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CD21 and CD24 Co-expression: A Translational Model between Mouse and Human

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CD21 and CD24 Co-expression: A Translational Model between Mouse and Human

by

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the requirements for the degree
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ABBREVIATIONS

BCR-	B CELL RECEPTOR
HSCs –	HEMATOPOIETIC STEM CELLS
BM –	BONE MARROW
MZ –	MARGINAL ZONE
FM –	FOLLICULAR MATURE
T1 –	TRANSITIONAL 1
T2 –	TRANSITIONAL 2
CLP-	COMMON LYMPHOID PROGENITOR
EBF-	EARLY B LYMPHOCYTE FACTOR
IgHC-	IMMUNOGLOBULIN HEAVY CHAIN
SLC-	SURROGATE LIGHT CHAIN
BAFF-	B CELL ACTIVATING FACTOR
APRIL-	ANTIGEN PROLIFERATING LIGAND
HP-	HOMEOSTATIC PROLIFERATION

ABSTRACT FOR DISSERTATION

CD21 and CD24 Co-expression: A Translational Model between Mouse and Human

by

Abigail Benitez

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, June 2014
Dr. Kimberly J. Payne, Chairperson

Systemic Lupus Erythematosus and Rheumatoid Arthritis are B cell-mediated autoimmune diseases that afflict millions of people worldwide. B cell-targeted therapies for these diseases result in variable clinical outcomes. Thus, a need exists to better understand the dynamics of human B cell production and function. The mouse model has provided a foundation for understanding the mechanisms involved in human B cell development and autoimmune disease. However, differences in mouse and human B cells are not fully understood. Our work shows that the co-expression of CD21 and CD24, determined by 7-color flow cytometry, can be used to demarcate developmental subsets of B cells. A comparison of analogous B cell subsets in mice and humans showed that the B cell subsets distribution differs between the species suggesting differences exist in mechanisms that regulate and maintain the specific B cell. This work provides a foundation for understanding human B cell development using the mouse model.

CHAPTER ONE

INTRODUCTION

The immune system must balance between protection of the host against foreign pathogens and guarding the host from its own antibody arsenal. The immune system utilizes negative selection processes to safeguard against the generation of lymphocytes with the potential to recognize and attack the host. The mechanism by which auto-reactive B cells can bypass negative selection and mature to become autoantibody-secreting cells is not understood (1). Failure to eliminate auto-reactive B cells can lead to B cell-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (2-4).

B Cell-Mediated Diseases

The incidence of SLE and RA diseases is also characterized by unique gender and ethnic differences. Indeed, both diseases are more prevalent in females compared to males (5). Hispanics and African-Americans display a younger age at onset of disease and higher morbidity than Caucasians. Moreover, epidemiology studies indicate that SLE is more prevalent in African-Americans, Hispanics, Afro-Caribbeans, Native North American Indians, Asian Indians, and Chinese compared to Caucasian populations (6). The clinical manifestations of SLE are very heterogeneous with respect to cellular and physiological factors. Some of the manifestations observed are: antibody production against ds-DNA, nuclear proteins, butterfly rash, immune complex deposition in kidneys,

joint malformation. SLE displays phenotypic heterogeneity among patients and is reflected in the variable immunopathological manifestations of the disease. These diverse phenotypes can be attributed to different genetic contributions of susceptibility genes as well as environmental factors (7).

RA is another B cell-mediated autoimmune disease that primarily afflicts women. The incidence of RA is similar among Caucasians, African-Americans and Hispanics (5). The disease primarily targets the synovial membrane of diarthrodial joints. Historically, RA was considered T cell/macrophage-driven pathology, but successful use of B-cell targeted therapies to treat the disease implicate B cells as the main culprits in the pathology (8). RA is unique compared to other inflammatory and degenerative diseases due to the presence of autoantibodies. These autoantibodies recognize self-antigens that include Fc regions (rheumatoid factors), type II collagen, and cyclic citrullinated peptides. These antibodies are present in the serum of RA patients well before the onset of the disease (9).

