Receptors, subcellular compartments and the regulation of peripheral B cell responses: the illuminating state of anergy

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Abstract

Signals through the B cell antigen receptor (BCR) are necessary but not sufficient for cellular activation. Co-stimulatory signals must be provided through other immune recognition receptor systems, such as MHC class II/CD40 and the toll-like receptor (TLR) 9 that can only productively acquire their ligands in the processive environment of specialized late endosomes (MHC class II containing compartment or MIIC). It has long been appreciated that the BCR, by effectively capturing complex antigens and delivering them to late endosomes, is the link between activation events on the cell surface and those dependent on late endosomes. However, it has become increasingly apparent that the BCR also directs the translocation of MHC class II and TLR9 into the MIIC and that the endocytic flow of these receptors coincides with that of the BCR. This likely ensures close apposition of receptor complexes within the MIIC and the efficient transfer of ligands from the BCR to MHC class II and TLR9. This complex orchestration of receptor endocytic movement is dependent upon the quality of signals elicited through the BCR. Failure to activate specific signaling pathways, such as occurs in anergic B cells, prevents the entry of the BCR and TLR9 into the MIIC and abrogates TLR9 activation. Like anergy, this block in endocytic trafficking is rapidly reversible. These findings indicate that cellular responsiveness can be determined by mechanisms that control the subcellular location of important immune recognition receptors.

Introduction

For most peripheral immune responses, lymphocytes require two signals to become fully activated. The first is elicited by antigen recognition while the second can be provided by a number of receptors that assess the context in which antigen is recognized. For T cells, the second signal is usually provided through surface co-stimulatory receptors expressed on activated antigen presenting cells (APCs)(Greenwald et al., 2005). The integration of antigen receptor and co-stimulatory receptors, at the cell surface, determine cell fate. In contrast, B cells are dependent upon processes exclusive to late endosomes for most second signals required for cellular activation (Clark et al., 2004; Herlands et al., 2008; Krieg and Vollmer, 2007; Watts, 1997).

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It has long been appreciated that the recruitment of T cell help requires antigens to be delivered to late endosomes (Ferrari et al., 1997; Qiu et al., 1994) which provide an environment where polypeptides can be processed and efficiently loaded onto MHC class II (Drake et al., 1999; Kleijmeer et al., 1997; Watts, 2001). Recently it has become apparent that the Toll-like receptors 7 and 9, which can effectively provide activation signals, are restricted to endocytic compartments. TLR activation plays a role in normal peripheral B cell responses, by detecting single stranded (ss) RNA (TLR7) and unmethylated CpG motifs within ssDNA (TLR9)(Krieg and Vollmer, 2007). They also are obligatory for many autoimmune humoral responses (Christensen et al., 2006; Leadbetter et al., 2002; Sadanaga et al., 2007).

In contrast to other APCs, non-specific mechanisms for the delivery of antigen to late endosomes, such as pinocytosis and phagocytosis, are not efficient in B cells (Pierce et al., 1988; Siemasko and Clark, 2001; Song et al., 1995). Rather, the MHC class II and TLR rich late endosomes of B cells are privileged environments preferentially targeted by immune receptors such as the BCR (Clark et al., 2004; Hess et al., 2000). The primacy of antigen receptors as portals for entry ensure the recruitment of second signals, and cellular activation, is dependent upon the nature of the antigenic complexes that are captured and delivered to late endosomes.

Herein, we will review our current understanding of how BCRs are selected for internalization and what are the molecular processes that direct the endocytosed BCR, and the TLRs, to late endosomes. Furthermore, we will review recent findings from our laboratory that receptor endocytic trafficking is abrogated in anergic cells. These latter data reveal a new level of regulation in which the subcellular location of important immune response receptors determine how B cells respond to immunogenic and tolerogenic ligands.

BCR Internalization: retention of signaling complexes on the cell surface

Recent *in vivo* studies have revealed several mechanisms by which antigen is provided to B lymphocytes (Batista and Harwood, 2009) including capture and presentation by macrophages (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2009), dendritic cells (Qi et al., 2006) and directly by diffusion through a conduit network composed of collagen bundles surrounded by follicular reticular cells (Gretz et al., 2000; Sixt et al., 2005). Non-cognate marginal zone B cells can also shuttle antigen to follicular dendritic cells for presentation to B cells (Batista and Harwood, 2009; Phan et al., 2009). Through most of these mechanisms, antigen is provided to B cells in membrane-bound arrays that induce receptor activation and clustering (Liu et al., 2010).

