Molecular & Cellular Immunology

ARTICLES

nature immunology

The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer

Pavel Tolar, Hae Won Sohn & Susan K Pierce

Binding of antigen to the B cell antigen receptor (BCR) triggers signaling that ultimately leads to B cell activation. Using quantitative fluorescence resonance energy transfer imaging, we provide evidence here that the BCR is a monomer on the surface of resting cells. Binding of multivalent antigen clustered the BCR, resulting in the simultaneous phosphorylation of and a conformational change in the BCR cytoplasmic domains from a closed to an open form. Notably, the open conformation required immunoreceptor tyrosine-activation motif and continuous Src family kinase activity but not binding of the kinase Syk. Thus, the initiation of BCR signaling is a very dynamic process accompanied by reversible conformational changes induced by Src family kinase activity.

VOLUME 6 NUMBER 11 NOVEMBER 2005 NATURE IMMUNOLOGY

Jonathan Irish, Ph.D.

‘Big Picture’ Concepts and Questions

1. How does antigen receptor signaling program diverse cellular outcomes (death, division, differentiation)?

2. What mechanisms initiate and control antigen receptor signaling? ‘Goldilocks’ signaling.

3. How does antigen receptor signaling change during development?

4. What are the ‘upstream’ and ‘downstream’ events in antigen receptor signaling?

5. What happens when antigen receptor signaling is dysregulated?

Outline

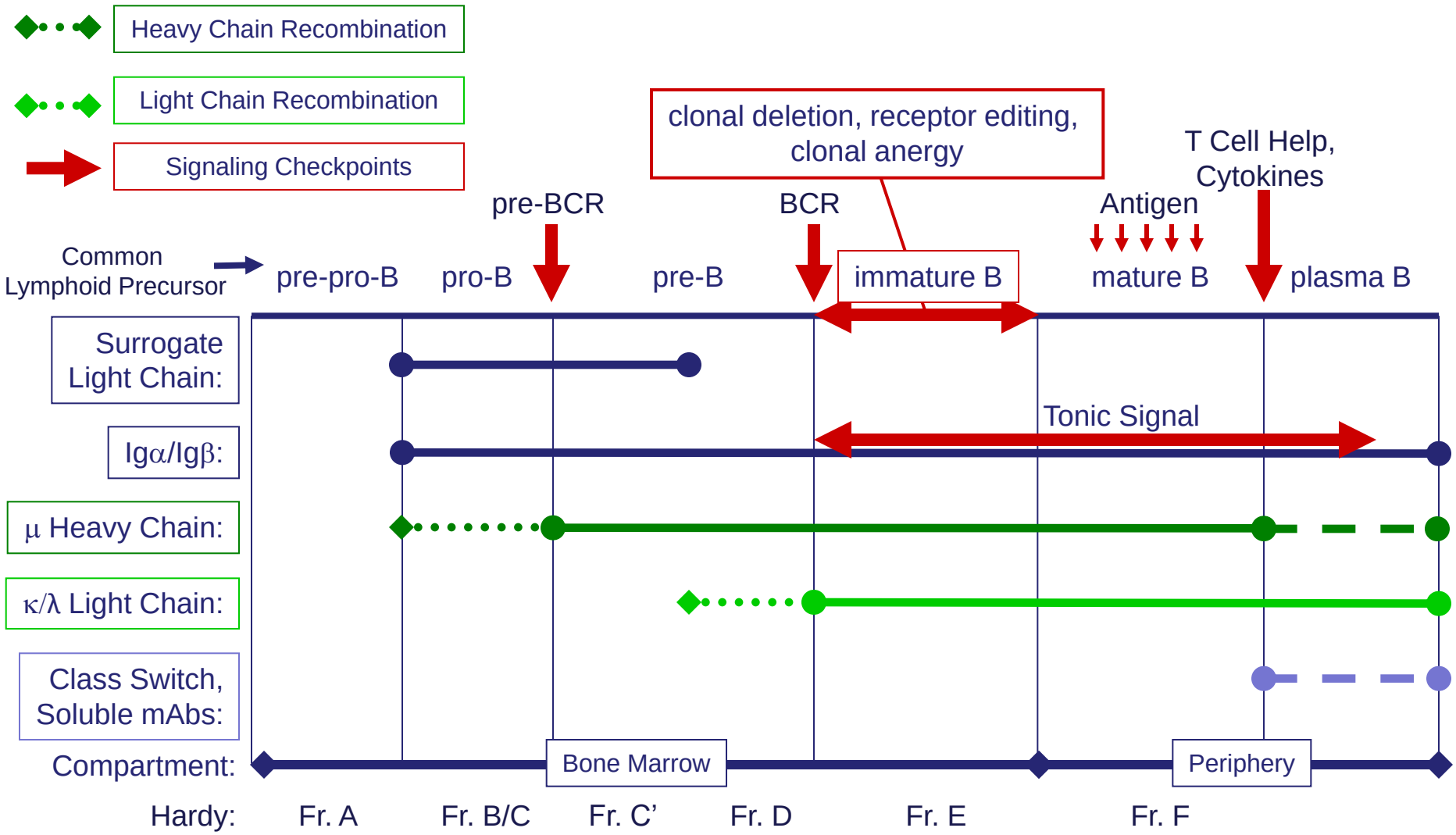
BCR Signaling
In Development

BCR Signaling Events
And Regulation

FRET Technology &
Other Applications

Other Tools
& Background

BCR Expression & Signaling in Development



Cellular Evolution: Central to Cancer and Immunity

- Immune cells undergo programmatic somatic translocations and mutations, generating a diverse pool of cells for selection.

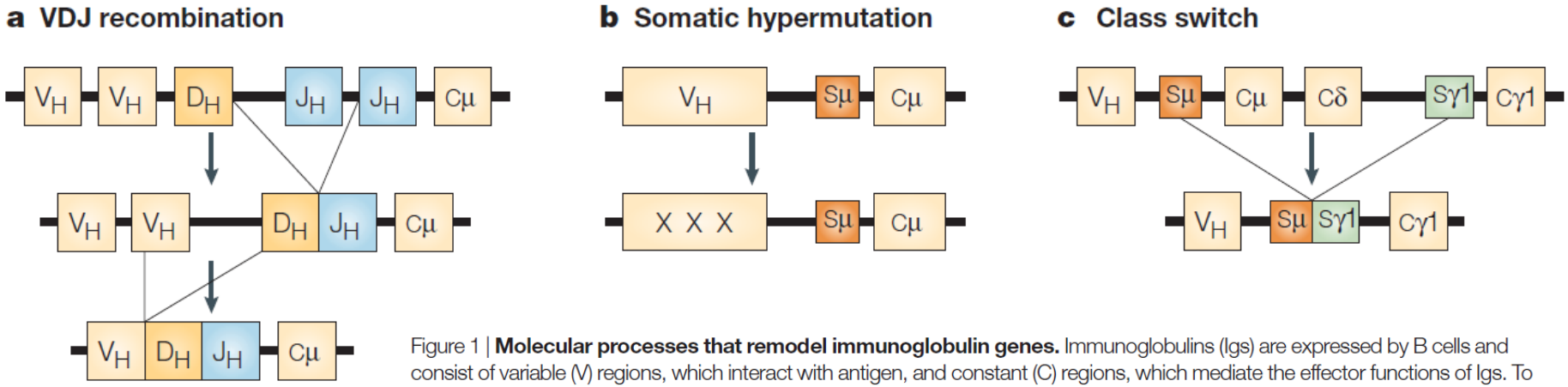
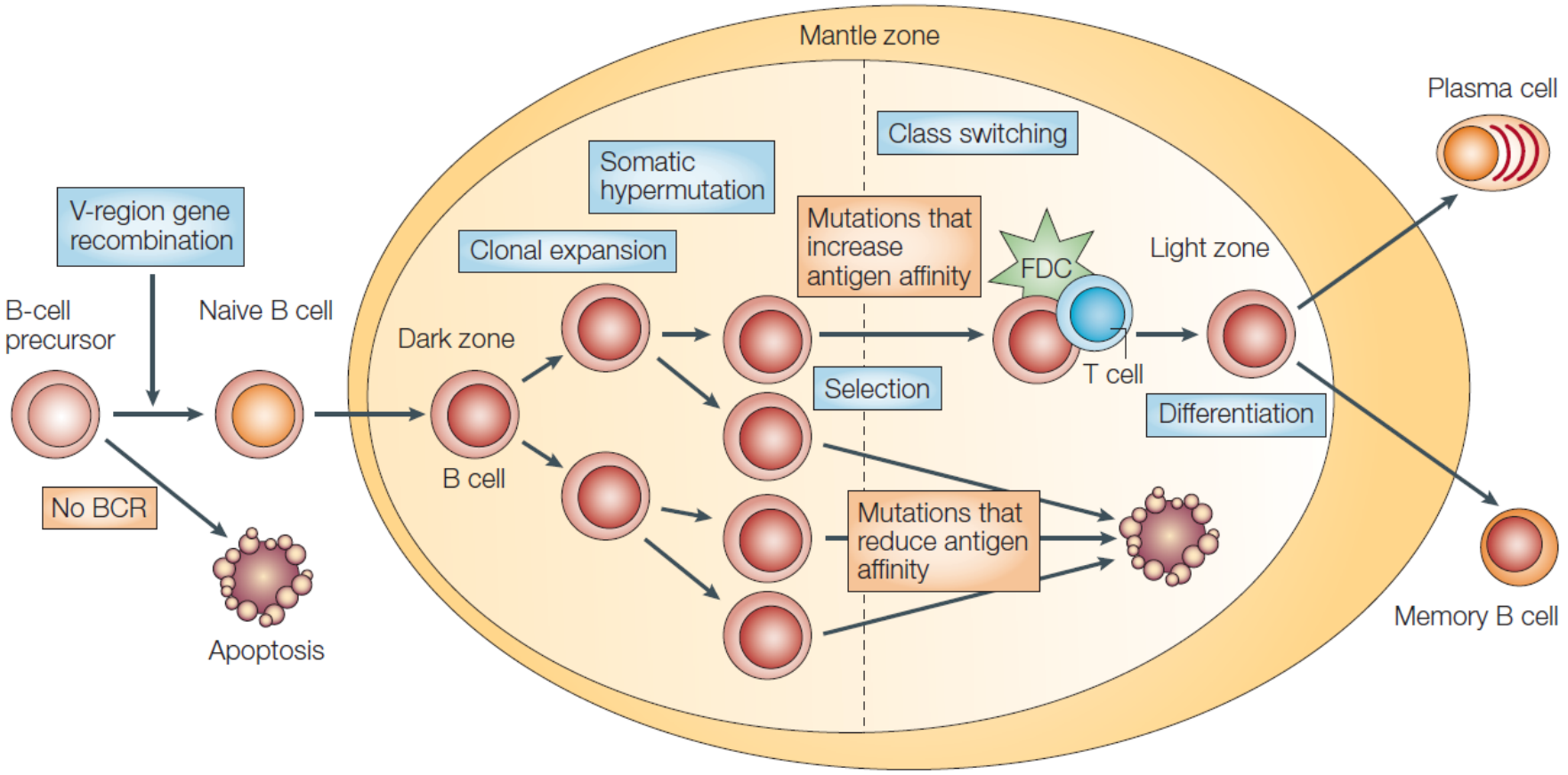


Figure 1 | **Molecular processes that remodel immunoglobulin genes.** Immunoglobulins (Igs) are expressed by B cells and consist of variable (V) regions, which interact with antigen, and constant (C) regions, which mediate the effector functions of Igs. To create a functional Ig, B cells must rearrange DNA segments that encode the heavy (H)- and light-chain (not shown) regions of the variable genes. **a** | First, through a process called 'V(D)J recombination', three gene segments, V_H, D_H and J_H, are joined to encode the H-chain variable region. The V regions of the κ- and λ-light chains, alternatively, are each encoded by two gene segments — the V_L and J_L genes (not shown). B-cell precursors first carry out D_H–J_H rearrangements in H-chain genes. These D_H–J_H rearrangements are followed by V_H–D_HJ_H rearrangements, resulting in the expression of a pre-B-cell receptor if the rearrangement is productive[3]. About 50 functional V_H gene segments, 27 D_H segments and 6 J_H segments are available in the germline, allowing the generation of a diverse repertoire of V_H gene rearrangements. The diversity is further increased by the addition or removal of nucleotides at the joining sites of the gene segments[3]. The cells then carry out rearrangements at their L-chain loci (not shown). The V-region of the Ig gene is ultimately connected to the C-region of the Ig gene (Cμ of IgM in diagram) **b** | The process of somatic hypermutation is activated when B cells reach the germinal centre (GC, shown in more details in FIG. 2). This process leads to the introduction of point mutations, deletions or duplications in the rearranged V-region of Ig genes (denoted by 'Xs' in the figure)[102]. These mutations occur in the V-region of Ig genes — not in the downstream Cμ region. **c** | Class switching results in the replacement of the originally expressed H-chain C-region gene with that of another Ig gene. In the diagram, the C-region for IgM (Cμ) and IgD (Cδ) are exchanged for the C-region of IgG (Cγ1) by recombination at the switch regions for these genes (Sμ and Sγ1, respectively). This results in an antibody with different effector functions but the same antigen-binding domain.

