3

Phenotypic Characteristics of Neonatal B Cells

1. INTRODUCTION

Macromolecules associated with the membrane of B cells mediate three major functions of B cells. The first function is recognition of antigen, leading to the activation and proliferation of resting B cells as well as the differentiation of plasma and memory cells. The second function is binding and internalization of antigen, followed by its processing for presentation of peptides to T cells in association with MHC molecules. The third function consists of delivering signal via cytokines and costimultory molecules.

Repetitive epitopes comprise T-independent antigen, which are recognized by the B-cell receptor (BCR), resulting in crosslinking of the BCR and subsequent activation and differentiation of B cells. Stimulation of B cells by T-dependent antigen requires not only the binding of antigen to BCRs but also the interaction of costimulatory molecules with their counterparts on T cells.

Polyclonal activators (B-cell mitogens) can induce the activation and differentiation of resting B cells by circumventing the binding of antigen to BCRs, subsequent to activator binding to mitogen or Tol receptors.

2. MOLECULES ASSOCIATED WITH B-CELL MEMBRANE

2.1. Structure and Function of the BCR

The BCR is composed of a macromolecular complex with surface Ig (sIg) that is able to bind the antigen and macromolecules involved in signal transduction, which leads to the activation or repression of B-cell genes. CD79a and CD79b, which are encoded by *mb-1* and *B29* genes, respectively, are directly associated with sIg, whereas other molecules such as CD19, CD21, CD22, and CD81 are associated with the sIg/CD79 BCR complex.

CD79 is a disulfide-bonded heterodimer, composed of CD79a (Igα) and CD79b (Igβ), which mediates signal transduction via the pre-BCR during the development of B cells and via the BCR in immature and mature B cells. Both molecules are type I transmembrane proteins belonging to the Ig superfamily *(350)*.

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In the BCR, one sIg is always paired with one CD79a and one CD79b molecule at a stoichiometry of 1:2. The ITAM motifs found in the intracellular domain of CD79a and CD79b are critical for the transduction signals. The first step in BCR signal transduction is the activation of protein tyrosine kinase (PTK) family members phosphorelays, which phosphorylate CD79. At least four PTKs act as BCR phosphorelays: Src9 Lyn, Fyn, Syk p85, and Btk. Btk is critical at the pre-B-cell stage in humans but not in mice. In humans, null mutations in Btk cause X-linked agammaglobulinemia. SLP-65 has been identified as the immediate downstream substrate of Syk and contains an SH2 domain *(351)*. SLP-65 belongs to a family of adaptor proteins including Grb2, Vav, Nck *(351,352)*, and CrkL *(353)*. Fully activated CD79 is required for the coordinate synthesis of second messengers (PI3 and PIP3), which are generated by enzymatic activation of phospholipase C and PI3K. P13K is required for the production of PIP3 and the recruitment of Btk on the membrane via the PH domain. Btk also contributes to PKCβ activation The Bkt-PKCβ pathway is a significant survival factor for B cells subsequent to ligation of the BCR by increasing the production of growth factors such as c-rel and Bcl-X_L. Bkt is also required for the activation of PLCγ on BCR crosslinking and mediates the production of diacylglycerol (DAG) and IP3.

These second messengers (P13, PIP3, and DAG) trigger the downstream activation of mitogen-acivated protein (MAP) kinases *(354,355)* and nuclear translocation of transcription factors such NF-κB and NF-AT. SLP-65 and BLNK adaptor proteins regulate this process and appear to be master elements in the signals transduced subsequent to the binding of antigen to the BCR *(356)*.

BCR signaling in mature B cells induces translocation of the BCR into cholesterol and sphingolipid membrane microdomains (rafts) that harbor the Src family kinase Lyn, which becomes phosphorylated upon BCR crosslinking *(357)*. Multiple coreceptor molecules are associated with the BCR and are expressed throughout B-cell differentiation and maturation; these factors typically upregulate the threshold of BCR signaling.

2.1.1. CD45

CD45 is a transmembrane protein tyrosine phosphatase that deactivates the Src family of kinases. CD45 is required for normal B-cell development and antigen stimulation by maintaining Src kinases in a partially activated state. This concept is well-supported by studies with two strains of CD45 knockout mice; one strain possesses a targeted mutation in exon 6 and the other in exon 9. B cells of these mice display phenotypic alterations characterized by a decreased number of IgD^{high}, IgM^{low}, CD23, and class II molecules *(358,359)*. B cells from the CD45 mutant mice cannot proliferate subsequent to crosslinking of the BCR with anti-IgM or anti-IgD antibodies, whereas they respond normally to lipopolysaccharide (LPS), IL-4, and anti-CD40 antibody *(359,360)*.

Loss of CD45 results in hyperphosphorylation of Src kinase and a concomitant decrease in its function. Hyperphosphorylation of Lyn prevents binding to the CD79a cytoplasmic domain. Therefore, CD45 is required to maintain Lyn and other Src kinase family members in a partially phosphorylated state, enabling the BCR to respond optimally to antigen stimulation. Finally, CD45 is required for the activation of ERK-MAP kinase pathway *(358)*.