Both SLE and RA have a high prevalence of auto-reactive B cells. To date, the factors that permit the presence of these auto-reactive B cells have not been fully elucidated. These factors are related to B cell tolerance processes that are active throughout B cell development. Normally, B cell tolerance mechanisms eliminate auto-reactive B cells as immature B cells differentiate into mature naïve B cells (1). The immature B cell stage begins with the expression of the B-cell receptor (BCR) on the surface of a B lineage committed cell. Negative selection mechanisms, a B cell tolerance process, operate via the BCR to eliminate self-reactive B cells (4). Hence, understanding the mechanisms that operate at the transitional stage of B cell development is important

for developing treatment strategies to treat B cell-mediated autoimmune diseases. To date, a human transitional B cell developmental model has not been characterized and studies of human transitional B cell development are few.

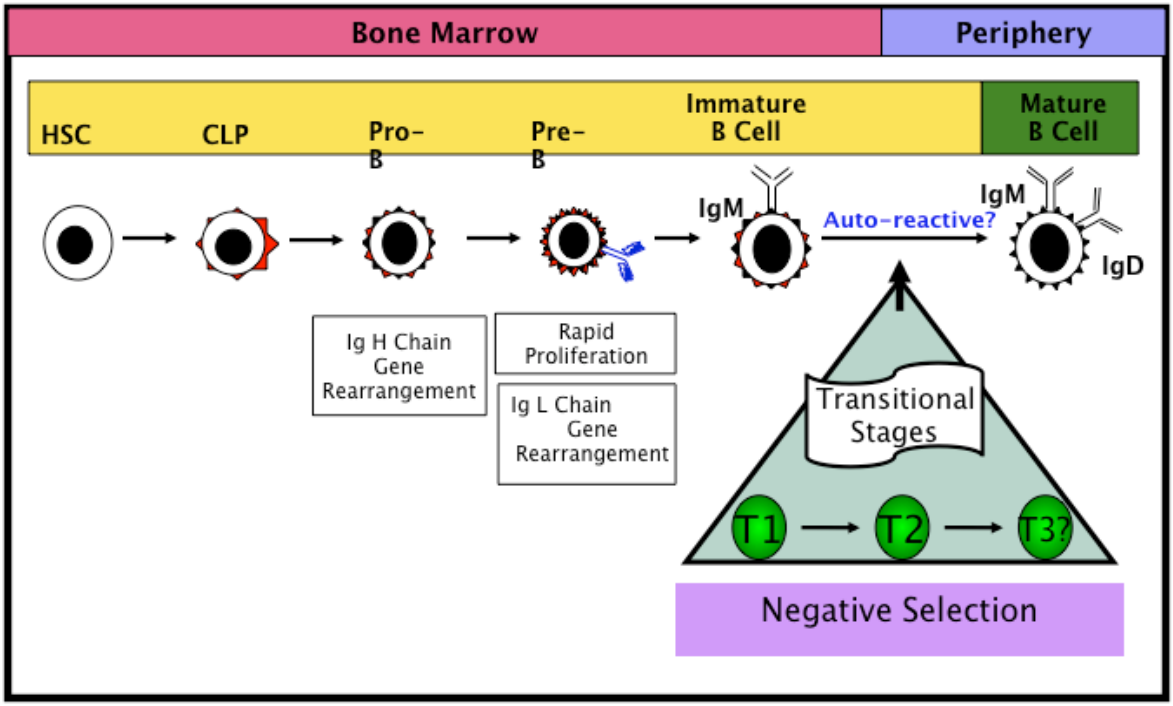

Figure 1. A model of human B cell development based on murine studies. The earliest B lineage committed cells are in the pro-B stage that is marked by heavy but not light chain immunoglobulin rearrangement of the future B cell receptor. Pro-B cells enter the pre-B stage as the immunoglobulin (Ig) heavy chain associates with the surrogate light chain producing a pre-BCR that facilitates rapid proliferation. The late pre-B stage is marked by loss of proliferation, reactivation of recombinatorial machinery with subsequent light chain rearrangement and expression. Pre-B cell entry into the immature B cell compartment is defined by the expression of fully formed B cell receptor (IgM) expression (10, 11). By the immature stage, B cells can recognize antigen through BCR. The immature stage is subdivided into transitional stages where negative selection processes function to eliminate auto-reactive B cells. Entry into the mature naïve B cell stage has traditionally been defined by expression of IgD, in addition to IgM, on the surface of the cell.

