Akt upon stimuli[87, 90].

1.13.6 Physiologic function of Gab proteins

Studies of Gab1 and Gab2 knockout mice have delineated distinct functions for individual Gab proteins. Gab1 was shown to have an essential role in heart, placenta, liver skin and muscle development. Gab1$^{-/-}$ mice die early in the embryonic stage[91]. Gab2$^{-/-}$ mice are viable, the most obviously affected cell linage seems to be mast cells. Gab2$^{-/-}$ mast cells show impaired degranulation and cytokine gene expression, mice have severe defects in their allergic response[77]. Little is known about the role of Gab proteins in B cells.

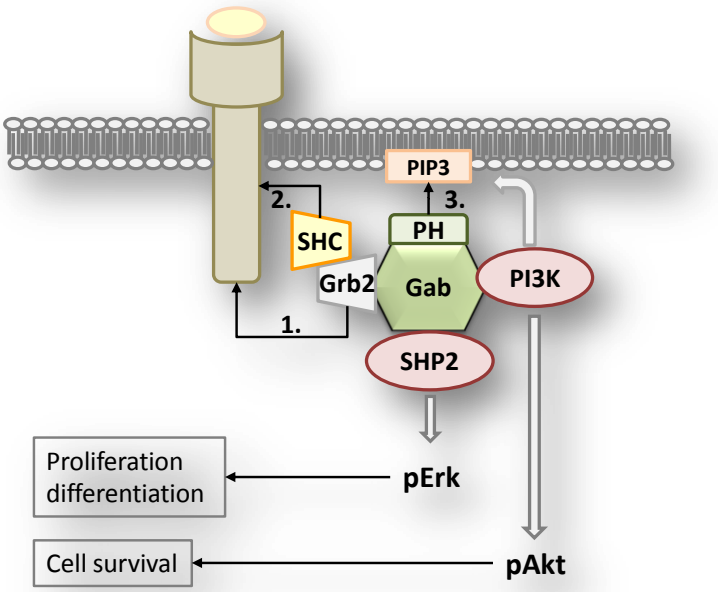

Figure 7. Gab proteins can integrate survival pathways PI3K/Akt and the MAPK cascade. *(1) Gab proteins can be recruited to the signalosome indirectly binding the receptor via Grb2 or SHC or through PH domain PIP3 interaction. (2) Major binding partners of Gab proteins are SHP2 and PI3K. (3) SHP2-Gab interaction may enhance the MAPK cascade, and thereby shift the cell towards proliferation and differentiation. (4) PI3K-Gab interaction may enhance Akt phosphorylation and thereby prevent apoptosis.*

2. Objectives

Microcluster formation in B cells is an actin dependent process. It requires actin reorganization with an initial depolymerization phase and a subsequent repolymerization phase. Microclusters form early during B cell activation, thereby regulation of this process needs to be mediated by a very upstream signal. Calcium regulates several actin binding proteins, its subcellular distribution is heterogeneous and its local concentration can vary on a millisecond timescale. These features make calcium a potent regulator of actin rearrangements and thereby microcluster formation.

2.1 Broadly speaking, our goal was to test for the existence of a specific interaction between calcium and actin dynamics.

2.2 In more detail, we aimed to investigate how raising or lowering the cytoplasmic calcium concentration affects the morphology of the cell.

2.3 How the same effect influenced the structure and density of the actin cytoskeleton.

2.4 And whether proposed interconnection between calcium and actin dynamics is functional and physiologic.

2.5 We aimed to decide whether theoretically such an interaction between calcium and actin could explain microcluster formation in B cells.

When microclusters are formed and the BCR is merged with the signalosomes, a central requirement of B cell activation and BCR induced cell survival is the successful recruitment of PI3K. PI3K activity in the signalosome is necessary for the activation of the PI3K/Akt pathway, for the recruitment of PLCγ and thereby activation of calcium signaling and subsequent NF-κB nuclear translocation. Thereby PI3K is a central player of B cell survival. We have seen that CD19 was suggested to be the main but not exclusive adaptor of PI3K in B cells. Gab family members and especially Gab2 are good candidates to be responsible for the recruitment of PI3K beside CD19.

2.6 Our goal in this case was to define the role of Gab2 in BCR signaling.

2.7 We aimed to investigate whether Gab2 is expressed and becomes phosphorylated in B cells.

2.8 Does Gab2 recruit PI3K and eventually SHP2?

2.9 Which kinase or kinases are responsible for its phosphorylation?

2.10 How is Gab2 recruited to the signalosome?

2.11 Does Gab2 contribute to the PI3K/Akt pathway or eventually to the SHP2/Erk pathway and thereby to cell survival?

3. Materials and methods

3.1 Reagents and antibodies

The used primary antibodies were anti-Gab2, anti p-Tyr, anti-SHP2, anti-CD79a, anti-CD79b, anti-Grb2, anti-phospho-Akt, anti-NCX (Santa Cruz), anti-actin, anti-phospho-Erk1/2, anti-IgM (Sigma Aldrich), anti-Lyn, anti-p85 (Upstate), anti-Fas, anti-Fas-biotin (clone JO2), anti-pan-Erk (Becton Dickinson/Transduction Laboratories). Secondary antibodies used in our experiments were Alexa488- or Alexa647- conjugated anti-mouse IgG (Invitrogen) Alexa488-, Alexa546- or Alexa647- conjugated anti-rabbit IgG (Invitrogen), HRP-conjugated anti-rabbit IgG (Dako). The anti-Gab2 was a kind gift from Dr. Gu, 2.4G2-biotin, anti-Thy-1.2 Ab and rabbit complement were kind gifts from Dr. Prechl. Alexa488 and Alexa555 conjugated Phalloidin (Invitrogen) was used for F-actin staining. Fluo-4 AM (Invitrogen) was used for calcium imaging. Rabbit anti-mouse IgG (Jackson) and rabbit $F(ab')_2$ anti-mouse IgG (Jackson) were used for the stimulation of A20 cells. Ionomycin calcium salt from *Streptomyces conglobatus* (#I0634) and Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid(EGTA) (E-4378) and Latrunculin-B were purchased from Sigma Aldrich Corporation. The horseradish peroxidase-conjugated anti-rabbit or anti-mouse Abs were from Dako Cytomation, protein A/G Plus-agarose beads from Santa Cruz, Alexa647 conjugated streptavidin was purchased from Invitrogen.PI3K inhibitor LY294002 was a product of Sigma, while Syk inhibitor III. was manufactured by Calbiochem. Peroxidase- labeled antibodies were detected using the Pierce ECL reagent.

3.2 Cell lines

IP12-7 T-cell hybridoma of helper phenotype was developed from BALB/c mice pre-immunized with the HA317 – 341 peptide and subsequently infected with the A/ PR/8/34 human influenza A virus. A20 (ATCC TIB208, I-Ad/Ed) cells are murine B-cell lymphomas. Cell lines 38C13, A20, X16C and IP12-7 were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 50 AM 2-mercaptoethanol, antibiotics and 10 % FCS. HeLa cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. All cells were kept at 37 ^{o} C, 5% CO_2 in a humidified atmosphere.

3.3 Mice and isolation of B lymphocytes

Adult (6–8 weeks old) C57Bl/6 mice, and Lyn, Hck, Fgr triple knockout mice, a kind gift of Dr. C.A. Lowell, University of San Francisco, (San Francisco, CA) were used. B-cells were purified as previously described[92]. Briefly, spleens were removed from mice killed by cervical dislocation and the cells were collected and washed in RPMI 1640 culture medium containing 5% fetal calf serum,1mMNa-pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5!10-5 M mercaptoethanol. Red blood cells were lysed in 5 ml Gey's solution for 1 min. The remaining cells were washed twice and resuspended in RPMI 1640 culture medium containing anti-Thy-1.2 antibodies for 20 min at room temperature and washed. The cells were further incubated with HBSS solution containing rabbit complement for 45 min at 37 °C then washed twice in RPMI 1640 culture medium containing 5% fetal calf serum. The percentage of B-cells was analyzed by flow-cytometer using fluorescently labeled anti-B220 and anti-CD3 Abs.

3.4 Constructs

pMSCV Gab2-IRES-GFP, pMSCV ΔPH Gab2-IRES-GFP (PH domain deficient construct) and negative control pMSCV-IRES-GFP were kindly provided by Dr. Haihua Gu, Harvard Medical School, Boston. The genetically encoded Ca^{2+} sensor, GCaMP2 fused to chick actin [93] was used for calcium imaging near the actin cytoskeleton. The construct pCAGGS-GCaMP2-actin (Addgene plasmid 18928) was obtained from Addgene (www.addgene.org) mCherry-actin was cloned between BamHI and EcoRI sites of the retroviral expression vector pMOWS that contains a puromycin resistance gene for selection (ref1). mCherry was amplified with the primers : gattggatccgccaccatggtgagcaagggcgag and cctgcatatgacccttgtacagctcgtccat. β-actin was amplified with the primers: ggtcatatgggaatggatgacgatatcgctg and cttagaattcctagaagcacttgcggtgcac. The insertion was done sequentially using the NdeI site built in the relevant primers.

3.5 Retroviral transfection

A20 cells were retrovirally transduced in all experiments as described previously[94]. In short, the Phoenix retroviral producer cell line was transfected using GeneJuice (Novagen) according to the manufacturer's instructions. Retroviral supernatants were harvested after 36 and 60 h post-transfection. 2×10^5 cells were mixed with 500μl retroviral supernatant and centrifuged at 300g at 37°C for 3 h. Transduced cells were cultured in RPMI medium.

Puromycin (10μg/ml) was added to the culture medium 24 hours later. Three days later positive cells were sorted by MoFlo cell sorter.

3.6 Lipofection

For transient transfection HeLa cells were cultured to reach 40-50% confluency and transfected with plasmid DNA pCAGGS-GCaMP2-actin (Addgene plasmid 18928) using FuGene 6 Transfection Reagent according to the manufacturer's protocol.

3.7 Single cell calcium imaging

A20 or HELA cells were grown on 8-well chamber coverglasses (Lab-Tek) ($2x10^4$ cells per chamber). After 12 hours in culture cells were washed and incubated for 15 minutes at 37 ^oC in 10 μg/ml Fluo 4-AM solution prepared in RPMI1640 medium (Sigma Aldrich). After staining, samples were washed six times. Images were taken with an Olympus Fluoview 500 (Olympus, Hamburg, Germany) confocal laser-scanning microscope using an avarage time resolution of 1,375 second per frame. An Argon/Ion laser (488 nm) was used for the excitation of Fluo-4 AM. EGTA was used in 2mM, while ionomycin in 2 μg/ml concentration.

3.8 Calcium measurement by flow cytometry

A20 cells were loaded with Fluo-4 AM at 10 μg/ml final concentration in RPMI 1640 medium at 37 ^oC for 20 minutes. The cells were diluted 10 times and further incubated at 37 ^oC for 20 minutes. After washing, the cells were resuspended in RPMI 1640 and treated with 1 μg/ml ionomycin or 2 mM EGTA at the indicated time points. Fluorescence measurements were performed using a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) flow cytometer equipped with an air-cooled argon ion laser (488 nm emission wavelength). Data collection and analysis were done with CellQuest Pro software. During data analysis, dead cells were excluded based on propidium-iodide uptake.

3.9 F-actin measurement

A20 cells ($5*10^5$ per tube) were collected in 300 μl RPMI1640, and treated at 37 ^oC with ionomycin, EGTA or both for different time intervals. Samples in a measurement were synchronized to minimize the failure due to spontaneous F-actin depolymerization. Immediately after treatment 100 μl of a solution containing 10% paraformaldehyde, 0,05% Triton-X and 2 units of Alexa488-conjugated phalloidin was mixed to the samples. After

labeling on ice for 10 minutes, samples were carefully washed and analyzed on a FACS Calibur flow cytometer with the CellQuest Pro software.

3.10 Immunofluorescence

A20 cells were grown on 8-well chamber coverglasses. After 12 hours in culture, cells were washed in RPMI 1640 stimulated either with 2mM EGTA or 2 μg/ml ionomycin or both. Immediately after stimulation cells were fixed in 3% paraformaldehyde for 5 minutes at 37 ^{o}C, washed two times with PBS, and permeabilized in 0,1% Triton X-100 (Sigma Aldrich) at room temperature for 5 minutes. After three times of washing with PBS, cells were stained with Alexa448- or ALexa555- conjugated phalloidin or anti-NCX, and secondary reagents Alexa488- or Alexa647- conjugated anti-rabbit IgG using concentrations provided by the manufacturer.

3.11 Stimulation of B cells and preparation of cell lysates

A total of $2x10^{6}$ cells were treated with 10 μg/ml F(ab')2 fragment of mouse IgG specific rabbit antibodies for 5, 15, 30, 60 and 90 min, respectively, at 37 ^{o}C. Cells were pelleted for 20 s and immediately frozen in liquid nitrogen. Then cells were solubilized in 20 μl of lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 100 mM NaF, 250 mM NaCl, 10 mM EDTA, 2 mM sodium-o-vanadate, 10 mM sodium pyrophosphate, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 5 μg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride. After 30 min of incubation on ice, cell lysates were centrifuged at 15,000 x g for 15 min, at 4 ^{o}C, and the supernatants were used in subsequent experiments.

3.12 Immunoprecipitation and immunoblotting

The post-nuclear supernatant was subjected to immunoprecipitation for 1 h at 4 °C with the indicated precipitating antibodies coupled to proteinA/G-agarose beads. The beads were washed four times with lysis buffer, and the immunoprecipitates were eluted from beads by heating in 30 μl of reducing SDS-PAGE sample buffer at 95 °C for 5 min. Samples were subjected to electrophoresis through 10% SDS-PAGE gel, the proteins were blotted onto nitro-cellulose membranes (BioRad), probed with different antibodies and developed by HRP-conjugated species specific IgG followed by enhanced chemiluminescence detection.

