CHAPTER-1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction to the Study

Immature B-lymphocytes have been widely studied in terms of their responses to antigen. Stimulation of these cells through their surface antigen receptors (BCR) leads to late G1 arrest followed by apoptosis (Banerji et al., 2001; Niiro and Clark, 2002; Scott et al., 1986). A variety of signaling molecules that include MAP kinases, receptor tyrosine kinases, and phosphatases, as well as transcription factors such as FOXO, NFKB, JUN, ATF3, EGR1, c-Myc have been implicated to play a role in this process (Gilchrist et al., 2006; Gururajan et al., 2005; Sutherland et al., 1996; Wu et al., 1996; Yang et al., 2001; Yusuf et al., 2004). Thus, for example, the BCR-mediated arrest in cell cycle has been attributed to the transient nature of ERK activation obtained (Gauld et al., 2002). In contrast, sustained ERK activation was observed in BCR-stimulated mature B cells, thereby leading to a proliferation response (Gauld et al., 2002). In addition to this, however, there are studies that suggest a key role for MEK-1/2 during G1-S transition (Richards et al., 2001), while yet others have implicated another MAP kinase, p38, during the BCR-induced G1 arrest of immature B cells (Swart et al., 2000; Swart and Chiles, 2000). Finally, in addition to these molecular intermediates, factors such as signal strength, amplitude and duration of the signaling response have also been shown to function as
determinants of cell fate (Kemp et al., 2007; Kumar et al., 2007; Niiro and Clark, 2002). However, although several signaling molecules have been identified to play a significant role in determining the outcome of BCR-dependent stimulation of immature B cells, an understanding of how these pathways eventually interact to enforce the G1 arrest has remained elusive.

The initial conceptualization of the signaling machinery as being composed of discrete biochemical cascades has now yielded to the view that it is in fact best represented as a complex network. The properties that emerge from this network, following activation of a given cell surface receptor then influence other functional modules of the cell, thereby driving cell-fate decisions (Kumar et al., 2007; Miller-Jensen et al., 2007). At one level, this is mediated through dynamical influences that are exerted on the downstream transcription factor network. This ensures a unique gene expression profile that enforces the phenotypic response. This revised perspective on both the topological features of the signaling machinery and its mode of function, therefore, prescribes that an understanding of the etiology of receptor-defined cellular responses will require a more global analysis of the resultant perturbations in the signaling network, and the consequent influences on the downstream transcription regulatory module. Consequently, in the present study we undertook a systems biology approach to decipher the mechanisms involved in mediating the BCR-induced G1 arrest of CH1 cells, a murine lymphoma cell line that is prototypic of the transitional stage of immature B cells (Lanier et al., 1982; Lanier et al., 1978). A combination of experimental and in silico approaches allowed us to map the network of pathways emanating from the BCR, and leading up to the induction of genes responsible for the G1 arrest. This network was characterized by the limited and transient activation of signaling intermediates, with the consequent activation of a handful of transcription factors that, together, induced expression of only those genes involved in the cell death pathway. Interestingly,
whereas a dense overlapping regulatory motif facilitated transcription factor mediated expression of these genes, this process was centrally controlled by the MAP kinase signaling intermediate p38. This control of p38 over the receptor-induced cellular response was mediated through a receptor-associated phosphatase, and involved the feedback regulation of Lyn, the kinase that initiates signaling from the BCR. This, in turn, influenced transmission of signals to the downstream intermediates. Importantly, our studies also reveal how changes in the basal activation state of early signaling intermediates defines sensitivity of the signaling machinery to a given cell surface receptor. We subsequently derived a mathematical model that yielded insights into the etiology of cell type-specific responses to a given stimulus.

The B cell Antigen Receptor (BCR) signaling Pathways

Each B cell expresses a unique receptor protein (B cell antigen receptor, or BCR) on its surface. The role of BCR is to identify intact antigens in order to eventually initiate an antibody response. The BCR complex is made up of immunoglobulin heavy (IgH) and light (IgL) chains associated with two signaling components, Igα and Igβ. After BCR ligation by antigen, both the protein tyrosine kinase (PTK) SYK, and SRC-family PTKs such as LYN, are activated initially. Then, LYN phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Igα and Igβ, which, in turn, recruit and facilitate the activation of SYK and the TEC-family PTK BTK5. Although B cells also express other SRC-family kinases, including FYN, BLK, FGR and HCK, mutations of FYN, BLK and FGR have little effect on B-cell function (Lowell and Soriano, 1996; Texido et al., 2000). This might be explained by redundancy of these kinases in BCR signaling. SYK is a key B-cell signaling molecule, because disruption of SYK prevents most downstream BCR signaling (Jiang et al., 1998; Takata et al.,
1994) and leads to a marked block in B-cell development (Turner et al., 1995). The VAV family of RHO-family GTPases, which consists of at least three isoforms VAV1, VAV2 and VAV3 (Turner and Billadeau, 2002) also has a crucial role in BCR signaling. VAV proteins activate RAC1 and regulate cytoskeletal structures after activation (Holsinger et al., 1998; Turner and Billadeau, 2002) and BCR-induced proliferation is defective in VAV1-deficient B cells (Texido et al., 2000; Zhang et al., 1995). In addition, recent studies of Vav1−/− and Vav2−/− mice showed clearly that VAV proteins have a crucial role in B-cell signaling, including calcium mobilization (Doody et al., 2001; Tedford et al., 2001). Although, VAV proteins have been proposed to function downstream of phosphatidylinositol 3-kinase (PI3K) because of their phospholipid-binding pleckstrin-homology (PH) domain, this proposal has recently been challenged by finding in VAV3 deficient cells. Indeed, SYK phosphorylates VAV3 and this, in turn, might regulate the activation of PI3K in B cells (Inabe et al., 2002). However, it remains to be proven if the same is true for VAV1 or VAV2 also. The VAV family might function both upstream and downstream of PI3K in B cells. BTK, together with SYK, phosphorylates and activates phospholipase Cγ2 (PLCγ2) after stimulation. PI3K and PLCγ2 are both crucial effector enzymes that generate key second messengers in BCR signaling (Marshall et al., 2000b). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdInsP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PtdInsP3), which, in turn, recruits some BCR signaling molecules to the membrane through PH domains. PLCγ2 uses PtdInsP2 as the same substrate as PI3K but generates inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG), which are required for the release of intracellular calcium (Ca2+) and activation of protein kinase C (PKC), respectively (Marshall et al., 2000b). Subsequently, Ca2+ flux and PKC activation induce the activation of mitogen-activated protein kinase (MAPK) family kinases extracellular signal
regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38 MAPK and transcription factors, including nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT). It is probable that the profile of these activated transcription factors then determines B-cell fate. Non-enzymatic adaptor proteins are important also for regulating BCR signaling. Among them, the B-cell linker (BLNK) efficiently connects SYK and BTK with PLCγ2 (Fu et al., 1998; Hashimoto et al., 1999). Disruption of the BLNK gene leads to impaired activation of PLCγ2 in B cells (Ishiai et al., 1999). However, BLNK also associates with VAV and NCK19, both of which regulate cytoskeletal organization in B cells. So, the defect in B-cell development is more severe in BLNK-deficient mice than in PLCγ2-deficient mice (Pappu et al., 1999; Xu et al., 2000). B-cell adaptor for PI3K (BCAP) was isolated originally as a new molecule that associates with the SRC-homology 2 (SH2) domain of PI3K p85 subunits, and disruption of BCAP in DT40 CELLS leads to impaired BCR-induced activation of PI3K and the protein serine/threonine kinase AKT (also known as PKB) (Okada et al., 2000). Another B-cell adaptor, B-lymphocyte adaptor molecule of 32 kDa (BAM32), also binds PLCγ2 and regulates its activation (Marshall et al., 2000a). As it is recruited to the membrane in a PI3K-dependent manner (Marshall et al., 2000a), BAM32 integrates the PI3K and PLCγ2 pathways. Intriguingly, BAM32 is expressed at a high level in germinal center (GC) B cells, whereas other adaptors, such as GRB2-related protein of the lymphoid system (GRPL; also known as GRB2-related adaptor downstream of SHC, GADS) and SH2-domain protein 1A (SH2D1A), are not expressed in GC and memory B cells, respectively (Shlapatska et al., 2001). This indicates that the selective or relative expression of key adaptors during B-cell development might influence BCR-induced signaling pathways and cell fate. The B-cell-specific coreceptor CD19 can function as an adaptor for PI3K in B cells also (Tuveson et al., 1993). Negative regulators of BCR signaling include B-cell co-receptors, such as
paired immunoglobulin-like receptor B (PIRB) and FcγRIIB, which have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails that recruit the phosphatases SH2-domain containing protein tyrosine phosphatase 1 (SHP1) and SH2-domain-containing inositol polyphosphate 5′ phosphatase (SHIP), thereby inhibiting BCR signaling (Chacko et al., 1996). The CD22 co-receptor might regulate BCR signaling both positively and negatively; recruitment of the adaptor protein GRB2 to the CD22 cytoplasmic tail might lead to MAPK activation, whereas recruitment of SHP1 to cytoplasmic ITIMs of CD22 blocks BCR signaling (Poe et al., 2000). Together, the adaptors and co-receptors finely modulate the quality and quantity of BCR.