Receptor clustering elicits a series of signaling events that greatly accelerate the internalization of the BCR and its delivery to late endosomes. These events can be organized into an afferent signaling pathway, which activates the endocytic machinery and an efferent limb that allows primed endocytic machinery to discriminate between surface BCR complexes destined for internalization and those that will assemble a signalsome at the cell surface (A in Figure 1). The afferent signaling pathway emanating from the BCR (Figure 2) involves proximal activation of one or more Src-family tyrosine kinases (SFTKs) and is associated with the phosphorylation of clathrin heavy chain and actin polymerization (Guagliardi et al., 1990;Salisbury et al., 1980;Stoddart et al., 2002;Stoddart et al., 2005). Internalization also requires the B lymphocyte adaptor protein LAB which is inductively phosphorylated and then recruits Grb2-dynamin and Vav (Malhotra et al., 2009a;Malhotra et al., 2009b). Alternatively, BLNK may serve to link Grb2 to BCR-mediated kinase activation {Kabak, 2002#2870}. Downstream of Vav, Rac1/2 is required for BCR endocytosis.

Once activated, the endocytic machinery must be targeted to the fraction of receptors fated for internalization (Figure 2). How is this accomplished? It had been assumed that active signaling complexes are efficiently or even preferentially targeted for degradation and that this would serve to directly down modulate BCR signaling. However, this is not the case. The BCR motifs that are recognized by the endocytic machinery are the same ones that, when phosphorylated, initiate BCR-dependent signaling cascades (Gazumyan et al., 2006;Hou et al., 2006)(A in Figure 1 and Figure 2). In the non-phosphorylated state, the tyrosines of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the BCR subcomponents Igα/Igβ and the Igα non-ITAM motif direct internalization of the receptor. Available data indicate that all of the conserved tyrosine-based motifs in both Igα and Igβ additively contribute to BCR internalization (Gazumyan et al., 2006;Hou et al., 2006). The exact mechanism by which this occurs is not clear. However, the ITAMs are predicted to recruit the adaptor AP2 (Traub, 2004) which has been demonstrated to couple receptors to internalization through clathrin-coated pits. Recognition by AP2 requires an intact phenyl group that can be provided by either a tyrosine or phenylalanine. Nonconservative mutations or phosphorylation of the AP2 motif tyrosines on the receptor ablates recognition by AP2. Such a mechanism is consistent with observations that mutation of Igα ITAM and non-ITAM tyrosines to alanine impedes internalization and that following ligation, phosphorylated BCR complexes are selectively retained on the cell surface (Hou et al., 2006).

These findings indicate that BCR internalization attenuates signaling by limiting the number of BCR complexes available for phosphorylation rather than targeting already phosphorylated complexes for degradation. Mathematical modeling predicts that selective internalization of non-phosphorylated BCRs, as opposed to non-selective internalization of all surface BCR complexes, would both increase the sensitivity of the receptor to low avidity ligands and limit the maximal response that can be elicited through the BCR (Hou et al., 2006). Selective internalization could also provide a mechanism of quality control, as hypophosphorylated BCR complexes would also be removed from the cell surface thereby increasing the efficiency of signal initiation.

If only non-phosphorylated BCR complexes are internalized, how are polyvalent ligands, those best at inducing BCR phosphorylation, delivered to late endosomes? There are several possibilities. Even with saturating ligation, only a small fraction of BCR complexes (<10%) are inducibly phosphorylated within two minutes (Hou et al., 2006). This fraction is then rapidly dephosphorylated such that only a few percent of BCR complexes remain phosphorylated ten minutes after receptor clustering. Therefore, at most, BCR phosphorylation might delay the internalization of a small fraction of antigen-bound receptors. While this would not limit the capture of prevalent antigens it might impede the capture of rare epitopes. Alternatively, it has been demonstrated that in anergic B cells, and in B cell lines, receptor ligation induces the dissociation of the antigen recognition subunit, membrane bound immunoglobulin (mIg), from the signaling subunits Igα/Igβ (termed receptor destabilization), allowing each to be internalized through different mechanisms (Kim et al., 2005; Vilen et al., 1999). While receptor destabilization has been demonstrated in biochemical and immunoelectron micrographic studies, it has not been observed in separate fluorescent resonance energy-transfer (FRET) studies (Tolar et al., 2005). However, this latter technique might not be sensitive to small subpopulations of receptors undergoing destabilization.