Küppers, *Nat Rev Cancer* 2005

B Cell Development

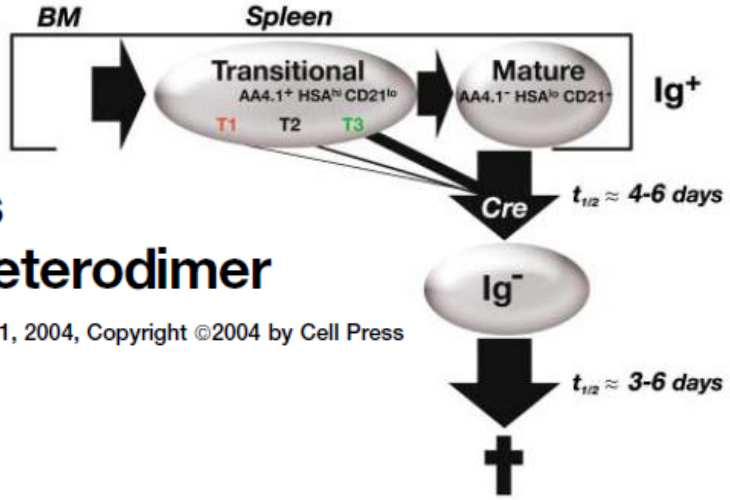


Küppers, *Nat Rev Cancer* 2005

Continuous ‘Tonic’ BCR Survival Signaling

In Vivo Ablation of Surface Immunoglobulin on Mature B Cells by Inducible Gene Targeting Results in Rapid Cell Death

Cell, Vol. 90, 1073–1083, September 19, 1997, Copyright ©1997 by Cell Press



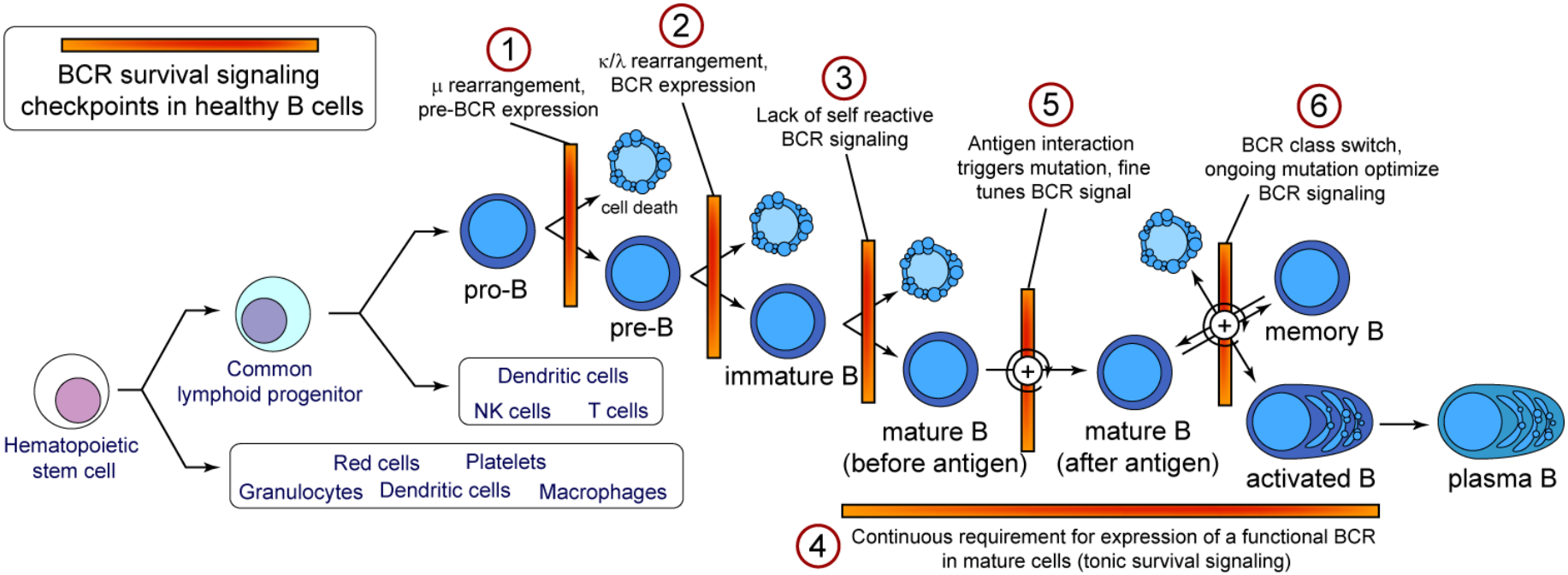
Survival of Resting Mature B Lymphocytes Depends on BCR Signaling via the Igα/β Heterodimer

Cell, Vol. 117, 787–800, June 11, 2004, Copyright ©2004 by Cell Press

PI3 Kinase Signals BCR-Dependent Mature B Cell Survival

Cell *139*, 573–586, October 30, 2009 ©2009 Elsevier Inc.

BCR Signaling Checkpoints in Development



Cellular Evolution: Central to Immunity and Cancer

- Immune cells undergo programmatic somatic translocation and mutation generating a diverse pool that undergoes selection.
- For both cancer and immunity, cells need to acquire new, heritable cellular features.
- When immune developmental checkpoints are dysregulated we see cancer, allergy, and autoimmunity.
- Examples of heritable cellular features
  - Genetic
    - Mutations (DNA basepair changes)
    - Amplifications / deletions (copy number changes)
    - Translocations (might include viruses, retrotransposons)
  - Epigenetic
    - Methylation / acetylation of DNA, histones
    - Prions
    - Infection by intracellular pathogens
    - Reprogramming, as with iPS cells (Oct4 + Sox2 + Nanog + Klf4 +/- Myc)

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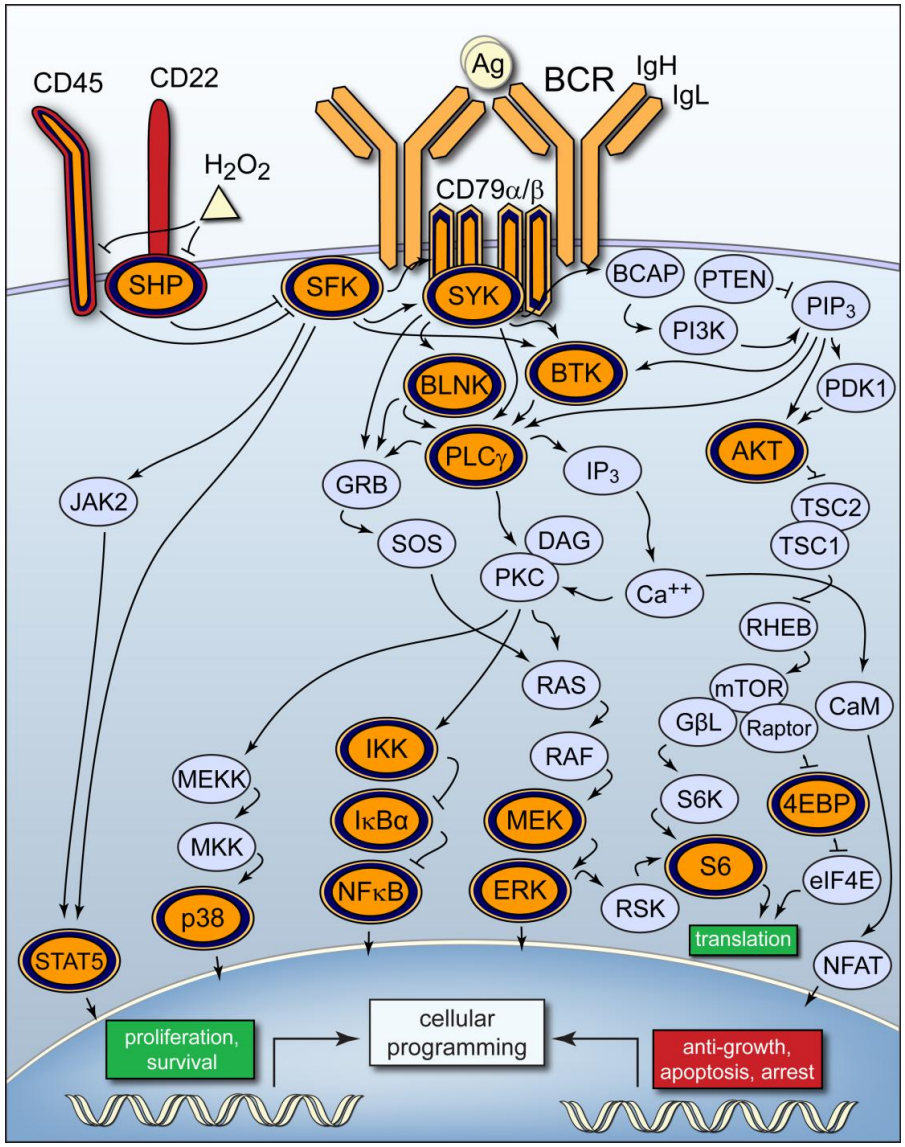
B Cell Receptor Signaling



Regulation of BCR Signaling: ITAMs vs. ITIMs

REGULATION OF B-CELL SIGNAL TRANSDUCTION BY ADAPTOR PROTEINS

Tomohiro Kurosaki

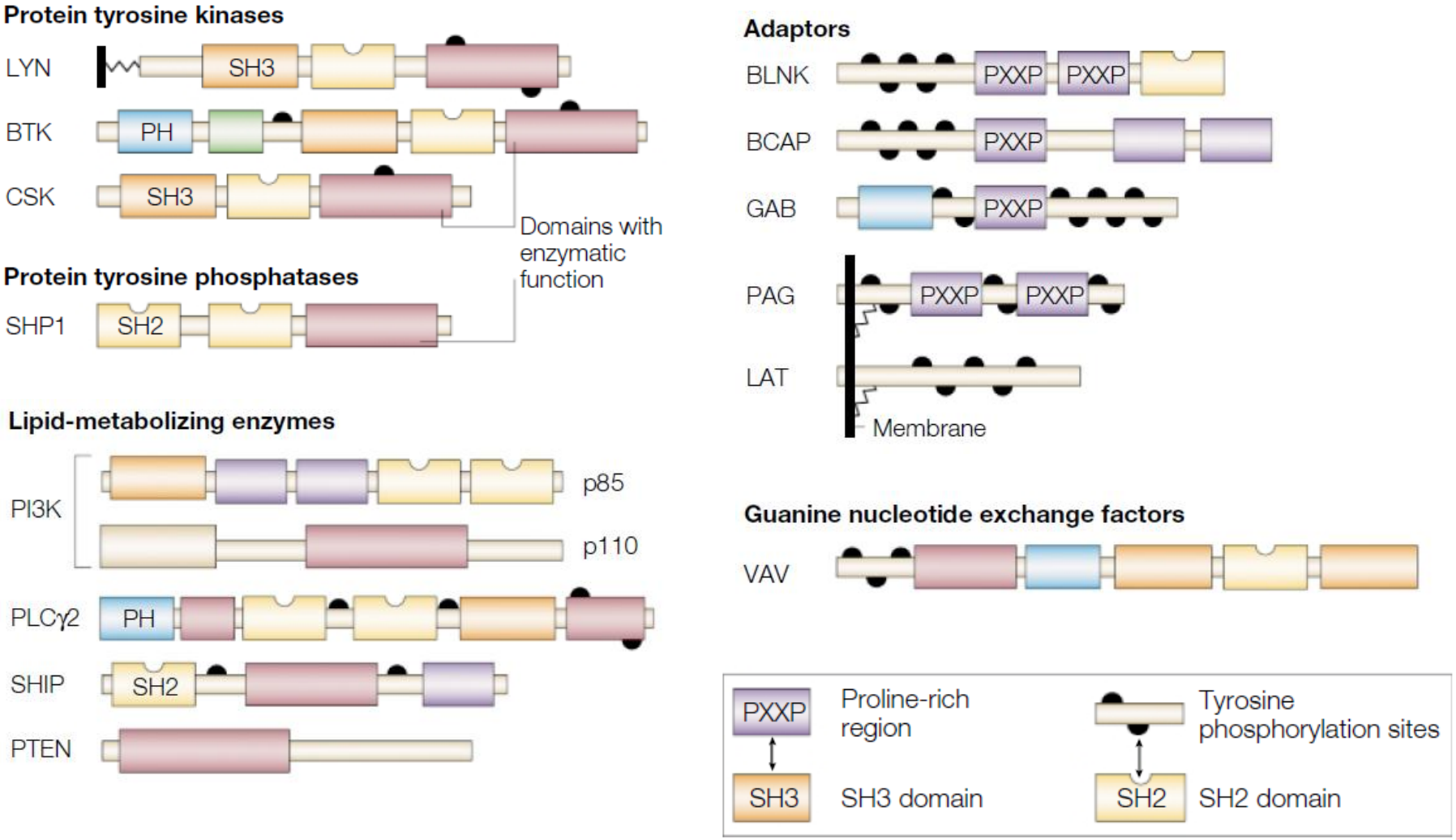
An important role has emerged for adaptor molecules in linking cell-surface receptors, such as the B-cell antigen receptor, with effector enzymes. Adaptor proteins direct the appropriate subcellular localization of effectors and regulate their activity by inducing conformational changes, both of which, in turn, contribute to the spatio-temporal precision of B-cell signal-transduction events. In addition, adaptor molecules participate in establishing negative- or positive-feedback regulatory loops in signalling networks, thereby fine-tuning the B-cell response.

IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). A structural motif containing tyrosine residues that is found in the cytoplasmic tails of several activating receptors, such as the BCR. The motif has the form Tyr-Xaa-Xaa-Leu/Ile, and the tyrosine is a target for phosphorylation by SRC tyrosine kinases and subsequent binding of proteins containing SH2 domains.