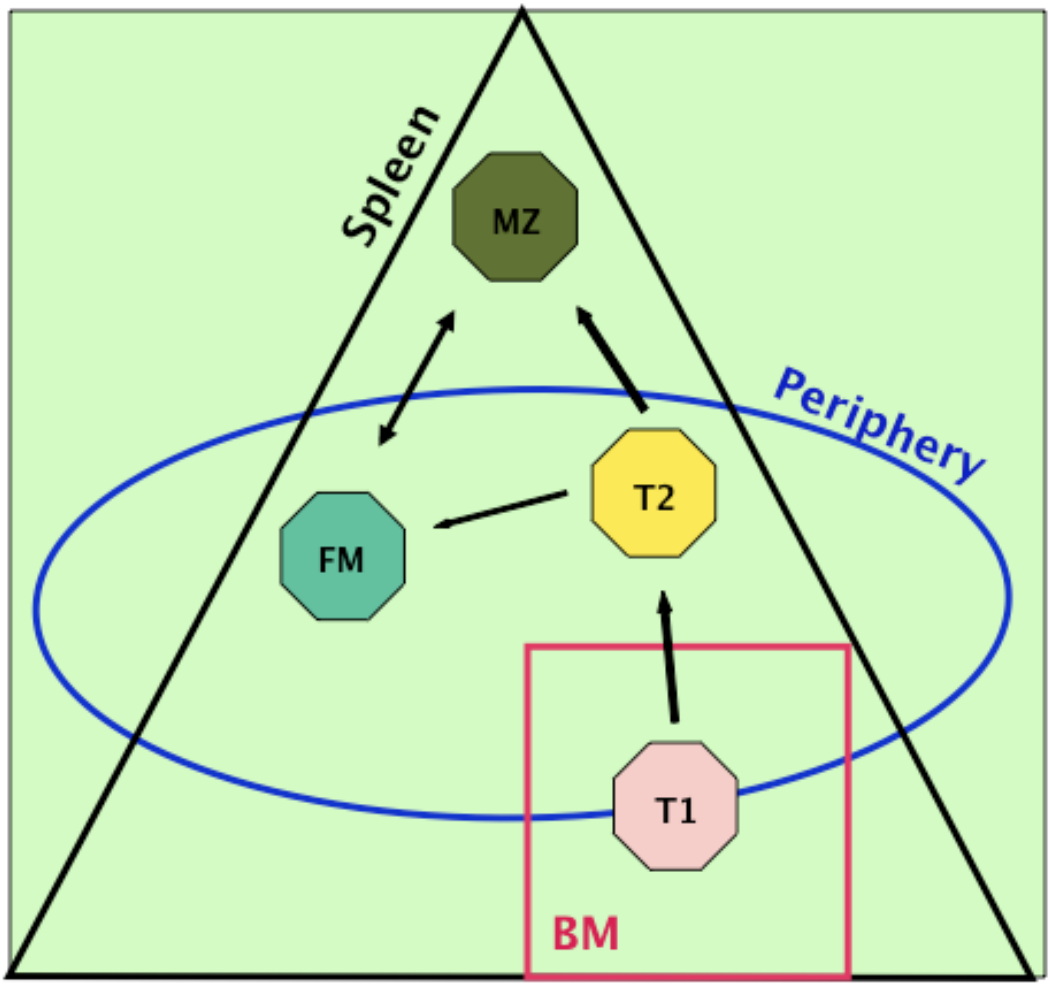

Figure 2. Distribution of developing B cells in hematopoietic tissues. Mouse B cell precursors become immature B cells in the BM and development continues as immature cells move into PB in transit to the spleen where they matured into either marginal zone (MZ) and follicular mature (FM). Based on surface marker expression, transitional B cells are delineated into developmentally sequential T1 and T2 subsets. T1 cells exit the BM into the periphery and develop into T2 cells when they reach the spleen. In the spleen T2 cells mature into MZ or FM (12, 13). Extrasplenic development of T2 into FM can occur in the periphery, but T2 cell maturation in the spleen remains the canonical pathway for B cell development (14).

B Cell Development

In adult mice, B cell development begins with multi-potential hematopoeitic stem cells (HSCs) that differentiate into B lineage committed cells in the bone endosteum of the BM. B lineage committed cells pass through a series of developmentally sequential stages that will give rise to immature B cells with a functional BCR on the surface (15, 16). Cell progression through these developmental stages will be dependent on proper recombination of immunoglobulin genes on the BCR (Figure 1) (16).. An immature B cell will leave the BM and travel to the spleen to mature into either a naïve marginal zone B cell (MZ) or a naïve follicular mature (FM) B cell (Figure 2). On their way to becoming mature B cells, immature B cells pass through several developmentally sequential "transitional B cell" stages (12). Murine studies suggest the presence of at least two functionally distinct transitional populations where negative selection processes operate (13). Transitional 1 or (T1) B cells are susceptible to apoptosis if their BCRs encounter activation/selection stimuli. Transitional 2 (T2) B cells can upregulate survival genes enabling them to better survive upon encounter with activation/selection stimuli (12, 17).