Targeting Late Endosomes: Mechanisms of BCR Ubiquitinylation and Endocytic Trafficking

Once the ligated BCR is internalized, it rapidly sorts through early endosomes to late endosomes. In APCs, late endosomes are specialized for antigen processing. They are rich in MHC class II and contain machineries for processing peptides and MHC loading (MIIC) (Watts, 1997; Watts, 2001). Ultrastructurally, typical MIIC vesicles consist of a limiting membrane studded with lysosomal associated membrane protein-1 (Lamp-1) and a lumen containing intraluminal multivesicular bodies (IMBs) (Ferrari et al., 1997; Siemasko and Clark, 2001; West et al., 1994). The IMBs are derived from transport vesicles that have gained access to the MIIC compartment (Railborg et al., 2003; Riberdy et al., 1994).

Work from several laboratories has provided a general model for how endocytosed receptor complexes are sorted through early endosomes and delivered into late endosomes (Railborg and Stenmark, 2009). Central to the model is the monoubiquitinylation of receptors (Hicke and Dunn, 2003; Stahl and Barbieri, 2002; Umebayashi, 2003) and the recognition of these ubiquitin modifications by a protein complex containing Hrs, Eps15 and STAM (collectively termed the endosomal complex required for transport or ESCRT-0)(Railborg and Stenmark, 2009; Slagsvold et al., 2006). ESCRT-0 engaged receptors are retained within the endosomal pathway while unbound receptors recycle to the cell surface. Retained receptors then bind Tsg 101, a subunit of ESCRT-I. Successive recruitment of the multimeric complexes ESCRT-II and ESCRT-III target receptors to late endosomes. These receptors are then sorted into intraluminal multivesicular bodies where they are degraded (Rodahl et al., 2009). Ubiquitinylation can also facilitate receptor internalization (Belouzard and Rouille, 2006). However, there are examples in which receptor ubiquitinylation has no role in receptor internalization (Duan et al., 2003; Huang et al., 2006).

This general model of receptor endocytic trafficking can be applied to the BCR (B in Figure 1). Within the BCR, Igβ ubiquitinylation is required for sorting through early endosomes and for trafficking into late endosomes (Zhang et al., 2007). Igα can become ubiquitinylated but is detectable only well after receptor ligation when most endocytosed complexes have been delivered to the MIIC. Mutation of Igβ cytosolic lysines to arginines, which ablates ubiquitinylation, yields a receptor that enters the recycling early endosomal pool upon internalization [(Zhang et al., 2007) and unpublished observations] and does not target to late endosomes. Igβ ubiquitinylation does not facilitate BCR internalization indicating that the mechanisms mediating BCR internalization are different from those directing endocytic trafficking.

In resting B cells, Igβ is constitutively ubiquitinylated and this does not change with receptor ligation. Therefore, receptor clustering, and the initiation of tyrosine kinase- mediated signal cascades, are not necessary for tagging the receptor with ubiquitin. This is consistent with earlier studies demonstrating that, once internalized, both ligated and unligated BCR complexes target late endosomes (Brown et al., 1999; Song et al., 1995). In normal B cells expressing wild-type BCR complexes, we have not observed significant recycling, or early endosomal retention, of clustered, endocytosed BCR complexes (unpublished observations). This suggests that the majority of surface BCRs are tagged with ubiquitin. One of the E3 ligases involved in Igβ ubiqutinylation is the HECT family member Itch (Zhang et al., 2007). It is not clear how Itch is recruited to the BCR or even if it directly ubiquitinylates Igβ. However, mutation of the Igβ ITAM tyrosines to alanines *in vivo* is associated with decreased Igβ ubiquitinylation of the resting receptor (Gazumyan et al., 2006). This observation suggests a role for the Igβ ITAM in coupling the BCR to Itch.