2.1.2. CD19

CD19 also is associated with the B-cell membrane and contains nine highly conserved tyrosine residues in its cytoplasmic domain. CD19 is phosphorylated at low levels in basal state and forms a complex with Lyn and Vav *(361)*. The antigen ligation of BCRs leads to increased Lyn phosphorylation and thereby to activation of CD19 and CD79. Phosphorylated CD19 enhances the activity of other members of the Src kinase family, such as PCLγ2, which is downstream of Syk and PI3K *(362–365)*. CD19 also appears to be a phosphorelay to Grb2, Sos, C-Abl, and PTK *(366–368)*. Thus, CD19 is central to regulation of intrinsic and BCR-induced Src family PTK activity *(362)*.

2.1.3. CD22

CD22 is a transmembrane glycoprotein restricted to the B cell but present in the cytoplasm of pro-B , pre-B, and IgD^+ B cells. CD22 controls signal transduction thresholds initiated upon antigen binding to the BCR. CD22 is associated with the BCR *(369)* and its natural ligand, which is a sialic protein. The cytoplasmic domain of CD22 contains six tyrosine residues, which are rapidly phosphorylated following ligand binding to the BCR. Phosphorylated CD22 physically interacts with positive regulatory molecules such as Syk, PI3K, PCLγ2, Grb2, and Sos *(370–372)*. Lajaunias et al. *(373)* showed that the expression of CD22 in conventional B2 cells is downregulated after crosslinking of the BCR with anti-IgM antibodies but is upregulated after stimulation with LPS, interleukin (IL)-4, or anti-CD40 antibody. By contrast, CD22 expression is barely altered in B1 cells after incubation with anti-IgM antibodies. These observations suggest that CD22 is differentially regulated subsequent to activation of B1 and B2 subsets of B cells.

In summary, current evidence demonstrates that the ligation of the BCR induces several specific phosphorelays, leading to activation of coreceptors and various transcription factors that activate B-cell-specific genes. Mutations affecting components of BCR signaling (e.g., CD79a, SLP-65, BTK, PKTβ p85, PI3 kinase, Vav, CD45, and CD19) have been reported to affect development of the B1 subset *(374–376)*.

2.1.4. LCK

LCK is another enzyme involved in BCR signaling. It is expressed only in B1 cells *(377)*. In LCK knockout mice, impaired phosphorylation and activation of

the MAP kinase ERK was observed during BCR crosslinking in B1 cells, whereas no differences were observed in the frequency of $CD5^+$ IgM^+ B1 cells *(377)*. The impairment in BCR signaling in B1 cells of LCK knockout mice suggests that LCK is required for BCR mediated signaling in this B-cell subset.

2.2. *Costimulatory Molecules Associated With the B-Cell Membrane*

Binding of antigen to the BCR activates signaling cascades that induce transcription of genes for B-cell activation and differentiation. However, except for ligands that crosslink the BCR (such as anti-IgM antibodies and T-independent antigen) the engagement of BCRs by T-dependent antigen or hapten-carrier complexes alone is not sufficient to activate B cells. Full activation requires the interaction of T cells with costimulatory molecules (e.g., *CD80, CD86*, and CD40) associated with the B-cell membrane. *CD80* and *CD86* bind *CD28* and CTLA-4, which are associated with the membrane of T cells, respectively, and the CD40 with CD40L (CD154).

2.2.1. CD80 and CD86

Both *CD80* and *CD86* belong to the Ig gene superfamily and are composed of an extracellular domain of 220 amino acids, a hydrophobic transmembrane domain of 23 amino acids, and a tail of 60 amino acids. Both are expressed on the surface of B cells as well as other antigen-presenting cells, such as dendridic cells or macrophages.

CD80 and *CD86* genes display only 26% structural homology *(378)*, which may explain their differential binding to CD28 and CTLA-4. For example, *CD80* displays 20 times higher binding affinity to CTLA-4 as it does to CD28. A large body of information is related to the activation/inhibition and signaling pathways in T cells subsequent to binding of *CD80* and *CD86* to *CD28* and CTLA-4 coreceptors, but few data concern the molecular alterations in B cells. Utilization of monoclonal antibodies specific for *CD80* and *CD86* recently permitted revealing experiments. Crosslinking of *CD86* enhances the proliferation of B cells and promotes the synthesis of IgG2a and IgG2b. The crosslinking of CD80 blocked the proliferation of B cells. Thus, whereas *CD80* provides a negative signal for the proliferation of B cells, *CD86* stimulates the activity of B cells *(379)*.

CD80 and *CD86* possess markedly different cytoplasmic domains. The cytoplasmic domain of CD80 has a short tail that lacks tyrosine residues, whereas CD86 contains three tyrosine residues as potential protein kinase C phosphorylation sites *(381)*. The cytoplasmic tail of *CD80* contains a tetrapeptide motif called RRNE at position 275–278 and a highly conserved serine at position 284 that can be phosphorylated. *CD80* from cells stimulated with ionomycin coprecipitates with a 39 kDa phosphoprotein that can contribute to the phosphorylation of *CD80 (382)*. Thus, *CD80* and *CD86* can mediate differential signal transduction and regulate the function of B cells differently *(379)*.