3.13 SDS-PAGE and Western blotting

Post-nuclear supernatants (20 µl) of detergent extract obtained from $2x10^6$ control and anti-IgG-treated A20 cells were incubated with 20 µl of reducing SDS–PAGE sample buffer for 5 min at 95 ^oC. The samples were subjected to electrophoresis through 10% SDS–PAGE gel, the proteins were transferred onto nitro-cellulose membranes (BioRad), probed with different antibodies and developed by HRP conjugated anti-rabbit IgG, followed by enhanced chemiluminescence detection (ECL system, Amersham International, Amersham, UK).

3.14 BCR internalization assay

A20 cells ($5x10^5$ per sample) were washed and treated with 10 µg/ml F(ab')2 fragment of mouse IgG specific rabbit antibodies for 5, 15, 30, 60 and 90 min, respectively, at 37 ^oC. Cells were pelleted for 20 s and stained with Alexa488-conjugated anti-rabbit IgG and with Alexa647-conjugated anti-mouse IgG. After labeling on ice for 10 minutes samples were carefully washed and analyzed on a FACS Calibur flow cytometer with the CellQuest Pro software.

3.15 Immunological synapse generation

A20 cells were grown on 8-well chamber coverglasses ($2x10^4$ cells per chamber). After culturing for 12 hours cells were washed and incubated in serum-free media containing 20 µM HA317 – 341 peptide [95]. After washing cells were coincubated with IP12-7 helper T-cells ($4x10^4$ cells/chamber) at 37 ^oC for 15 minutes. If single cell calcium imaging was carried out IP12-7 cells were previously loaded with Fluo-4 according to 2.6 and the synapse formation was recorded with an Olympus Fluoview 500 confocal laser-scanning microscope If immunofluorescent detection was used then cells were washed, fixed with 3% paraformaldehyde and stained according to 2.9 after the synapse formation.

3.16 Cell death experiments

The sensitivity of A20 cells to Fas mediated cell death was examined after incubating the 106 cells with 100 ng/ml of JO2 anti-Fas at 37 °C for 2 h or 3 h. Cells were incubated with 5 µg/ml of goat anti-IgG at 37 °C for 10 min before addition of JO2 anti-Fas to examine their BCR-mediated escape from apoptosis. After the indicated time, the apoptotic rate of the cells was detected by flow cytometric analysis[96]. Briefly, cells were mixed in ice-cold 70% ethanol, washed in 38 mM sodium citrate (pH 7.4) and stained for 20 min at 37 °C with 69 mM propidium iodide (Sigma) and 5 mg/ml RNase A (Sigma) in 38 mM sodium citrate (pH 7.4).

Cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson), and the proportion of apoptotic cells represented by the subG1 peak was determined using WinMDI software.

3.17 Image processing

3D surface images and 3D image reconstruction was performed with Imaris 4.0.3 (Bitplane). Calcium imaging data during synapse formation was reanalyzed based on a method used in spatial informatics. Our application loads the raw image data into a suitable database schema. A double filter is set up manually for the frame where the synapse formation is supposed to occur. The thresholds of the two filters divides the intensity scale into three ranges and assigns a color to every pixel, in our case low calcium was represented by white, medium calcium by gray and high calcium by black. Finally, we applied a median based search through the histograms of all images to set up the appropriate filters from frame to frame.

3.18 Statistical analysis of Ca^{2+} and cortical actin time curves

Cortical actin data were obtained from calcium imaging images by defining a region of interest around the plasma membrane from frame to frame manually. Experimental data was calculated from 4 different measurements.

Since both Ca^{2+} and cortical actin signals were contaminated by measurement noise spline smoothing was first applied as preprocessing. Thereafter the very low frequency oscillations were eliminated using trend removal procedures. Instantaneous phases were calculated by Hilbert transform. In order to detect and quantify synchronization, normalized mutual information (nMI) was applied. Entropy based synchronization index has important advantages over other synchronicity measures, most of all its ability to grasp nonlinear interdependence (beyond linear dependency) and providing a general characterization of all possible frequency locking. Due to the normalization of the mutual entropy the range of the synchronization indices is the [0,1] interval. In order to expose the dynamics of the mutual interaction windowed ("running") entropies were estimated within 15 consecutive, non-overlapping 52 seconds length windows.

High synchronicity indices were found of about 0.7 when estimated from experimental data. The relevance of this strong phase locking was tested with the aim of surrogate bootstrap technique and a subsequent adaptive Neyman's test. Surrogates were generated under two null hypotheses proposing that the data show both extreme, the possible lowest and highest synchronization. Surrogates of low synchronicity was created by shuffling of the original

phases (using permutation), while “fully synchronized” surrogates were the realizations of experimental data reproduced with identical phases.

For testing the differences between the experimental and surrogate data the Neyman’s smooth statistics was used making it data driven by applying the Schwarz’s selection rule.

Mathematical calculations were accomplished in Matlab 6.5 software environment (the MathWorks Inc., Natick, MA, USA) by the application of self devised scripts for preprocessing, entropy measures, surrogate analysis and adaptive Neyman’s test.

4. Results I.: Subcellular distribution of calcium regulates site-specific actin rearrangements

We have seen how important regulated rearrangements of actin cytoskeleton are in order to support proper B cell activation in germinal centers. But actin dynamics are not only important during antigen encounter or signaling; they are required for cell movement, cell-cell interactions, cell-shape development and support lymphocyte function in many ways. Lymphocytes have dramatically different cytoarchitecture depending on whether they are circulating in the bloodstream, migrating through the tissues, or interacting in an immunological synapse[97]. Circulating lymphocytes rolling along the vascular endothelium are covered by short microvilli, dense bundles of actin filaments enclosed in the plasma membrane [98]. Once a lymphocyte encounters the appropriate chemokine and integrin signal, a rapid collapse of the cortical actin network occurs allowing lymphocyte migration across the endothelial wall [34]. Formation of immunological synapses (IS) is another example for rapid actin rearrangement in lymphocytes. Following integrin associated binding to the antigen-presenting cell (APC), T-cells form a dense actin structure at the cell-cell contact zone [99]. All of these processes require regulated rearrangement of the actin cytoskeleton [97] that makes lymphocytes good candidates to study actin dynamics.

Actin filaments are major cytoskeletal components of eukaryotic cells. The dynamic assembly and disassembly of filaments and the formation of larger scale filament structures are crucial to the function of actin [19].

As mentioned in the introduction several actin-binding proteins and signal-transduction pathways are known to tightly regulate actin structure and dynamics[12, 19, 100], but little is known about how this regulation is controlled in time and space. A number of actin regulating proteins are sensitive to changes in the cytoplasmic calcium concentration. Earlier studies have indicated that a large and rapid rise in the intracellular Ca^{2+} level has a substantial impact on the actin cytoskeleton [101, 102], but still little is known about the specific role of calcium in the regulation of actin dynamics.

Calcium is one of the major intracellular messengers regulating many different cellular functions. The cytoplasmic concentration of Ca^{2+} is under the tight control of the calcium releasing and sequestering machinery [66]. The spatial distribution of Ca^{2+} along the cytoplasm is inhomogeneous with localized increases and decreases, recently termed as calcium microdomains [103].

In the following experiments, we approached to understand and describe the mechanism of the site specific regulation of actin growth and decay in order to better understand the

happenings during B cell activation. Our goal was to reveal the interaction between calcium and actin dynamics.

4.1 Huge elevations in the intracellular calcium concentration result in the loss of lymphocyte cell structure

First we investigated how a large increase in the cytoplasmic calcium concentration acts on lymphocyte cell-shape. Adherent B-lymphoma cells (A20) were grown on solid surface in an 8 well chamber for 12 hours over night. 4 x 10^4 cells/chamber was found to be the optimum density in order to allow single cell monitoring. After 12 hours of growing cells were washed and loaded with fluo-4 AM, a plasma membrane permeable green-fluorescent calcium indicator used to measure calcium levels in living cells. Cells were investigated with confocal microscopy to perform single cell calcium imaging.

We exposed cells to ionomycin, a calcium-specific ionophore, which allows the influx of extracellular Ca^{2+}, and followed the changes in the intracellular calcium levels and cell morphology. Ionomycin (2 μg/ml) had a dramatic effect, it induced a 3 fold increase in the intracellular calcium level followed by the instant loss of cell structure. Cells began to round up and released wheir residual membranes in the form of membrane vesicles (Fig. 1A).

We excluded a calcium-independent side effect of ionomycin, by exposing cells to ionomycin and ethylene glycol tetraacetic acid (EGTA) together. EGTA chelates calcium ions in the media and blocks ionomycin induced calcium entry. When EGTA (2 mM) and ionomycin were used together, cells showed a transient elevation in the intracellular calcium concentration followed by a subsequent decrease far below the resting level, cell structure however, remained normal (Fig. 1B).

Using EGTA alone evoked an oscillatory decrease in the intracellular calcium concentration without any obvious change in cellular morphology (Fig.1C). Thus, increases in the cellular calcium level have a specific destructive effect on cell structure.

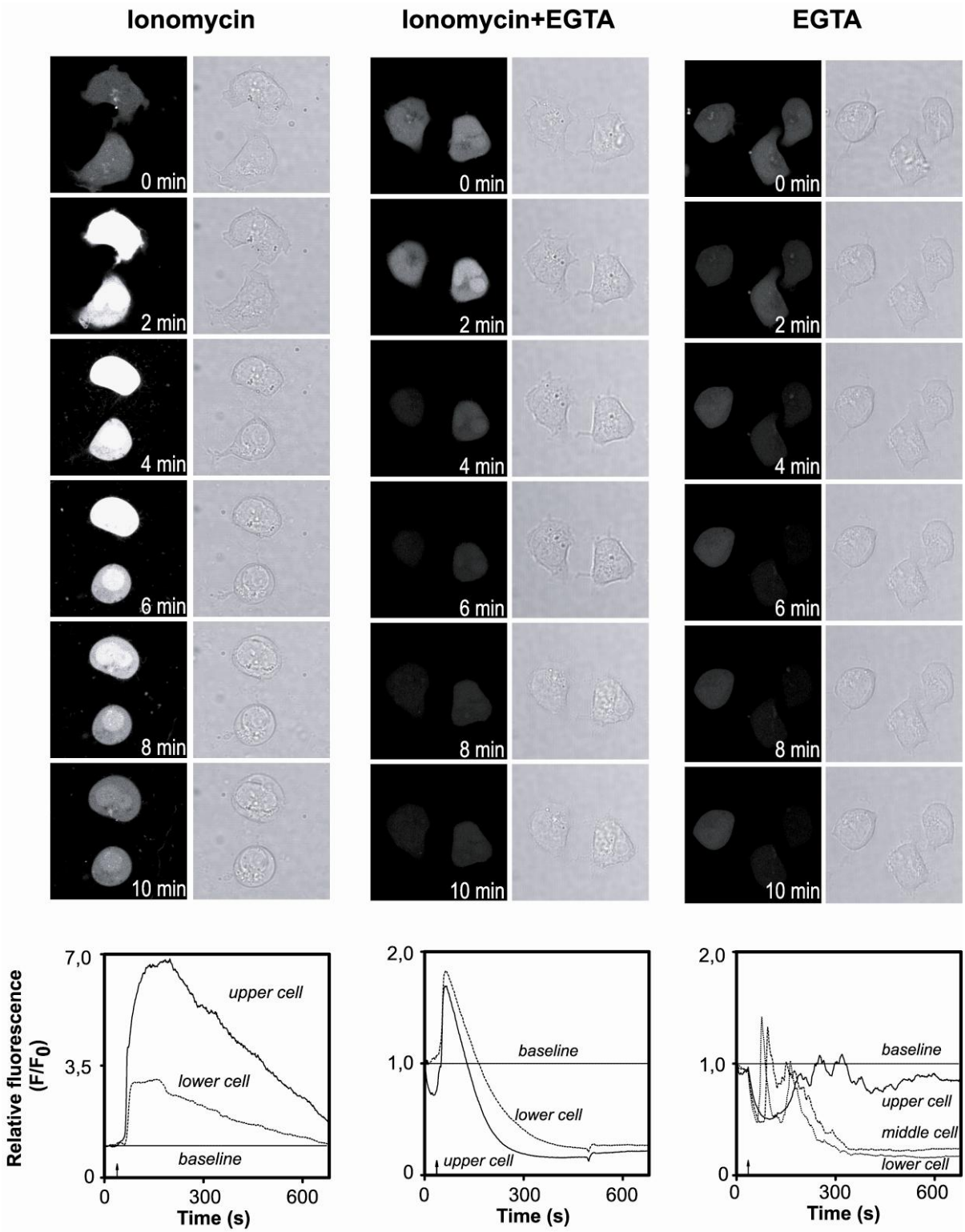

Figure 8. Large increases in the cytoplasmic calcium concentration induce the loss of cell structure. *Calcium imaging of adherent A20 cells labled with fluo-4 AM treated with (A) ionomycin (left) EGTA+ionomycin (middle) or EGTA alone (right) by confocal microscopy. Left panels show the fluorescent images color mapped with a gradient from white (high fluorescence) to black (low fluorescence), right panels the DIC images. Relative fluorescence*

as a function of time is indicated on the bottom side. Arrows on the diagrams indicate the time point of exposure.

4.2 High intracellular calcium destroys peripheral actin structures, while in regions with locally lower calcium level F-actin growth is favored

Lymphocyte cell shape is primary determined by the actin cytoskeleton. To relate the observed loss of morphology upon large calcium increases to the actin cytoskeleton, cells from the previous experiment were stained with Phalloidin-Alexa488 a fungal toxin, which specifically binds filamentary actin and further investigated with confocal microscopy. EGTA or EGTA+ionomycin treatments did not cause any recognizable change in cell morphology (Fig. 2). The unaltered cell structure suggests that if there was any change in the filamentary actin ratio it was mostly concentrated to the cortical actin network.

Ionomycin however induced the collapse of cortical actin network with the apparent loss of peripheral protrusive actin structures (Fig. 2A). However, a well recognizable ring-like actin meshwork appeared around the nucleus (Fig. 2A, Fig 2B).