Early B Cell Development

HSCs are a self-renewing population that is constantly generating more blood cell progenitors. They will differentiate to become blood cell lineages which home to the venous sinusoids and there associate with a network of sessile stromal cells (16). Stromal cells play a role in regulating blood cell development via the secretion of different cytokines that influence progenitor cell growth, differentiation, and/or survival (16).

Blood progenitor fate decision is controlled by the activation of signaling pathways, the interplay of multiple transcription factors, and expression of cytokine receptors. B lineage cells are derived from the common lymphoid progenitor (CLP), characterized by the expression of IL-7 receptor and AA4.1 marker as well as increased expression of *RAG1/2*. In the mouse, stromal cell-derived IL-7 and its receptor are necessary for differentiation and maintenance of B lineage cells (16).

CLP commitment into the B lineage is dependent on various factors. First, the process consists of down-regulation of Inhibitor of DNA-binding (ID) proteins and up-regulation of *E2A* gene. Signaling via the IL-7 receptor (IL-7R) along with E2A proteins and transcription factor PU.1 control the expression of early B lymphocyte factor (EBF) (16). Expression of EBF along with Pax5 is critical for a progenitor's commitment into the B lineage. Pax5 suppresses the expression of non-B lineage genes in cell signaling, cell adhesion, cell migration, transcription, and cellular metabolism. In turn, Pax5 activates B lineage genes that include *mb-1* and *cd19* (16).

Once the CLP has become committed to B lineage the various subsequent stages can be defined by unique cell surface phenotypes that are associated with status of immunoglobulin (Ig) gene rearrangements (16). Ig genes will produce two types of Ig chains that will form the B cell receptor (BCR). Beginning from the CLP stage to pre-pro-B and to the pro-B cell stage, the Ig heavy chains (IgHC) will undergo rearrangement. If rearrangement is effective, the pro-B cell will progress into the more mature pre-B cell stage. Pre-B cells display IgμHC protein and the pre-BCR on the cell surface. During pre-B cell stage, the μHC proteins combine with the surrogate light chain

(SLC) proteins. After functional IgLC rearrangement on the pre-BCR, pre-B cells begin to express IgM and are called immature (16).

Mouse B Cell Transitional and Mature Identification Schemas

Recently emigrated immature B cells from the BM to the spleen were first termed "transitional B cells" by Allman et al. (14, 18) as these cells exhibited distinct phenotypic and functional characteristics from mature B cells. Transitional B cells have been subdivided into subpopulations termed transitional 1 (T1) and transitional 2 (T2) on the basis of surface phenotype and functional characteristics (Figure 4) (12, 13, 18, 19). While there is general agreement regarding the use of some markers to distinguish subsets of transitional B cells (IgD, IgM, CD21), different laboratories have used various combinations of markers to study transitional B cell development in the mouse (4, 17, 20). One problem with the use of IgD and IgM is that antibody binding to these molecules has the potential to mimic BCR engagement and therefore limits the use of these markers in isolating B cell subsets for subsequent functional studies (17).