The negative signal transduction induced by ligation of *CD80* inhibits B-cell proliferation by upregulating proapoptotic genes such as *caspase-3*, *caspase-8*, *Fas*, *Fas ligand*, *Bax*, and *Bac*. In contrast, the signal delivered via *CD86* enhances B-cell proliferation and activates antiapoptotic genes such as *Bcl-x(L) (379)*.

2.2.2. CD40

CD40 is a member of the nerve growth factor family and is expressed on B cells and dendritic cells. The binding of CD40 to the CD40 ligand expressed on T cells (called CD154) exerts various effects, including increased interaction between adhesion molecules LFA1/ICAM-1, proliferation of B cells, enhanced expression of costimulatory molecules CD80 and CD86 *(382–385)*, and class switching to IgE and IgG1 *(386)*. Optimal activation of B cells requires a synergistic effect between the activation of BCRs and CD40. CD40 does not possess intrinsic kinase activity, probably because of absence of conserved tyrosine residues in its cytoplasmic tail. However, CD40 does possess several residues potentially available for serine and threonine phosphorylation, allowing for interaction with tumor necrosis factor (TNF)-receptor-associated factor (TRAF), which serves as an adaptor protein in the CD40 signaling pathway *(386)*.

In B lymphocytes, cooperation of multiple signaling pathways initiated by the binding of CD40 to CD154 leads to the activation of transcription factors and subsequently to gene expression.

Both BCR and CD40 signaling stimulate MAP kinase modules, leading to the activation of transcription factors such as c-Jun NF-κB, NFAT, and AP-1 *(387,388)*. Among three MAP kinase modules studied by Dadgastor et al. *(389)*, it appears that the ERK-MAP kinase pathway has a minor role in CD40-mediated gene expression. The role of the p38 MAP kinase pathway appears to be small and almost entirely cooperative with other pathways. The p38 pathway may contribute to the downregulated expression of *Ndr1*, *Rb2*, and *SPA-1* genes, which inhibit cell growth. By contrast, the PI3K pathway contributes significantly to the gene induction of CD40 via downregulation of *Rb1* and *BTG-2* genes with antiproliferative properties.

CD40 plays an important role in the expression of CD80 and CD86 costimulatory molecules. Two functional domains in CD40, threonines at position 227 and 234, regulate induction of CD80 expression *(390)*. The phosphorylation of threonine in the PXQXT motifs of TNFR-2 is necessary for the synergy between BCRs and CD40 *(391)*. TRAF molecules associated with CD40 are required for class switching because they induce *IgG1* and *IgE* promoter transcription activity. This concept is supported by experiments demonstrating that mutation of TRAF-binding motifs within the CD40 cytoplasmic tail lead to significant decreases of promoter activity within *IgG1* and *IgE* constant region genes *(392)*.

Taken together, these data demonstrate that the binding of specific ligands to costimulatory molecules *CD80*, *CD86*, and CD40 triggers the activation of B-cell growth, cell interaction, Ig synthesis, and class switching, which augments the binding affinity of antibodies.

2.3. Fc and Complement Receptors Associated With the B-Cell Membrane

The receptors that mediate antigen internalization are the FcγR (CD16, CD32, CD64), FcεR (CD23), and complement (CD21 and CD35) receptors. Among the receptors expressed on various cells, CD32, CD23, and CD21 are expressed on B cells.

2.3.1. CD32

FcγIIRB is an inhibitory, single-chain, low-affinity receptor with an extracellular and a cytoplasmic domain containing a tyrosine inhibitory motif (ITIM) *(393)*. CD23 is encoded by a single gene, but alternative splicing of pro-messenger RNA (mRNA) generates two isoforms: FcγRIIb1 and FcγRIIb2 *(394)*. FcγRIIb1 is preferentially expressed on B cells and is involved in the negative regulation of antibody response, BCR-generated calcium mobilization, and cell proliferation. The inhibitory activity is related to an inhibitory sequence of 13 amino acids (AENTITYSLLKHP) embedded in the cytoplasmic domain *(395)*. Co-ligation of BCRs and CD23 leads to tyrosine phosphorylation but also rapid abrogation of ITAM activation signaling by hydrolyzing membrane inositol phosphate PIP3, a product of CD23 activation. In the absence of PIP3, the protein bound to the PH domain is released, leading to a blockage of calcium signaling and to arrest of B-cell proliferation triggered by the BCR *(394)*.

2.3.2. CD21 and CD35

These receptors for complement bind to C3b, C3d, or C3g. They are expressed on B cells and follicular dendritic cells. CD21 contains an extracellular domain of 15–16 repetitive units called SCR, a transmembrane domain, and a 34-amino acid cytoplasmic domain. The cytoplasmic domain is devoid of signaling motifs, but it is required for the internalization of polymerized C3b and C3g *(365)*. CD35 serves as cofactor for the hydrolysis of C3b-antigen complexes, allowing the binding of C3b to CD21. CD21, the receptor for C3d fragment of complement, is associated with CD19 and CD81 (TAPA-1), forming a signaling complex that can activate BCR signaling. The role of CD21 and CD53 in B-cell development is supported by data indicating a 40% reduction of peritoneal B1 cells, a 30–60% reduction of serum levels of IgG, and a reduction of germinal center formation in CD21/CD35 knockout mice (reviewed in ref. *362*).