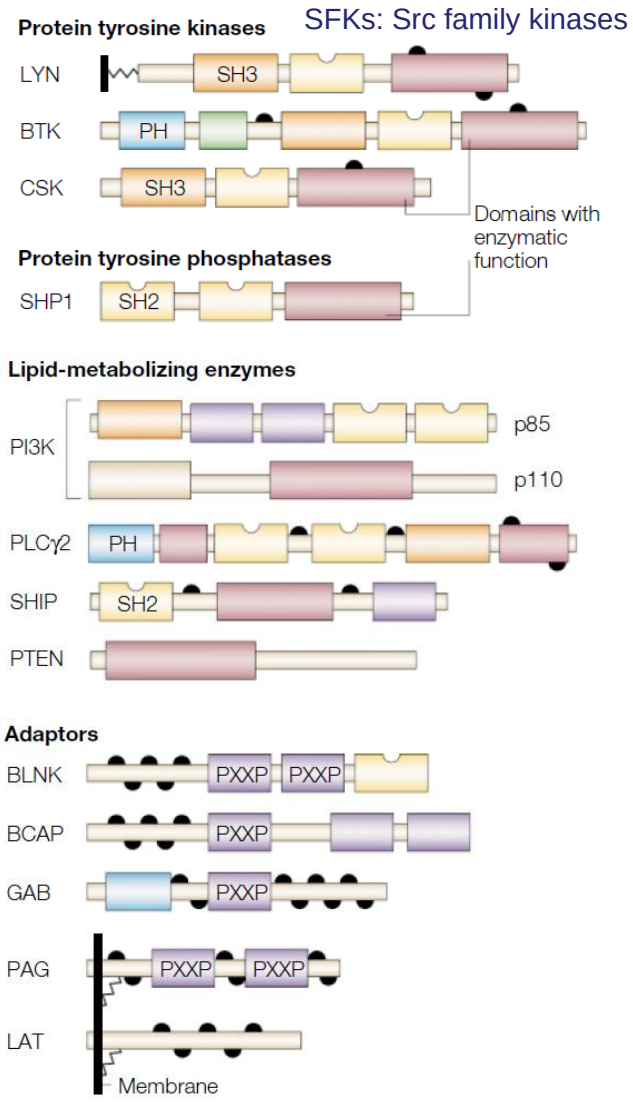
IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF (ITIM). A structural motif containing tyrosine residues that is found in the cytoplasmic tails of several inhibitory receptors, such as FcγRIIB and PIRB. The prototype six-amino-acid sequence is (Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-Leu/Val. Ligand-induced clustering of these inhibitory receptors results in tyrosine phosphorylation, often by SRC-family tyrosine kinases, which provides a docking site for the recruitment of cytoplasmic phosphatases that have an SH2 domain.

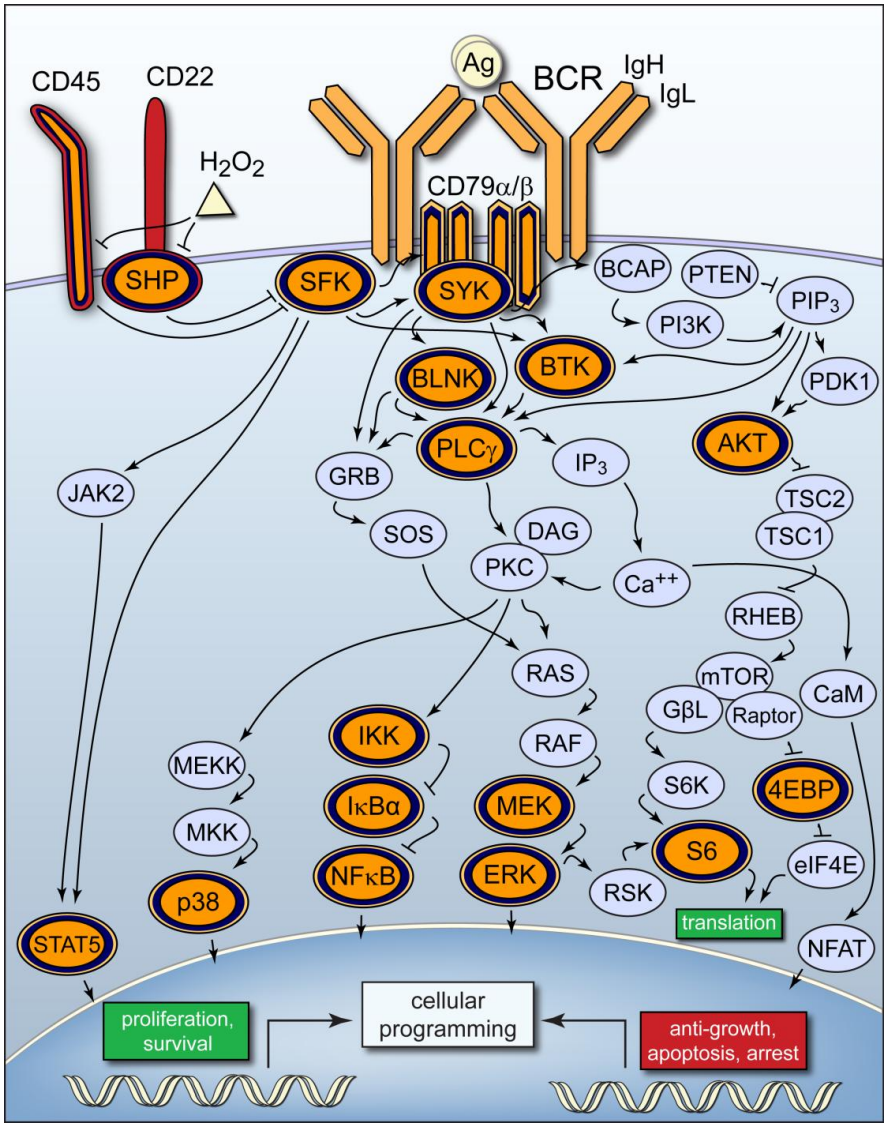
Kurosaki, *Nat Rev Immunol* 2002

BCR Signaling 'LEGO Blocks'

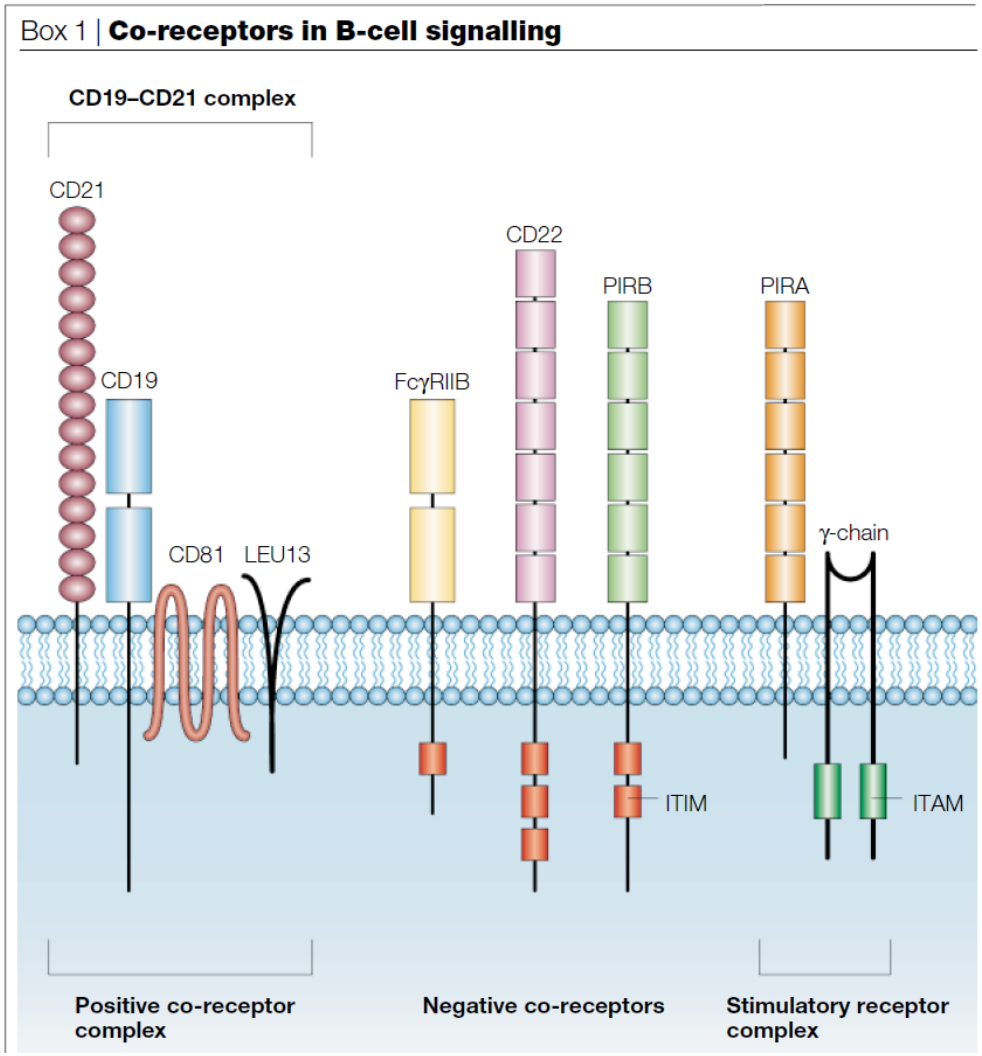


B Cell Receptor Signaling





BCR Co-Receptors



CD19–CD21

CD19 is a B-cell-specific transmembrane glycoprotein that is expressed from the pro-B-cell to the plasma-cell stage. On mature B cells, CD19 associates with three different molecules to form a tetrameric complex comprising CD21 (complement receptor type 2), CD81 (transporter for antigen processing 1) and LEU13 (interferon-induced transmembrane protein 1). On B-cell receptor (BCR) activation, the cytoplasmic tail of CD19 is phosphorylated by LYN, and provides binding sites for the SRC-homology 2 (SH2) domains of phosphoinositide 3-kinase (PI3K) and VAV.

FcγRIIB

FcγRIIB, an inhibitory receptor for the Fc of immunoglobulin G, contains one immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. When FcγRIIB is co-ligated with BCR, SH2-domain-containing inositol 5-phosphatase (SHIP) is recruited, leading to the abrogation of BCR signalling by the hydrolysis of phosphatidylinositol-3,4,5-triphosphate ($PtdInsP_3$).

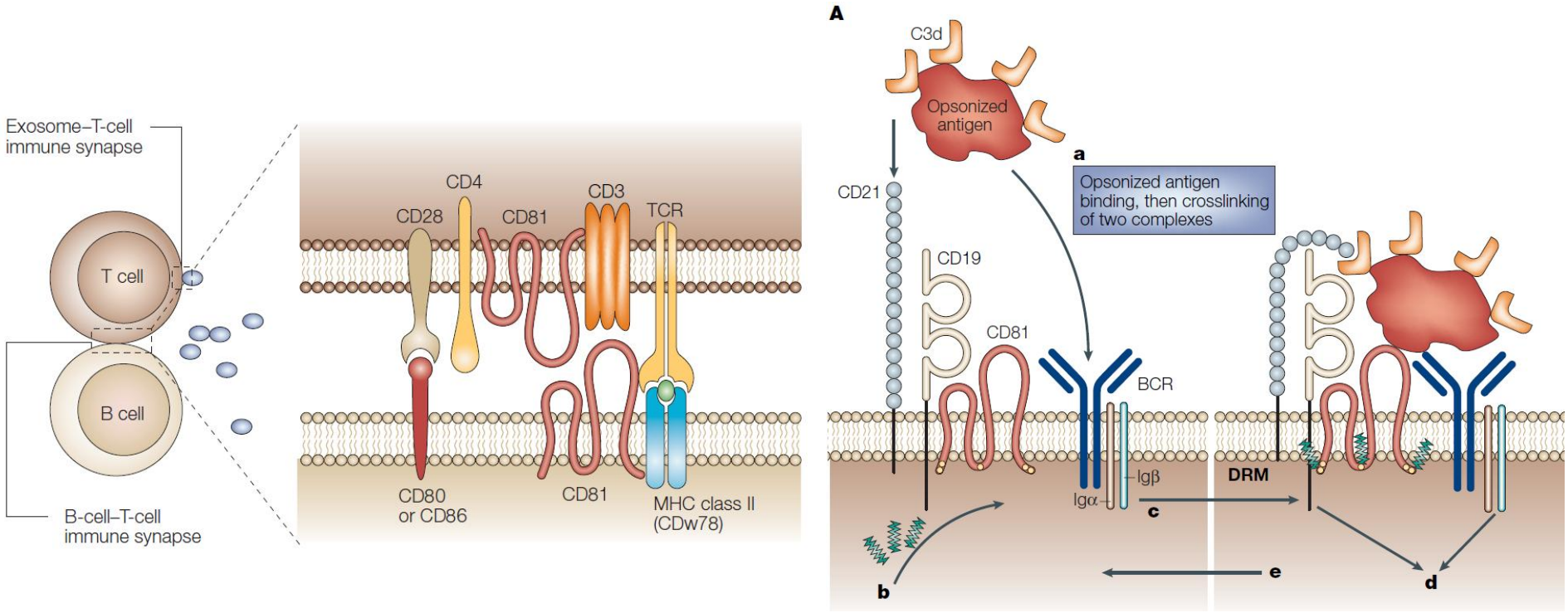
CD22

CD22 is an ITIM-containing surface molecule that interacts with sialic-acid-bearing ligands. It is a member of the sialoadhesin class of immunoglobulin-superfamily receptors. The ubiquitous distribution of sialic-acid-bearing ligands might account for the generalized inhibitory property of CD22 on B cells in regulating activation through the BCR. The ITIMs in CD22 interact with SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), thereby opposing the activation that is mediated by the BCR.

PIRA/PIRB

Paired immunoglobulin-like receptor A (PIRA) and PIRB have similar extracellular domains, which indicates that they have the same ligand specificity, although their ligand is still unknown. PIRB has two ITIMs in its cytoplamic domain, phosphorylation of which recruits SHP1. This attenuates BCR-triggered activation responses through the dephosphorylation of several intracellular substrates, including SYK and Bruton's tyrosine kinase (BTK). In contrast to PIRB, PIRA functions as a stimulatory receptor through its association with the immunoreceptor tyrosine-based activation motif (ITAM)-bearing FcR γ-chain.