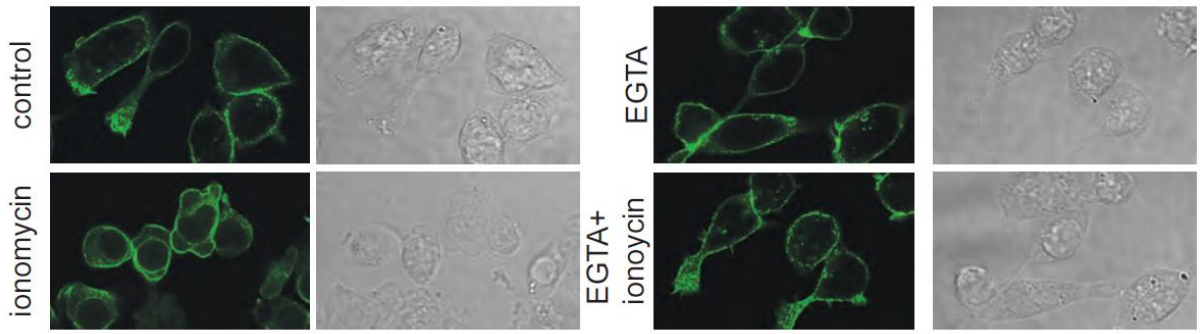

Figure 9. Large increases in the cytoplasmic calcium concentration destroy peripheral actin structures. *Fluorescent (right side) and DIC images (left side) of control, ionomycin, EGTA or EGTA+ionomycin treated adherent A20 cells labeled with Phalloidin-Alexa488 obtained by confocal microscopy [filamentous-actin (green)].*

However, a well recognizable ring-like actin meshwork appeared around the nucleus (Fig. 2A, Fig 2B).

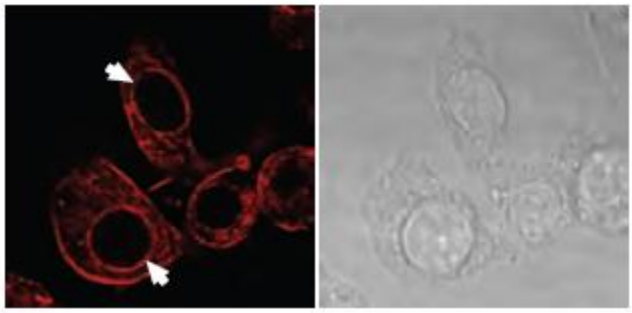

Figure 10. A perinuclear actin meshwork appears in cells upon ionomycin iduced calcium elevation. *Ionomycin treated cells from the experiment descried in Fig.8. White arrows indicate the actin meshwork around the nucleus [filamentous-actin (red)].*

We further analyzed the calcium imaging data from 4.1 to find an explanation for the appearance of the perinuclear F-actin structure.

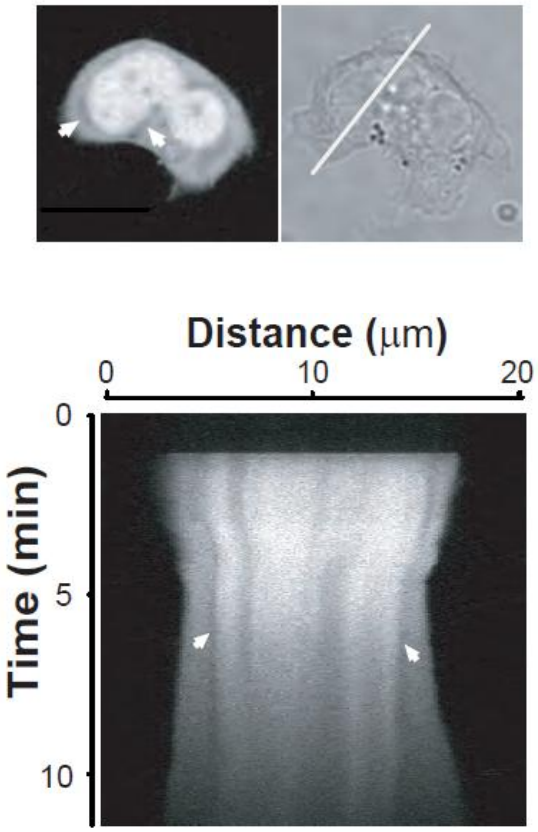

Figure 11. F-actin growth is favored in cytoplasmic regions with locally lower calcium concentration. *Single time-point image of the calcium imaging performed on ionomycin*

treated A20 cell. Fluorescent image (upper left) is color mapped with a gradient from white (high fluorescence) to black (low fluorescence). White arrows indicate low calcium level areas around the nucleus. Line-scan imaging (bottom panel) was performed around the white line indicated on the DIC image (upper rigth). White arrows indicate the low calcium level areas around the nucleus.

Single time point images showed an unequal distribution of Ca^{2+}, with cytoplasmic regions of low calcium concentration around the nucleus (Fig. 2C). These regions remained intact in time (Fig. 2D), and were most possibly resulted by the calcium sequestering ER network around the nucleus, suggesting that while high intracellular calcium disrupts actin filaments, F-actin growth is still favored in regions with locally lower calcium concentration.

4.3 The amount of F-actin shows an inverse correlation with the cytoplasmic concentration of calcium

We quantified the qualitative and morphological changes in lymphocytes induced by large elevations in the cytoplasmic calcium concentration by measuring relative F-actin amount. A20 cells were treated with ionomycin, EGTA, EGTA+ionomycin until time points indicated on the figure (Fig. 11.), and subsequently permeabilized with Triton-X, fixed with paraformaldehyde and stained with phalloidin-Alexa488. Measurement was performed in a flow-cytometer.

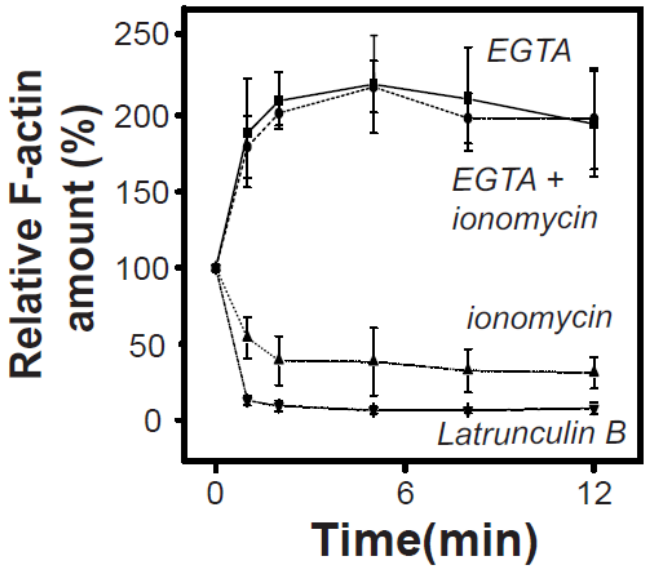

Figure 12. Large elevations in the cytoplasmic calcium concentration induce actin decay, while low calcium concentration leads to actin polymerization. *Quantification of relative F-*

actin amount as a function of time in A20 cells upon different treatments [squares, EGTA; circles, EGTA+ionomycin; triangles, ionomycin; inverted triangles, Latrunculin B].

Ionomycin induced a disintegration of actin filaments (Fig. 2E) comparable to that of Latrunculin B, a compound widely used to disrupt microfilament organization. About 60% of the resting F-actin amount was still intact after 1-minute of treatment, while after 8 minutes it decreased to only 35%. Lowering the calcium concentration by EGTA or EGTA+ionomycin had an opposite effect; it induced a saturation-curve-like growth in the amount of actin filaments. After 2 minutes of treatment the relative amount of F-actin had doubled and reached its saturation. Thus, we could show an inverse correlation between the amount of cytoplasmic calcium and F-actin.

4.4 Low cytoplasmic calcium concentration induced actin growth enhances actin-dependent internalization

We next tested whether EGTA induced growth in the cortical actin network is functional. Following antigen stimulation B cells internalize their B cell receptors (BCR) in an actin-dependent process, thus we assumed that if low calcium level induced actin growth is functional, we should see an enhanced rate in internalization. We stimulated EGTA pretreated (1 hour, 37^{o}C) and control B cells with anti-mouse IgG at 37^{o}C until time points indicated on the figure (Fig. 12.), subsequently washed them, fixed them with paraformaldehyd and stained with anti-mouse IgG-Alexa488. Data was obtained by flow-cytometry.
EGTA obviously speeded up receptor internalization. After 15 minutes of stimulation control cells still bore 75% of their surface IgGs, when pretreated cells have already internalized 50% of their receptors (Fig. 2I). This marked difference implies a functional effect for low calcium induced actin growth.

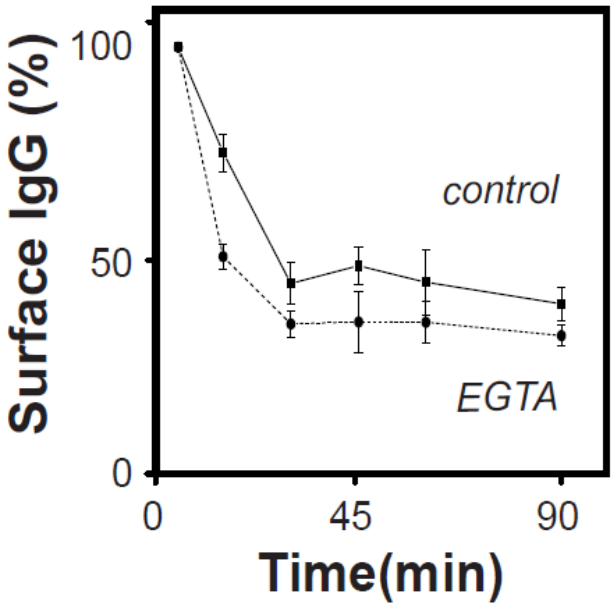

Figure 13. EGTA pretreatment enhances B cell receptor internalization in anti-IgG stimulated B lymphocytes. *The relative amount of surface IgGs as a function of time in control (square) and EGTA pretreated (circle) A20 cells upon BCR engagement with anti-IgG. Data was obtained by flow-cytometry.*

4.5 EGTA pretreatment increases BCR induced Akt phosphorylation

To confirm the previous result we investigated Akt phosphorylation upon BCR engagement. The serine/threonine kinase Akt plays an important role in B cell survival by inhibiting apoptotic processes [75]. Akt was shown to bind filamentary actin through its PH-domain [104], in a process that might be prerequisite to its phosphorylation, since depolymerization of actin filaments markedly reduces the stimulus-dependent activation of Akt [105]. Consequently we asked whether EGTA induced low intracellular Ca^{2+} concentration could selectively modulate Akt phosphorylation through its actin polymerizing effect. We stimulated EGTA pretreated (1 hour, 37^{o}C) and control B cells with anti-IgG until timepoints indicated on the figure (Fig. 12). Subsequently they were washed and lysed, and further investigated with Western blot (WB).

EGTA pretreatment dramatically increased BCR induced Akt phosphorylation, while actin-independent Erk phosphorylation remained unaffected. These results denote that low calcium concentration induced actin growth enhances actin-dependent cell-functions.

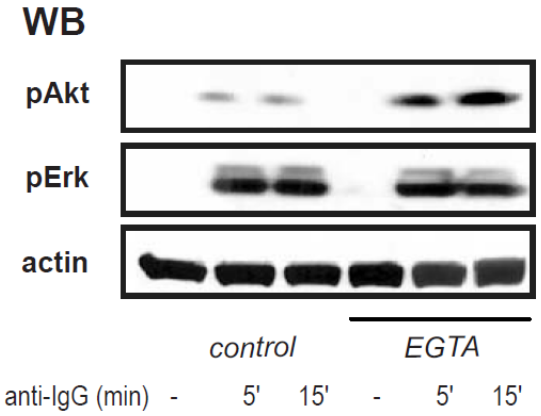

Figure 14. EGTA pretreatment enhances B cell receptor induced Akt phosphorylation. *Phospho-Akt and phospho-Erk immunoblot of control and EGTA pretreated A20 stimulated with anti-IgG for 0, 5, 15 minutes as indicated.*

4.6 In vivo imaging reveals the breakdown of cortical actin upon ionomycin exposure

We next explored the effect of intracellular calcium increases on the actin cytoskeleton *in vivo*. To do so, HeLa cells were transfected with a plasmid containing an actin targeted Ca^{2+} sensor, GCaMP2-actin [94] (Addgene plasmid 18928). Transient transfection was performed with lipofection. The GCaMP2-actin is expressed, becomes built in actin filaments (Fig. 3A) and indicates changes in the nearby calcium concentration.

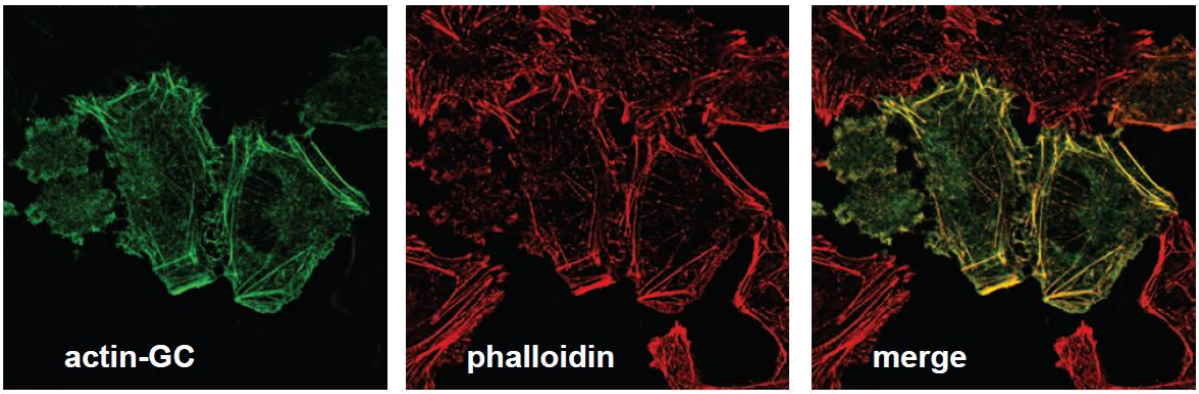

Figure 15. GCaMP2-actin becomes built in actin filaments. *Fluorescent images of HELA cells expressing GCAMP2-actin fusion protein (green) stained with phalloidin (red). (Experiment was performed by Kata Pászthy and Ágnes Enyedi)*

Addition of ionomycin to positive HELA cells induced a large transient increase in GCaMP2-actin fluorescence followed by a sustained phase.