3. EXPRESSION OF THE BCR AND OTHER MEMBRANE-ASSOCIATED RECEPTORS IN NEONATAL B LYMPHOCYTES

Classically, newborns were considered deficient in mounting a humoral immune response as a result of immaturity in their B cells. The immaturity of neonatal B cells implies difficulties in the induction of B-cell differentiation as a result of genetic and phenotypic differences between neonatal and adult B lymphocytes. More recently, a thorough analysis of the BCR and the phenotype of neonatal B cells did not reveal important differences, indicating that the poor responses of neonatal B cells to T-dependent antigen might be related to the immaturity of T cells and antigen-presenting cells in neonates.

3.1. Expression of the BCR Complex on Neonatal B Cells

As described earlier, the BCR exists as a protein complex on the B-cell surface. This complex is composed of surface Ig associated with CD79a and CD79b. Surface Ig differs from secreted Ig, because surface Ig contains two additional segments: a transmembrane domain and a cytoplasmic region. The Ig receptor and secreted Ig are encoded by V_H and V_L ($V\kappa$ or $V\lambda$) genes. Several exons comprise these genes: V_H (D) J_H (V_L, J_L), and constant region (Cκ or Cλ). The surface Ig component of the BCR contains a transmembrane domain and a cytoplasmic region encoded by two additional exons: M1 and M2. CD79a is encoded by the *mb-1* gene, and CD79b is encoded by the *B29* gene.

In the common lymphocyte progenitor from which B-cell progenitors derive, the exons encoding surface Ig are in germline configuration. During B-cell development, the exons encoding Ig are assembled by a process of somatic recombination *(396)*, leading to a complete surface IgM molecule expressed on immature B cells, which are predominant in the neonatal immune system. After birth, the immature B cells mature and express both surface IgM and IgD. With subsequent antigen stimulation, the V_H genes can recombine with other constant region exons, leading to the surface expression of IgG, IgA, or IgE. The somatic recombination is mediated by RAG enzymes, which are activated during various stages of B-cell development.

The VDJ recombination process is of fundamental importance to the generation of antibody diversity because of multiple possible combinations of joining events and also because of allelic exclusion, because the VDJ of a single chromosome can be productively rearranged.

VDJ recombination is a site-specific recombination process that occurs only between exons flanked by conserved recombination signal sequences (RS), each of which is composed of a conserved palindromic heptamer and an AT-

rich nonamer separated by a 12- or 23-bp spacer. These two sequence blocks comprise the recognition sites for the joining of the D segment to J_H, the V_H to DJ, and the V_L to J_L exons *(397–399)*. Joining results either from inversion or deletion of intervening sequences, depending on the relative orientation of the recombining exons *(396)*. The recombination process is initiated and mediated by RAG enzymes, which bind to RS. The recombination process is initiated via double-strand DNA breaks between Ig exons and RS. Then, RS ends are joined, and TdT can modify the coding ends by addition or deletion of nucleotides, which contributes to the diversity of BCRs (reviewed in ref. *400*). In addition to RAG enzymes, other proteins participate in the repair of RAG-initiated double-strand breaks. Three proteins are subunits of DNA-dependent protein kinase consisting of the Ku70 and Ku80 subunits and a catalytic subunit related to PI3 kinase *(401)*.

The recombination process during development of the B-cell lineage begins in pro-B cells expressing V-pre-BCR and then continues with the rearrangement of D and J exons of heavy chain in pre-B1 cells, which exhibit the phenotype $B200^+$ $CD45^+$ $CD25^-$ $c\text{-}kit^+$ *(402,403)*. The joining of V_H exon to rearranged DJ segment and to μ-constant region exons occurs in pre-B-II large cells. The pre-B cells have cytoplasmic IgM heavy chain and express B220, CD43, CD25 c-Kit markers, and TdT. In the pre-B-II small lymphocytes, the rearrangement of exons encoding the light chain occurs. These cells express sIgM but cease to express CD45, CD25, c-kit, and TdT. From pre-B-II small cells arise the immature lymphocytes ($B220^+$ $sIgM^+$), which are predominant during the first weeks after birth. The *RAG* genes are first expressed in the pro-B cell ($CD220^+$ $CD43^+$ HSA^- $CD4^-$) and increase as B cells mature and the VDJ recombination process occurs. Their expression decreases in pre-B-II large cells and reoccurs in pre-B-II small cells during recombination of V_L and J_L exons but ceases in immature B cells *(404,405)*. CD79a and CD79b comprise an integral component of BCRs that is required for both surface expression and signaling. In particular, they play a role in the transition of immature IgM^{high} IgD^{low} B cells to mature B cells. Mice expressing the IgM/CD79b transgenic receptor exhibit normal maturation. However, mice harboring a truncation of the CD79d intracellular domain exhibit impaired B lymphopoiesis, in particular during the transition phase of $B200^+$ $c\text{-}kit^+$ to $B220^+$ $CD25^+$ pre-B cells, with a three- to sixfold reduction of IgM^+ immature B cells. In these mice, the immature B cells exhibit a reduced migration efficiency, resulting in an over 100-fold reduction of mature B cells in peripheral organs *(406)*.