**PI3K pathway.** PI3K is a primary candidate for mediating B-cell survival. Treatment of B cells with inhibitors of PI3K leads to an increase in BCR-induced cell death (Fruman et al., 1999). Of the downstream molecules that are activated after PI3K activation, AKT is of particular interest. In mice that are deficient for the p110δ subunit of PI3K, the activation of AKT in B cells is impaired significantly (Okkenhaug et al., 2002). Generally, AKT phosphorylates cellular targets that are involved in cell survival, including factors that regulate apoptosis (Datta et al., 1997) and glycogen metabolism (Cross et al., 1995). BCR signalling activates AKT even in PLCγ2-deficient B cells, whereas inhibitors of PI3K block this activation completely (Craxton et al., 1999; Pogue et al., 2000), which indicates that AKT is downstream of PI3K, but not PLCγ2. The finding that disruption of AKT leads to the death of DT40 cells underscores a role for AKT in B-cell survival (Pogue et al., 2000). So, AKT functions not only to protect against BCR-induced cell death, but also to promote B-cell survival in the resting state. One function of AKT is to inhibit activation of the pro-apoptotic BCL-2-family member BAD (BCL-2 antagonist of cell death) (Datta et al., 1997). AKT phosphorylates and inhibits glycogen synthase kinase 3 (GSK3)
kinase in B cells (Gold et al., 1999). In unstimulated cells, GSK3 is active constitutively, and it phosphorylates and destabilizes MYC and cyclin D, both of which are required for cell-cycle progression (Sears and Nevins, 2002). So, it seems that AKT functions to promote BCR-induced cell proliferation, as well as survival.

**NF-κB pathway:** NF-κB, a family of dimeric transcription factors, has been shown to protect many cells from proapoptotic signals (Barkett and Gilmore, 1999). Activation of PLCγ2 is absolutely required for BCR-induced NF-κB activation in B cells (Petro and Khan, 2001). PLCγ2-induced Ca2+ flux and PKC activation are both required for NF-κB activation (Petro and Khan, 2001), which indicates that the activation of Ca2+-dependent PKC isoforms might regulate the activation of NF-κB. Recent studies found that PKCβ is required to activate IKKα/β, an upstream kinase of NF-κB activation (Saijo et al., 2002). The NF-κB transcription-factor family consists of heterodimers or homodimers of the subunits NF-κB1 (p50), NF-κB2 (p52), c-REL, RELA (p65) and RELB78. Different pairs of these subunits function at different stages of B-cell development. NF-κB induces the expression of anti-apoptotic BCL-2-family members, such as BCL-2, BCL-XL and A1 (Grumont et al., 1998). These molecules counteract the effects of pro-apoptotic BCL-2-family molecules, thereby protecting cells from BCR induced death. Recently, BCL-XL was reported to block expression of the InsP3 receptor (InsP3R) in a B-cell line (Li et al., 2002), which indicates that BCL-XL might also regulate BCR-induced cell death by inhibiting the release of Ca2+ through the InsP3R. Another NF-κB-regulated gene product is cyclin D2 (Joyce et al., 2001). The results indicate that a Ca2+-dependent PKC regulates the expression of cyclin D2 through activation of NF-κB. However, BCR-induced induction of cyclin D2 is intact in PKCβ-deficient B cells, in which IKKα/β is not activated (Su et al., 2002).
So, an NF-κB-independent pathway (for example, activation of ERK) induced by other PKCs or RAS–RAF1-induced ERK activation might regulate the expression of cyclin D2 by B cells.

**MAP Kinase pathway:** Mitogen activated protein kinase (MAPK) pathways are highly conserved pathways (Widmann et al., 1999)(Lewis et al., 1998; Widmann et al., 1999). There are six different groups of MAPKs have been characterized in mammals; Out of these, B cells express three major MAPK including ERK1/2, p38 and JNK (Kurosaki, 1999; Kurosaki, 2000; Kurosaki, 2002). The ERK pathway is the best studied so far in the mammalian MAPK pathways. The upstream signals that are required for the activation of ERK in B cells are distinct from those that are required for the activation of other MAPKs, such as JNK and p38 MAPK. For example, in PLCγ2-deficient B cells, BCR-mediated JNK and p38 MAPK activity are not detectable, whereas ERK activation is significantly impaired, but still induced. This indicates that ERK is also regulated in a PLCγ2-independent manner, most probably through the RAS–RAF pathway(Hashimoto et al., 1998). Although it is clear that ERK is involved in the survival of other cell types(Ballif and Blenis, 2001), its role in immature B cells is unclear. An inhibitor of ERK activation does not affect BCR-induced cell death in immature B-cell lines(Richards et al., 2001). However, this inhibitor does block BCR-induced proliferation of mature B cells. The RAF1–ERK pathway is involved also in BCR-induced expression of cyclin D2 in B cells(Piatelli et al., 2002). Disruption of the Raf1 gene in mice has shown a unique ERK-independent function for Raf1 in cell survival; in these mice, the activation of ERK is as normal, but cell death is enhanced (Huser et al., 2001). Raf1 can translocate to mitochondria and regulate mitochondrial function directly(Wang et al., 1996), and so it is likely to be a pro-survival molecule in B cells.

**p38 MAP kinase pathway:** Regulation of the p38 pathway is controlled by different upstream signals, which lead to p38 activation. These signals may be p38 specific (M KK3/6), general
MAPKKs (MKK4), or MAPKK independent signals (TAB1). Downstream signaling pathways of p38 are quite divergent and each component may interact with other cellular components, both upstream and downstream, to coordinate cellular processes such as feedback mechanisms. Furthermore, in vivo p38 is not an isolated event and exists in the presence of other MAP kinases and a plethora of other signaling pathways. The sub-cellular location of p38 activation may also play a critical role determining the resulting effect and may add yet another order of complexity to the understanding of p38 function.

Numerous genes regulated by the p38 MAP kinase pathway have been identified by the use of mutant or inhibitor (SB203580) strategy, which includes families of cytokines, transcription factors and cell surface receptors. Among them about half of p38 substrates identified so far are transcription factors. Examples include activating transcription factor (Han et al., 1993; Lechner et al., 1996; Rouse et al., 1994), (ATF-1/2/6), SRF accessory protein (Sap1), CHOP (growth arrest and DNA damage inducible gene 153, or GADD153), p53, C/EBPβ, myocyte enhance factor 2C (MEF2C), MEF2A, MITF1, DDIT3, ELK1, NFAT, and high mobility group-box protein 1 (HBP1) (Galibert et al., 2001; Gomez del Arco et al., 2000; Han et al., 1997; Huang et al., 1999; Janknecht and Hunter, 1997; Pereira et al., 2004; Raingeaud et al., 1995; Tan et al., 1996; Wang and Ron, 1996; Whitmarsh et al., 1997; Yee et al., 2004; Zhao et al., 1999). Recently, the HBP1 transcription factor has been identified as a substrate for p38. HBP1 has been linked to G1 cell cycle arrest and inhibition of p38 has been shown to decrease HBP1 protein levels (Yee et al., 2004). So, it is obvious that p38 has a role in regulating gene expression at the transcriptional level. Post-transcriptional regulation of inflammatory gene expression has also been linked with the p38 pathway (Kotlyarov et al., 1999; Prichett et al., 1995).
There is evidence for p38 involvement in apoptosis also. Cysteine proteases (caspases) are central to the apoptotic pathway and are expressed as inactivezymogens (Cahill et al., 1996; Fernandes-Alnemri et al., 1996). Caspase inhibitors then can block p38 activation through Fas crosslinking, suggesting p38 functions downstream of caspase activation (Henkart, 1996; Huang et al., 1997).

There are other reports which suggest p38 role in G1 and G2/M phases of the cell cycle (Molnar et al., 1997; Wang et al., 2000; Yee et al., 2004). G1 arrest of NIH3T3 cells caused by microinjection of Cdc42 was found to be p38α-dependent (Wang et al., 2000). Also, as mentioned earlier, a link between p38 and G1 cell cycle control has been proposed through the regulation of p38 substrates HBP1 and p21 (Yee et al., 2004). HBP1 is thought to have a role in regulating G1 cell cycle progression through repression of cell cycle regulatory genes, similar in function to retinoblast protein (RB) while the p21 CDK inhibitor is established as a crucial factor in preventing G1 progression through blockage of CDK activity.