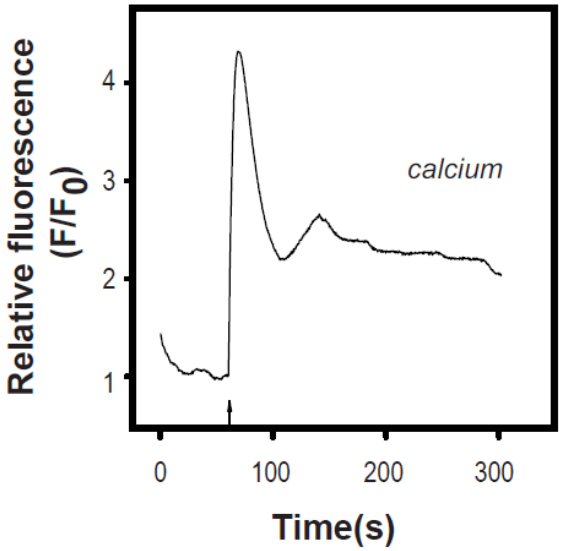

Figure 16. Relative fluorescence of GCaMP2-actin as a function of time upon ionomycin exposure. *(Experiment was performed by Kata Pászthy and Ágnes Enyedi)*

During this sustained phase a complete disorganization of the actin cytoskeleton was detected, accompanied by the termination of the plasma membrane cytoskeleton coupling, followed by intensive membrane blebbing (Fig. 3B).

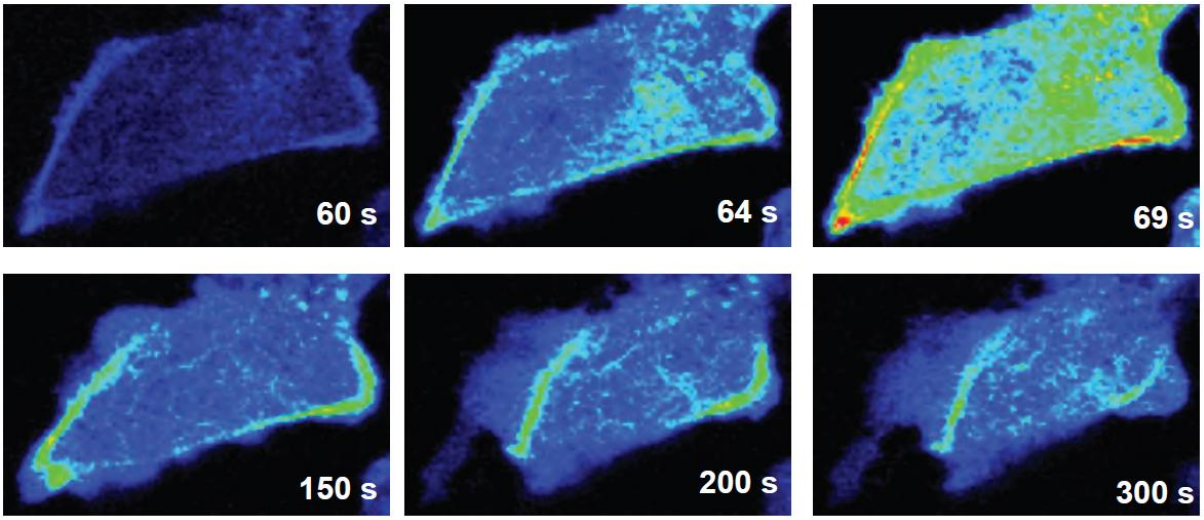

Figure 17. Large calcium increase induces the breakdown of actin cytoskeleton in vivo. Time lapse of GCAMP2-actin expressing HELA cells treated with ionomycin. Relative

fluorescence is color mapped with a gradient from blue (low fluorescence) to red (high fluorescence). *(Experiment was performed by Kata Pászthy and Ágnes Enyedi).*

4.7 Polymerization and decay of actin filaments are strongly coupled to local calcium concentration *in vivo*

To show the *in vivo* effect of the decreased calcium level on the actin cytoskeleton we generated stable mCherry-actin expressing A20 cells. A20 cells were transduced retroviraly with a plasmid containing the mCherry-actin fusion protein genetically encoded. Stable, positive clones were collected by cell-sorting (*retroviral transduction and cell sorting was performed by David Medgyesi*).

Positive A20 cells were grown on 8 well chamber plates ($4x10^4$/chamber) overnight, then washed and loaded with fluo-4 AM. Imaging data was obtained by confocal microscopy. Exposing cells to 2 mM EGTA induced a rapid decrease in cell calcium followed by an oscillatory Ca^{2+} signal. As a result the amount of cortical actin began to fluctuate synchronously with calcium producing maximum peaks where calcium had its minimums (Fig. 17.).

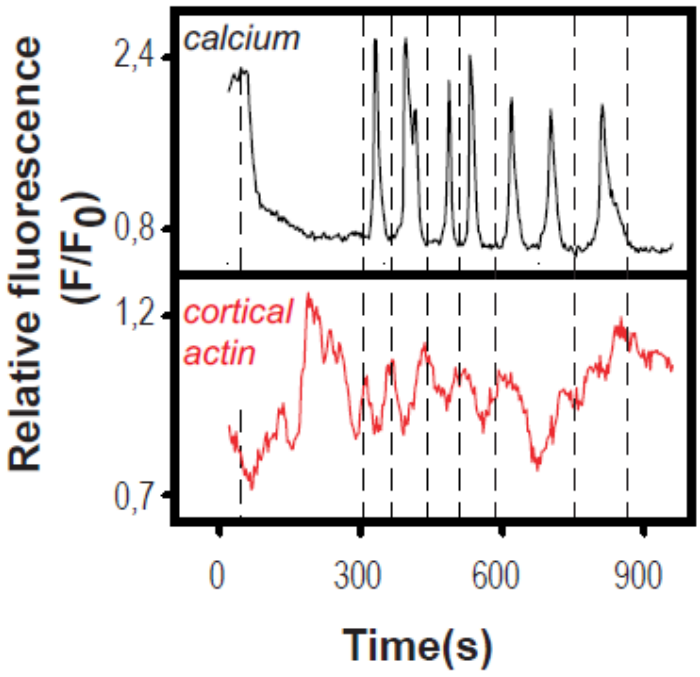

Figure 18. Calcium oscillation induces fluoctuation in the amount of cortical actin, with maximum peaks where calcium produces its minimum. *Relative fluorescence of cortical*

actin (red line) and calcium (black line) as a function of time in mCherry-actin expressing A20 cells loaded with fluo-4 AM exposed to EGTA. The amount of cortical actin was determined by defining a region of interest around the plasma membrane for every image individually.

We quantified the interdependency of Ca^{2+} and actin signals by determining the normalized mutual information (nMI), a number that measures the interdependence of two variables in the [0,1] interval.

Entropy based synchronization indices were calculated on noise reduced data. Very low frequency oscillations were eliminated. Normalized mutual information was determined within 15 consecutive, non-overlapping 52 seconds length windows.

High synchronicity indices were found of about 0.7 for the experimental data (Fig. 18.) that proved to be highly significant in comparison with the phase permuted asynchronous surrogate data (p=0,000001) and differed much less from fully synchronized surrogates (p= 0,0013). Significance was calculated with the Neymann's smooth statistics making it data driven by the Schwartz's selection rule. *(nMI calculation and statistical analysis was performed by Nora Szilagyi).*

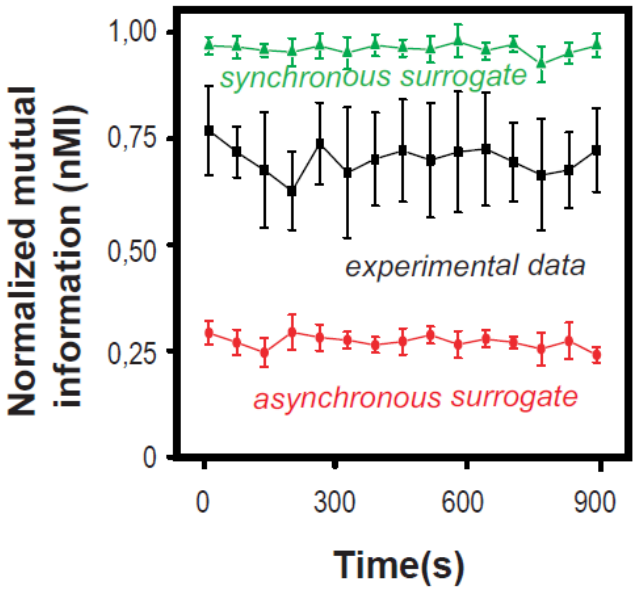

Figure 19. Calcium oscillation induces fluctuation in the amount of cortical actin, with maximum peaks where calcium produces its minimum. *Relative fluorescence of cortical actin (red line) and calcium (black line) as a function of time in mCherry-actin expressing*

A20 cells loaded with fluo-4 AM exposed to EGTA. The amount of cortical actin was determined by defining a region of interest around the plasma membrane for every image individually.

Thus the polymerization and decay of actin filaments are strongly coupled to the local calcium concentration and show a phase locking during oscillations *in vivo*.

4.8 Immunological synapse formation is a suitable physiologic process to test the predictions of our hypothesis of calcium-regulated actin dynamics (CRAD)

The previous experiments pronounce that the density of the actin cytoskeleton can be modified by lowering or raising the cytoplasmic Ca^{2+} concentration artificially and that the local amount of filamentary actin is in an inverse correlation with the Ca^{2+} concentration. We asked the question whether cells themselves use this regulatory mechanism to control site-specific actin rearrangements.

Immunological synapses (IS) are surface interactions between antigen specific T-lymphocytes and antigen presenting cells or target cells displaying the appropriate antigen (MHC/peptide complex) on their surface. An early and requisite step in synapse formation is a massive actin polymerization on the T-cell side [106] that has a key role in maintaining cell-cell adhesion by organizing surface integrins at the periphery of the synapse [107].

Our hypothesis of calcium-regulated actin dynamics (CRAD) predicts the maintenance of low local calcium concentration compared to the surrounding as a prerequisite for actin polymerization at the IS site. To test our prediction, we investigated the distribution of filamentary actin and the calcium sequestering machinery during synapse formation.

4.9 T cells form a ring-like actin network at the APC/T-cell contact zone when IS is formed

A20 cells were grown on 8 well chamber plates ($4x10^4$/chamber) overnight, then washed and pulsed with the antigen HA317 – 341. Cloned IP12-7 helper T cells, specific for the influenza peptide antigen HA317 – 341[95] and adherent A20 cells used as APCs were coincubated on the plates for 15 minutes, then washed, fixed with 3% paraformaldehyde, and stained with anti-IgG-Alexa647 for the B cell receptor to distinguish APCs. After primary staining, cells were

permeabilized with 0,1% Triton-X and stained with Phalloidin-Alexa488 for filamentous actin.

3D confocal images showed (Fig. 19.) the formation of the dense actin meshwork at the cell-cell contact zone on the T-cell side. Rotating the 3-dimensional pictures revealed the ring-like structure of the meshwork.

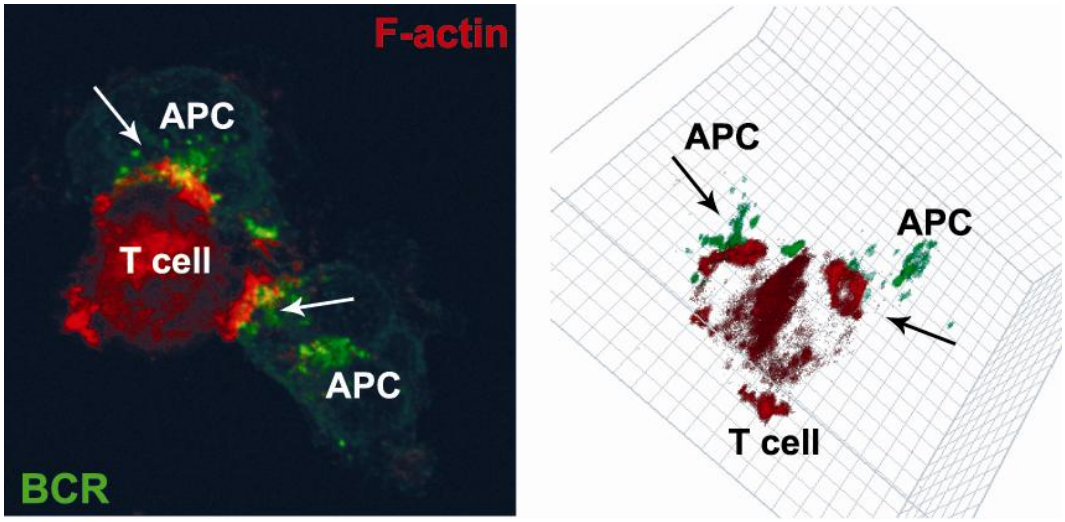

Figure 20. Ring-shaped actin network at the IS-site. *3D fluorescent images (left side) and 3D surface images rotated in space (right side) of immunological synapses between A20 cells functioning as APCs pulsed with the antigen HA317 – 341 and IP12-7 helper T cells specific for the antigen stained for filamentous-actin (red) and IgG (green).*

4.10 The calcium sequestering machinery translocates to the IS-site during synapse formation

One key step in T cell activation is the global rise in cytoplasmic calcium concentration upon interaction with the APC [108]. In order to maintain lower calcium concentration at IS-site as predicted by the CRAD model, calcium needs to be sequestered locally. The Na^{+}/Ca^{2+} exchangers (NCX) and mitochondrial uniporters are known to have the highest transport rates for calcium [66]. The redistribution of mitochondria to the close vicinity of the IS, followed by calcium reuptake was shown to be prerequisite in synapse formation between T cells and anti-CD3 loaded microbeads [109]. We assumed that mitochondrial translocation and calcium reuptake could also occur during synapse formation between APCs and T cells.