A major characteristic of the neonatal immune system consists of induction of an immune response to a restricted range of antigen, producing largely IgM antibodies. In humans, neonatal B cells differ from adult B cells by a series of phenotypic features. Phenotypic analysis of cord blood B cells showed a pre-

dominance of $CD5^+$ B cells and more consistent expression of high levels of IgM than adult cells. Whereas the adult B cells expressed 12,000 to 48,000 IgM molecules per cell, the cord blood B cells express 63,000 to 240,000 IgM molecules per cell. No quantitative differences were observed in the sIgM density between cord blood B1 and B2 cells. In contrast to adult B cells-in which the majority of IgM is located within cytoplasm-most of the IgM for cord blood cells is located on the membrane *(407)*. The high density of IgM BCRs in neonatal B cells may increase the sensitivity of the response to polymeric antigen, causing the crosslinking of BCRs. This concept is supported by observations that neonatal B cells can make antibodies subsequent to stimulation with trinitrophenyl (TNP)-*Brucella abortus*, a TI-1 independent antigen *(408)*.

CD22 is a molecule associated with BCRs and is known to be a negative regulator of BCR signaling. Comparison of the expression of CD22 on cord blood and adult peripheral lymphocytes showed a significantly lower percentage of $CD22^+CD5^-$ B cells in the neonatal lymphocyte population. The stimulation of neonatal lymphocytes with anti-IgM antibodies (which crosslink the BCR) resulted in a dramatically reduced number of both $CD22^+$ $CD5^+$ and $CD22^+$ $CD5^-$ B cells. Meanwhile, the T-cell-dependent stimulation with anti-CD40 monoclonal antibody and IL-4 resulted in a dramatically increased number of $CD22^+$ neonatal B cells *(409)*. These data suggest that whereas polyclonal stimulation of neonatal B cells lowers the threshold of BCR signaling after crosslinking, the T-cell-dependent stimulation might increase the inhibiting effect of CD22 on BCR signaling of neonatal B cells.

The binding of antigen to BCR induces a rapid translocation into cholesterol and the sphingomyelin-rich microdomains, called lipid rafts. Lipid rafts provide the platforms for BCR signaling and the phosphorylation of Src kinase family members such as Lyn. These events lead to the internalization of BCR in mature B cells *(357)*. Comparison of BCR crosslinking by anti-IgM antibody on two B-cell lines (WEHI-231, an immature B-cell line, and CH27, a mature phenotype) revealed strikingly different responses to BCR crosslinking. After crosslinking, the cell lysates (obtained by incubation with Triton X-100 detergent) were subjected to density gradient centrifugation. In the case of CH27, about 40% of the BCR was present within lipid raft, whereas in the case of WEHI-231 only 3% of BCR was present. This result clearly showed that in the case of immature B cells, the BCR and CD79a did not translocate significantly in the lipid rafts subsequent to the crosslinking with anti-IgM antibodies. Both BCRs and CD79a were detected in the soluble fraction. In contrast to CH27 cells, which exhibited a significant internalization of BCRs on crosslinking (25% by 40 min), in the case of WEHI-213 cells, the constitutive internalization was not affected by crosslinking *(410)*. These elegant experiments provided evidence that the lipid raft in immature B cells may be differ-

ent compared to mature B cells in either ability to translocate BCRs or to stabilize BCR localization in lipid rafts.

The transition from immature to mature B cells is associated with a series of signaling processes through BCRs. The signaling via IgH is necessary for allelic exclusion and differentiation of pro-B cells in pre-B cells. This concept is supported by data originating from transgenic mice harboring an IgM-bearing mutation in the transmembrane domain. These mice cannot express sIgM and lack pre-B cells and allelic exclusion *(411)*. The same effect was observed in mice expressing an IgM heavy chain and also with mutation in transmembrane domain that prevented the association of BCRs with CD79 *(412)*. Crosslinking of BCRs triggers sequential activation of at least three phosphorelays required for the generation of second message.

Study of syk phosphorylation and Src-related kinases (Lyn, Fyn, fgr, Blk, and LCK) did not show differences between adult and neonatal lymphocytes isolated from 2- to 3-d-old mice. By contrast, purified neonatal B cells, consisting of more than 90% IgM^+ cells, expressed low levels of p59 Fyn and p55 fgr. These two elements attain the levels found in adult B cells by 2–4 wk after birth *(413)*. The reduced expression of Fyn and fgr in neonatal B cells might account for the differences in IgM signaling noted in immature B cells. Neonatal B cells are deficient in the expression of p56, which is another BCR-associated protein *(414)*.

Another difference between neonatal and adult B cells is their ability to hydrolyze phospholipids in response to crosslinking of BCRs.