The unphosphorylated TCR is not ubiquitinylated in thymocytes. Ubiquitinylation requires receptor phosphorylation and the recruitment of the E3 ligase c-Cbl (Dragone et al., 2009; Myers et al., 2006). C-Cbl has also been implicated in controlling BCR surface expression and in mediating the ubiquitinylation and degradation of internalized receptor complexes (Dragone et al., 2009; Dragone et al., 2006). This function is dependent upon co-expression of the SFTK-associated adaptor protein (SLAP). However, we could not detect c-Cbl recruitment to the ligated BCR in peripheral naïve B cells (manuscript in preparation). Therefore, it is unclear how c-Cbl and SLAP are contributing to BCR degradation in peripheral B cells. While these data indicate that both the BCR and TCR are ubiquitinylated, and this is required for normal endocytic transit, the mechanisms by which each receptor is tagged with ubiquitin are different.

Coordination of BCR endocytic trafficking with MHC class II and TLR9

Stimulation of the BCR regulates and coordinates the endocytic transit of other immune receptors destined for the MIIC. The most well described example is newly synthesized MHC class II that, in resting cells, primarily resides outside the MIIC. Stimulation of the BCR induces the rapid targeting of MHC class II into the MIIC (Lankar et al., 2002; Siemasko et al., 1998). The activation of specific BCR-dependent signaling pathways is required for the trafficking of MHC class II and the biogenesis of IMBs (Lankar et al., 2002; Le Roux et al., 2007; Siemasko et al., 1998; Siemasko et al., 2002). However, it is also likely that normal IMB biogenesis requires the physical transit of the BCR into the MIIC. This latter point is suggested by studies of chimeric receptors containing only the cytosolic tail of Igα, and not Igβ, that can activate signaling pathways but arrest in early endosomes (Siemasko et al., 1999). In these cells, intracellular MHC class II co-localizes with the endocytosed Igα receptor and is not detectable in late endosomes (unpublished observations).

In addition to the BCR and BCR-dependent signaling pathways, cathespin S and invariant chain (Ii) are required to form IMBs (Boes et al., 2005) implicating MHC class II endocytic transit and/or processing in IMB biogenesis. A physical requirement for both MHC class II and the BCR to form IMBs would ensure that the MIIC intraluminal vesicles containing endocytosed BCR complexes were also decorated with MHC class II. Such close juxtaposition of receptors would enhance the loading of peptides derived from BCR-captured antigens onto MHC class II.

The BCR also controls and coordinates the transit of TLR9 into the MIIC (Chaturvedi et al., 2008; O'Neill et al., 2009). In resting cells, TLR9 is resident outside late endosomes, presumably in endoplasmic reticulum (Latz et al., 2004). Significant surface expression cannot be detected (Trinchieri and Sher, 2006). Upon BCR ligation, and the initiation of BCR-dependent signaling cascades, TLR9 transits into the MIIC. Like MHC class II, BCR and TLR9 transit are concurrent. Furthermore, as described below, analysis of anergic B cells indicates that the BCR and TLR9 enter the MIIC together, on common transport vesicles (O'Neill et al., 2009). Like MHC class II, segregation of TLR9 into the same IMBs as ligand-bearing BCRs should facilitate the productive transfer of ligand to TLR9. This juxtaposition of BCR and TLR9 occurs in the permissive environment of late endosomes where TLR9 is cleaved to its active form by acid-dependent cathepsins (Barton and Kagan, 2009; Park et al., 2008).

It is not clear if similar or different BCR-dependent signaling pathways control the endocytic transit of MHC class II and TLR9. Proximally, B cell linker protein (BLNK or SLP65)(Ishiai et al., 1999) and the downstream activation of one or more protein kinase Cs (PKCs) have been implicated in MHC class II endocytic trafficking (Siemasko et al., 1999;

Siemasko et al., 2002). For TLR9 trafficking, MAP kinase (MAPK), c-Jun N-terminal kinase (JNK)(O'Neill et al., 2009) and phospholipase D (Chaturvedi et al., 2008) activation have been demonstrated to be important. However, there has been no direct comparison of the signaling requirements for targeting MHC class II and TLR9 to the MIIC. There are several possible downstream effectors that could have a role in controlling the entry of the BCR, MHC class II and TLR9 into late endosomes. The Rab family of GTPases are possible targets as they are important for the internalization and endocytic targeting of a variety of receptors and they can be regulated by the MAPKs (Cavalli et al., 2001; Zwang and Yarden, 2006). Other possibilities include the adaptor protein Cbl-interacting protein of 85 kDa (CIN85) and/or its homolog the CD2 adaptor protein (CD2AP). CIN85 has mostly been studied in the context of EGF receptor trafficking where it co-localizes with late endosomes (Zhang et al., 2009) and is important for receptor degradation (Haglund et al., 2002). CD2AP plays a prominent role in TCR function (Lee et al., 2003).