Tetraspanin CD81 Enhances BCR/TCR Signaling



Regulation of antigen receptor signaling complexes by the 'tetraspanin web' (CD81)

Lipid Rafts Help Control BCR Signaling

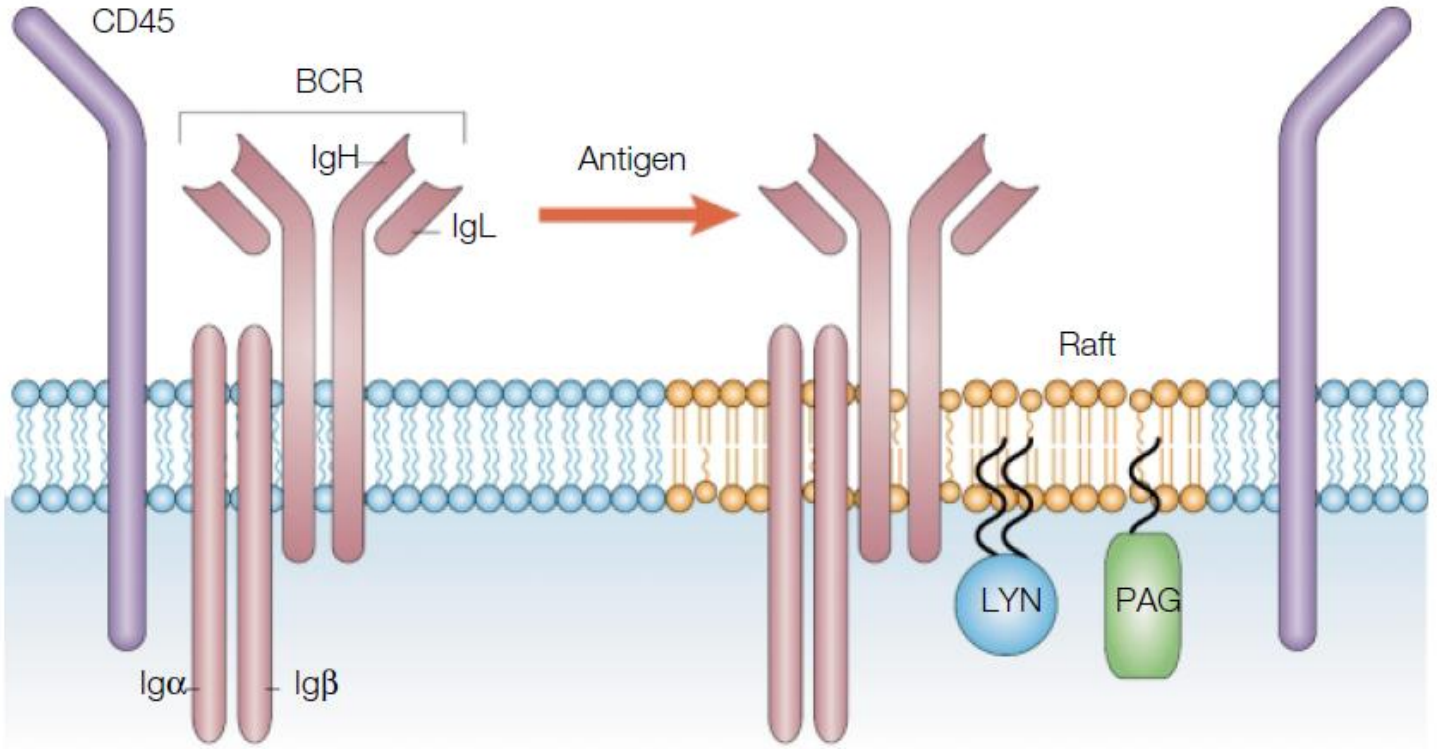
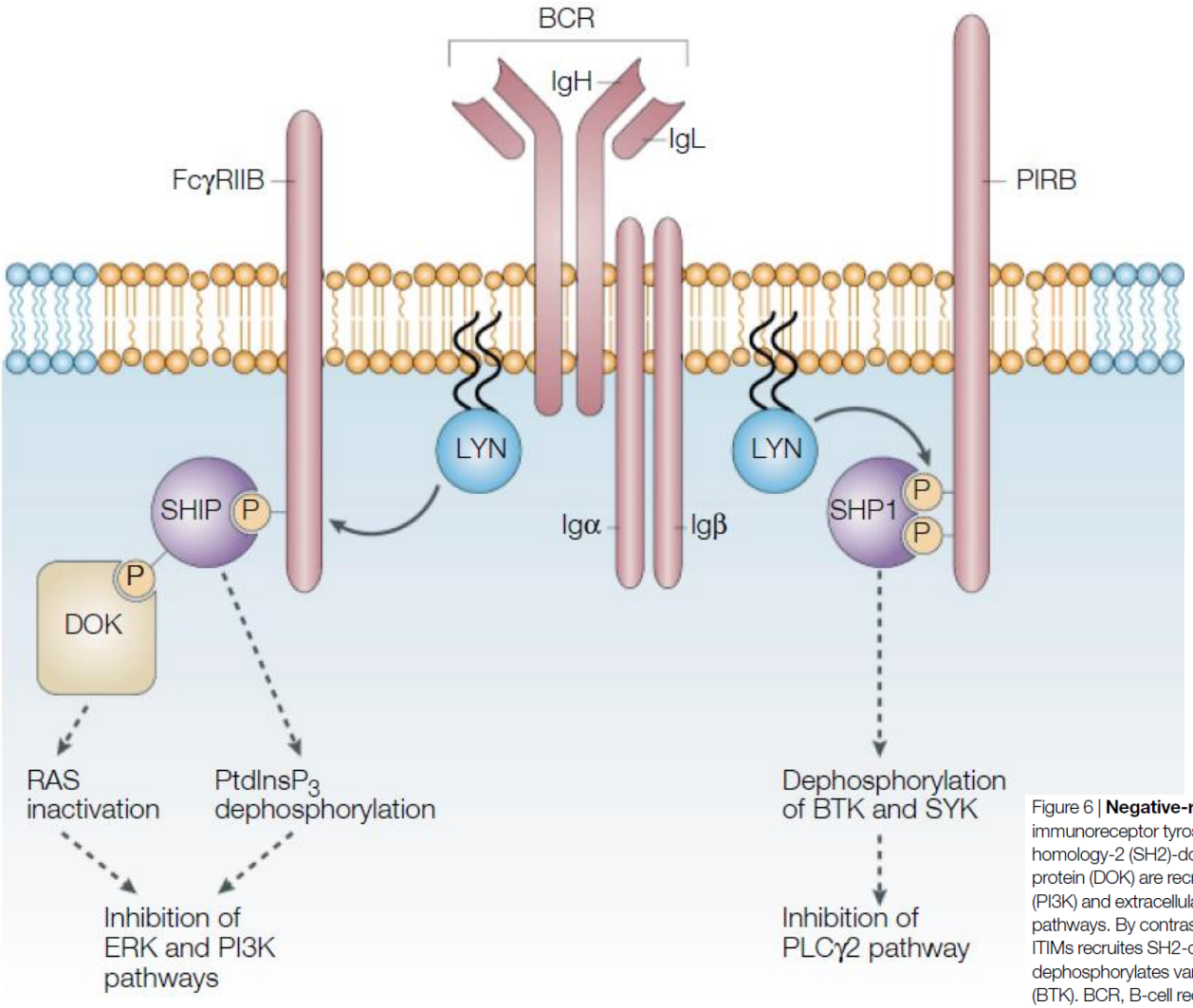


Figure 2 | **Raft translocation of the BCR by ligand binding.** In resting B cells, the B-cell receptor (BCR) is excluded from lipid rafts, as are most plasma-membrane proteins, including CD45. The rafts concentrate glycosylphosphatidylinositol (GPI)-linked proteins and myristylated proteins, such as LYN and phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG). After antigen engagement, the BCR relocates within rafts. IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain.

Kurosaki, *Nat Rev Immunol* 2002

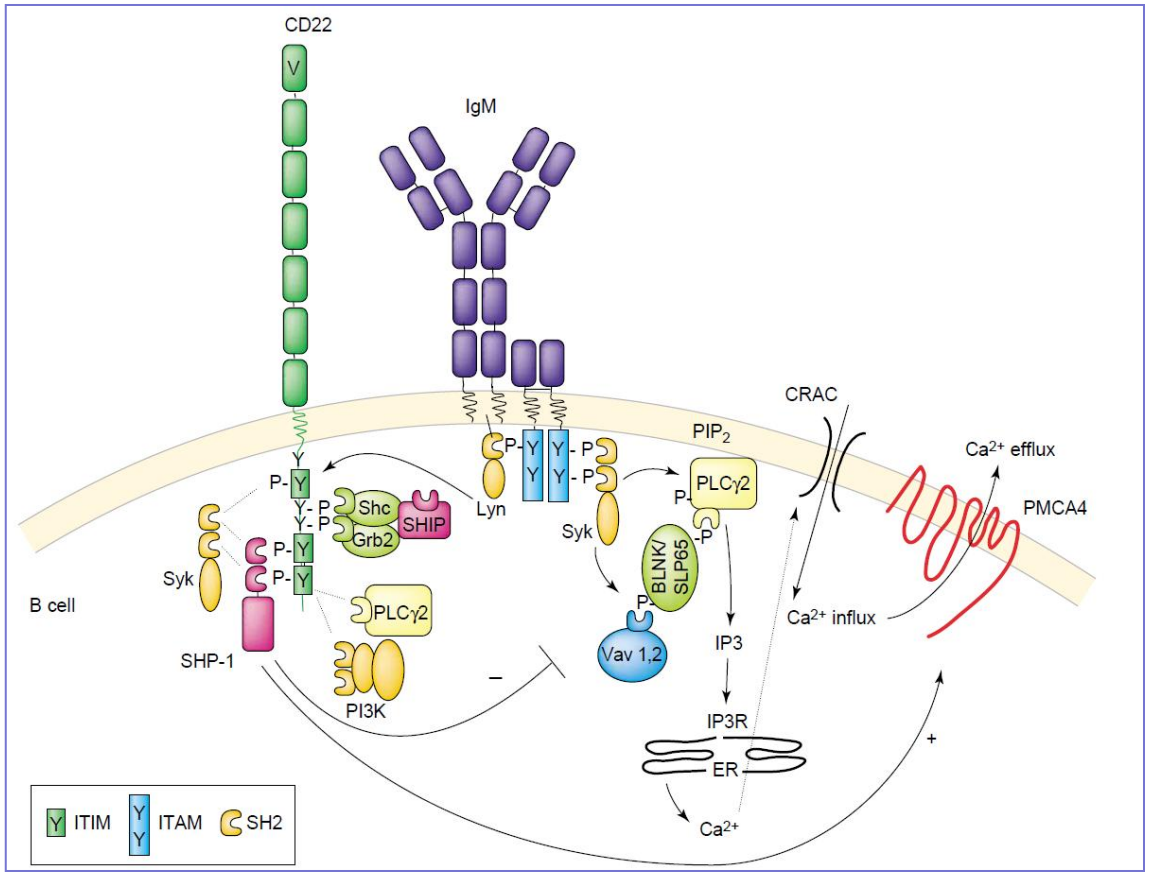
ITIMs in Co-Receptors Fine Tune Signaling



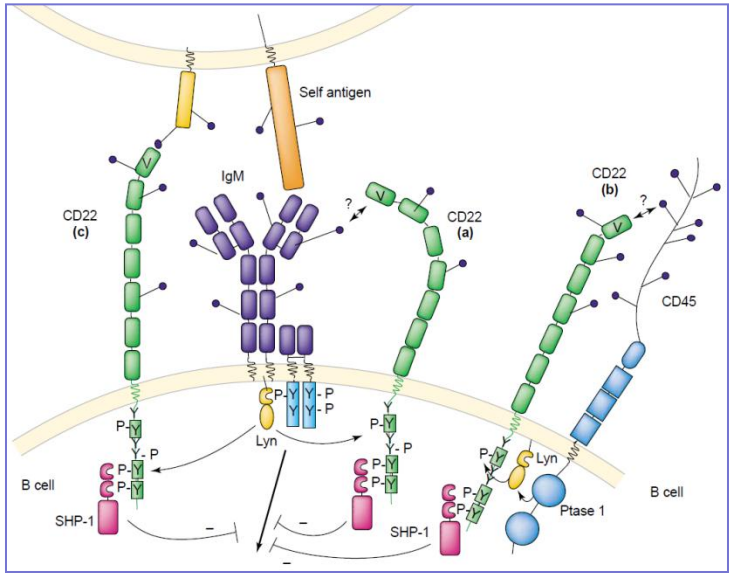
IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF (ITIM). A structural motif containing tyrosine residues that is found in the cytoplasmic tails of several inhibitory receptors, such as FcγRIIB and PIRB. The prototype six-amino-acid sequence is (Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-Leu/Val. Ligand-induced clustering of these inhibitory receptors results in tyrosine phosphorylation, often by SRC-family tyrosine kinases, which provides a docking site for the recruitment of cytoplasmic phosphatases that have an SH2 domain.