Hashimoto et al. *(415)*, using a gene-targeted approach, generated phospholipase C-γ2-deficient mice with an interferon (IFN)-induced Cre recombinase transgene. The inactivation of *PLC-γ2* gene in these mice 2 d after birth resulted in a two- to threefold reduction of $B220^+IgM^+$ cells in all peripheral organs as well as defects in B-cell development at the pre-B-cell stage. Taken together, these results demonstrated that there are subtle differences in the BCR signaling pathways between neonatal and adult B cells, which may also explain the meager neonatal response to immunization.

3.2. Costimulatory Molecules Expressed on Neonatal B Cells

The BCR and the majority of antigen associated with the membrane of immature and mature B cells are not expressed on hematopoietic stem cells (HSCs) or B-cell progenitors. However, CD80 and CD86 are expressed on $CD45^+$ and embryoid bodies by d 12 of gestation. HSCs were stained with antibodies specific for CD80 and CD86, and stain data were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting. These experiments showed the presence of corresponding transcripts of both genes. CD80 and CD86 are expressed on 10–20% and 30–50% of $CD45^+$

HSCs, respectively. In addition, it was shown that CTLA-4-Ig, but not CD28-Ig fusion protein, binds to CD80 *(416)*. The binding of CTLA-4 to *CD80* indicates that the CD80 molecule is functional on HSCs. Presently, there is no information regarding the function of CD80 and CD86 molecules during the embryonic development.

Comparing expression of CD80 and CD86 on lymphocytes from cord blood, young children (2–20 mo), and adults showed no detectable CD80 expression on resting B cells. By contrast, CD86 was expressed at the same level of density on neonatal and adult B lymphocytes. Stimulation of neonatal B cells with PMA and ionomycin induced CD80 expression. No significant differences were observed in the fluorescence intensity of the expression of CD80 and CD86 on stimulated neonatal and adult B cells *(417)*.

The limited ability of neonatal B cells to respond to T-dependent antigen and to generate memory cells can be related to the defective expression of CD40 or other costimulatory molecules. CD40 expression is equivalent between neonatal and adult B cells *(418)*. Elliot et al. *(419)* studied the expression of CD40 in peripheral blood lymphocytes (PBLs) from cord blood, young children (2–12 mo), and adults. They reported that in some healthy neonates, the expression of CD40 was higher than in adults. However, the fluorescence intensity after stimulation with PMA and ionomycin was identical on cord blood and adult lymphocytes. Therefore, reduced synthesis subsequent to binding of CD40L by neonatal B cells cannot be related to the expression of CD40 but rather to differences in CD40 signaling pathways in neonatal B cells.

These observations taken together show that limited response of neonatal B cells is not related to lack or poor expression of costimulatory molecules but possibly relates to immature signal transduction.

3.3. Expression of Fc Receptors on Neonatal B Cells

Binding of immune complexes to B cells (by co-engaging the BCR and FcγRII-CD32) induces inhibitory pathways that may lead to apoptosis *(420,421)*. This action may be beneficial for neonatal B cells, because the maternal antibodies (IgG) can form immune complexes with environmental antigen after birth. Jessup et al. *(422)* compared the expression of two CD32 isoforms (FcγRIIa and FcγRIIb) in neonatal and adult B lymphocytes. Most cord blood and adult B lymphocytes expressed both isoforms, yet cord blood lymphocytes expressed lower levels of CD32. The reduced expression of CD32 on neonatal B cells may make them more resistant to the Fc-mediated inhibition seen in adult B cells.

The production of IgE is very low in neonates, although VDJCε transcripts can be detected in fetal liver by 20 wk of gestation *(423)*. However, the increased concentration of IgE specific for allergens or parasites *(424,425)* indi-

cates that neonatal lymphocytes are able to synthesize IgE. It has been shown that CD23 and CD21 have an important role in IgE production *(426)*. CD23 is a low-affinity receptor for IgE that is constitutively expressed on B cells. CD21 expressed on B cells and follicular dendritic cells is a high-affinity receptor for CD23. CD23 downregulates IgE production *(427)*, which may partially explain the poor synthesis of IgE in neonates. Studies of the expression of CD21 and CD23 on cord blood and adult B lymphocytes demonstrated that the percentage of $CD23^+$ and $CD21^+$ B cells was comparable in neonatal and adult B cells *(428)*. Therefore, equivalent expression of CD23 and CD21 on neonatal and adult B cells cannot explain the poor IgE synthesis subsequent to downregulation by CD23.

3.4. Expression of CD72 and CD38 in Neonatal B Cells

CD72 is a C-type lectin receptor that binds CD5, a ligand expressed on all T cells as well as the B1 cell subset. CD72 ligation plays an important role in the induction of primary response by naïve B cells and increases the sensitivity to IL-10 stimulation (implicated in class switching and apparently defective in newborn B cells). Strong support for these conclusions came from observations that combined stimulation with anti-CD72 and IL-10 increased the synthesis of Ig by neonatal but not adult B cells *(429)*.