**Immunobiology of the Immature and mature B cell**

B-cell development proceeds in an ordered and sequential manner in which the stages of maturation are defined by the rearrangement status of the immunoglobulin (Ig) heavy and light chain genes as well as by the expression of developmentally regulated phenotypic markers (Burrows and Cooper, 1990; Loffert et al., 1994). During adult B-cell development, immature stage B cells developing in the bone marrow are the first cells to express the prototypic form of the B-cell antigen receptor (BCR). Consequently, the immature stage B cell is the first representative of the B-cell lineage to recognize and respond to antigen in a clonotypically restricted manner. Phenotypically, immature B cells within the bone marrow are IgM$^{hi}$ IgD$^{neg}$
Expression of IgM on the cell surface distinguishes the immature B cell from pre-B-cell progenitors. Immature B cells also differ phenotypically from mature B cells in that they express relatively higher levels of HSA (CD24) and are positive for the surface marker defined by the 493 antibody (Rolink et al., 1998). Immature B cells exit the bone marrow and enter the peripheral lymphoid tissues, where they progressively upregulate mature B-cell markers such as IgD, CD22, and CD23 (Monroe, 2000). Peripheral immature B cells are referred to as transitional immature B cells (Carsetti et al., 1995). After a series of yet to be defined processes, a subset of transitional immature B cells become long-lived mature B cells, defining the pool of potentially immunoreactive B cells (Levine et al., 2000).

Unlike the apoptosis induced directly by anti-Fas antibodies that occurs relatively rapidly in these cells (~2–4 h) (Lenczowski et al., 1997), apoptosis induced by antigen receptor crosslinking generally takes longer (10–14 h) due to the time required to upregulate FasL and subsequently induce Fas-mediated death. The expression of at least low levels of Fas on immature B cells and a similar time lag between BCR ligation and the induction of cell death (Norvell et al., 1995; Sandel and Monroe, 1999; Sater et al., 1998) suggested that a Fas/FasL mechanism might be responsible for the BCR-induced cell death of immature B cells.

To address this question, L. B. King, Z. Zhou, A. Norvell, J. G. Monroe, have shown from Fas-deficient MRL lpr/lpr mouse strain were capable of undergoing BCR-induced apoptosis. Induction of apoptosis following BCR ligation appeared relatively normal in immature B cells obtained from lpr/lpr mice, suggesting that Fas did not play a major role in this process. In support of this conclusion, addition of a Fas-Fc fusion protein that functions as a competitive inhibitor for FasL does not prevent the BCR-induced death of immature B cells obtained from BALB/c mice. Such results are consistent with data obtained in Fas-deficient mouse systems that
suggest that Fas may play a role in maintaining peripheral tolerance of both activated and anergized B cells (Rathmell et al., 1995; Rothstein et al., 1995) but that central tolerance is relatively Fas independent (Adachi et al., 1996; Rathmell and Goodnow, 1994; Rubio et al., 1996). While the experiments described above suggest that Fas itself does not play an obvious role in the induction of immature B-cell death following receptor cross-linking, it is still possible that other members of this family do function in this regard like TNF/TNFR families play a role in BCR induced apoptosis.

On the other hand molecular examination revealed that immature B cells upregulate cyclin D2, activate the cyclin-dependent kinase CDK4, and enter G1 following BCR engagement, but they do not upregulate the late G1 cyclin, cyclin E, or form cyclin E/cdk2 complexes. The inability to generate functional cyclin E/cdk2 complexes blocks their progression to the S phase of the cell cycle (Carman et al., 1996). It is not clear at this time if the abortive entry into the cell cycle and the induction of apoptosis are functionally related or are the consequence of two separate and distinct signaling events. However, the inability to enter the cell cycle does not appear to be caused directly by the induction of apoptosis.

The differential responsiveness observed between immature and mature B cells is not limited to proliferative vs apoptotic fate decisions. For example, while mature B cells upregulate the co-stimulatory molecule CD86 and hyperexpress major histocompatibility complex class II (MHC-II) molecules in response to BCR ligation, immature B cells do not (Benschop et al., 1999; Tasker and Marshall-Clarke, 1997). The relative inability of immature B cells to express CD86 following BCR engagement, coupled with their inability to proliferate even in cases where they have been rescued from BCR induced death, provides a compelling argument for developmentally regulated intrinsic differences in signal transduction between immature and
mature B cells. The intrinsic signaling differences regulating this functional dichotomy could be quantitative, with a "strong" signal resulting in apoptosis and a "weak" signal leading to activation (Lam and Rajewsky, 1998; Parry et al., 1994). Thus, the strength of BCR signal transduction may dictate the functional response of the B cell by determining whether an apoptotic or an activation response is elicited following receptor engagement. Quantitative differences in BCR-induced signal transduction could potentially be mediated by developmentally regulated differences in the expression of B-cell coreceptors. CD22 is expressed at relatively lower levels on immature B cells than on mature B cells (Erickson et al., 1996; Stoddart et al., 1997), and it is possible that the relative lack of this negative regulator could result in "stronger" BCR signal transduction, as a result it enhances the sensitivity of immature B cells to apoptotic signals by lowering the antigen threshold required to elicit such a response. In order to test whether altering the threshold of responsiveness following BCR engagement played a determining role in the fate decision of the mature B cell, R. Sater, and J. G. Monroe, sequestered CD22 with anti-CD22-coated beads and stimulated mature B cells with anti-BCR. As was the case in the studies by Doody et al. (Doody et al., 1995), sequestration of CD22 resulted in an enhanced proliferative response. However, despite the increased BCR sensitivity, there was no measurable increase in the frequency of cells undergoing apoptosis. These results suggest that neither the relative expression levels of CD22 on immature and mature B cells nor the ability of CD22 to associate with the BCR is likely to play a defining role in determining the ultimate functional response of the cell.

The BCR signal transduction in immature B cells is more sensitive than in mature B cells. In support of this hypothesis, King et al reported that immature B cells undergo apoptosis at approximately 30-fold lower concentration of anti-BCR than is required for the induction of
mature B-cell proliferation, despite having similar levels of surface Ig (IgM+IgD) (Sater et al., 1998). The reports found that the apoptotic response of immature B cells can occur following short term engagement of the BCR (as little as 20 min) (Sater et al., 1998), while the proliferative response of mature B cells is known to require prolonged receptor engagement (DeFranco et al., 1985). It is likely that the negative selection of immature B cells following a brief exposure to antigen concentrations or avidities that are below the threshold required to induce an activation response in mature B cells is critical for immunologic self tolerance. Such a mechanism would obviate concerns that potentially autoreactive cells would be activated by self antigens in the periphery.

In addition to quantitative differences in signal transduction, there are many differences in BCR-induced signal transduction in immature and mature B cells. For example, a phosphatidylinositol-specific phospholipase D appears to be preferentially activated in mature B cells following BCR engagement (Gilbert et al., 1998), while cytosolic phospholipase A2 is expressed predominantly in immature B cells (Gilbert et al., 1996). It has been proposed that the differential expression/activation of enzymes intimately involved in signal transduction in immature and mature B cells demonstrates the utilization of qualitatively distinct signaling pathways in each cell type. Apart from BCR proximal molecule there are a lot of evidences for more distal effector molecules upon which BCR-induced signals impinge (i.e. transcription factors, anti- or pro-apoptotic molecules). For example, the anti-apoptotic molecule A1 is expressed at relatively high levels in long-lived mature B cells but not immature B cells (Tomayko and Cancro, 1998). In addition, mice deficient in c-rel (a member of the nuclear factor (NF-κB family) were unable to upregulate A1 following receptor ligation may be at least partially responsible for their susceptibility to BCR-induced death (Grumont et al., 1999).
Overexpression of Bcl-2 does lead to enhanced survival in the apoptotic-prone c-rel-deficient B cells, it does not allow for cell cycle progression (Grumont et al., 1998). Grumont RJ, 1991 reported that while mature B cells increase intracellular Ca2+ and hydrolyze phosphatidylinositol-4, 5-bisphosphate (PIP2) in response to BCR cross-linking, immature B cells increase intracellular Ca2+ levels in the relative absence of PIP2 hydrolysis (Yellen et al., 1991). Since hydrolysis of PIP2 results in the production of diacylglycerol (DAG), an essential co-factor for the activation of the conventional isoenzymes of protein kinase C (PKC), signal consisting of an increase in intracellular Ca2+ levels that is unopposed by activation of PKC may potentiate immature B cell apoptosis. It appears that a developmentally regulated, functional coupling of PKC to BCR-induced signal transduction may play a role in determining the apoptotic response of immature B cells from the activation response of mature B cells.