To decide whether this was the case IP12-7 helper T cells were stained with MitoTracker and conincubated with adherent A20 cells used as APCs and previously pulsed with the antigen HA317 – 341. After synapse formation cells were fixed and stained for filamentary actin. We observed a marked enrichment of mitochondria in the vicinity of the IS in both APCs and T cells, and a mitochondria-rich domain in the actin-rich uropodium of T cells. Rotating the 3 dimensional images revealed the ring-shaped distribution of mitochondria at the IS (Fig. 20.) anticipating the later forming actin ring.

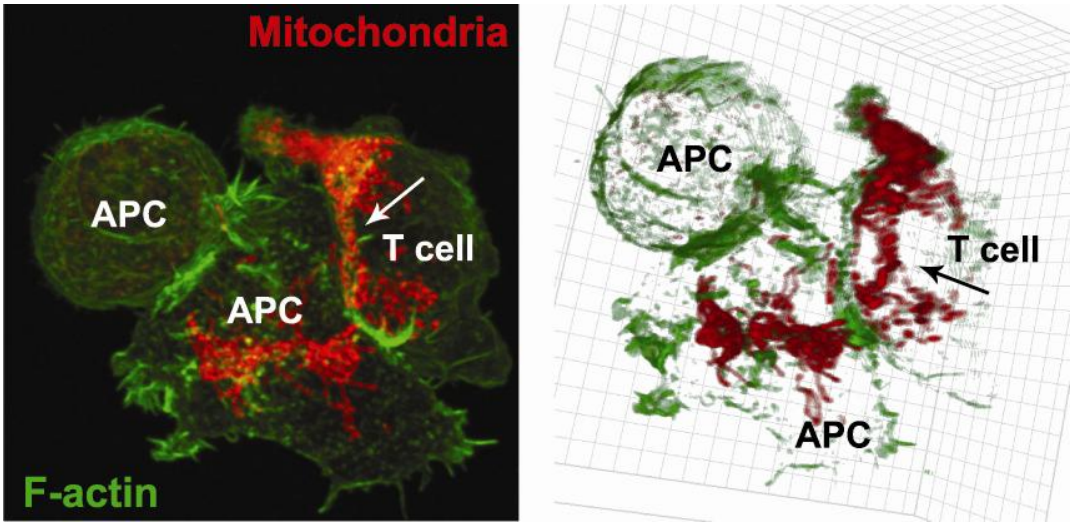

Figure 21. Ring-shaped mitochondria network at the IS-site. *3D fluorescent images (left side) and 3D surface images rotated in space (right side) of immunological synapses between A20 cells functioning as APCs pulsed with the antigen HA317 – 341 and IP12-7 helper T cells specific for the antigen stained for filamentous-actin (green) and mitochondria (red).*

We carried out the same experiment for NCX and found a well recognizable enrichment of the exchanger at the IS site (Fig. 21.).

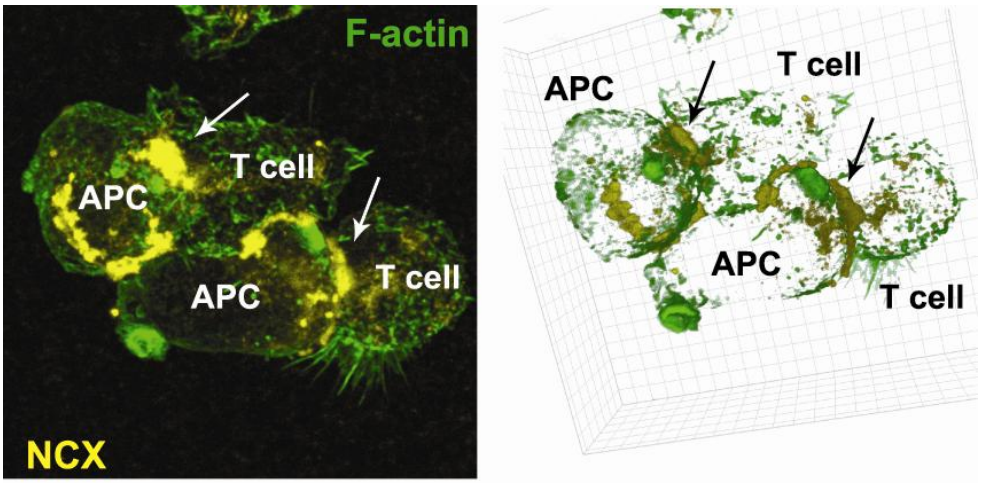

Figure 22. Ring-shaped mitochondria network at the IS-site. *3D fluorescent images (left side) and 3D surface images rotated in space (right side) of immunological synapses between A20 cells functioning as APCs pulsed with the antigen HA317 – 341 and IP12-7 helper T cells specific for the antigen stained for filamentous-actin (green) and NCX (yellow).*

Thus the calcium sequestering machinery concentrates at the IS, during synapse formation.

4.11 T cells maintain lower calcium at the IS-site where actin polymerization will occur

To decide whether T-cells indeed maintain lower calcium in the vicinity of the forming actin ring calcium imaging was performed. T cells were loaded with the calcium indicator Fluo4-AM and put together with adherent APCs pulsed with the antigen. Imaging was performed by confocal microscopy.

As soon as T cells met the APCs, they began to scan for the appropriate antigen. A calcium release event indicated when the TCR/MHCII/peptide interaction was supposed to occur, which was followed by the slowing down of the T cell. Cytoplasmic calcium at this stage showed an asymmetric distribution with lower calcium concentrations at the IS site.

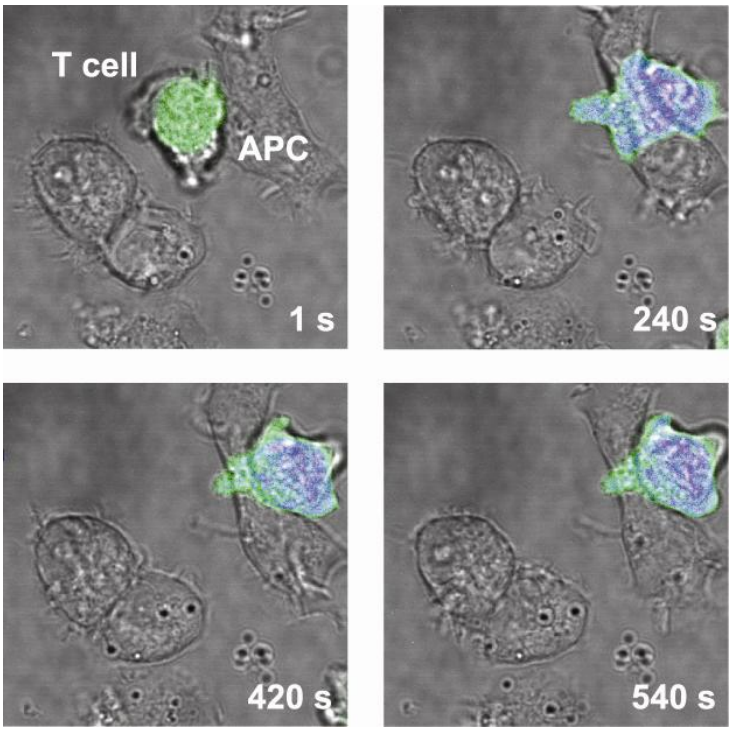

Figure 23. Calcium shows an asymmetric distribution in T cells during immunological synapse formation. *Time-lapse of a calcium imaging experiment performed during synapse formation between an adherent APC and a helper T cell obtained by confocal microscopy.*

To get a better view, we optimized the color mapping based on a spatial informatics motivated algorithm. This method revealed (Fig. 4D) a well recognizable low calcium area at the APC/T-cell contact zone with the same ring shape as seen in the case of mitochondria or in the later forming actin ring.

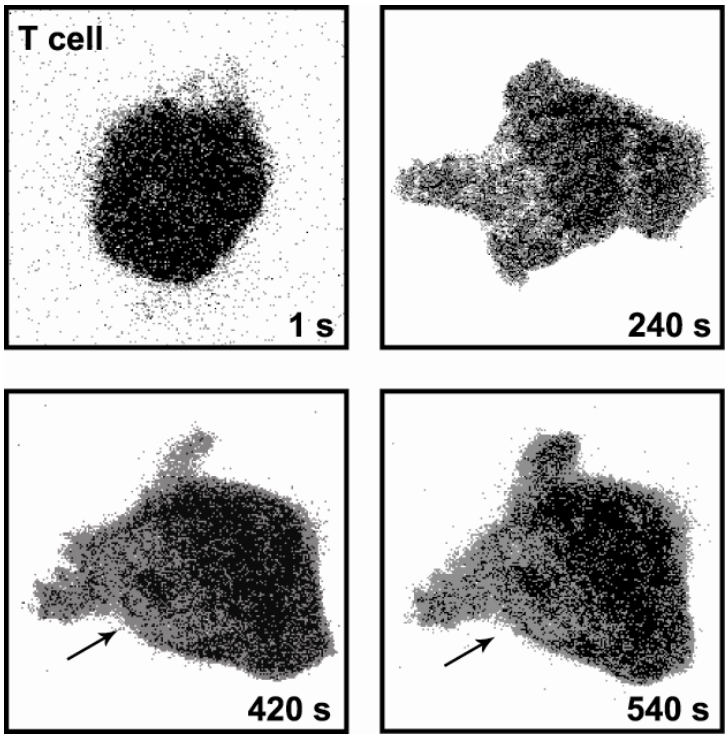

Figure 24. Cytoplasmic calcium forms a ring-shape calcium low area at the at the IS-site in T cells. *Color mapping (white: low [Ca^{2+}]; grey: medium [Ca^{2+}]; black: high [Ca^{2+}]) of the experiment described in Fig. 23. based on a median based search algorithm. Black arrows indicate the ring-like low calcium level microdomain at the IS-site.*

The model of calcium regulated actin dynamics interpret the ring-shaped low calcium area as the necessary driving force for actin polymerization maintained by the calcium sequestering machinery.

4.12 The CRAD model and its predictions

Studying the immunological synapse formation allowed us to show in a physiologic system, that cells lower local calcium concentration at sites of actin growth.

The CRAD model suggests a major role for calcium in the decision between polymerization versus depolymerization of actin filaments. It links the spatio-temporal control of actin reorganization to elementary calcium release and sequestering events in the cell. The CRAD model predicts that at cell-sites with locally higher calcium concentration actin

depolimerization and thus loss of structure will dominate, while at sites with locally lower calcium concentration actin-growth and gain of structure will govern.

How might high calcium-level in the cell induce actin depolimerization? Calcium regulates the activity of several molecules involved in actin rearrangements. Actin cross-linking proteins such as fodrin [110] and myristoylated, alanine-rich C kinase substrate (MARCKS) [111] exhibit decreased cross-linking activity in the presence of high calcium. Actin severing proteins [24] gelsolin, villin and scinderin can become active upon calcium release and enhance the disassembly of actin filaments. Calcium-dependent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC) can prevent actin growth and shorten the half-life of filaments [16]. Members of the calcium-regulated proteases, calpains can cleave proteins, such as ezrin and radixin that couple the actin cytoskeleton to the plasma membrane upon calcium release [112]. Cleavage of actin filaments, uncoupling the actin cytoskeleton from the plasma membrane and inhibition of actin growth and actin bundling, these could be the direct effects how high intracellular calcium targets the actin cytoskeleton.

Then again, why is actin-polymerization favored in cytoplasmic regions where Ca^{2+} concentration is lower as the surrounding? A possible explanation could be that as a result of calcium-induced actin depolymerization at cell-sites of high calcium levels, monomer G-actin concentration increases in the cytoplasm, which on the other hand is the main driving force of filamentary actin growth [19], and promotes actin growth in calcium low cytoplasmic regions.

The CRAD model explains observations such as why circulating lymphocytes lose their microvilli upon integrin-binding at the endothelial wall [34], and interprets the phenomenon as the result of integrin-binding induced huge elevation in the cytoplasmic Ca^{2+} concentration, or why F-actin breakdown is preceded by a calcium release event, in spermatozoa during acrosome reaction, and can also be induced by calcium ionophores [113]. Migrating cells maintain a calcium gradient in their cytoplasm with the lowest Ca^{2+} concentration in the leading lamellipodium [114]. The CRAD model explains this in accordance with the current model of motility [19]. Low calcium concentration in the leading edge allows actin polymerization that produces the driving force to push forward the plasma membrane, while sites of the cytoplasm with higher calcium-level ensure G-actin supply through calcium-induced actin depolymerization.

The CRAD model predicts a major role for the spatio-termporal distribution of calcium channels and pumps in the regulation of actin rearrangements, and expects that at cell-sites where actin growth is initiated calcium channels are excluded, while calcium pumps become recruited, and where actin decay happens the opposite tendency occurs.

5. Results II.: Grb2 associated binder 2 couples B-cell receptor to cell survival

Regulated actin rearrangements are required for proper lymphocyte function. In B cells encounter of the antigen, subsequent translocation of the BCR to the raft compartment and microluster formation require an early depolimerization and a subsequent repolymeriztion phase of actin filaments[39]. We have seen that calcium is a potent regulator of these procedures, its explicit role however remain to be elucidated.

Once BCR translocation occurred to the raft compartment and microclusters are formed downstream signaling takes over the place to decide between live or die. B cells are programmed to die, which means in case they do not encounter enough survival signals, they undergo apoptosis. In B cells the BCR is the main source of survival signals. B cell clones capable to recognize foreign antigens through their B cell receptors are able to escape apoptosis and undergo proliferation with subsequent maturation to plasma cells or memory B cells.

One key step in survival is the downregulation of pro-apoptotic Bcl-2 family members through activation of the PI3K/Akt survival pathways. PI3K becomes recruited to the plasma membrane, and catalyzes the synthesis of PIP3. PIP3 in the membrane allows binding of the serine/threonine kinase Akt and its upstream kinases PDPK1 and PDPK2. PDPK activated Akt inhibits function of pro-apoptotic Bcl-2 molecules.