CD38 is an ectoenzyme marker expressed on the surface of B cells. Its extracellular domain mediates the conversion of nicotinamide adenine dinucleotide (NAD) to cyclic- adenonine dinucleo-phosphate (ADP)-ribose, producing nicotin acid dinucleotide and nicotinic acid. Both products are powerful calcium messengers in various cells *(430,431)*. Anti-CD38 induces the proliferation of B cells *(432)*. Immature B lymphocytes from young (1-wk-old) mice that express significant levels of CD38 still fail to proliferate with anti-CD38 plus anti-IgM antibodies. Proliferation was observed only in older mice (2 wk old) and reached adult levels by age 4 wk *(433)*. The role of CD38 in the development of B cells is not known, but possibly, CD38 delivers signals that are integrated in transduction pathways initiated by the ligation of BCRs.

The data illustrated in Table 15 summarizes the major phenotypic differences between neonatal and adult B cells.

Analysis of phenotype of neonatal and adult B cells shows some important differences that may explain why neonates produce mainly IgM antibodies, which display low affinity for antigen, multispecificity, and high connectivity. The major B-cell species in neonates is the B1 cell, whereas in adults, the B2 subset constitutes the majority. The B2 subset is able to synthesize high-affinity antibodies and exhibits class switching and somatic mutations. Lack of anti-

Table 15
Phenotypic Characteristics of Neonatal and Adult B Cells

Trait	Neonatal	Adult
Predominance of B-cell subset		
	B1	B2
Surface Ig	IgM	IgM IgD
Ig synthesis	Mmainly IgM	All subclasses
CD80	Not expressed	Not expressed
CD86	Comparable	
CD32	Expressed	Lower expressed
CD40	Expressed	Higher expressed
CD21	Comparable	
CD22	High CD22+ $CD5^-$	Low $CD22^+$ $CD5^-$
CD72	Normal ligation	Defective ligation
$p50^{fyn}$, $p55^{frg}$	Normal	Low level

body diversity may be related to low expression of TdT, which is responsible for the increase of diversity resulting from N-addition process, low expression of molecules associated with BCRs, and activation of BCR signal transduction.

Apparently, the inability of neonatal B2 cells to mount a vigorous response to T-cell-dependent antigen is not related to low expression of costimulatory molecules but rather to immaturity of T cells. Neonatal B cells do produce antibodies after stimulation with polyclonal activators such as Epstein–Barr virus, pockweed mitogen, *Nocardia opaca* mitogen, protein A, and LPC in murine B cells. Neonatal B cells do not exhibit class switching, a defect related perhaps to defective expression of activation-induced cytidine deaminase, an RNA-editing enzyme involved in the process of class switching and somatic mutation *(434)*. Finally, subtle differences in Src-tyrosine kinases noted in neonatal B cells, as compared to adult B cells, may explain differences in signaling pathway resulting from BCR occupancy.

Investigations of the development of the B1 subset, carried out in transgenic mice, suggest that specific transcription factors and enzymes are critical for the neonatal development of this B-cell subset. B1 cells were not detected in mice bearing null mutations of CD45, CD19, Btk, PKCβ, or the p85a subunit of PI3K (reviewed in ref. *374*). A decreased number of B1 cells were observed in CD21/CD35 or CD81/TAPA-1 knockout mice. However, an increased number of B1 cells was observed in moth-eaten mice, which lack functional SHP-1 *(435)* or CD22 *(436)*, the recruiter of SHP-1 to the BCR that leads to enhanced proliferation of B1 cells.

4. POSITIVE AND NEGATIVE SELECTION OF B CELLS DURING DEVELOPMENT

After birth, the bone marrow generates approx 100 million B cells daily, *(437)* and about 5×10^7 exhibit the phenotype of immature B cells *(438)*. The majority of neonatal immature B cells display a short half-life between 2 and 4 d, and approx $2–3 \times 10^6$ are found in the peripheral lymphoid organs, where they mature into antigen-responsive B cells. These B cells have a longer half-life *(439)*.

These quantitative differences suggest that only a small fraction of B cells produced in the bone marrow survive to colonize peripheral lymphoid organs after birth. B cells arising from the differentiation of B-cell progenitors display BCRs that can recognize the entire antigen dictionary (about 1 billion epitopes) of foreign and and slef-antigen.

Ehrlich *(43)*, a classic immunologist, proposed that the immune system must avoid the response to self-antigen, a concept he termed as *horor autotoxicus*. This concept implies that the immune system possesses the intrinsic ability to distinguish self from nonself. Ehrlich's concept implies that during the development of immune system, the B cells are censored. Those bearing a BCR specific for foreign antigen are positively selected, but those bearing a BCR specific for self-antigen are negatively selected.

During B-cell development, the first positive selection takes place in the bone marrow at the level of pre-B cells. Only pre-B cells displaying in-frame rearrangements of the V_H gene survive and eventually differentiate into immature B cells. Thus, the BCR expressed on the pre-B and immature B cells, which predominate after birth, is involved in the positive selection. The concept of positive selection is supported by two groups of findings. First, the repertoire of neonatal peripheral B cells is more limited vs adults *(440)*. Second, only a small proportion of B cells produced in the bone marrow emigrate and colonize the peripheral lymphoid organs *(439)*. This suggests that the immature B cells, which leave the bone marrow after birth and later during adult life, were properly and positively selected and received adequate signals that permitted their survival.