The mechanism by which PKC may promote the survival of immature B cells is not yet clear. The pharmacological agents such as ionomycin and thapsigargin, which increase intracellular Ca2+ levels but bypass the BCR completely, also lead to the apoptosis of immature B cells (L.B. King, A. Norvell, J.G. Monroe, manuscript in preparation). PKC activation by PMA is able to reverse both ionomycin and thapsigargin-induced death. Therefore, it is likely that PKC activation is able to rescue immature B cells from BCR-induced apoptosis at a point distal to that of receptor desensitization. Addition of PKC activators reverses BCR-induced apoptosis in immature B cells (King et al., 1999) and allows them to upregulate CD86 following receptor ligation. However, a combination of phorbol ester and either anti-BCR or Ca2+ ionophore is not sufficient to drive immature B-cell proliferation (Allman et al., 1992). These results suggest an additional difference in signal transduction downstream of PKC activation which results in an inability of immature B cells to proliferate in response to receptor ligation. Thus, it appears that
there are at least two distinct differences in signal transduction taking place in immature B cells following BCR ligation: 1) an inefficient coupling of PKC activation to BCR aggregation and 2) a more distal defect that prevents the induction of a proliferative response.

**PKC-dependent downstream effectors pathways: NF-κB /c-Myc**

The apparent inability of immature B cells to activate PKC following BCR engagement suggested that the induction of effector pathways downstream of PKC activation may be compromised in these cells. In mature B cells, activation of PKC leads to an increase in activity of the NF-κB family of transcription factors (Liu et al., 1991). Consistent with our hypothesis that PKC is inefficiently coupled to BCR signal transduction in immature B cells, we find that BCR-induced NF-κB binding activity is not upregulated to the same extent in immature B cells as it is in mature B cells. However, treatment of immature B cells with PMA results in a substantial increase in NF-κB activation, suggesting that a PKC dependent NF-κB activation pathway is intact in these cells but is not efficiently coupled to BCR ligation.

A potential link between the activation of NF-κB and the regulation of apoptosis has been observed in WEHI-231, a B cell line that is susceptible to BCR-induced apoptosis. In WEHI-231, various heterodimeric or homodimeric complexes of NF-κB family members have been shown to regulate the expression of the proto-oncogene *c-Myc* (Lee et al., 1995). Following treatment of WEHI-231 cells with anti-BCR antibody, there is an initial transient increase in c-Myc expression that is followed by a gradual decrease back to basal levels. It has been suggested that an inability to maintain high levels of c-Myc protein following BCR engagement in WEHI-231 cells results in an increase in the expression of the cyclin-dependent kinase inhibitor p27Kip.
In turn, increased expression of p27Kip contributes to a cell cycle arrest that is followed by apoptosis (Wu et al., 1996; Wu et al., 1999). Unlike mature B cells, in which a BCR-induced increase in c-Myc protein expression is maintained for at least 6 h, immature B cells exhibit a transient increase in c-Myc that returns to basal levels (or lower) within 6 h of stimulation. In addition, treatment of anti-BCR-stimulated mature B cells with either the PKC inhibitor Ro 32-0432 (Wilkinson et al., 1993) or the NF-κB inhibitor cyclosporine A (Holschermann et al., 1996) prevents sustained c-Myc expression and allows for the induction of apoptosis. These results suggest that a sustained, PKC-dependent increase in c-Myc expression is associated with prevention of cell death. However, the situation is likely to be more complicated, for although interleukin (IL)-4, anti-CD40, and PMA are all capable of rescuing immature B cells from BCR-induced death, only anti-CD40 maintains c-Myc expression. Importantly, immature B cells treated with a combination of anti-BCR and anti-CD40 are capable of proliferating, whereas cells treated with anti-BCR and either IL-4 or PMA are not (Sater et al., 1998). Thus, findings suggest that sustained expression of c-Myc may be more closely associated with the induction of a BCR-induced proliferative response than it is with the prevention of BCR-induced apoptosis. Despite the ability of primary c-rel-deficient B cells to upregulate endogenous levels of c-Myc following BCR engagement, they still undergo cell cycle arrest and die by apoptosis. These results suggest that a sustained increase in c-Myc expression does not prevent BCR-induced apoptosis, and that high levels of c-Myc may be necessary, but are not sufficient, for the induction of a proliferative response in primary B cells. In this regard, it is important to note that comparisons of normal and transformed B cells may be misleading. For example, in WEHI-231 cells that are actively cycling, BCR engagement leads to a cell cycle arrest and a subsequent induction of apoptosis (24–48 h after BCR engagement). In contrast, immature B cells (primarily in G0) exhibit an
abortive entry into the cell cycle and rapidly undergo apoptosis (14–16 h after BCR engagement). Thus, it is possible that transformed B cells and primary immature B cells undergo BCR-induced cell cycle arrest followed by apoptosis via distinct mechanisms; one that is a consequence of cell cycle arrest and one that is accompanied by an abortive entry into the cell cycle.

The eukaryotic cell cycle:

Animal development from a single-cell zygote to fertile adult requires many rounds of cell division. During each division, cells complete an ordered series of events that collectively form the "cell cycle". This cycle includes accurate duplication of the genome during the DNA synthesis phase (S phase) (Sherlock, 2006), and segregation of complete sets of chromosomes to each of the daughter cells in M phase. The somatic cell cycle also contains "Gap" phases, known as G1 which connects the completion of M phase to initiation of S phase in the next cycle, and G0 which separates the S and M phases. Dependent on environmental and developmental signals, cells in G1 may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase known as G0. Cell external signals and cell intrinsic information together determine whether cells enter a division cycle. In general, external signals affect this decision only until cells commit to go through the entire cycle, at a time in G1 known as "START" in yeast and "Restriction point" in mammals (Hartwell et al., 1974; Nurse, 1975). From there on, progression through the cell cycle is controlled intrinsically by the cell-cycle machinery. The basic components of this machinery are conserved in all eukaryotes. Consequently, findings based on genetics in yeast, biochemistry in frog eggs and tissue culture of mammalian cells have all come together and generated a substantial molecular understanding of cell-cycle regulation.
Here we have tried to reveal the signaling as well as transcriptional regulatory network by perturbing strategy. Our results demonstrate that MAP Kinase pathway subunit p38 act as key regulator of early B cell signaling in CH1, an immature B cell line (Lanier et al., 1982; Lanier et al., 1978). Much of what is known about the regulated transition of cells through the cell cycle comes from genetic and biochemical evidence obtained through traditional studies carried out in lower organisms and cell culture based experiments. Apart from the traditional studies we now need to establish the link between traditional and system level oriented studies to readout the cellular outputs.

**Regulation of eukaryotic cell cycle:** Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. The molecular events that control the cell cycle are ordered and directional; that is, each process occurs in a sequential fashion and it is impossible to "reverse" the cycle.

**Role of cyclins and CDKs:** Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse won the 2001 Nobel Prize in Physiology or Medicine for their discovery of two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), which determine a cell's progress through the cell cycle (Nigg, 1995). Many of the genes encoding cyclins and CDKs are conserved among all eukaryotes, but in general more complex organisms have more elaborate cell cycle control systems that incorporate more individual components. Many of the relevant genes were first identified by studying yeast, especially *Saccharomyces cerevisiae*; (Spellman et al., 1998) Genetic nomenclature in yeast dubs many these genes *cdc* (for "cell division cycle") followed by an identifying number, e.g., *cdc25*. 
Cyclins form the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer; cyclins have no catalytic activity and CDKs are inactive in the absence of a partner cyclin. When activated by a bound cyclin, CDKs perform a common biochemical reaction called phosphorylation that activates or inactivates target proteins to orchestrate coordinated entry into the next phase of the cell cycle. Different cyclin-CDK combinations determine the downstream proteins targeted. CDKs are constitutively expressed in cells whereas cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals.

**General mechanism of cyclin-CDK interaction:** Upon receiving a pro-mitotic extracellular signal, G₁ cyclin-CDK complexes become active to prepare the cell for S phase, promoting the expression of transcription factors that in turn promote the expression of S cyclins and of enzymes required for DNA replication. The G₁ cyclin-CDK complexes also promote the degradation of molecules that function as S phase inhibitors by targeting them for ubiquitination. Once a protein has been ubiquitinated, it is targeted for proteolytic degradation by the proteasome. Active S cyclin-CDK complexes phosphorylate proteins that make up the pre-replication complexes assembled during G₁ phase on DNA replication origins. The phosphorylation serves two purposes: to activate each already-assembled pre-replication complex, and to prevent new complexes from forming. This ensures that every portion of the cell's genome will be replicated once and only once. The reason for prevention of gaps in replication is fairly clear, because daughter cells that are missing all or part of crucial genes will die. However, for reasons related to gene copy number effects, possession of extra copies of certain genes would also prove deleterious to the daughter cells. Mitotic cyclin-CDK complexes, which are synthesized but inactivated during S and G₂ phases, promote the initiation of mitosis by stimulating downstream proteins involved in chromosome condensation and mitotic spindle
assembly. A critical complex activated during this process is a ubiquitin ligase known as the anaphase-promoting complex (APC), which promotes degradation of structural proteins associated with the chromosomal kinetochore. APC also targets the mitotic cyclins for degradation, ensuring that telophase and cytokinesis can proceed. Interphase: Interphase generally lasts at least 12 to 24 hours in mammalian tissue. During this period, the cell is constantly synthesizing RNA, producing protein and growing in size. By studying molecular events in cells, scientists have determined that interphase can be divided into 4 steps: Gap 0 (G₀), Gap 1 (G₁), S (synthesis) phase, Gap 2 (G₂).