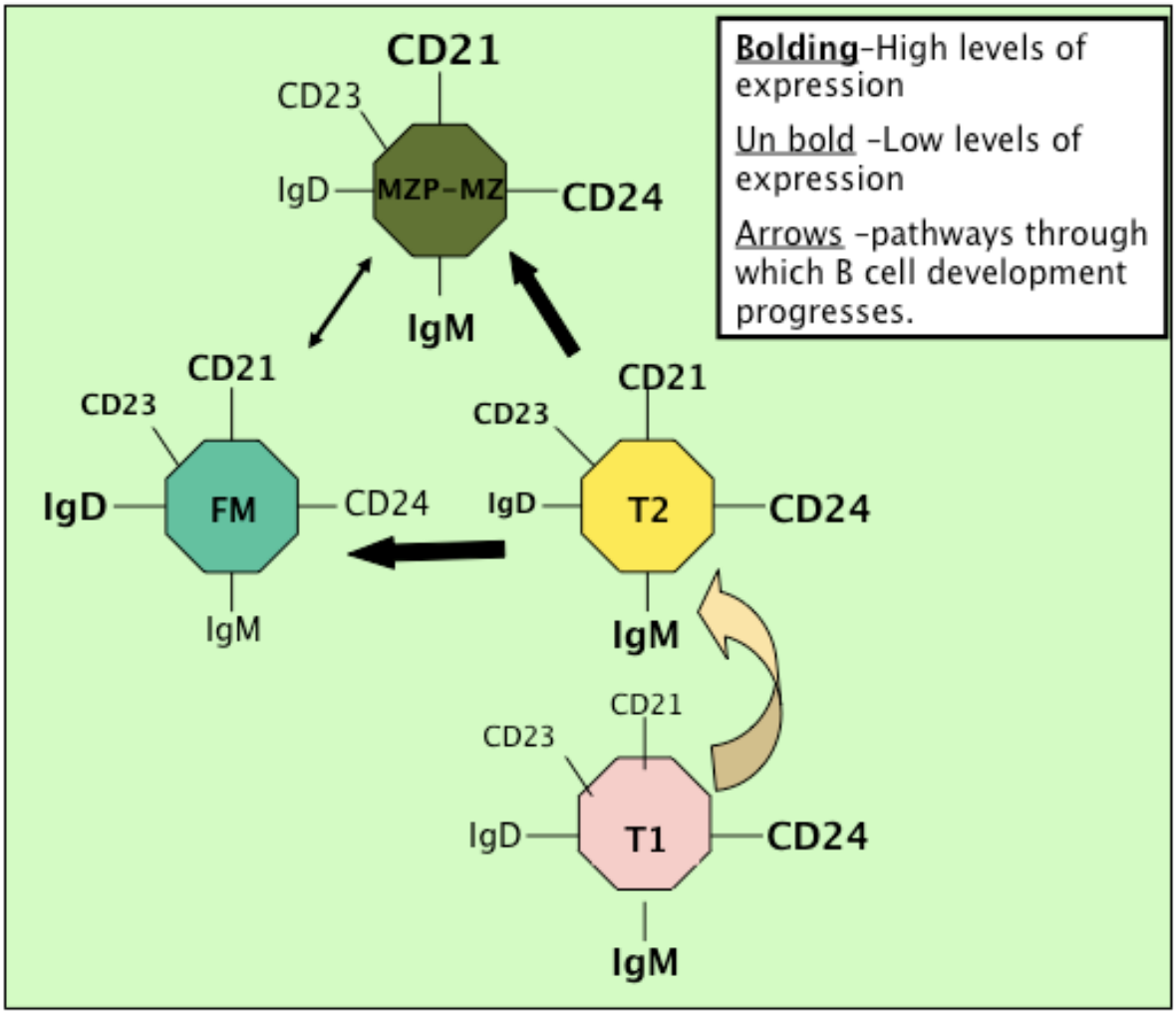

Figure 3. Schematic of murine transitional B cell development. Surface marker co-expression as shown has been used to identify developmentally sequential populations during murine transitional B cell development: transitional (T1 and T2) B cells, follicular mature (FM), and marginal zone B cells and their precursors (MZ) (14, 17). High levels of surface marker expression are indicated by bolding. Arrows indicate pathways through which B cell development progresses.

Mouse Transitional B cell Phenotype

Loder et al. (13) first delineated transitional subsets into two subsets, transitional 1 (T1) and transitional 2 (T2) in murine spleen using surface markers IgM, IgD, CD21, CD24, and CD23. However, identification of transitional B cells has been controversial and several developmental schemas have been proposed. The main differences between the various models of transitional B cells is 1) use or non-use of AA4.1 as marker to identify transitional and mature subsets, 2) the T2 population is more hetererogenous if AA4.1, CD23, IgD, and CD21 are used to identify transitional and mature subsets, 3) inconsistency in identification of transitional subsets results in conflicting data on what is the functional response to activation stimuli by transitional subsets (14).