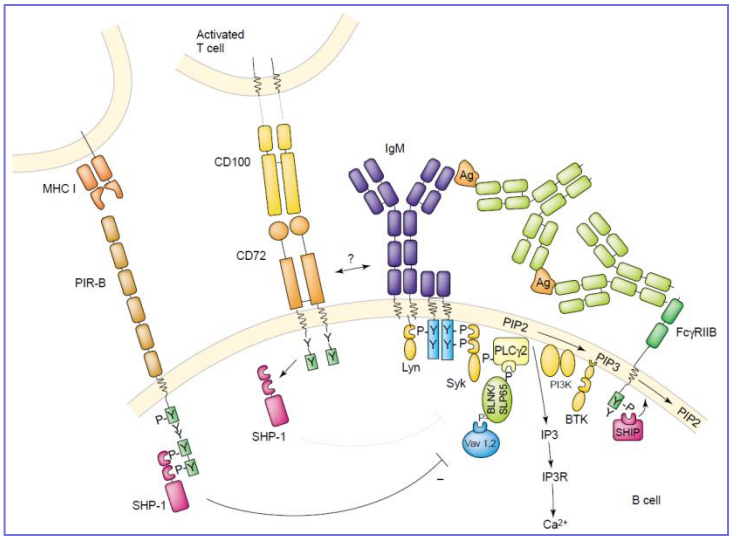
Figure 6 | **Negative-regulatory loops mediated by inhibitory receptors on B cells.** Once the immunoreceptor tyrosine-based inhibitory motif (ITIM) of FcγRIIB is phosphorylated, SRC-homology-2 (SH2)-domain-containing inositol 5-phosphatase (SHIP) and the associated docking protein (DOK) are recruited, which, in turn, have negative influences on phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK; mitogen-activated protein kinase 1) pathways. By contrast, tyrosine phosphorylation of paired immunoglobulin-like receptor B (PIRB) ITIMs recruites SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), which, in turn, dephosphorylates various protein-tyrosine kinases, including SYK and Bruton's tyrosine kinase (BTK). BCR, B-cell receptor; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; PLCγ2, phospholipase Cγ2; $PtdInsP_3$, phosphatidylinositol-3,4,5-triphosphate.

ITIMs in Co-Receptors Dampen Signaling



Regulation of B-cell receptor signalling by CD22. Upon ligation of mIgM tyrosines of the CD22 cytoplasmic tail are phosphorylated by the BCR-associated kinase Lyn. The tyrosines of CD22 that have been mapped as interaction sites are indicated with intracellular binding proteins. A clear function has only been shown for binding of tyrosine phosphatase SHP-1. SHP-1 dephosphorylates intracellular signalling molecules (indicated by –). BCR signalling triggers depletion of intracellular Ca^{2+} stores of the endoplasmic reticulum (ER). This activates opening of Ca^{2+}-release-activated channels (CRACs) and triggers Ca^{2+} influx. CD22 (via SHP-1) activates (indicated by +) the Ca^{2+} pump PMCA4 and thereby controls Ca^{2+} efflux. Abbreviations: IP3, inositol-1,4,5-trisphosphate; IP3R, IP3 receptor.





Redox (H_2O_2) May Also Control BCR Signaling

Amplification of B Cell Antigen Receptor Signaling by a Syk/ITAM Positive Feedback Loop

Molecular Cell, Vol. 10, 1057–1069, November, 2002, Copyright ©2002 by Cell Press

Hydrogen peroxide as second messenger in lymphocyte activation

Michael Reth

Regulation of PTPs. In resting cells, PTPs are active and carry a deprotonated cysteine in their active center. Upon signaling, NADPH oxidases (NOXs) become active and produce, in conjunction with superoxide dismutase (SOD), H_2O_2 that oxidizes the cysteine to sulfenic acid (C-SOH) and renders PTP inactive. The reducing environment of the cytosol contains many redox regulators—such as glutathione (GSH) and thioredoxin (Trx)—which reduce sulfenic acid to cysteine, thereby reactivating the PTP.

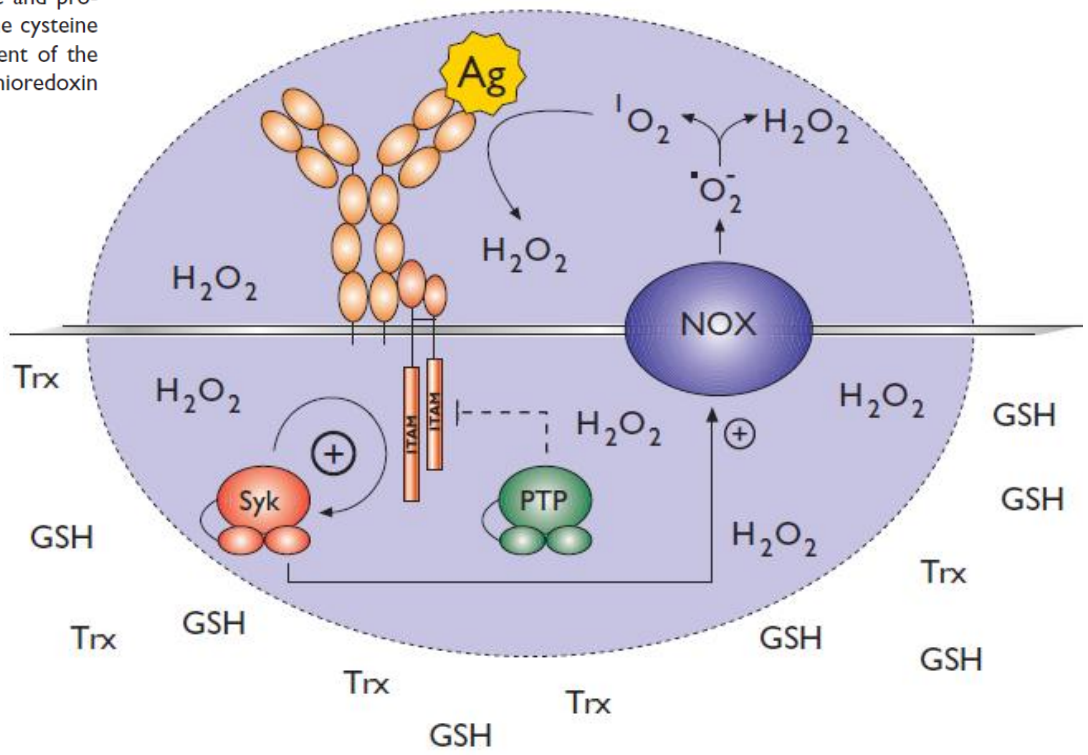


Figure 2. Model of the redox regulation of BCR signaling. (**a**) In the resting state of the BCR (a complex between membrane IgM (mIgM) and the Igαβ heterodimer), the signal-transducing kinase Syk cannot become activated at the ITAM, as any ITAM phosphorylation is prevented by dominant PTP activity. (**b**) Upon antigen (Ag) binding, the BCR is localized closed to a ROS-producing NADPH oxidase. The increased H_2O_2 production generates around the BCR an oxidizing environment or domain (dashed circle) that inhibits PTP, thus allowing Syk to become active. Signals through Syk and Lyn (data not shown) can further activate the NADPH oxidase, resulting in increased H_2O_2 production and spreading of the signal. During the conversion of $^{\bullet}O_2^-$ into H_2O_2, singlet oxygen ($^1O_2$) is produced that is reduced by the catalytic activity of Ig (here, mIgM) into H_2O_2. This process may help increase the oxidizing domain range around the BCR.

Redox Signals May Provide BCR Signal Stability

The Strength of Receptor Signaling Is Centrally Controlled through a Cooperative Loop between Ca^{2+} and an Oxidant Signal

Cell, Vol. 121, 281–293, April 22, 2005,

Dinesh Kumar Singh, Dhiraj Kumar, Zaved Siddiqui, Sandip Kumar Basu, Vikas Kumar, and Kanury V.S. Rao*
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India

In this study we examined the dynamics and possible regulatory mechanisms of signal propagation from the BCR. We provide evidence to suggest that it is the strength of the initial signal generated that controls both the rate and extent of its subsequent progression through the downstream pathways. The former, in turn, is shown to depend upon a positive feedback loop that was rapidly established between two BCR-dependent second messengers, Ca_i^{2+} and reactive oxygen species (ROS). A rheostat-like function could be assigned to this loop, in terms of regulating the amplitude and duration of BCR signaling.

The predominant oxidant species produced in response to stimulation of A20 cells with anti-IgG appears to be H_2O_2, at least as deduced from the experiments described here. Importantly, ROS was rapidly induced, being detectable within seconds of addition of anti-IgG. Furthermore, the effect of Ca_i^{2+} on ROS levels was also evident from the earliest time point of detection of ROS. In this context our present results identify *DUOX1* as at least one of the enzymatic components responsible for the early BCR-dependent ROS production. Our experiments employing siRNA revealed that *DUOX1* is critical for establishing the BCR-dependent Ca_i^{2+}-ROS feedback loop and, thereby, ensuring optimal phosphorylation of Lyn following BCR activation.

Differential Regulation of IgG vs. IgM BCRs

A Distinct Signaling Pathway Used by the IgG-Containing B Cell Antigen Receptor

Chisato Wakabayashi,[1]* Takahiro Adachi,[1]*
Jürgen Wienands,[2]† Takeshi Tsubata[1]‡

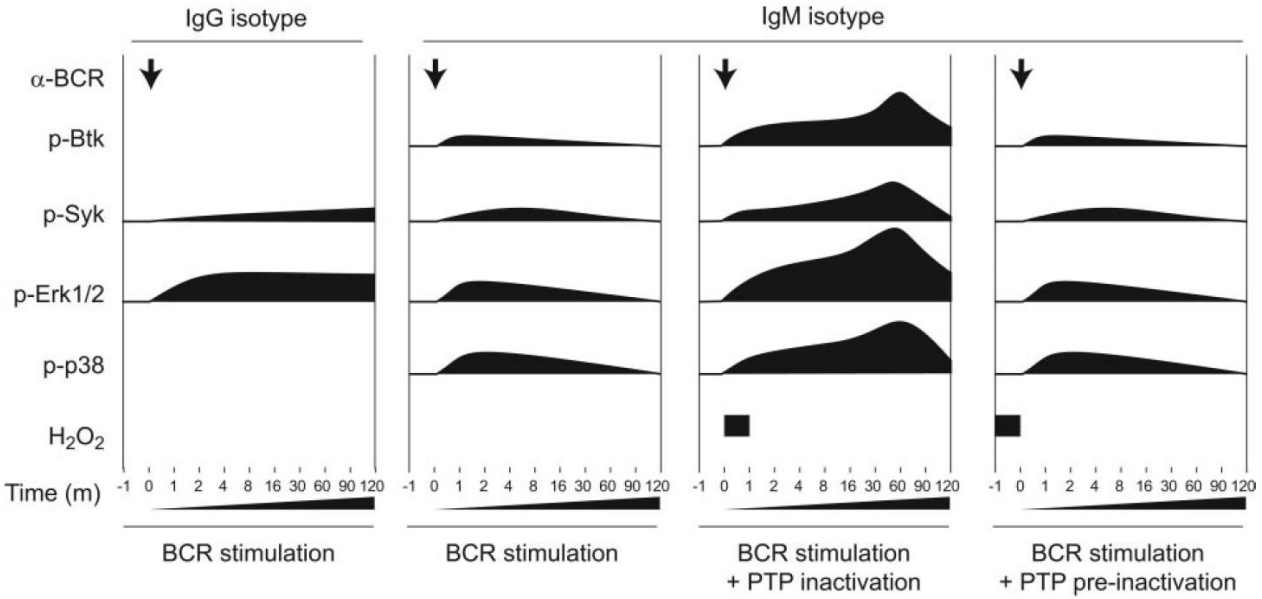
The immunoglobulin G (IgG)–containing B lymphocyte antigen receptor (IgG-BCR) transmits a signal distinct from that of IgM-BCR or IgD-BCR, although all three use the same signal-transducing component, Igα/Igβ. Here we demonstrate that the inhibitory coreceptor CD22 down-modulates signaling through IgM-BCR and IgD-BCR, but not that through IgG-BCR, because of the IgG cytoplasmic tail, which prevents CD22 phosphorylation. These results suggest that the cytoplasmic tail of IgG specifically enhances IgG-BCR signaling by preventing CD22-mediated signal inhibition. Enhanced signaling through IgG-BCR may be involved in efficient IgG production, which is crucial for immunity to pathogens.

20 DECEMBER 2002 VOL 298 SCIENCE www.sciencemag.org

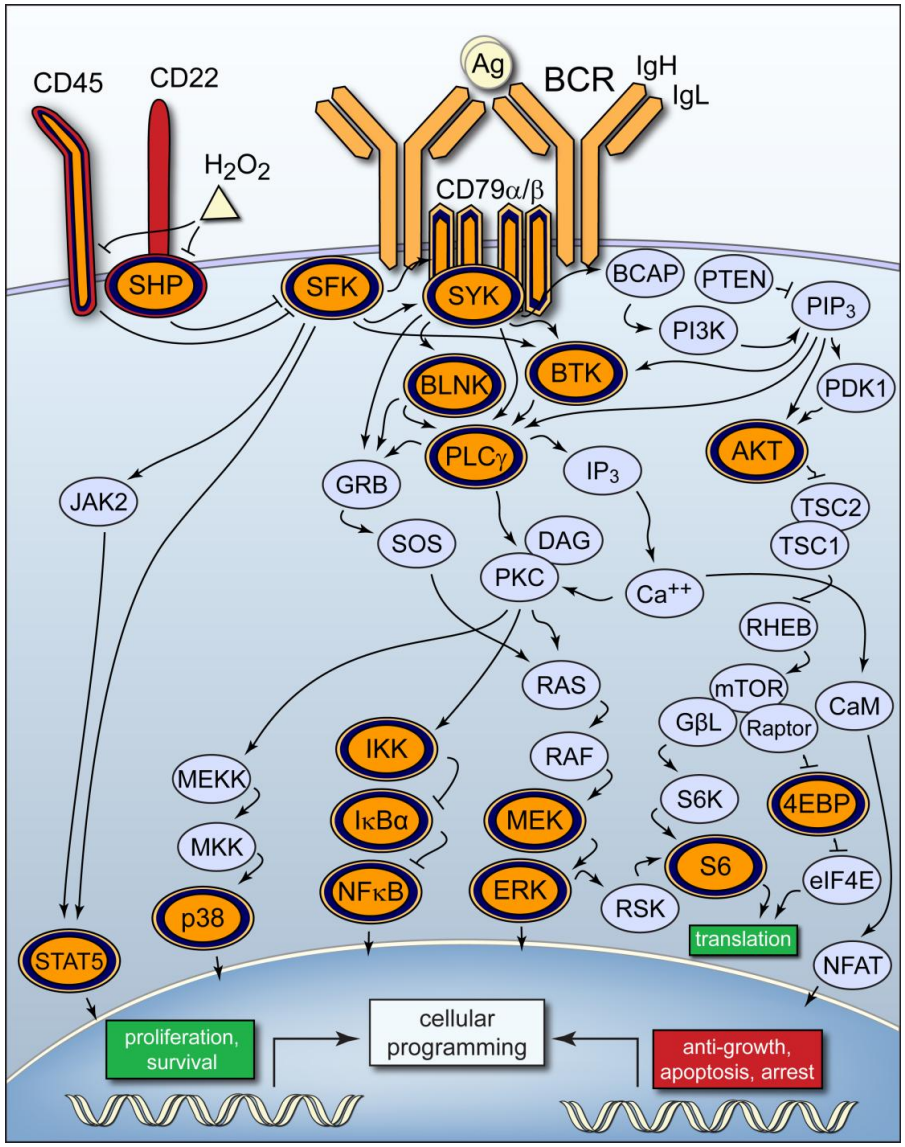
Kinetics of B Cell Receptor Signaling in Human B Cell Subsets Mapped by Phosphospecific Flow Cytometry[1]