Specific action of cyclin-CDK complexes: Cyclin D is the first cyclin produced in the cell cycle, in response to extracellular signals (e.g., growth factors). Cyclin D binds to existing CDK4, forming the active cyclin D-CDK4 complex. Cyclin D-CDK4 complex in turn phosphorylates the retinoblastoma susceptibility protein (Rb). The hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex (which was bound to the E2F responsive genes, effectively "blocking" them from transcription), activating E2F. Activation of E2F results in transcription of various genes like cyclin E, cyclin A, DNA polymerase, thymidine kinase, etc. Cyclin E thus produced binds to CDK2, forming the cyclin E-CDK2 complex, which pushes the cell from G₁ to S phase (G₁/S transition). Cyclin B along with cdc2 (cdc2 - fission yeasts (CDK1 - mammalia)) forms the cyclin B-cdc2 complex, which initiates the G₂/M transition (Norbury et al; 1995). Cyclin B-cdc2 complex activation causes breakdown of nuclear envelope and initiation of prophase, and subsequently, its deactivation causes the cell to exit mitosis (Robbins and Cotran; et al 2004)

CDK Inhibitors: Two families of genes, the cip/kip family and the INK4a/ARF (Inhibitor of Kinase 4/Alternative Reading Frame) prevent the progression of the cell cycle. Because these genes are instrumental in prevention of tumor formation, they are known as tumor suppressors.
The *cip/kip* family includes the genes p21, p27 and p57. They halt cell cycle in G1 phase, by binding to, and inactivating, cyclin-CDK complexes. p21 is activated by p53 (which, in turn, is triggered by DNA damage eg. due to radiation). p27 is activated by Transforming Growth Factor β (TGF β), a growth inhibitor. The INK4a/ARF family includes p16INK4a, which binds to CDK4 and arrests the cell cycle in G1 phase, and p14arf which prevents p53 degradation.

**Checkpoints:** Cell cycle checkpoints are used by the cell to monitor and regulate the progress of the cell cycle. Checkpoints prevent cell cycle progression at specific points, allowing verification of necessary phase processes and repair of DNA damage. The cell cannot proceed to the next phase until checkpoint requirements have been met. Several checkpoints are designed to ensure that damaged or incomplete DNA is not passed on to daughter cells. Two main checkpoints exist: the G1/S checkpoint and the G2/M checkpoint. G1/S transition is a rate-limiting step in the cell cycle and is also known as restriction point. An alternative model of the cell cycle response to DNA damage has also been proposed, known as the post replication checkpoint.

**Regulation of Gene Expression**

In order to survive, cells must be able to interpret the environmental and developmental signals. (Karin, 1992). For all living cells, regulation of gene expression by extracellular signals is a fundamental mechanism of development, homeostasis, and adaptation to the environment. The phenotypic response represents the end product of unique processing events within the signal transduction network which then translate, through the coordinated activation of transcription factors (TFs), into a defined pattern of gene expression. Mechanisms that underlie the control of gene expression are becoming increasingly well understood. Every conceivable step in the process is subject to dynamic regulation in the cell. This includes structural changes in the
chromatin to make a particular gene accessible for transcription, transcription of DNA into RNA, splicing of RNA into mRNA, editing and other covalent modifications of the mRNA, translation of mRNA into protein, and finally post-translational modification of the protein into its mature and functional form.

Among the above mentioned regulatory steps, the process of transcription initiations has been well studied. Where a host of proteins crucial to transcription initiation are assembled into the RNA polymerase, the general transcription factors, coactivators, corepressors, chromatin remodelers, histone acetylases, deacetylases, kinases, and methylases, to list the main participants (Jenuwein and Allis, 2001; Jones and Kadonaga, 2000; Malik and Roeder, 2000; Monteiro, 2000; Naar et al., 2001; Tan and Khachigian, 2009). These crucial proteins are present in all eukaryotic cells and contribute to the initiation of every RNA polymerase II primary transcript that eventually becomes messenger RNA. Around, 200 to 300 proteins constitute the coactivators and the transcriptional machinery. The regulation of the choice of specific initiation sites for transcription is not vested in these proteins. Rather, transcriptional regulation depends on members of an even larger number of proteins, in mammals perhaps 2000 to 3000 (Venter et al., 2001), with two characteristic domains: a DNA binding domain that binds gene-specific regulatory sites directly, and a second domain that exhibits transcriptional activation potential. The site specific transcription factors recruit coactivators and the transcription machinery to initiate gene-specific transcription. As development and cell specialization occurs, selection among these 2000 transcription factors for the regulation of cell-specific gene expression involves (i) a cascade of transcriptional control of transcription factor genes, and (ii) signals from outside the cell that activate, post-transcriptionally, already formed transcription factors.
One of most important step of gene expression is mediated by transcription factors by the virtue of their reading the signals in response to the extracellular signals to alter gene expression for certain biological outputs. Indeed, the ultimate step in many signal transduction pathways is the modification of transcription factors (TFs) that can alter the expression of specific sets of genes. There is various mode of activation of TFs, few of them is like phosphorylation, dephosphorylation, nuclear translocation and complex formation. But at least we have not come across any report showing that how the signaling machinery regulate these TFs at the global level for a given biological output and how the globally activated pool of TFs function in combinatorial manner to answer the environmental cues. A brief account of some the important transcription factor is as follows.

**Constitutively active nuclear factors:** A sizable group of site-specific DNA binding proteins are present in the cell nucleus of all cells at all times, and they have transcriptional activating potential. They play an important facilitating role in the transcription of many chromosomal genes, possibly in genes that seem to be always transcribed [e.g., structural proteins like tubulin and actin, and ubiquitous metabolic enzymes such as glyceraldehyde phosphate dehydrogenase (GAPDH)]. These constitutively active transcription factors can also participate in composing enhanceosomes together with transcription factors that are regulated. This group includes Sp1 (Tan and Khachigian, 2009) CCAAT binding protein (Buratowski et al., 1988) NF1 (Nye and Graves, 1990; Rejman et al., 1991) and many others.

**Regulatory transcription factors:** There are two broad classes of this group of TF. Among them first is Developmental ("cell type–specific") transcription factors. The cell-specific accumulation during development of this group of factors is dependent largely on sequential waves of regulated transcription of the genes encoding these transcription factors (Ingham, 1990;
St Johnston and Nusslein-Volhard, 1992; Xanthopoulos et al., 1989). Examples include many early embryonic factors in *Drosophila* e.g., bicoid (St Johnston and Nusslein-Volhard, 1992), the Hox cluster of homeobox genes sequentially expressed in the anteroposterior axis of vertebrates (Lufkin, 1996), a series of helix-loop-helix factors (MyoD, Myf5, and myogenin) that appear in sequence in the control of muscle differentiation (Erck and Seidl, 1998; Yun and Wold, 1996). Developmental factors are not strictly cell, tissue or region-specific. Rather, combinatorial distribution of groups of these factors in different cell types helps to direct cell determination (choice of cell fate) and differentiation (synthesis of recognized cell-specific proteins). These TFs automatically enter the nucleus upon being made and thereby immediately contribute to transcription, a number of developmental factors for example Mef-2, a MADS box protein, and at least several different forkhead (winged helix) proteins (Brunet et al., 1999; Kops et al., 1999; Naya and Olson, 1999) do shuttle in and out of the nucleus and can be phosphorylated in the cytoplasm, leading to a block in their reentry to the nucleus. In addition, some factors (e.g., MyoD and myogenin) can be phosphorylated on serine while in the nucleus, resulting in decreased transcriptional activation potential (Li et al., 1992). And the second group of regulatory TFs is Signal-dependent transcription factors. This is of three types, first is

**The steroid receptor superfamily:** Steroids dissolve in the lipid bilayer of the plasma membrane and enter cells, where they bind and activate one of the many, 50 in humans specific steroid receptors that then participate in activating specific gene transcription (Fisher et al., 1995; Ringold et al., 1977). All except the glucocorticoid receptor (GR) are found primarily in the nucleus before the appearance of their cognate hormone. GR is held in a cytoplasmic complex until glucocorticoids bind it and release the GR dimers, which then enter the nucleus (Htun et al., 1996; Mackem et al., 2001).
Transcription factors activated by internal (cell-autonomous) signals: Internal sterol concentrations regulate the proteolysis of a membrane protein precursor within the membrane to release sterol response element binding protein (SREBP) (Brown and Goldstein, 1999). In yeast, low internal unsaturated fatty acid concentration leads to the juxta-membrane cleavage of precursors to Spt23 and MGA2, liberating for nuclear entry of these two transcription factors that regulate genes of unsaturated fatty acid synthesis.