Entry into the MIIC by the BCR, TLR9 and MHC class II is a highly coordinated process that has specific signaling requirements and likely involves the physical delivery of one or more of these receptors. It is also a process critical for coupling BCR recognition to the recruitment of additional signaling pathways, ultimately downstream of MHC class II/CD40 and endocytic TLRs, necessary for full B cell activation. Therefore, processes that disrupt the signaling pathways necessary for normal receptor endocytic trafficking would be expected to abrogate B cell activation. Indeed, this is what happens in anergic B cells.

BCR endocytic trafficking in B cell tolerance

Receptor editing and clonal deletion irreversibly eliminate high affinity autoreactive B cell clones from the naïve repertoire (Erikson et al., 1991; Gay et al., 1993; Goodnow et al., 1989; Goodnow et al., 2010; Nemazee and Buerki, 1989; Tiegs et al., 1993). However, these mechanisms of tolerance inefficiently eliminate clones with relatively low affinity for self-antigen. Up to 20% of B cells that survive central tolerance, and that are selected into the peripheral B cell pool, express self-reactive immunoglobulins (Yurasov et al., 2005). However, this large and potentially dangerous pool of lymphocytes does not contribute to normal immune responses. In murine models of B cell tolerance, most of these peripheral B cell populations are intrinsically unresponsive to antigen, or anergic (Andrews and Wilson, 2010; Benschop et al., 2001; Borrero and Clarke, 2002; Nguyen et al., 1997). While anergy has been most convincingly demonstrated in immunoglobulin transgenic mice, populations of cells with features of anergy have been identified in both unmanipulated mice (Merrell et al., 2006) and in humans (Duty et al., 2009; Isnardi et al., 2010). Therefore, anergy appears to be a common and important mechanism of B cell tolerance.

In contrast to deletion and receptor editing, anergy is a reversible state that must be reinforced by chronic antigen exposure and signaling through the BCR (Cambier et al., 2007; Cornall et al., 1995; Cyster and Goodnow, 1995; Goodnow et al., 1991). This has been most convincingly demonstrated in the Ars/A1 transgenic model in which Ars-Tyr hapten effectively competes with single-stranded DNA for BCR binding and rapidly reverses anergy (Gauld et al., 2005). These data suggest that one way that B cells discriminate self from non-self is by the chronicity of antigenic exposure.

Immunogenic and tolerogenic stimuli induce different proximal BCR signaling responses (Jun and Goodnow, 2003). Immunogenicity is associated with the coordinated activation of a network of signaling pathways, including those leading to rapid intracellular calcium elevation as well as extracellular signal related kinase (ERK) and JNK activation (Skaggs and Clark, 2004). Most anergic B cells manifest aberrant BCR-induced calcium responses and attenuated JNK activation (Cambier et al., 2007; Healy et al., 1997). In contrast, it has

been difficult to identify a characteristic transcriptional profile for anergic B cells (Cambier et al., 2007; Merrell et al., 2006). The apparent lack of correlation between transcriptional events and B cell anergy is not surprising given the rapidity with which anergy can be reversed.

If the proximal signaling aberrancies of anergy are not inducing tolergenic transcriptional programs, how are they attenuating cellular activation? We propose that a major function of BCR anergic signaling is to prevent the subsequent solicitation of the second signals needed for cellular activation (C in Figure 1). In anergic, ssDNA-specific B cells this is accomplished by excluding both the BCR and TLR9 from the MIIC (O'Neill et al., 2009). Without access to the processive environment of the MIIC DNA ligands captured by the BCR cannot be productively transferred to TLR9 (Park et al., 2008) (Barton and Kagan, 2009). This is particularly important for preventing the secretion of anti-DNA antibodies, which requires TLR9 (Christensen et al., 2006) and the downstream activation of MyD88 (Sadanaga et al., 2007).

The failure of both the BCR and TLR9 to enter the MIIC is a consequence of aberrant proximal BCR signaling in anergic cells. Pharmacologically complementing BCR signaling rapidly restores BCR and TLR9 endocytic trafficking within 30 minutes as does reversing BCR anergic signaling by competing away self-ligand (O'Neill et al., 2009). The kinetics of regulation implicates mechanisms of control that do not depend upon changes in transcription or protein translation. Such mechanisms are in keeping with the observed reversibility of anergy.