The first step of the pathway, recruitment of the PI3K molecule to the signalosome remains controversial in B cells. CD19 was suggested to be the adaptor of PI3K in B cells[74], however $CD19^{-/-}$ mice still present Akt phosphorylation upon BCR engagement[75].

Gab family members and especially Gab2 was suggested to provide an alternative way for PI3K recruitment.

5.1 Gab2 is expressed in B cells and becomes phosphorylated upon BCR crosslinking to recruit PI3K and SHP2

Our goal was to explore the role of Gab2 in B cell survival. To do so, first we have defined its binding partners in 38C13 cells. Cell lysates were prepared of control and anti-IgM treated cells, Gab2 was immunoprecipitated with an anti-Gab2 antibody and transferred to a nitrocellulose membrane subsequently probed with different antibodies.

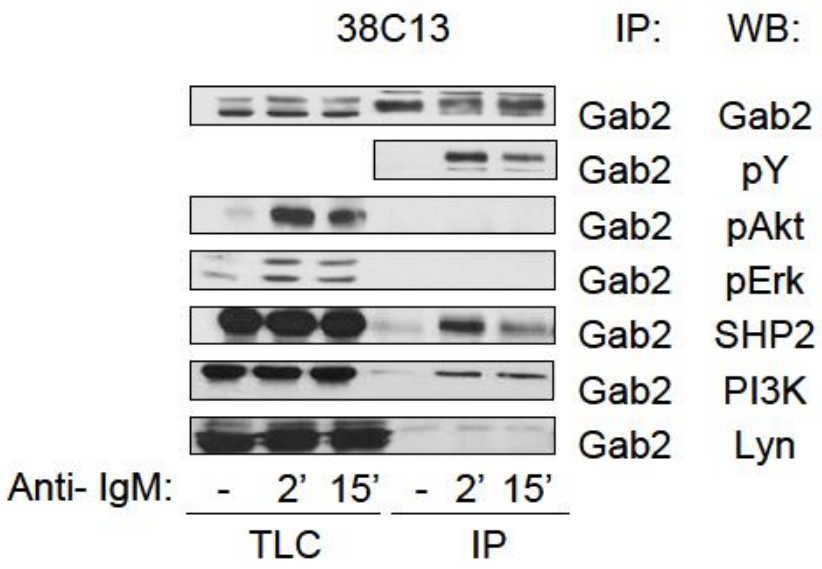

Figure 25. Gab2 becomes phosphorylated and recruits PI3K and SHP2 upon BCR engagement. *Total lysate controls (TLC) and immunoprecipitates (IP) of 38C13 cells stimulated with anti-IgM for 0, 2 or 15 minutes. Gab2 was isolated from the TLC fraction by immunoprcipitation with specific antibody. The cell lysate and immunprecipitate samples were subjected to electrophoresis followed by immunoblotting with anti-phospho-tyrosine (pY), anti-pAkt, anti-pErk, anti-SHP2, anti-p85 regulatory subunit of PI3K, anti-Lyn and anti-Gab2.*

We found that Gab2 is expressed in B cells and upon BCR cross-linking it becomes phosphorylated and recruits binding partners PI3K, and SHP2. A low level of constitutive Lyn association was detected, while pAkt and pErk were not present in the complex.

5.2 Gab2 phosphorylation is only partially dependent on PH domain mediated PIP3 binding

It was suggested that PH domain of Gab2 binds PIP3 in the cell membrane and that this binding is required for its r ıent[81]. Therefore, we tested whether inhibiting PIP3 production upon BCR signaling by the specific PI3K inhibitor LY294002 has any effect on Gab2 phosphorylation. Cell lysates were prepared of anti-Ig stimulated and control 38C13, A20 cell lines and primary spleen B cells pre-incubated with LY294002 if indicated. Gab2 immunoprecipitates were probed with anti-pY.

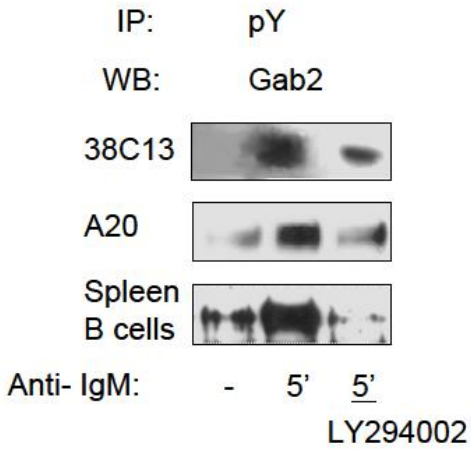

Figure 26. Gab2 phosphorylation is only partially dependent on PIP3 production in 38C13 and A20 B cell lines. *38C13, A20 or spleen B cells were pre-incubated with LY294002 if indicated. Cell lysate samples of stimulated and control cells were immunoprecipitated by a phospho-tyrosine specific antibody, then subjected to immunoblot analysis with anti-Gab2 antibody.*

We found that Gab2 phosphorylation is fully inhibited in spleen B cells pre-incubated with LY294002, however in 38C13 and A20 cell lines phosphorylation still occurred when PI3K function was inhibited, however it was considerably lower as in control cells.
Thus, we concluded that an alternative, PH-domain independent recruitment of Gab2 might exist in these B cell lines.

5.3 Phosphorylation of PI3K binding site 452Tyr is PH-domain independent in 38C13 and A20 cell lines

PI3K binding to Gab2 occurs via the recruitment of the regulatory subunit p85. Three binding sites exist for p85 in mammalian Gab2; one of them is the 452Tyr. Phosphorylation of this binding site allows recruitment of PI3K to the signalosome through Gab2 and thereby could function to enhance Akt phosphorylation and cell survival.
We investigated the phosphorylation of 452Tyr and in its dependence of PH domain-PIP3 interaction by stimulating control and LY294002 treated 38C13 and A20 cells as well as primary spleen B cells with anti-Ig and probing cell lysates with an antibody specific for the phosphorylated form 452Tyr.

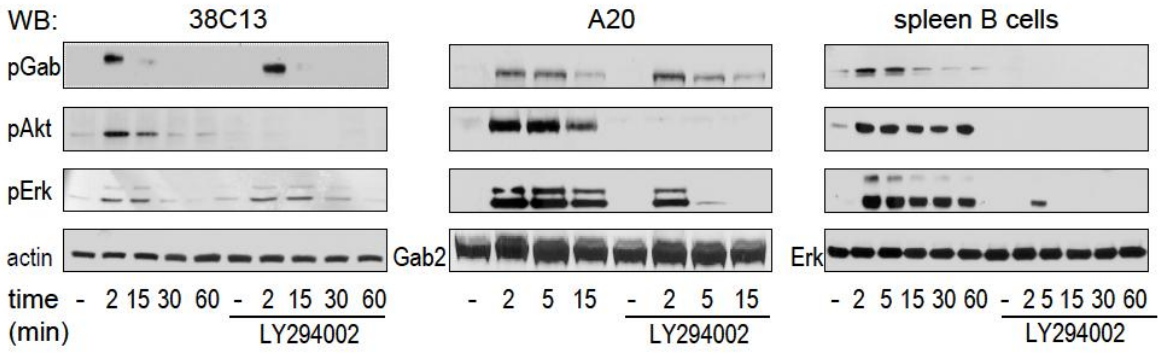

Figure 27. Phosphorylation of PI3K binding-site 452Tyr does not depend on PIP3 production in 38C13 and A20 cell lines. *38C13, A20 or spleen B cells were pre-incubated with LY294002 if indicated. Cell lysate samples of stimulated (anti-Ig) and control cells were subjected to immunoblot analysis with anti-pGab2 (p452Tyr), pAkt and pErk antibodies. Loading controls were actin, Gab2 or Erk as indicated in the figure.*

We found that LY294002 inhibitor had no effect on the phosphorylation of 452Tyr in 38C13 and A20 cells, but fully inhibited phosphorylation in the case of primary B cells. Disappearance of the pAkt signal upon LY294002 treatment shows, that inhibition of PI3K was fully functioning in all three cell types. Partial or no inhibition of the Erk signal is in accordance with the current findings, that Erk phosphorylation is only partially or none dependent on PI3K function, and suggests that inhibition was specific.

Thus we concluded that phosphorylation of the PI3K binding-site is independent of PIP3 amount in the plasma membrane in the case of the investigated cell lines, and thus recruitment independent of the PH-domain must exist in these cells.

5.4 Recruitment of SHP2 to Gab2 is fully dependent, while recruitment of PI3K is only partially dependent on the PIP3 amount in the plasma membrane

Since inhibition of PI3K activity in A20 and 38C13 cell lines had no effect on Tyr452 phosphorylation, consequently we asked the question whether the inhibitor affects the recruitment of Gab2 binding partners PI3K and SHP2.

Therefore cell lysates were prepared of anti-Ig stimulated and control 38C13 and A20 cells pre-incubated with LY294002 if indicated. Gab2 immunoprecipitates were probed with anti-PI3K, anti-SHP2.

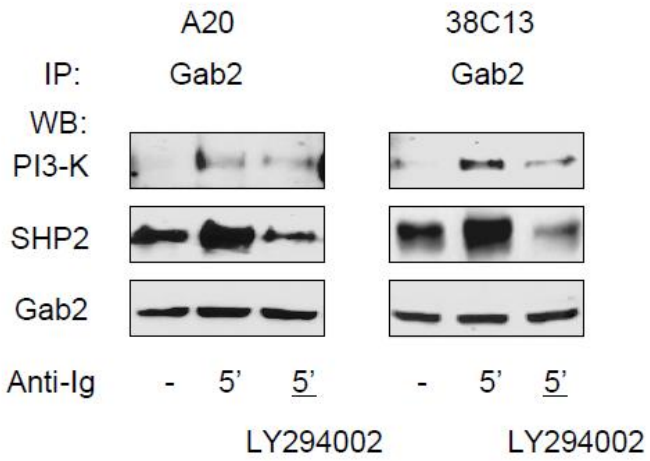

Figure 28. PI3K catalytic activity is a prerequisite of SHP2 recruitment, while partially dispensable for PI3K recruitment to the Gab2 complex. *38C13 and A20 cells were pre-incubated with LY294002 if indicated. Gab2 was immunoprecipitated from cell lysates of stimulated (anti-Ig) and control cells. Immunoprecipitates were subjected to immunoblot analysis with anti-PI3K and anti-SHP2 antibodies.*

We found that in the absence of PI3K activity the amount Gab2 bound PI3K was only partially reduced, while the level of bound SHP2 decreased below the level of control.
These data suggests that PIP3 synthesis and thereby PH-domain binding of Gab2 is necessary for SHP2 recruitment, but is dispensable for PI3K recruitment.

5.5 An alternative pathway of Gab2 recruitment to the signalosome occurs via the Igα/Igβ complex

PI3K-activity is necessary for the production of PIP3 in the membrane, and thereby for PH-domain mediated protein recruitment to the signalosome. In case of Gab2 however both PI3K binding-site phosphorylation and PI3K recruitment proved to be partially independent of PI3K-activity in A20 and 38C13 cell lines. Gab adaptor proteins were shown to have different types of recruitment depending on cell type and signal. Recruitment to the activated receptor can occur via direct or indirect mechanisms.

We assumed that in case of B cells the B cell receptor complex itself may function to recruit Gab2 to the signalosome. To decide whether this was the case cell lysates were prepared of anti-Ig stimulated and control A20 and primary spleen B cells pre-incubated with LY294002 or Syk inhibitor, if indicated. Igα and Igβ immunoprecipitates were probed with anti-Gab2.

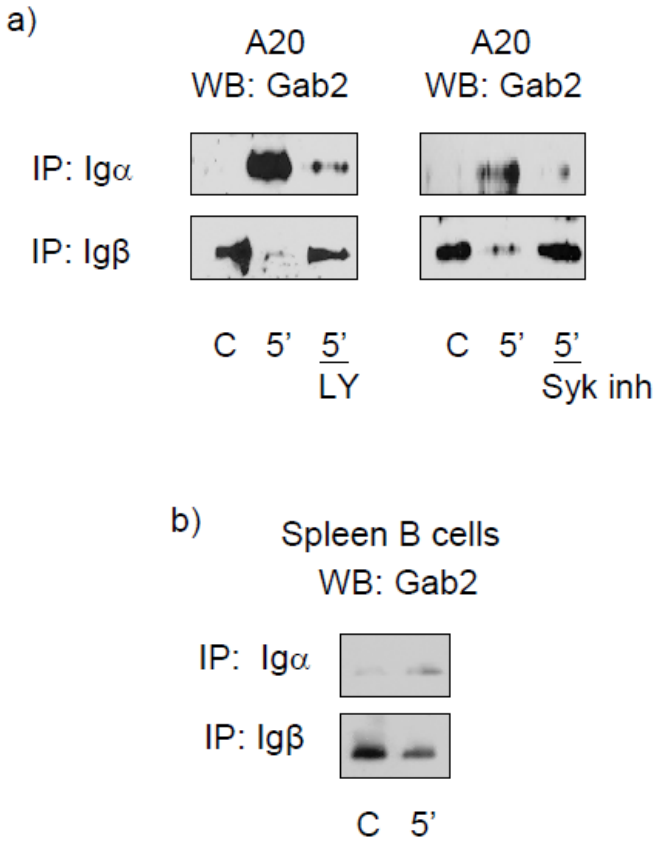

Figure 29. Igα and Igβ serve as docking sites for Gab2. ***(a)*** *A20 cells or* ***(b)*** *primary spleen B cells were pre-incubated with LY294002 or Syk inhibitor if indicated. Igα and Igβ was immunoprecipitated from cell lysates of stimulated (anti-Ig) and control cells. Immunoprecipitates were subjected to immunoblot analysis with anti-Gab2 antibody.*

Results revealed a constitutive binding of Gab2 to Igβ in resting B cells. Upon BCR cross-linking Gab2 became released from Igβ and translocated to Igα. Interestingly both PI3K inhibitor and Syk inhibitor could inhibit this translocation.