Transcription factors activated by cell surface receptor–ligand interactions: The G coupled cell surface receptor protein (GCRP) and (receptor tyrosine kinase RTK) regulate transcription through a multitude of serine kinase cascades that finally terminate in serine phosphorylation of the abundant resident nuclear transcription factors. The substrate proteins include the Ets family, the c-Jun–c-Fos–ATM family, the cAMP response element binding protein (CREB) cAMP response element modulator (CREM) families, and the MADS box family of transcription factors including serum response factor (SRF), to list some of the better studied groups. Many of these target proteins are not only constitutively nuclear but are bound to DNA at all times (Cavigelli et al., 1995; Gille et al., 1995a; Gille et al., 1995b; Janknecht and Hunter, 1997; Janknecht and Nordheim, 1993).

Serine phosphorylation of resident nuclear factors is evolutionarily ancient and occurs in fungi, plants, and single-celled organisms such as Saccharomyces cerevisiae (Wasylyk et al., 1998; Whitmarsh and Davis, 1998). For example, mammalian CREB protein must be phosphorylated on Ser133 for maximal activity(Pawson and Scott, 1997; Shaywitz and Greenberg, 1999). Phosphorylation allows binding to CREB of a coactivator CBP (CREB binding protein, or a relative p300). At least five different kinases cAMP-dependent protein kinase (PKA), ribosome S6 kinase, and multiple mitogen-activated protein (MAP) kinases and calcium/calmodulin–
dependent (CAM) kinases can catalyze this phosphorylation. Other examples of proteins that can be substrates of multiple kinases are the Ets and MADS box families (Janknecht, 1995; Shaywitz and Greenberg, 1999; Wasylyk et al., 1998).

**Latent cytoplasmic factors:** The hallmark of this group of transcription factors is that they reside in an inactive form in cytoplasm until they are activated by proteins that bind to cell surface receptors.

**Serine phosphorylation in the SMAD pathway:** The SMAD proteins are the only known latent cytoplasmic transcription factors that become directly activated by serine phosphorylation at their cognate receptors. This family of transcription factors transduces signals on behalf of the transforming growth factor-β (TGF-β) superfamily of ligands (Massague, 2000). There are three types of SMADs, most extensively studied in mammalian cells. The effector SMADs (also called the R-SMADs) become serine-phosphorylated in the COOH terminal domain by the activated receptor. Smad1, Smad5, Smad8, and Smad9 become phosphorylated in response to bone morphogenetic protein (BMP) and growth and differentiation factor (GDF), and Smad2 and Smad3 become phosphorylated in response to the activin/nodal branch of the TGF-β pathway. The second group in this family are called regulatory or co-SMADs. There are two regulatory SMADs: Smad4 and Smad4b (also called Smad10) (Howell et al., 1999; Masuyama et al., 1999). Smad4 binds to, and is essential for, the function of Smad1 and Smad2 (Lagna et al., 1996). The regulatory Smad4 binds to all effector SMADs in the formation of transcriptional complexes (Lagna et al., 1996; Masuyama et al., 1999), but it does not appear to be required for nuclear translocation of the effector molecules (Liu et al., 1997). Finally, two inhibitory SMADs, Smad6 and Smad7 provide negative regulation of the pathway (Hata et al., 1998) by blocking Smad4 binding. Once an activated, serine-phosphorylated effector SMAD binds Smad4 and escapes the
negative influences of Smad6 and Smad7, nuclear accumulation and regulation of specific target genes can occur. In most cases, SMADs require partner transcription factors with strong DNA binding capacity that determine the gene to be activated. The specificity of response among different ligands can be partially explained by the choice of DNA binding partner proteins.

**Tyrosine phosphorylation of the STATs:** The STATs (signal transducers and activators of transcription) are the only known transcription factors that become activated from a latent state by phosphorylation on tyrosine (Darnell, 1997; Stark et al., 1998). They are activated by more than 20 different cytokines, the receptors for which are associated with Janus kinases (JAKs) that tyrosine-phosphorylate the liganded receptor and then the associated STAT. The STATs can also be activated by RTKs such as epidermal growth factor (EGF), platelet-derived growth factor, and Eyk; by non-RTKs such as Src and Abl; and through GCRPs (Bromberg and Darnell, 2000; Rane and Reddy, 2000). Tyrosine phosphorylation of a STAT is followed by dimerization through reciprocal SH2 (SRC homology 2) phosphotyrosine interaction. The natural activation-deactivation cycle of STAT molecules is quite short, about 15 min for an individual molecule (Haspel and Darnell, 1999)(73). There are seven known STATs in mammals, each of which has separate in vivo functions, as revealed by knockout experiments (Darnell, 1997; Stark et al., 1998).

**The Rel/ NF-κB family:** There are five Rel/ NF-κB transcription factors in mammals: NF-κB 1, NF-κB 2, c-Rel, Rel-a, and Rel-b. This family of factors can be activated by a large array of extracellular products, including tumor necrosis factor–α, interleukin-1, growth factors, bacterial and viral infections, oxidative stress, and a variety of pharmaceutical compounds (Baeuerle and Baltimore, 1996). A cytoplasmic inhibitor of active NF-κB called IkB binds to a subunit of NF-κB(Baeuerle and Baltimore, 1996; Karin, 1999; Perkins, 2000). The IkB protein has ankyrin
repeats that bind to the actin cytoskeleton, both tethering the bound NF-κB in the cytoplasm and blocking the nuclear localization signal of NF-κB. Two serine residues in IκB when phosphorylated lead to destruction of IκB by proteosomes (Karin, 1999; Perkins, 2000) with NF-κB then moving to the nucleus. Thus, two proteolytic events—the cleavage of p105 to p50 and the destruction of IκB governed by serine phosphorylation—are required to produce the proteins and then activate them. At present it is not clear how long an NF-κB molecule continues to function once it is in the nucleus. However, IκB is a target gene of NF-κB. Thus, an increase in IκB may help to again sequester NF-κB in the cytoplasm. Finally, it seems likely that different serine kinases are responsible in different cells in the physiologic activation of NFκB (Baeuerle and Baltimore, 1996; Karin, 1999; Perkins, 2000).

**NFAT activation and Ca2+ increase:** An important subdivision of the NFκB/Rel transcription factor family based on sequence similarity is the NFAT (nuclear factors in activated T cells) proteins (Crabtree, 1999; Rao et al., 1997), but their regulation is by an entirely different mechanism. The cytoplasmic NFAT molecules are heavily phosphorylated in resting cells, but binding of cell surface receptors (the T cell receptor by a cognate surface-expressed immunoglobulin on a B cell) causes a cyclical fluctuation in internal Ca2+ concentration. The increase in Ca2+ concentration activates the phosphatase calcineurin, which dephosphorylates NFAT, leading to an accumulation of NFAT in the nucleus (Okamura et al., 2000; Rao et al., 1997). More than 10 different NFAT proteins are expressed in a variety of different tissues (Rao et al., 1997). The NFATs represent the only known case of a cell surface protein interaction resulting in an internal Ca2+ ion increase that triggers activation of specific latent cytoplasmic transcription factors. The NFAT proteins have a weak affinity for DNA and usually associate with other factors such as AP-1.
EVOLUTION OF SYSTEMS BIOLOGY

Although, System biology seems to be originated at the time of quantitative modeling of enzyme kinetics that flourished during 1900-1970, Ludwig von Bertalanffy is considered as precursor of systems biology as he gave general systems theory in his book titled "General Systems Theory in Physics and Biology", published in 1950. One of the first numerical simulations in biology was published in 1952 by the British neurophysiologists and Nobel prize winners Alan Lloyd Hodgkin and Andrew Fielding Huxley, who constructed a mathematical model that explained the action potential propagating along the axon of a neuronal cell.(Hodgkin and Huxley, 1952) Their model described a cellular function emerging from the interaction between two different molecular components, a potassium and a sodium channels, and can therefore be seen as the beginning of computational systems biology(Le Novere, 2007). In 1960, Denis Noble developed the first computer model of the heart pacemaker(Noble, 1960). The formal study of systems biology, as a distinct discipline, was launched by systems theorist Mihajlo Mesarovic in 1966 with an international symposium at the Case Institute of Technology in Cleveland, Ohio entitled "Systems Theory and Biology." The 1960s and 1970s saw the development of several approaches to study complex molecular systems, such as the Metabolic Control Analysis and the biochemical systems theory. The successes of molecular biology throughout the 1980s, coupled with skepticism toward theoretical biology, that then promised more than it achieved, caused the quantitative modeling of biological processes to become a somewhat minor field. However the birth of functional genomics in the 1990s meant that large quantities of high quality data became available, while the computing power exploded, making more realistic models possible. In 1997,
the group of Masaru Tomita published the first quantitative model of the metabolism of a whole (hypothetical) cell. Around the year 2000, when Institutes of Systems Biology were established in Seattle and Tokyo, systems biology emerged as a movement in its own right, spurred on by the completion of various genome projects, the large increase in data from the omics (e.g. genomics and proteomics) and the accompanying advances in high-throughput experiments and bioinformatics. Since then, various research institutes dedicated to systems biology have been developed.