The block in receptor trafficking in anergic B cells appears to affect both the BCR and TLR9 receptors at the same level, along a common route into the MIIC. This is apparent from confocal images of anergic B cells in which the BCR and TLR9 co-localize in small vesicles lying outside Lamp-1$^+$ late endosomes. For the BCR, the endocytic block is after early endocytic sorting, consistent with regulation at the level of entry into late endosomes (O'Neill et al., 2009). However, it should be noted that these conclusions are based on fixed cell images so it is possible that instead of inducing a static block in receptor trafficking, anergy changes the balance or flow of receptors through the endocytic pathway. Also, it is not known if receptors that cannot enter the MIIC adopt an alternative endocytic fate (e.g., trafficking to terminal lysosomes). Resolution of these questions will require additional live cell imaging studies.

To induce and/or maintain the anergic state, signaling through other intrinsic immune receptors, such as the TLRs and CD40, must be attenuated. However, anergy is phenotypically and undoubtedly mechanistically heterogeneous (Cambier et al., 2007). For example, in anergic B cells expressing a high affinity BCR specific for HEL, there is not a substantial block in BCR endocytic transit (Blery et al., 2006; O'Neill et al., 2009). Rather, in these cells constitutive ERK activation induces a global inhibition in intrinsic TLR mediated activation (Rui et al., 2003). In contrast, constitutive ERK activation is not a feature of anergic anti-ssDNA B cells (O'Neill et al., 2009). However, these cells are also hyporesponsive to the TLR4 ligand lipopolysaccharide (Nguyen et al., 1997), even though this receptor should be available on the cell surface and therefore not regulated by endocytic transit (Kagan et al., 2008). Therefore, in different anergic cell populations, and probably within single anergic cells, multiple mechanisms ensure that cells do not become spuriously activated through intrinsic immune response receptors.

Conclusions and Perspectives

The findings discussed above illustrate that anergy cannot be adequately characterized by the biochemical or transcriptional state of the cell. In addition, cell biological processes, and the spatial relationships of immune recognition receptors, are important features of the anergic state. Such mechanisms of regulation, which can be rapidly reversed if the need arises, are consistent with current concepts that anergy must be constantly reinforced by the presence of self-antigen.

These mechanisms of receptor sequestration not only reveal something novel about anergy, they reveal how basic cell biological processes can be regulated in lymphocytes to determine peripheral responsiveness. The mechanisms that mediate endocytic vesicular trafficking have been studied in great detail and in a variety of models. However, they have been viewed as largely constitutive processes that ensure cellular homeostasis. The above observations indicate that they are also potential points of regulation that can be modulated to determine how a cell responses to environmental cues. From the perspectives of both the cell biologist and the immunologist, it would be revealing to understand how BCR signaling controlled the kinetics of immune receptor endocytic trafficking as well as docking and entry into the MIIC.

If anergy is potentially reversible, why are these autoreactive and potentially pathogenic cells allowed to persistent in the periphery? In addition to being a mechanism to sequester autoreactivity, anergic B cells could play active roles in some immune responses. Anergic cells could directly suppress autoreactivity, through unknown mechanisms, and/or they could give rise to regulatory B cells (Andrews and Wilson, 2010). Alternatively, it could be that the broader autoimmune "naïve" or starting repertoire potentially afforded by anergic cells might enable productive immune responses against some infections. This possibility is suggested by observations that polyreactivity with self-antigens is a feature of many broadly neutralizing HIV antibodies (Haynes et al., 2005; Mouquet et al., 2010). Therefore, preserving a degree of autoreactivity may enable some protective immune responses.