Jonathan M. Irish,[†‡] Debra K. Czerwinski,[†] Garry P. Nolan,[3‡] and Ronald Levy[2,3†]

Differences in BCR signaling may govern outcomes as diverse as proliferation and cell death. We profiled BCR signaling kinetics in subsets of primary human B cells using flow cytometry. In the predominant population expressing IgM, BCR cross-linking led to a quick burst of Syk, ERK1/2, and p38 signaling. In contrast, IgG B cells sustained higher per-cell ERK1/2 phosphorylation over time. This dichotomy suggested a mechanism for dampening signals transmitted by IgM. Regulatory phosphatase activity in IgM B cells was BCR-mediated and initiated more slowly than kinase activity. This BCR-mediated phosphatase activity was sensitive to inhibition by H_2O_2 and required to attenuate IgM BCR signaling. These results provide the first kinetic maps of BCR signaling in primary human B cell subsets and enable new studies of signaling in B cell disorders, such as autoimmunity and cancer. *The Journal of Immunology*, 2006, 177: 1581–1589.



B Cell Receptor Signaling



Controlling BCR Signaling is Critical to Development

REPORTS

B Cell Receptor Signal Transduction in the GC Is Short-Circuited by High Phosphatase Activity

Ashraf M. Khalil,[1] John C. Cambier,[3] Mark J. Shlomchik[1,2]*

Germinal centers (GCs) generate memory B and plasma cells, which are essential for long-lived humoral immunity. GC B cells with high-affinity B cell receptors (BCRs) are selectively expanded. To enable this selection, BCRs of such cells are thought to signal differently from those with lower affinity. We show that, surprisingly, most proliferating GC B cells did not demonstrate active BCR signaling. Rather, spontaneous and induced signaling was limited by increased phosphatase activity. Accordingly, both SH2 domain–containing phosphatase-1 (SHP-1) and SH2 domain–containing inositol 5 phosphatase were hyperphosphorylated in GC cells and remained colocalized with BCRs after ligation. Furthermore, SHP-1 was required for GC maintenance. Intriguingly, GC B cells in the cell-cycle G_2 period regained responsiveness to BCR stimulation. These data have implications for how higher-affinity B cells are selected in the GC.

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1 JUNE 2012 VOL 336 **SCIENCE** www.sciencemag.org

Khalil et al., *Science* 2012

What are consequences of inappropriate antigen receptor signaling?

Autoimmunity, allergy, cancer, and infections

B Cell Lymphoma: BCR Selection Failure

REVIEWS

MECHANISMS OF B-CELL LYMPHOMA PATHOGENESIS

Ralf Küppers

Abstract | Chromosomal translocations involving the immunoglobulin loci are a hallmark of many types of B-cell lymphoma. Other factors, however, also have important roles in the pathogenesis of B-cell malignancies. Most B-cell lymphomas depend on the expression of a B-cell receptor (BCR) for survival, and in several B-cell malignancies antigen activation of lymphoma cells through BCR signalling seems to be an important factor for lymphoma pathogenesis. Recent insights into the lymphomagenic role of factors supplied by the microenvironment also offer new therapeutic strategies.

Küppers, *Nat Rev Cancer* 2005

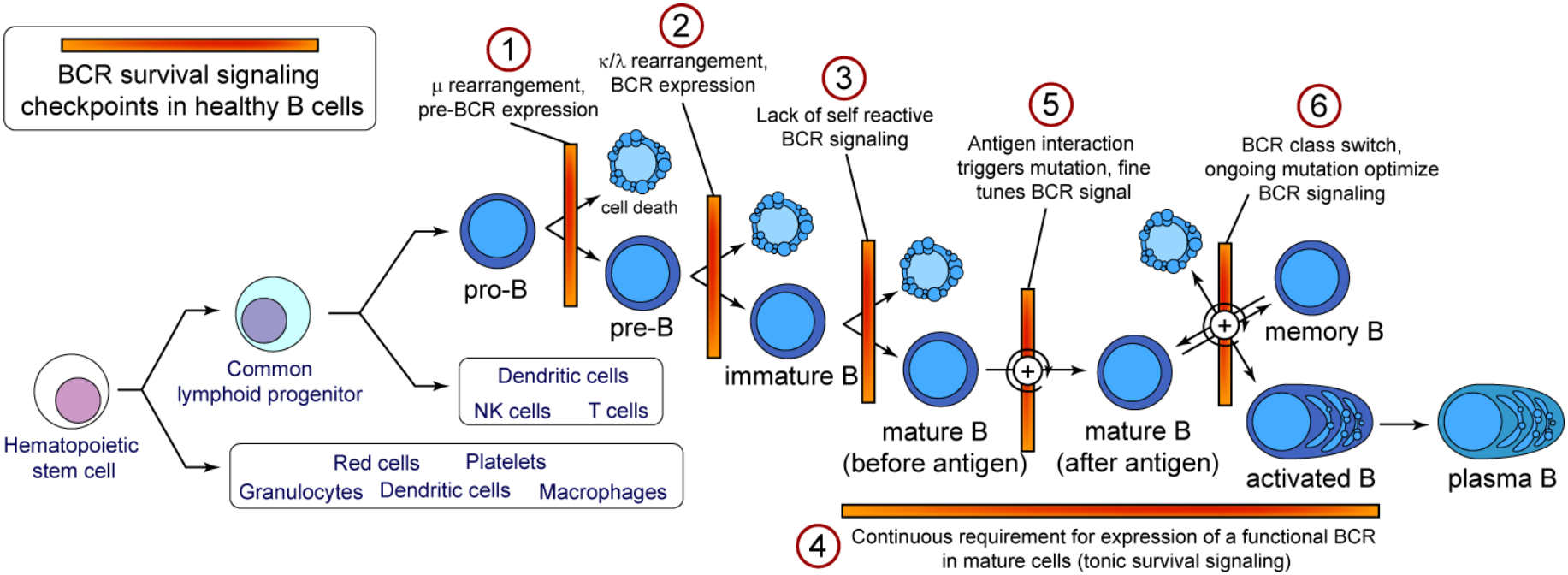
BCR Signaling as 'Oncogenic'

Lymphomas associated with BCR expression and indication for antigen activation

- Follicular lymphomas arise and grow in the germinal centre and in some patient samples the BCR is autoreactive. The BCR variable domain contains mutations that promote carbohydrate modification.
- Gastric mucosa-associated lymphoid tissue lymphomas are in many cases associated with autoreactive BCR, particularly with rheumatoid factors.
- B-cell chronic lymphocytic leukaemia has a restricted variable (V)-region gene repertoire and the BCR is often autoreactive. A BCR specific to human T-cell lymphotropic virus 1 has been identified in patients who are infected with this virus.
- In hepatitis C virus (HCV)-associated lymphomas, HCV-specificity of BCR has been reported in some cases. Disease regression occurs after antiviral therapy.
- In primary central nervous system lymphomas, about half the cases express the same heavy-chain (V_H) gene segment (VH4-34), whereas other genes of the BCR are diverse, indicating tumour-cell stimulation by superantigen binding to the BCR.

Küppers, *Nat Rev Cancer* 2005

BCR Signaling Checkpoints in Healthy Development



Immune Status Matters for Cancer

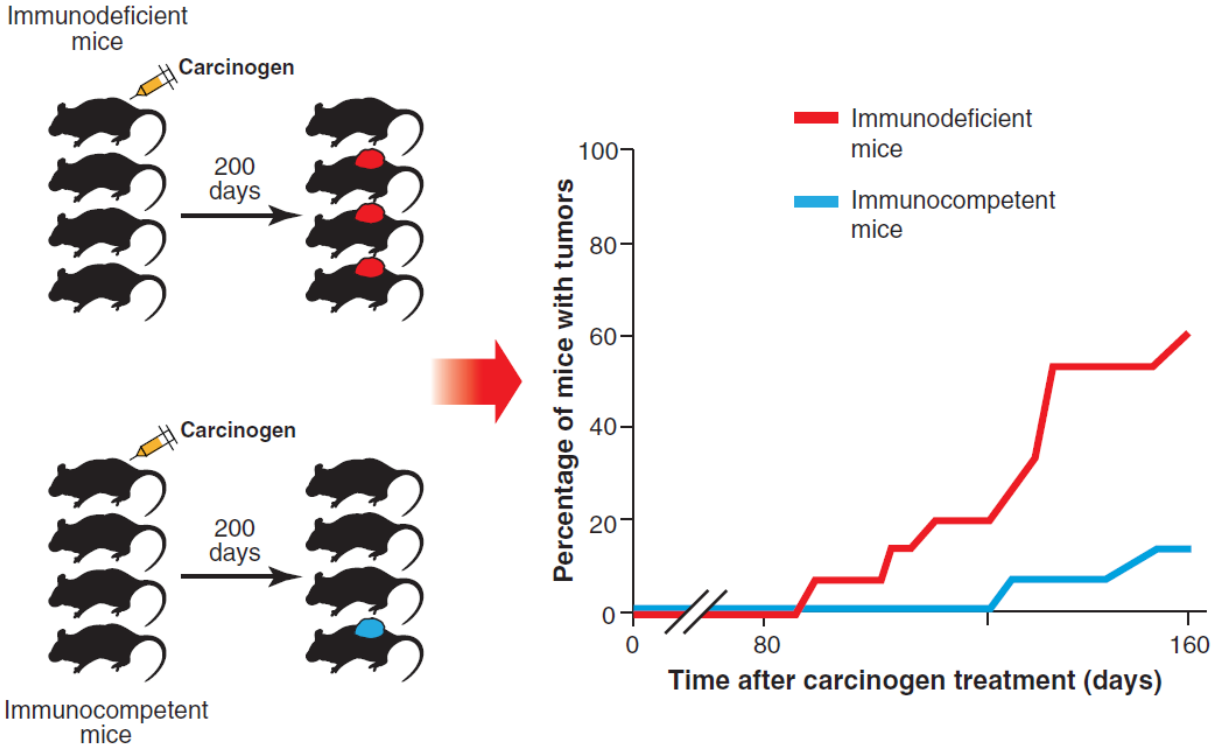
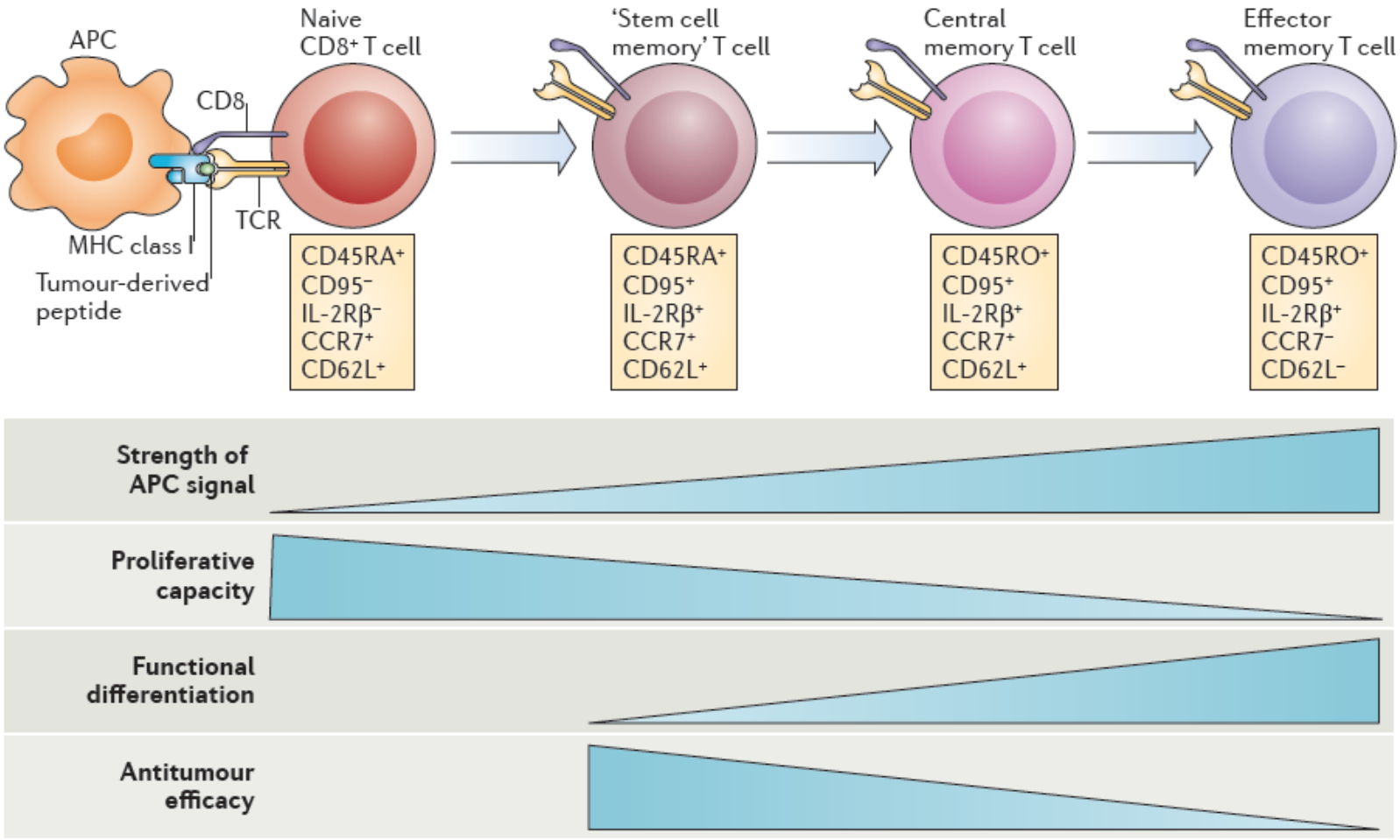


Fig. 1. The immune status of mice is a critical determinant of their susceptibility to tumors induced by chemical carcinogens. Over the past two decades, numerous studies have established that immunodeficient mice are more tumor prone than are immunocompetent mice after treatment with carcinogens such as MCA. The immunodeficient mice tested in such experiments include gene-targeted mice on pure genetic backgrounds with deficits of innate or adaptive immunity as well as wild-type mice rendered immunodeficient by chronic administration of monoclonal antibodies that, for example, deplete $CD4^+$ and $CD8^+$ T cells or interferon-γ. Immunodeficiency has also been found to increase the susceptibility of untreated mice to spontaneously arising tumors and to increase the incidence of tumor formation in mouse genetic models of cancer. Schematic is based on experiments described in (*13*).