Since the discovery of double helix structure of DNA and a series of efforts that gave a birth to molecular biology, astonishing progress has been made on our understanding on living forms as molecular machinery. The climax came as completion of human genome sequencing. With accumulating knowledge of genes and proteins, the next natural question to ask is how they are working together? What are principles that govern at the system level? With the progress of molecular biology, genomics, computer science and control theory, the old question is revisited with new concepts and methodologies. A system is not just an assembly of components. There are principles that govern at the system level. Unlike genes and proteins that are rather tangible objects, a system is no tangible. The essence of system lies in dynamics that is not tangible. This makes the game of system biology complex, and may sound alien to many molecular biologists who are accustomed to a molecular-oriented view of the world. Needless to say system level understanding has to be grounded onto molecular level so that a continuous spectrum of knowledge can be established.

Systems biology is driven partly by the curiosity of scientists, but even more so by its high potential of its applications. The exact formulation of the cellular networks and prediction of systems behavior in the areas of drug development, monitoring and treatment. For example,
epidermal growth factor receptor which is targeted by a new generation of cancer drugs belongs to a family of four related receptors. These receptors are turned on by more than 30 different molecules. Thus such a complex setup makes it necessary to derive the wiring diagram to understand how each component plays its role in responding to various stimuli. One a detailed model has been constructed; all effects of possible perturbation can be predicted fairly cheaply in silico. Furthermore, models gained by systems biology approach can be used for prediction of the behavior of the biological system even under conditions that are not easily accessible with experiments. A major topic of current system biology is the analysis of networks: gene networks, protein interaction networks, metabolic networks, transcription and signaling network, etc. Initially, investigation of abstract network was fashionable. However, it has become clear that it is necessary to study more realistic and detailed networks in order to uncover the peculiarities of biological regulation. Different theoretical attempts have been made to study the different types of network. For example gene regulatory network are sometime described by Boolean logic assigning to genes of one of two states, on or off; protein relation are mainly characterized by a static view of putative interaction measured by yeast two hybrid methods, and metabolic networks are determined by the set of catalyzing enzymes and the possible metabolite fluxes and intrinsic modes of regulations.

**Typical aspect of modeling in biological systems:** A number of notions have been introduced or applied in the context of systems biology or computational modeling of biological systems. Some of them that are helpful in understanding are as follows-

**Network versus Elements:** A system consists of individual elements that interact and thus form a network. The elements have certain properties. In the network, the elements have certain relations to each to each other (and, if appropriate, to the environment). The system has
properties that rely on the individual properties and the dynamic characteristics that often cannot be deduced from the individual properties of the elements.

**Modularity**: Modules are subsystems of complex networks that can be treated as functional units, which perform identifiable tasks (Lauffenburger, 2000). Typical examples for assignment of module are (1) the DNA-mRNA-enzyme-metabolism cascade and (2) signal transduction cascades consisting of covalent modification cycles. The reaction networks at each level are separated as modules by the criterion that mass transfer occurs internally but not between the modules, and they are linked by means of catalytic or regulatory effects of a chemical species of one module to reaction to another module (Hofmeyr and Westerhoff, 2001). Consideration of module has the advantage that modeling can be performed in hierarchical, nested, or sequential fashion. The properties of each module can be studied first in isolation and subsequently in a comprehensive, integrative attempt. The concept is appealing since it allows thinking in terms of classes of systems with common characteristics that can be handled with a common set of methods. The disadvantage is that a modular approach has to ignore or at least reduce the high level connectivity of cellular networks— in particular the variety of positive and negative feedback and feed-forward regulatory loops— which actually contradicts the basic idea of system biology.

**Robustness and sensitivity are the two sides of the same coin**: Robustness is an essential feature of biological systems. It characterizes the insensitivity of system properties to variations in parameters, structure, and environment or other uncertainties. Robust systems maintain their state and functions despite external and internal perturbations. An earlier observation for this notion is homeostasis. Robustness in biological systems is often achieved by high degree of complexity involving feedback, modularity, redundancy, and structural stability (Kitano, 2002).
On the other hand biological systems must protect their genetic information and their mode of living against perturbations; on the other hand, they must adapt to changes, sense and process internal and external signal, and react precisely depending on the type or strength of perturbation. Sensitivity or fragility characterizes the ability of living organism for adequately reacting on a certain stimulus. Note that in some areas sensitivity is more rigorously defined as ratio of the change of a variation by the change of quantity that caused the change in the variable.

**A need for modeling in systems biology:** Observation of the real world and, especially, of biological processes confronts us with many simple and complex processes that cannot be explained with elementary principles and the outcome of which cannot reliably be foreseen from experience. Mathematical modeling and computer simulation helps us to understand the internal nature and dynamics of these processes and to arrive at well founded predictions about their future development and the effect of interactions with the environment.

What is a model? The answer will differ among communities of researchers. In the broadest sense, a model is an abstract representation of objects or processes that explains features of objects or processes. For instance, the string composed of the letters A, C, G and T is used as a model for DNA sequence. In some cases a cartoon of reaction network showing dots for metabolites and arrows for reaction is a model, while in other cases a system of differential equations is employed to describe the dynamics of that network. In experimental biology the term model is also used to denote species that are especially suitable for experiments. For example the mouse Ts65DN serves as a model for human trisomy 21 (Reeves et al., 1995).

**Advantages of computational modeling**
Modeling derives conceptual clarification: It requires that verbal hypotheses be made specific and conceptually rigorous. Modeling also highlights gaps in knowledge or understanding. During the process of model formulation, unspecified component or interactions have to be determined.

Modeling provides independence of the modeled objects: Time and space may be stretched and compressed *ad libitum*. Solution algorithms and computer program can be used independently of the concrete system. Modeling is cheap compared to experiments. Models exert by themselves no harm on animals or plants and help to reduce it in experiments. They do not pollute the environment. Models interact neither with the environment nor with the modeled system.

Modeling can assist in experimentation: With an adequate model one may test different scenarios that are not accessible by experiments. One may follow time courses of compounds that cannot be measured in an experiment. One may impose perturbations that are not feasible in real system. One may cause precise perturbations without directly changing other system components, which is usually impossible in real system. Model simulation can be repeated often and for many different conditions. Model results often can be represented in precise mathematical terms that allow for generalization. Graphical representation and visualization make it easier to understand the system. Finally, modeling allows for making well founded and testable predictions.

**Understanding Biology through Mathematical Modeling:**

Mathematical biology or bioinformatics is an interdisciplinary field of academic study which models natural, biological processes using mathematical techniques. It has both theoretical and practical application in biological research. Applying mathematics to biology has a long history, but only recently there has been explosion of interest in the field. Researchers are becoming more interested in using mathematical tools to solve biological problems because of (i) an
explosion of data-rich information sets which are difficult to understand without the use of analytical tools, (ii) a recent development of mathematical tools to help understand complex nonlinear mechanisms in biology, and (iii) an increase in computing power which enables calculations and simulations to be performed that were not previously possible. Some of the areas of research in mathematical biology are population dynamics, epidemiology, evolutionary game theory, theoretical enzymology, modeling of neurons and carcinogenesis, modeling the movement of interacting cell population, modeling physiological systems and many more.

Mathematical models are very useful in cell biology, especially in understanding the control mechanisms of signal transduction pathways (Heinrich et al., 2002; Lee and Song, 2003). Cellular signals are not static but dynamic (Hunter, 2000; Pawson, 2004), and time courses are perhaps the most fundamental type of signaling data set. So we need mathematical models using system of ordinary differential equations with time as an independent variable for a better understanding of the insight mechanism. To solve these models we use tools like linear algebra and numerical simulations. The use of linear algebra also helps us in data analysis. We can accommodate theoretically infinite number of dimensions of data by vectors or the mathematical transformations. Moreover, algebraically restructuring the data space, it becomes possible to identify a small number of ‘optimal’ dimensions in the experimental observations. So, mathematical models and its solutions actually help us to identify the key parameter involves in a signaling process.