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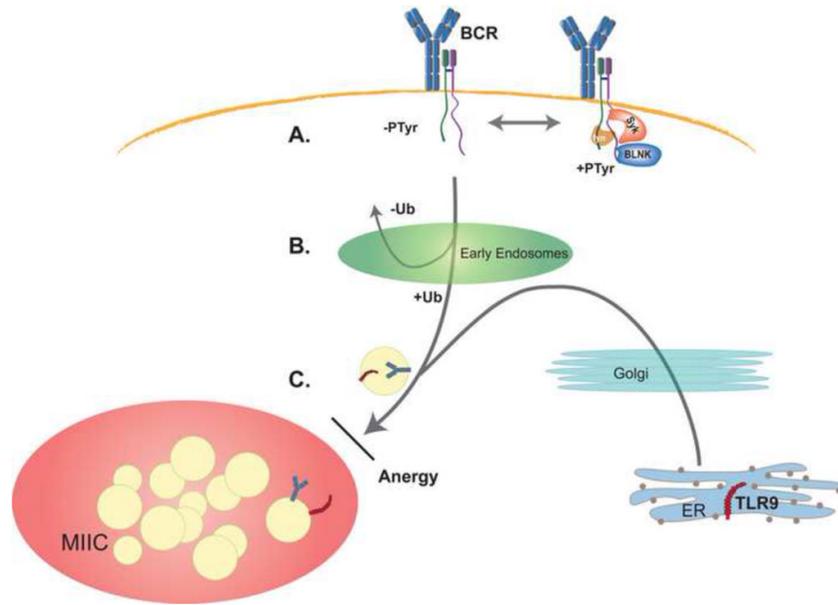
Figure 1. Decision points along the endocytic pathway to the MIIC
(A) Following recognition of antigen, only a small subfraction of surface BCR complexes
are tyrosine phosphorylated (PTyr) and these assemble a signalsome at the cell surface. The
majority of BCR complexes remain unphosphorylated and therefore are targeted for
internalization through tyrosine-based internalization motifs within the cytosolic tails of Igα
and Igβ. (B) The second decision point occurs in early endosomes. Receptors that are
ubiquitinylated (Ub) are sorted towards late endosomes while those that are not
ubiquitinylated recycle back to the cell surface. (C) Analysis of anergic B cells has revealed
a third decision point along the endocytic pathway, at entry into the MIIC. BCR signals also
control the transit of TLR9 and the block induced in anergy affects entry of both the BCR
and TLR9 into the MIIC. These and other observations indicate that the BCR and TLR9
share a common final pathway into the MIIC and that, in wild-type cells, they enter together.
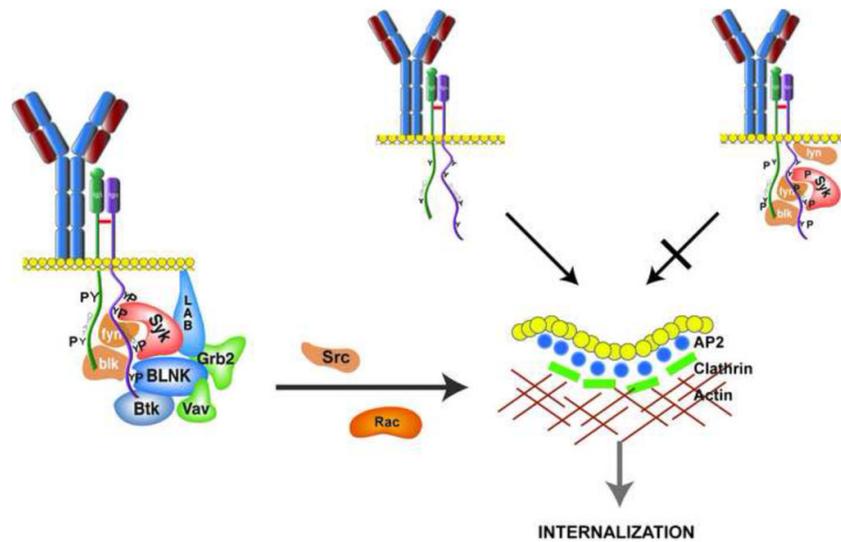
Figure 2. Selection of BCR complexes for internalization
Clustering of the BCR induces the assembly of a signalsome at the receptor containing Syk, BLNK and BLNK-associated molecules. The complex, in conjunction with the adaptor LAB activates signaling cascades that include the molecules Vav, Rac and Src-family kinases (such as fyn and blk). These signaling pathways activate endocytic machinery (the adaptor AP2, clathrin and actin) such that they can select cargo (receptors) for endocytosis and entry into the endocytic pathway. In our model, BCR complexes are selected for inclusion in clathrin-coated pits if they have AP2 docking sites available (YXXL). If these motifs are blocked because they are phosphoryated, or they are obscured by recruited signaling molecules, the receptor is retained on the cell surface. Through this mechanism, BCR complexes involved in signal propagation are retained on the plasma membrane and are not targeted for degradation.