Schreiber et al., *Science* 2011 / Nature 2001

Challenges in Making Effective Anti-tumor T Cells



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Förster resonance energy transfer (FRET)

Fluorescent protein FRET: the good, the bad and the ugly

David W. Piston and Gert-Jan Kremers

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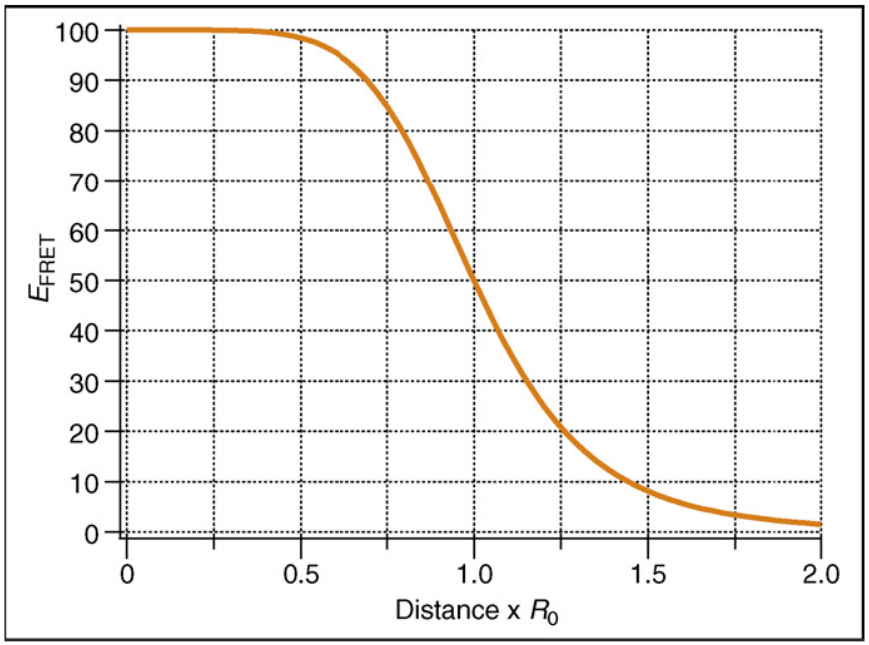
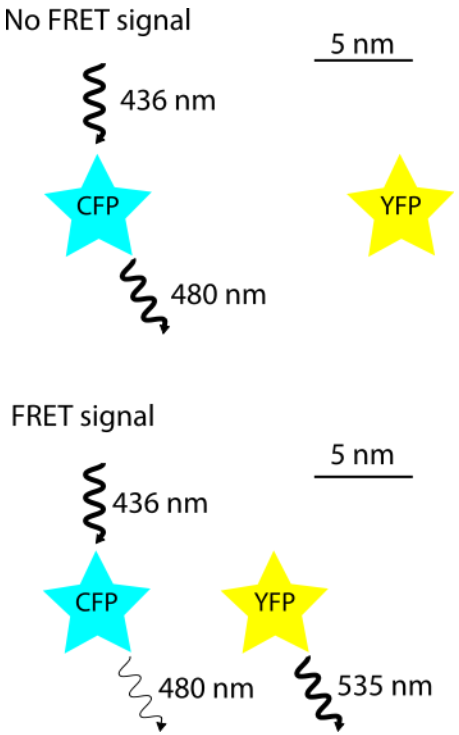


Figure 1. FRET efficiency versus distance. The FRET efficiency (E_{FRET}) varies with the sixth power of distance between donor and acceptor. As a result, there is a steep fall in E_{FRET} with increasing distance. The Förster radius (R_0) is the distance at which 50% FRET occurs. Owing to the strong distance dependence, FRET is usually detected only when the two fluorophores are closer than $1.5R_0$.



Example of FRET between CFP and YFP (Wavelength vs. Absorption): a fusion protein containing CFP and YFP excited at 440nm wavelength. The fluorescent emission peak of CFP overlaps the excitation peak of YFP. Because the two proteins are adjacent to each other, the energy transfer is significant–a large proportion of the energy from CFP is transferred to YFP and creates a much larger YFP emission peak.
http://en.wikipedia.org/wiki/F%C3%B6rster_resonance_energy_transfer

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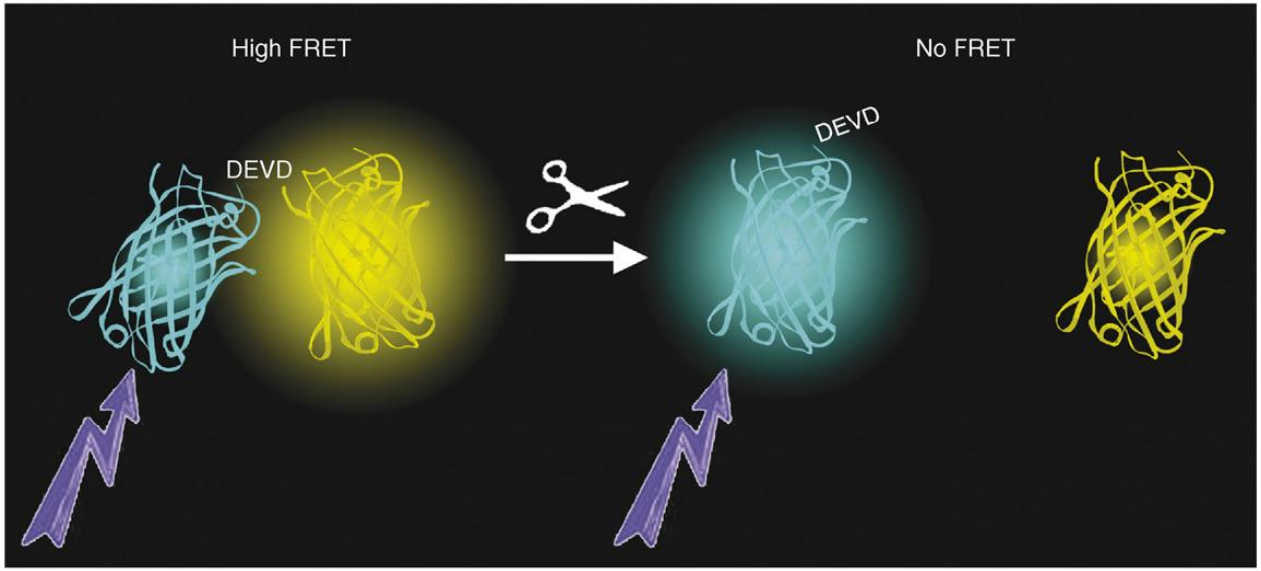

Figure 5. Biosensor for caspase-3 protease activity. FRET-based biosensors for protease activity can be constructed by directly fusing the donor and acceptor FPs through a protease sensitive linker with the sequence Asp-Glu-Val-Asp (DEVD). In the absence of protease activity, the biosensor will show a high FRET efficiency, owing to the short distance between donor and acceptor. Activation of Caspase3 (represented by scissors) results in cleavage of the linker, which enables donor and acceptor to diffuse away from each other, thereby abolishing FRET.

Viewing the Initiation of BCR Signaling

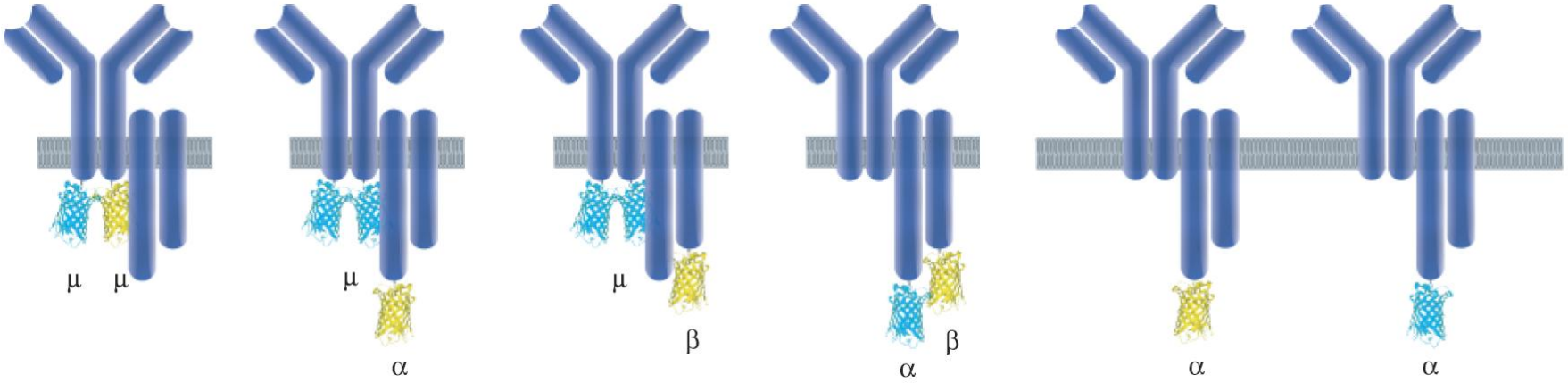

Fig. 1. Fluorescent BCR constructs used to probe the interactions of the BCR chains by FRET imaging. B-cell lines were generated that expressed all possible combinations of the BCR's Ig, Igα, and Igβ chains engineered to contain FRET donor CFP and FRET acceptor YFP fluorescent proteins to report on interchain distances within a BCR (Top). Depicted are constructs containing Igμ. Corresponding cell lines were generated using Igγ. A cell line was also generated that expressed both Igα-CFP and Igα-YFP in approximately equal amounts to report on interactions between BCRs (bottom). Images are of a cell expressing Igμ-CFP and Igα-YFP.

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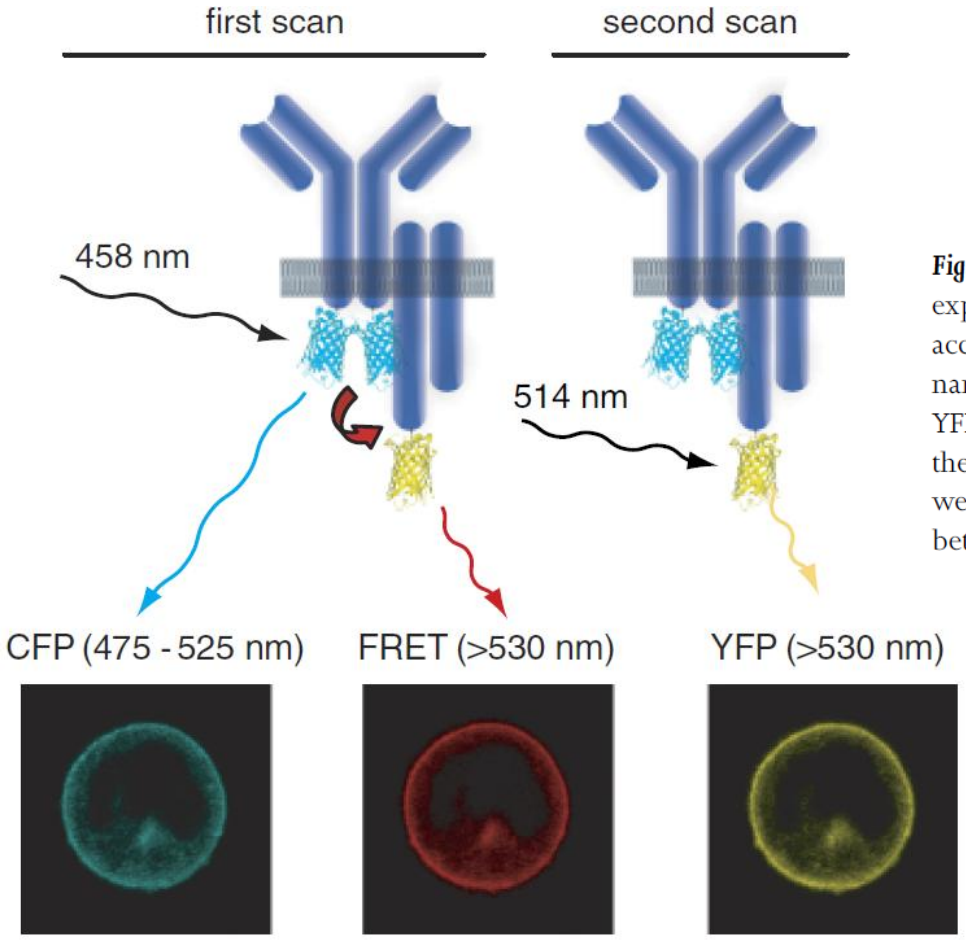

Fig. 2. FRET confocal microscopy in living B cells. Depicted is the experimental design for the measurement of FRET by calibrated sensitized acceptor emission. Fluorescence was collected from three channels, namely CFP (CFP excitation and CFP emissions), FRET (CFP excitation and YFP emissions), and YFP (YFP excitation and YFP emissions). Data from these three channels along with controls correcting for bleed through were sufficient to calculate FRET efficiencies and to estimate the distance between the chains of the B-cell receptor .