In the present study we were interested to compare the dynamical behavior between the mature and the immature cell. We have already discussed on matured and immature cells differential BCR signaling in our earlier section. It has been reported that levels of phospho-Lyn and other intermediates are significantly higher in immature B cell compared to the mature counterpart.
Another important characteristic feature of immature B cells is that they do not get activated strongly upon anti-IgM treatment. However, mature cells get activated strongly and the activity is sustained for longer levels. Since we had sufficient information on both mature and immature counterparts regarding their basal status and the way they respond to the antigen we built a mathematical model representing the fundamental differences in both mature and immature B cells in the initiation of the BCR signal transduction. The mathematical model was built with the help of a system of ordinary differential equations to analyze Lyn activation and its down-stream effect on Syk. The main aim behind the model formulation was to identify the key parameter(s) that might lead to weak activation in immature B cells while stronger activation was found for mature B cells, though immature state has higher basal values of the intermediate than the mature B cells.

**Emerging Concepts in Network Biology**

Elucidation of the mechanisms that connect extracellular signal inputs to the control of transcription factors was until recently restricted to small-scale biochemical, genetic and pharmacological techniques. Signal transduction pathways have traditionally been viewed as linear chains of biochemical reactions and protein-protein interactions, starting from signal-sensing molecules and reaching intracellular targets; however, the increasingly recognized abundance of components shared by several pathways indicates that an interconnected signaling network exists. The largest reconstructed signal transduction network contains 1259 interactions among 545 cellular components of the hippocampal CA1 neuron (Ma'ayan et al., 2005), based on more than 1200 articles in the experimental literature. This network exhibits impressive interconnectivity: its strongly connected component (the central signaling network) includes 60%
of the nodes, and the subgraphs that start from various ligand-occupied receptors reach most of the network within 15 steps. The average input-output path-length is near 4, which suggests that a very rapid response to signaling inputs is possible. Both the in and out-degree distributions of this network are consistent with a power-law that has an exponent of around 2, the highest degree nodes including four major protein kinases (MAPK, CaMKII, PKA and PKC).

In biochemical regulatory networks, the direct physical interactions between proteins, transcription factors binding to promoter sites, are represented through graph/network, vertices/nodes/components, and edges/links/interactions are used interchangeably but mostly have the same meaning. Using graph analyses, biochemical networks are found to be scale-free and small-world, indicating that these networks contain hubs, which are proteins that interact with many other molecules. These hubs may interact with many different types of proteins at the same time and location or at different times and locations, resulting in diverse biological responses. Groups of components in networks are organized in recurring patterns termed network motifs such as feedback and feed-forward loops. Graph theory-based analysis revealed that negative feedback loops are less common and are present mostly in proximity to the membrane, whereas positive feedback loops are highly nested in an architecture that promotes dynamical stability. Cell signaling networks have multiple pathways from some input receptors and few from others. Such topology is reminiscent of a classification system. Signaling networks display a bow-tie structure indicative of funneling information from extracellular signals and then dispatching information from a few specific central intracellular signaling nexuses.

Cell signaling pathways are commonly represented using mixed graphs in which arcs represent activation or inhibition relations, whereas edges represent physical protein-protein interactions without a clear-cut directionality such as binding to anchors and scaffolds (Ma'ayan et al., 2005).
Other sets in cell signaling graphs can represent other properties of edges such as interaction weights. Weights of arcs can be used to represent the kinetics of biochemical reactions (Bhalla and Iyengar, 1999). Vertices with high degree are informally called *hubs*. Analysis of protein-protein interaction networks demonstrated that hubs can be classified into "party" hubs and "date" hubs (Han et al., 2004). Party hubs are proteins that interact with their neighbors in the same place at the same time, whereas date hubs are proteins that interact at different times in different places within the cell. Another classification of hubs showed that hubs can be divided into single-domain or multidomain hubs (Kim et al., 2006). Some examples of single-domain date hubs are protein kinases A and C and the phosphatase PP2A, which have many known substrates. CASK is an example of a party hub with multiple domains. *Assortative mixing* is when the probability for interactions between nodes is biased due to nodes properties. For example, assortative mixing by valence is between two vertices in a network.

Finding the shortest path between a cell-surface receptor and downstream transcription factors in a cell signaling network can be used to identify important new signaling pathways. Such an approach was useful to hypothesize potential signaling mechanisms in Neuro2A cells downstream of CB1R receptors. Cells were stimulated with a CB1R agonist, and assessment of activity for hundreds of canonical transcription factors was performed. It was found that after 20 min, CB1R activation modulates the activity of 23 transcription factors (Bromberg et al., 2008).

Using known cell signaling and protein-protein interactions extracted from published experimental studies, new biological roles for pathways and co-regulators were identified. In another study, a global analysis of paths from receptors to effectors in a literature-based mammalian cell signaling network showed that from some receptors, e.g. the N-methyl-D-aspartate receptor, there are many paths to effectors, e.g. the transcription factor cAMP-
responsive element-binding protein (CREB), whereas from other receptors, there are only a few (26). This topological feature can be due to biased research (most data from popular proteins and pathways) but can also indicate a design that is commonly observed in learning classifier systems implemented in computer programs. The topology of signaling networks also displays a bow-tie structure, in which signals from many receptors converge on the same intermediate components and then are directed to regulate different transcription factor effectors. This type of organization is common for Toll-like receptors sharing adaptor proteins such as MyD88 (Oda and Kitano, 2006), G protein-coupled receptors sharing G-α and Gβγ (Neves et al., 2002), and growth factor receptors sharing adaptor proteins such as SOS1 and GRB2. The shortest path algorithm can be used to find automatically and display previously characterized interactions that “connect” genes and proteins (Berger et al., 2007) or to compute global network properties such as characteristic path length (Watts and Strogatz, 1998) or network diameter. Network diameter is simply the longest of the shortest paths among all possible shortest paths between all pairs of nodes in a network. The characteristic path length is the average shortest path across all possible pairs of nodes. Biochemical networks such as signal transduction networks and gene regulatory networks show similar patterns of network motifs. For example, the bifan motif (Lipshtat et al., 2008) is made of two upstream regulators both regulating the same two downstream effectors. This dual regulation structure was identified statistically as the most over-represented network motif in gene regulatory networks of yeast (Milo et al., 2002) and Escherichia coli (Milo et al., 2002; Shen-Orr et al., 2002) as well as in a mammalian neuronal cell signaling network (Ma'ayan et al., 2005). One example of a bifan motif in cell signaling networks is the regulation of transcription factors ATF2 and Elk by the kinases JNK (c-Jun N-terminal kinase) and p38 (Lipshtat et al., 2008). The abundance of bifans is most likely due to a large number of isoforms generated...
through gene duplication-divergence evolution. The bifan motif and other motifs such as feedback and feed-forward loops were found to act as noise filters (Ferrell, 2002; Hayot and Jayaprakash, 2005; Lipshtat et al., 2008). Two types of network motifs, namely feed-back and feed-forward loops, are very important for characterizing the dynamics of biochemical networks (Kramer and Fussenegger, 2005; Ma'ayan et al., 2008). Graph analysis of a large cell signaling network suggested that negative feedback loops are more prevalent than positive feedback loops near the cell surface (Ma'ayan et al., 2005), a design that could be helpful for dampening noise while amplifying persistent extracellular signals. A paucity of negative feedback and feed-forward loops in yeast, E. coli, and mammalian cell signaling networks was also observed (Ma'ayan et al., 2008). This feature of the topology suggests that negative loops have not been favored through evolution because of their potential to introduce dynamical instabilities. Hence, it appears that negative regulators are less regulated outgoing hubs, examples of which are known in cell signaling networks. For instance, phosphatases such as PP1 and PP2A are enzymes that deactivate most of their effectors through dephosphorylation. On the other hand, positive feedback loops are highly nested, where the same proteins function in many positive feedback loops, a topology that also favors dynamical stability (Kwon and Cho, 2008). Some regulatory motifs in biochemical networks have long been known, e.g. the negative feedback loop in the synthesis of branched chain amino acid from threonine to isoleucine. The concept of network motifs is illustrated by several examples from cell signaling. Those are PFBL, positive feedback loop; NFBL, negative feedback loop; PFFL, positive feed forward loop; NFFL, negative feed forward loop.

One of the limitations of graph theory applications in analyzing biochemical networks is the static quality of graphs. Biochemical networks are dynamical, and the abstraction to graphs can
mask temporal aspects of information flow. The nodes and links of biochemical networks change with time. Static graph representation of a system is, however, a prerequisite for building detailed dynamical models (Eungdamrong and Iyengar, 2004). Most dynamical modeling approaches, e.g. Boolean networks (Li et al., 2006), Petri nets (Hardy and Robillard, 2008), and event ontologies (INOH Pathway Database), can be used to simulate network dynamics while using the graph representation as the skeleton of the model. Modeling the dynamics of biochemical networks provides closer to reality recapitulation of the system’s behavior in silico, which can be useful for developing more quantitative hypotheses.