Thus beside recruitment via the PH-domain, a fraction of Gab2 is recruited via the Igα/Igβ signal transducing units to the signalosome.

5.6 Gab2 is phosphorylated by both Lyn and Syk

Several kinasese were reported to participate in the phosphorylation of Gab2 depending on the cell type. We used two approaches to address this question in B cells. First Gab2 phosphorylation was compared in B cells isolated from $Lyn^{-/-}$, $Hck^{-/-}$, $Fgr^{-/-}$ triple knock out[115] and wild type C57Bl/6 mice. B cells only express Lyn out of these three kinases, thereby any effect experienced can be interpreted as a result of the lacking Lyn kinase.

Cell lysates were prepared of triple knock out and wild type mice B cells, stimulated with anti-IgM, and pretreated with Syk inhibitor if indicated. Gab2 was immunoprecipitated and probed for phospho-tyrosine, PI3K and SHP2.

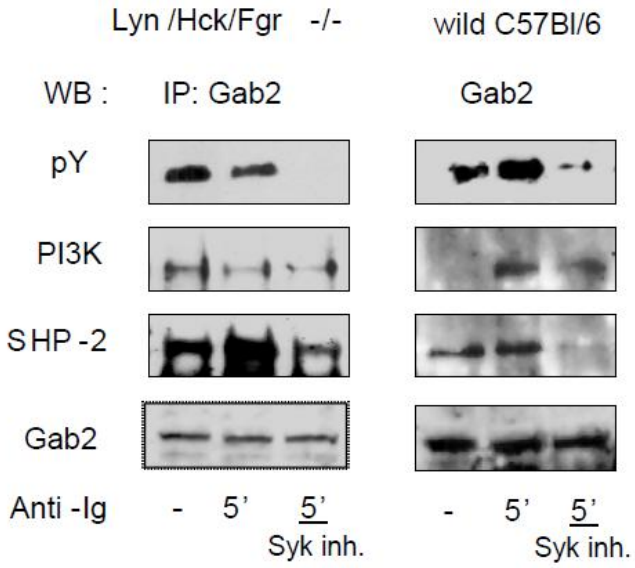

Figure 30. Lyn is required for PI3K binding site phosphorylation, while Syk for the SHP2 binding-site. *Cell lysates were prepared of triple knock out and wild type mice B cells, stimulated with anti-IgM, and pretreated with Syk inhibitor if indicated. Gab2 was immunoprecipitated and probed for phospho-tyrosine, PI3K and SHP2.*

We observed a constitutive phosphorylation of Gab2 in triple knockout mice. These could be a result of Lyn absence. Lyn is known to have a negative regulatory role in B cells besides initiating B cell signaling. SHP2 recruitment in these cells was intact, while PI3K failed to become recruited. Inhibition of Syk function made SHP2 recruitment diminish.

Both PI3K and SHP2 became recruited to Gab2 in wild type mice B cells. Inhibition of Syk opposed SHP2 recruitment but did not affect PI3K recruitment.

As a summary, these data suggests that Syk function is necessary for the phosphorylation of SHP2 binding-site, while Lyn is required for PI3K binding-site phosphorylation.

5.7 Overexpression of WT Gab2 enhances Akt phosphorylation and supports BCR mediated cell survival

The previous results have shown that Gab2 is a potent adaptor of PI3K in B cells. To test its contributions to the PI3K/Akt pathway and thereby to cell survival wild type (WT) and PH domain deficient (ΔPH) Gab2 was overexpressed in A20 cells. The Gab2 sequence was inserted into a pMSCV-IRES-GFP vector. Cells were transduced retrovirally. Positive cells were sorted with a cell-sorter.

Expression of the three vectors was comparable, and did not alter surface IgG expression. To show a functional effect, cells were stimulated with anti-IgG Akt and Erk phosphorylation was determined with western blotting.

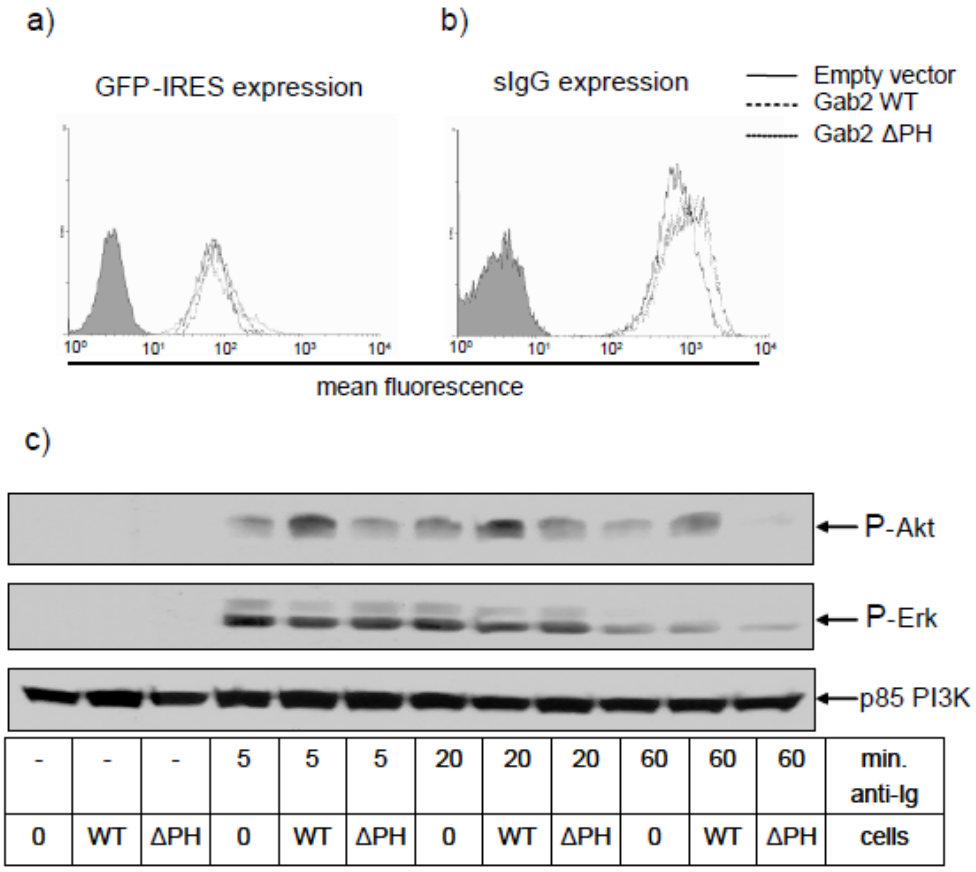

Figure 31. Overexpression of WT Gab2 enhances Akt phosphorylation upon BCR stimulus. *(A) Expression of the IRES-GFP sequence in retrovirally transduced A20 cells obtained by*

flow-cytometry. (B) Expression of surface-IgG obtained by flow-cytometry. (C) A20 cells expressing MSCV-IRES-GFP empty vector, WT Gab2, or PH domain deficient Gab2 (ΔPH) were stimulated with anti-IgG. Cell lysates were prepared, subjected to immunoblotting and probed with anti-pAkt and anti-pErk antibodies.

Cells overexpressing wild type Gab2 showed a marked enhancement in Akt phosphorylation, while Erk phoshporylation remained unaffected. Cells expressing PH domain deficient Gab2 had an Akt kinetic similar to control cells.

Enhanced Akt phosphorylation in Gab2 overexpressing cells suggests a phenotype less sensitive to undergo apoptosis. To test this prediction we tested Fas-induced apoptosis in transfected cells.

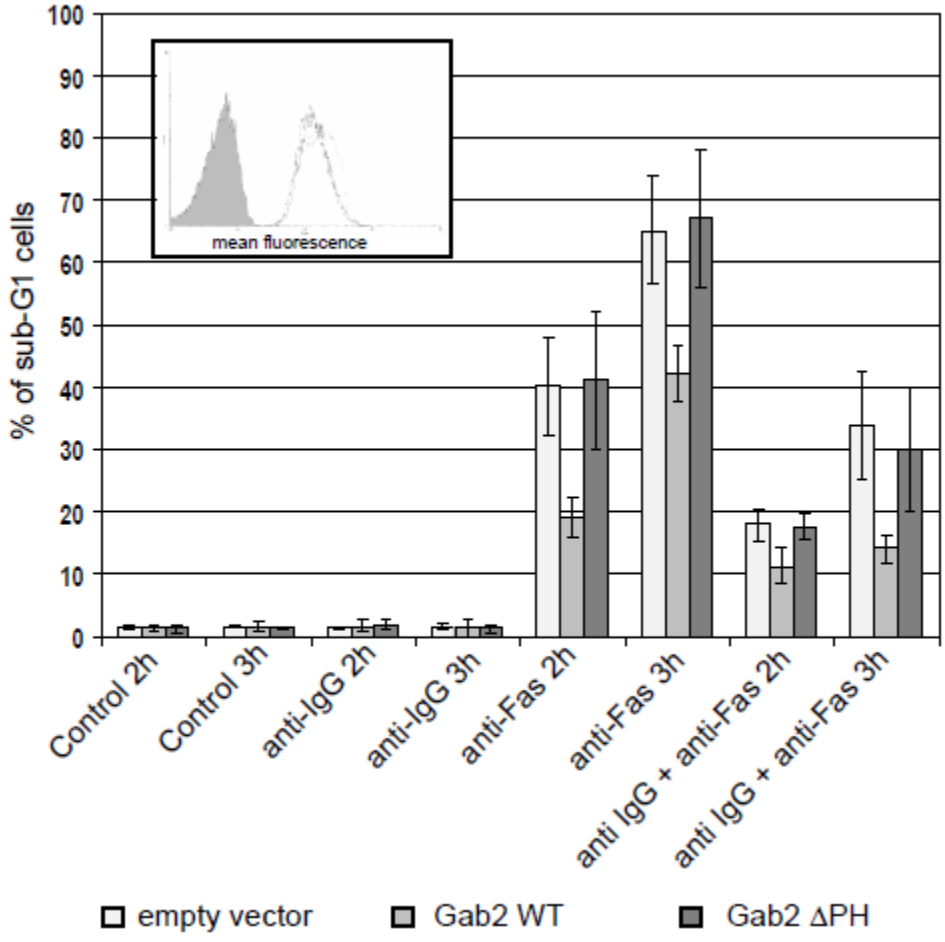

Figure 32. Overexpression of WT Gab2 protects cells from Fas induced cell death and supports BCR mediated cell survival. *A20 cells overexpressing WT Gab2, PH domain deficient Gab2, or only endogenous Gab2 were treated with 100 ng/ml JO2 anti-Fas antibody. 10 minutes prior to anti-Fas treatment cells were treated with 5 μg/ml anti-IgG if*

indicated. Fas expression of the transfectants (insert in the upper left) was compared by flow-cytometry.

Transfectant cells expressed CD95/Fas, the prototypic death receptor in similar amounts. Cells were treated with anti-Fas, the sub-G1 cell population, which corresponds to apoptotic cells, was determined by flow-cytometry. Three hours treatment resulted in an apoptotic death rate of about 60% in cells expressing the endogenous Gab2 only, while overexpression of WT Gab2 decreased the percentage of apoptotic cells by about 30-50%. PH domain deficient Gab2 transfectants have shown a similar death rate as control cells.

We tested how BCR cross-linking affects Fas-induced death rate in WT Gab2 and ΔPH Gab2 expressing cells. Anti-IgG stimulation had a protective effect on all cell types. 20-30% of cells died when expressing only endogenous Gab2, while overexpression of WT Gab2 decreased this number to only 10-15% of cell death. ΔPH Gab2 expressing cells again showed a similar death rate as control cells.

Thus, we could show that Gab2 is not just a potent adaptor of PI3K in B cells, but is a part of the PI3K/Akt survival pathway, and raises the threshold for apoptosis in B cells.

5.8 Gab2 in B cell signaling

Based on our results we could picture the schematic function and role of Gab2 in B cell signaling. In resting cells a fraction of Gab2 is attached directly or indirectly to the BCR complex via an interaction with the signal transducing unit Igβ. Antigen binding induces dephosphorylation and activation of src kinase Lyn, which subsequently phosphorylates Gab2 on the PI3K binding site. Recruitment of PI3K is the early step in the Gab2 complex formation, since neither PI3K nor Syk activity is necessary for its occurrence. A still unknown process induces the translocation of the Gab2/PI3K complex from the Igβ to the Igα chain, where Gab2 comes into the vicinity of Syk kinase. Syk phosphorylates the SHP2 binding-site and induces the recruitment of SHP2. The role of SHP2-Gab2 interaction remains to be elucidated, since unlike in other cell types, overexpression of Gab2 did not affect Erk phosphorylation in B cells.

The Gab2-PI3K interaction brings PI3K into proximal position with the plasma membrane and catalyzes the synthesis of PIP3 in the membrane. PIP3 allows Akt and new Gab2 molecules to bind the membrane via their PH domains and to become phosphorylated. Phosphorylated Akt suppresses apoptotic procedures and supports the rescue of B cells from apoptosis.

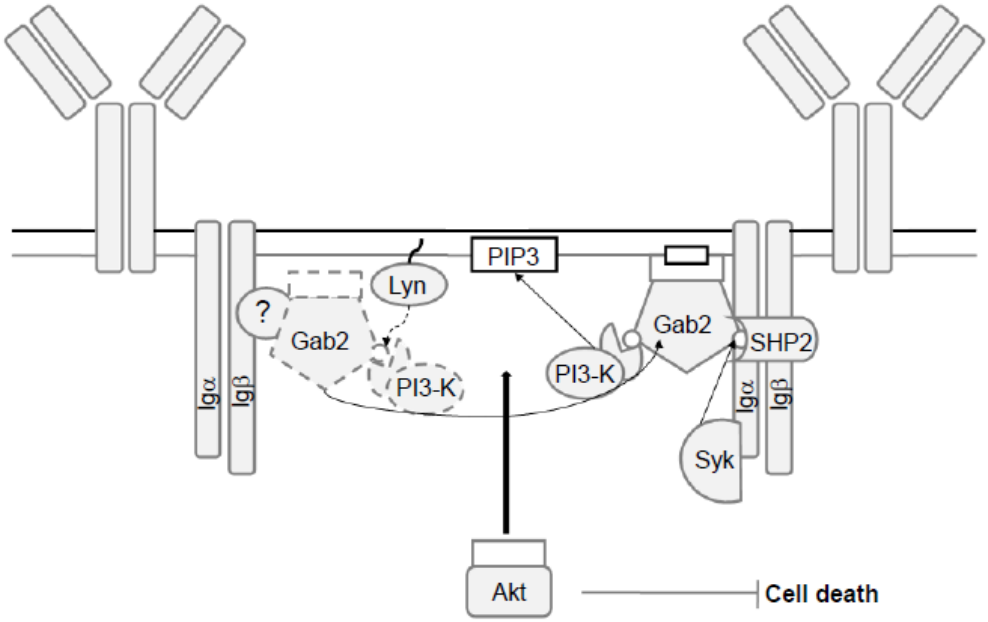

Figure 33. The schematic model for the role of Gab2 in B cell receptor signaling.