Two factors influence positive selection of B cells during primary development. One factor is the level of expression of BCRs at the B-cell surface. This was elegantly demonstrated by a study of the expression of BCRs in IgHμδ transgenic mice containing various numbers of copies of transgene *(441)*. The mice having 1–15 copies of transgene exhibited an increased number of a subset of B2 cells in marginal zone of the germinal center. This B2 subset, called MZ/T2, exhibits the following phenotype: $sIgD^{high}$, $sIgM^{high}$, $CD21/CD35^{high}$, $CD23^{low}$ $CD1d^{+}$ $CD24^{+}$. By contrast, mice containing 20–30 copies of

transgene have a reduced number of cells in bone marrow and spleen and essentially no B cells in the lymph nodes.

The form and localization of antigen also play an important role in the process of positive selection of B2 cells. This was shown in double transgenic mice containing VH-VL transgenes specific for hen egg lysozyme (HEL) plus lysozyme gene encoding for soluble or cell-bound HEL. In these mice, the B cells bind to HEL with affinities ranging from 10^{-5} to 10^{-9}. When HEL was expressed at concentration of 1 nM in soluble form in blood, the B cells with HEL low-binding affinity were positively selected, whereas those with high-binding affinity were deleted. Both B cells with low- or high-binding affinity were deleted in mice expressing the cell-bound form of HEL *(442)*.

Apparently, the mechanisms of positive selection of B1 subset are different. This subset expresses BCR specific for multivalent self-antigen such as immunoglobulins, erythrocytes, thymocytes, cytoplasmic antigen, DNA (reviewed in ref. *271*), and phosphatidyl choline *(443)*, or environmental antigen such as phosphoryl choline *(444)*. Binding specificity of antibodies produced by hybridomas expressing CD5 transcript *(445)* (obtained from moth-eaten mice, a strain in which more than 90% of cells are $CD5^+$) *(446,447)* were compared to other strains prone to autoimmune diseases *(445)* or B-cell lymphoma *(448)*. This study confirmed the differences in positive selection of the B1 subset across antigen/antibodies.

The idea of positive selection of B1 cells is based on the observation that the majority of murine antibromelin-treated red blood cell (RBC) autoantibodies produced by B1 cells are encoded by the restricted set of V_H and V_L genes (V_H11–V_k 9, V_{H12}–V_{k4}) *(449)*. Human cold agglutinin produced mainly by $CD5^+$ B cells are encoded by a single gene member of the V_{H4} family (data reviewed in ref. *109*). B1 cell precursors probably are positively selected by the self-antigen mentioned earlier, which induce the expression of CD5. The ligation of CD5 to CD75, as well as low-affinity binding of B1 BCRs to multimeric antigen, downregulates the BCR signaling and allows the survival of B1 cells. It is noteworthy that in B1 cells, the STAT3 is constitutively phosphorylated *(450)*. The autophosphorylation of STAT3 by unknown environmental stimuli may contribute to positive selection, because it was shown that targeted mutation of STAT 3 results in embryonic death.

The concept of negative selection has its roots in Burnet's clonal theory, which proposed that self-reactive B cells are deleted during B-cell development, leading to central tolerance. Central tolerance occurs during lymphocyte ontogeny as a result of negative selection in bone morrow. The concept of negative selection of B cells is well-supported by studies carried out in transgenic mice. In mice expressing V_H and V_L genes encoding antibodies spe-

cific for allogeneic MHC class I molecules *(46)*, HEL *(47)*, or an allelic form of the CD8 self-antigen *(451)*, B cells specific for these antigen are deleted. Deletion occurs in the bone marrow during the differentiation of pre-B (sIg^-) to immature ($sIgM^+$) B-cell stages *(451)*. Deletion of self-reactive clones may also occur in peripheral lymphoid organs, particularly in immature HSA^+ B cells exported from bone marrow to enter into the marginal zone of the white pulp of spleen *(452)*. Negative selection operates at the cellular level through alteration of signaling pathways or by apoptosis initiated by the interaction of high-affinity BCRs with antigen *(453)*.

Current evidence supports several conclusions about positive and negative selection of B cells during development.

First, positive selection of B2 cells occurs in the bone marrow at pre-B and immature B cells, which express BCRs with low affinity for unknown antigen. In contrast, the B1 subset is selected by virtue of their BCRs to interact with multivalent microenvironmental or self-antigen. The positive selection may be associated with downregulation of signaling pathways initiated by the binding of ligands to BCRs and associated molecules.

Second, central clonal deletion occurs in bone morrow during the transition from pre-B to immature B cells as a result of exposure to self-antigen. Central clonal deletion can also occur in the peripheral lymphoid organs and, in particular, within the marginal zone of white pulp. Negative selection depends on the threshold of BCR occupancy, either because of an increased density of membrane-associated BCRs or because of exposure with membrane-bound antigen.

Thus, the neonatal B cells found in peripheral lymphoid organs arise from both positive and negative selection.