Tolar et al., *Immunol Rev* 2008

Viewing the Initiation of BCR Signaling

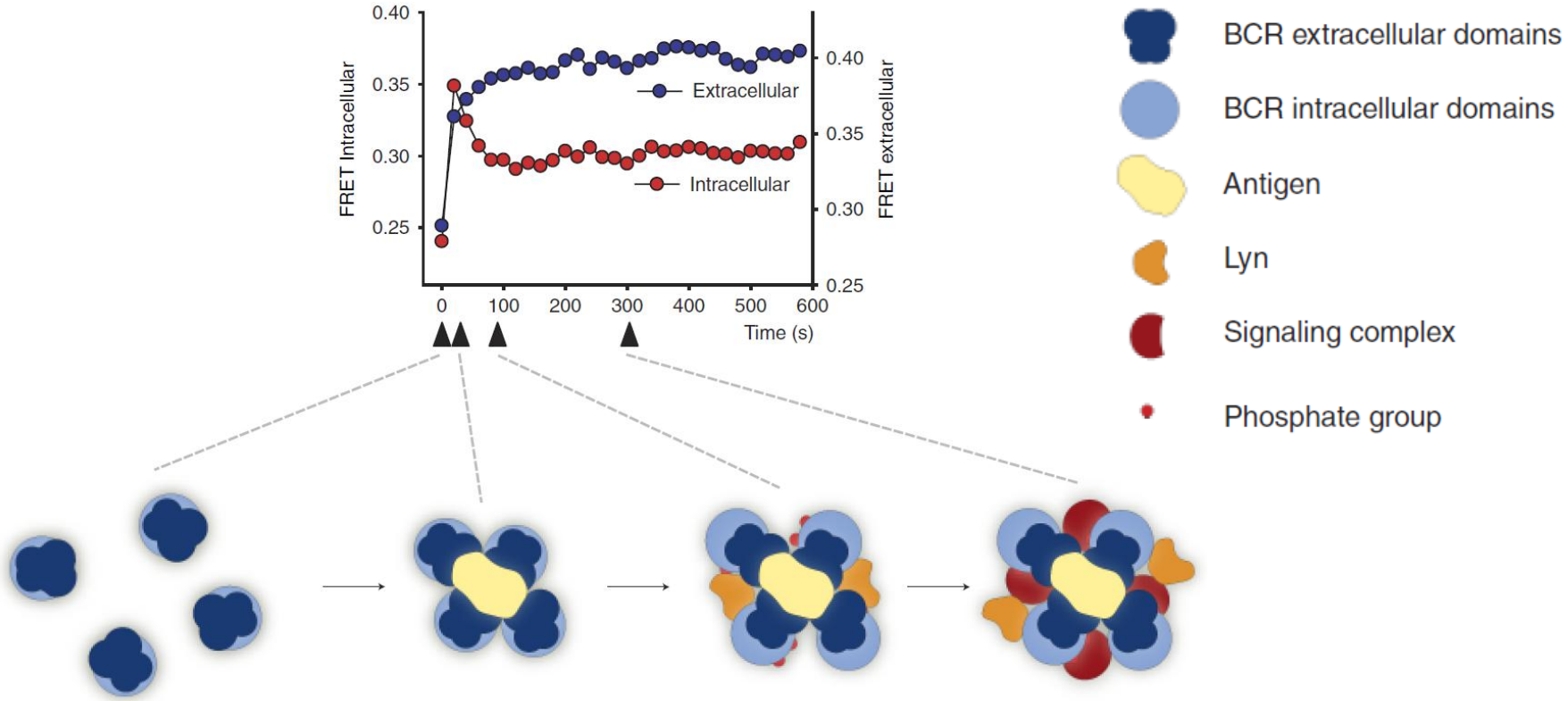

***Fig. 3.* FRET imaging of live B cells revealed a conformational change in the cytoplasmic domains that accompanied BCR clustering and signaling.** Shown are the results of an analysis of FRET from Ig-specific Fab probes containing FRET donor and acceptor fluorescent tags after the addition of the antigens to B cells. Also shown is an example of the FRET efficiencies for the cytoplasmic domains of a BCR containing Igγ-CFP and Igα-YFP. The interpretation of these data is depicted below. The FRET between the ectodomain of the BCR increases with the time, reaches a maximum, and is maintained at that level, indicating that the BCRs are brought into close molecular proximity in a stable cluster. The pattern of FRET between the cytoplasmic domains is strikingly different. FRET increases with the addition of antigen, peaks, and then decreases. Because this pattern was similar for all of the combinations of BCR CFP- and YFP-containing chains shown in *Fig. 1*, including the Igα-CFP and Igα-YFP expressing cell line that reports on the interactions between individual BCRs, we interpret this result to reflect an initial antigen-induced clustering of the BCR cytoplasmic domains and then an opening of the domains. The opening correlates with the phosphorylation of the BCR by Lyn and precedes the recruitment of Syk.

Tolar et al., *Immunol Rev* 2008

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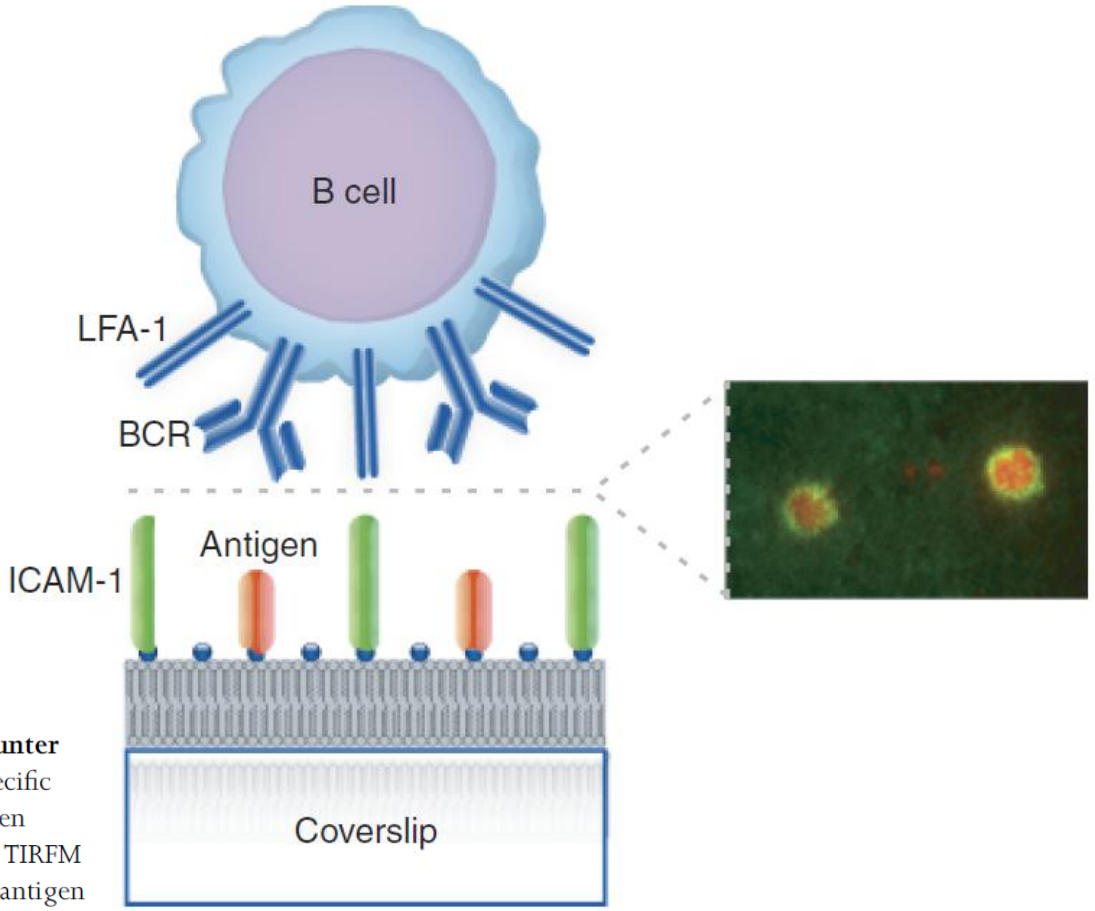

Fig. 4. TIRFM imaging of antigen-specific B cells as they encounter antigen- and ICAM-1-containing planar lipid bilayers. NIP-specific splenic B cells incubated on red-fluorescent NIP-antigen– and green fluorescent ICAM-1-containing lipid bilayers. Images obtained by TIRFM show that the B cells have formed immune synapses with the NIP-antigen clustered in the center surrounded by ICAM-1.

Tolar et al., *Immunol Rev* 2008

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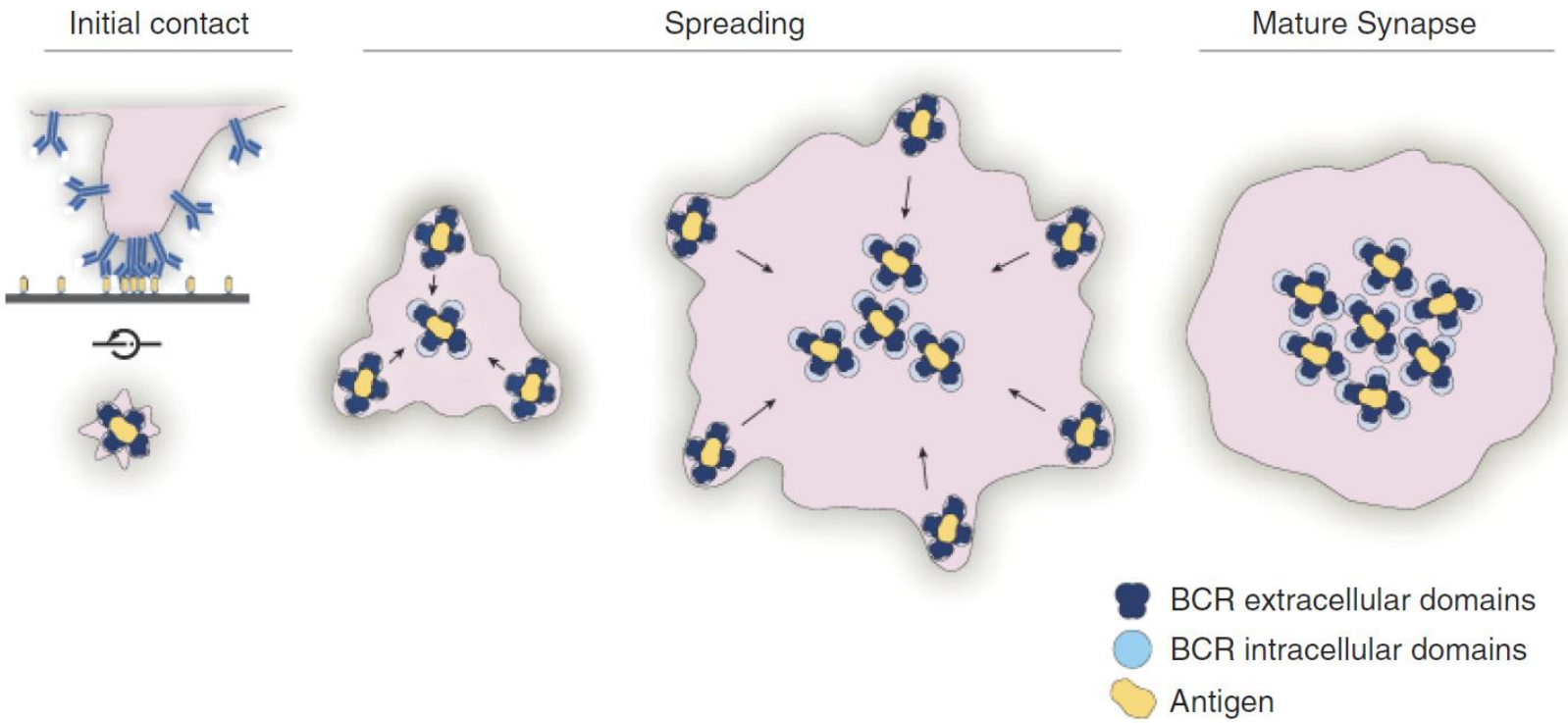

Fig. 5. A depiction of the results obtained by TIRFM and FRET of B cells encountering antigen on a planar lipid bilayer. Beginning from the left, the B cell first encounters the antigen-containing bilayer by small membrane protrusions that reach towards the bilayer. Depicted are both a TIRFM view and a side view of the B-cell protrusion touching the bilayer. The BCRs in these protrusions cluster following antigen binding and undergo a conformational change to a signaling open form. The formation of the clusters was similar for monovalent and multivalent antigens. The BCR signaling triggers a spreading response, and new BCR clusters are formed and open in the protrusions at the leading edge of the cell. These open clusters are actively transported toward the center of the contact area forming the immune synapse. The clusters continue to form and open in the ruffling membranes in the periphery of the spread cell and move to the center of the synapse.

Tolar et al., *Immunol Rev* 2008