6. Discussion: relevance of the CRAD model and the Gab2/PI3K/Akt pathway in B cell function and activation

6.1 A disadvantage of BCR diversity

B cells encode a large a variety of Ig genes. The stochastic nature of Ig gene rearrangement allows the assembly of at least 10^{11} different Igs. Somatic hypermutation further extends the available repertoire of specificity. This large variety ensures the reactivity to a wide array of antigens. The attendant disadvantage of such a great diversity is that it becomes unavoidable to generate autoreactive clones; B cells that express self-antigen recognizing BCRs. Signal transduction and signal strength of the BCR mediate the elimination of self-reactive clones at a series of checkpoints during B cell maturation and differentiation[116]. The outcome of BCR engagement during these processes is determined by a composite of factors, which include the affinity of the BCR for Ag, the capability of the cell to grab the antigen, the concentration and valency of the Ag, and a cohort of coreceptors, signaling molecules and adaptor molecules that modulate the strength of the BCR signal[117]. The lack of success in eliminating autoreactive clones can have its price. Autoimmune diseases, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) can emerge from conditions that lead to aberrant selection of self-reactive B cells.

6.2 Negative selection

There is a consensus in the existence of negative selection during B cell maturation. Immature B cells expressing autoreactive BCRs are barred from the entry into the mature immunocompetent B cell repertoire. In B cells expressing high affinity autoreactive B cell receptors this is accomplished by the induction of receptor editing through the reactivation of the recombinase machinery in order to generate a non-autoreactive phenotype[118]. However, if after receptor editing the autoreactive phenotype is still obtained, immature B cells will undergo apoptosis through a process, which is mediated by the B cell receptor[119]. Autoreactive B cells with lower affinity for self-antigens are rendered anergic[120] or escape negative selection.

It remains to be resolved how BCR ligation can have such an opposing effect in immature B cells and drive negative selection, while it can cause activation of mature B cells. The differences might be caused by distinct characteristics of signal transduction in the two

maturation stages. Subtle differences found were more sustained calcium level upon BCR engagement in immature B cells and enhanced expression of BLNK, Btk and PLCγ[121] and that BCRs of immature B cells are unable to translocate to lipid rafts[122].

6.3 Microcluster formation and the CRAD model

Coherence of the lipid raft microdomain largely depends on its linkage to the actin cytoskeleton. BCR stimulus induces an initial depolymerization phase in the actin cytoskeleton, which is followed by a repolymerization phase[39]. Gupta et al. have shown that during this depolymerization phase the ezrin mediated linkage between rafts and cytoskeleton diminished and only reappeared when BCR had already translocated to the raft compartment[38]. The CRAD model predicts that high expression of calcium signaling components such as BLNK, Btk and PLCγ and sustained calcium signal counteracts actin repolymerization. This in turn could explain why the BCR is unable to form stable clusters with the raft compartments in immature cells. However, further investigations are necessary to prove such a connection.

6.4 Negative selection and Gab2

Another difference found between mature and immature B cells was that cross-linking the BCR did not cause the phosphorylation of adaptor molecule Gab2 in immature B cells[123].
The results discussed in section 5. are in accordance with this finding. We have demonstrated that Gab2 was a part of the PI3K/Akt survival pathway in BCR signaling and served as an additional adaptor for PI3K beside CD19. We have shown that overexpression of Gab2 had a marked effect on Akt phosphorylation and supported BCR mediated survival upon Fas induced apoptosis. The lack of Gab2 to become phosphorylated in immature B cells, may cause reduced PI3K activity, that would explain reduced Akt phosphorylation[124] in these cells shown by Kovesdi et al. The missing function of Gab2 could thereby be one reason of elevated sensitivity for apoptosis in immature B cells.

6.5 Secondary lymphoid organs (SLOs) provide the milieu for antigen encounter

Lymphocytes, which have survived negative selection and gathered enough survival signals, enter the mature B cell pool. Their goal now is to gather antigen stimulus, or otherwise they die in only few days[125] and become replaced by the $2x10^7$ of newly formed B cells every

day[126] (data refers to measurements performed in young mice). Owing to their great antigenic diversity the event of stochastic spontaneous interaction between a B cell and the appropriate antigen would be unlikely. To maximize the probability of such an interaction, antigen encounter occurs in sites with highly organized microstructure optimized to promote the encounter of the antigen. These structures such as the spleen and lymph nodes are known as secondary lymphoide organs. SLOs are extremely well connected to blood and lymphatic vessels, allowing them the continual sampling and concentration of antigens circulating in the body[45].

6.6 Initial activation of a B lymphocyte in the SLOs

Naive B cells arriving to the SLOs spend 24 hours to find the appropriate antigen. In case of missing success they begin to recirculate throughout the body to increase their chances of encountering cognate antigen[127]. Antigen in the SLOs can arrive in soluble form or carried by antigen presenting cells, such as macrophages or dendritic cells. B cells that can encounter antigen can differentiate along two distinct pathways. They can either form extrafollicular plasmablasts that are essential for rapid antibody production and early protective immune responses or they can enter the germinal centers, where they differentiate to high-affinity antibody secreting plasma cells or memory B cells following affinity maturation[128].

6.7 Grabbing the antigen in the germinal centers

Germinal centers consist of rapidly proliferating B cells within a network of follicular dendritic cells. FDC bear antigens in the form of immune complexes on their surface, and B cells compete for them. Cells that are unable to grab the antigen, fail to gather survival signals through the BCR and undergo apoptosis. B cells migrate in the GC with an average high velocity of 6 μm/min, an scan FDCs for the antigen[99, 129]. Successfully grabbing a surface bound antigen requires B cells to go through a spreading and a contraction phase. They first spread over the antigen-bearing membrane and then contract thereby collecting the bound antigen in a central aggregate[130].

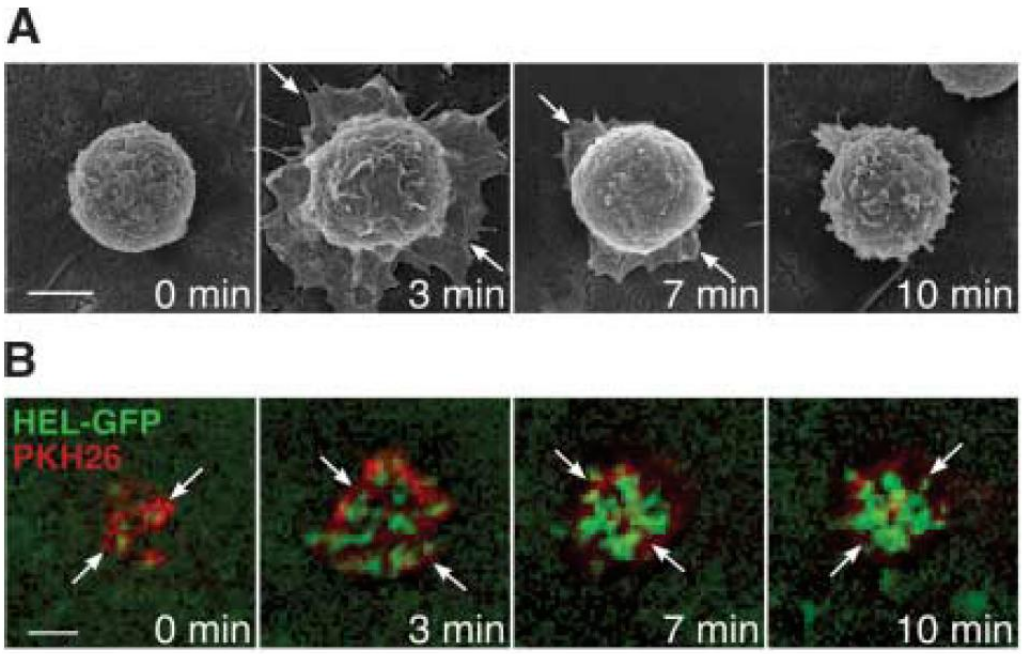

Figure 34. B cell spreading and contraction. *MD4 transgenic B cells interacting with COS-7 cells wxpressing a membrane form of HEL-GFP obtained using (A) SEM of fixed cells or (B) time-lapse wide-field fluorescence microscopy. [antigen (green) and B cell membrane (red)] (from Fleire and Batista)*

The spreading phase requires a rapid polymerization of actin filaments at the cell-cell contact zone of B cells and FDCs. The CRAD model predicts an asymmetric calcium distribution in the cytoplasm with lower calcium concentration close to the contact zone and higher calcium levels in the distal pole. Subsequent contraction phase would require the opposite tendency.
Collecting the most of antigens also means acquiring most of the BCR signals. The Gab2/PI3K/Akt survival pathway is a part of this signal. Gab2 by recruiting PI3K and enhancing Akt phosphorylation is able to suppress the apoptotic phenotype of GC B cells.

6.8 B cell T cell interaction in the germinal centers

B cell clones that acquire the most antigens have the highest chance to survive. They internalize and process the antigen to present it to $CD4^+$ T cells. Helper T cells that recognize the epitope/MHCII complex on the B cell surface form immunological synapses with the B cells and mediate thereby an additional survival signal. The CRAD model had proved its prediction in this scenario, and had shown how the subcellular distribution of calcium contributes to the successful IS formation.

B cells that are able to gather enough antigen and survival signals by performing the highest affinity receptor undergo proliferation and differentiation to mature to plasma cells or memory B cells and to finish off with the pathogen.

7. Main results

1. Large increases in the intracellular calcium concentration induce the collapse of the actin cytoskeleton.
2. Actin growth is favored in subcellular regions with local lower calcium concentration.
3. T cells maintain lower calcium at cell sites where actin growth will occur during immunological synapse formation.
4. Gab2 is expressed in B cells and recruits PI3K and SHP2 upon BCR engagement.
5. Both Lyn and Syk are required for the phosphorylation of Gab2.
6. Overexpression of Gab2 causes enhanced Akt phosphorylation and rescues BCR triggered B cells from Fas induced cell death.

Abbreviation

ΔPH	PH domain deficient
ADF	actin depolymerizing factor
Ag	antigen
ANK	ankyrin
APC	antigen presenting cell
Arp2/3	Actin related protein-2/3
Bak	Bcl-2 antagonist/killer-1
Bax	Bcl2-associated X-protein
Bcl-XL	Bcl-2-like 1
Bcl-w	Bcl-2-like 2
Bcl-2	B-cell CLL/Lymphoma 2
BCR	B cell receptor
Bfl-1	Bcl-2-related protein A1
BLNK	B-cell linker
CRAC	calcium-release activated calcium
CRAD	calcium-regulated actin dynamics
Csk	c-SRC tyrosine kinase
DAG	diacylglycerol
DC	dendritic cell
ER	endoplasmatic reticulum
ERM	ezrin/radixin/moezin

FA	focal adhesions
Fas	tumor necrosis factor receptor superfamily member 6
FasR	Fas receptor
FasL	Fas ligand
F-actin	filamentous actin
FDC	follicular dendritic cell
FERM-domain	(4.1, ezrin, radixin, moezin)-domain
FH2	Formin-homology domain-2
FOXO	Forkhead Box, Subgroup O family
Gab1/2/3	Grb2 associated binding protein 1/2/3
G-actin	globular actin
GC	germinal center
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
Ig	immunoglobulin
IKK	IκB kinase
IP	immunoprecipitate
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
ITAM	immunoreceptor tyrosine-based activation motif
LN	lymph node
Mcl-1	myeloid cell leukemia-1
MHC	major histocompatibility complex

mIg	membrane immonoglobulin
Myc	v-myc myelocytomatosis viral oncogene homolog (avian)
MZB	marginal-zone B cell
NCX	Sodium/calcium exchanger
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
N-WASP	neuronal Wiskott-Aldrich syndrome protein
Pag	phosphoprotein associated with GEMs
PDPK1	phosphoinositide-dependent protein kinase 1
PH-domain	Pleckstrin homology domain
PI	phosphoinositide
PKCβ	protein kinase C β
PLCγ	phospholipase C γ
PMCA	plasma membrane calcium ATPase
PTB	phosphor-tyrosine binding domain
PTK	protein tyrosine kinase
RA	rheumatoid arthritis
Scar/WAVE	suppressor of cAMP receptor/ WASP-family verprolin homology protein
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SHC	src-homology domain 2 containing transforming protein
SHP2	SH2-domain containing protein tyrosine phosphatase
SH1/2/3	src homology domain 1/2/3
Smac/Diablo	second mitochondria-derived activator of caspase
SLE	systemic lupus erythematosus
SLO	secondary lymphoid organ

SOCE	stre-operated calcium entry
Sos	son of sevenless homolog
Src kinase	Sarcoma kinase
STIM	stromal interaction molecule
Syk	spleen tyrosine kinase
TLC	total cell lysate
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
WASP	Wiskott-Aldrich syndrome protein
WB	Western blot
WT	wild type
WH1	Wiskott-Aldrich syndrome protein homology domain-1
WH2	Wiskott-Aldrich syndrome protein homology domain-2
ZAP-70	zeta-chain associated protein kinase 70 kDa

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