The Carsetti group (13) first proposed that IgM, IgD, and CD21 could be used to distinguish developmentally sequential T1 ($IgM^{HI}IgD^{-}CD21^{LO}$), T2 ($IgM^{HI}IgD^{+}CD21^{HI}$) B cells and the mature follicular naïve (FM) ($IgM^{+}IgD^{+}CD21^{LO}$) and marginal zone (MZ) ($IgM^{HI}IgD^{LO}CD21^{HI}$) B cells (Figure 4) (13). Hence, T1 precede T2, and T2 give rise to FM and MZ with the FM population having the ability to give rise to MZ. Transitional B cell subsets inhabit different micro-environmental niches. T1 cells are not present in the splenic follicular region, but T2 cells migrate to the splenic B cell follicles and display BCR-induced proliferation. MZ B cells, a functionally distinct mature B cell population, is unique to the spleen and are located in the marginal zone around the germinal center (13).

Using developmental marker CD93 (AA4.1) along with CD23 expression, Allman's group (21) identified a T2-like subsets in BM and a third transitional subset, named T3. The use of AA4.1, CD23, IgM, IgD, and CD21 markers provided an identification of follicular B cells that could be precursors or naïve mature B cells (22,

23). Along with T3 B cells, Allman's group proposed that there are two follicular B cell subsets (FO I and FO II) (24, 25). FO I subsets is more mature and its phenotypically similar to T3 cells, but lacks developmental marker, AA4.1. FO II cells precede FO I cells in development as well as marginal zone precursor cells and are phenotypically similar to T2 cells, with the exception of AA4.1 (23). Furthermore, the incorporation of CD1d is used to identify MZP and MZ subsets in combination with high CD21 expression (25).

Recent studies by Rawlings group utilizing CD21 and CD24 co-expression on immature B cells have given further insight into functional differences between transitional subsets (4, 13, 26). These two marker combinations allowed identification of four B cell subsets in the spleen 1) T1 cells, 2) CD21-intermediate T2 cells (which we will refer to as T2 cells), 3) T2-MZ Precursor-MZ, a population of MZ B cells and MZ B cell precursors that we will refer to as MZ, and 4) FM B cells. Subsets showed differences in response to B cell activating factor (BAFF) due to differences in BAFF-R expression. Increased BAFF-R expression in late transitional B cells was correlated with upregulation of survival genes (4). These markers are particularly useful for functional studies because they allow for the identification and isolation of B cell subsets with using surface markers that are not known to induce BCR signaling upon binding of labeling antibody.

Functional Properties of Mouse Transitional B cells

Transitional B cell subset maturation occurs in different anatomical sites in the spleen and exhibit different functional responses when activated. T1 cells enter the spleen through the perioarteriolar lymphoid sheath (PALS) (27). T1 cells are the main

targets of negative selection (27, 28). Upon BCR-engagement, they undergo apoptosis possibly due low threshold for BCR activation and partially functional BCR-signaling components (29). T1 cells differentiation into T2 cells is mediated with the help of BAFF since BAFF-/- mice show a block in T1 maturation into T2 (29, 30).

Whereas T1 cells seem to be the primary targets of negative selection, T2 cells are the targets of positive selection (29). BCR-engagement leads to proliferation and upregulation of survival proteins such as Bcl-xL. Signals for survival and maturation are dependent on Bruton's tyrosine kinase (Btk) and PKCβ signaling (29). T2 differentiation into FM B-cell pool is Btk-dependent and PKCβ-independent. Consequently, while strong BCR stimulation of T2 cells leads to the FM fate, BCR stimulation and toll-like receptor (TLR) stimulation leads to an FM fate, but TLR stimulation only leads to an MZ fate (29).

The existence of T3 B cells remains controversial (21). T3 cells phenotypically resemble T2 cells with the exception of decreased levels of IgM. They are non-proliferating and some studies suggest that they represent the T2 cells that have been exposed to antigen and are being positively selected (24). Other studies indicate that this subset is composed of anergic cells that have constantly been maintained by the constant exposure to antigen, but remain unresponsive. This population also contains a high number of self-reactive B cells (28).

Another key difference within transitional B cell schemas is whether transitional B cell maturation progresses in a linear or a non-linear fashion. Carsetti's group proposed that transitional maturation occurs in sequential steps from one subset to another (31, 32). T1 cells differentiate into T2 cells that then give rise to marginal zone (MZ) or follicular

mature B cells (FM) (32). This schema primarily applies to transitional maturation in the spleen. On the other hand, Allman's group proposed that progression through the transitional stages of development was not a linear process (23). Using a developmental marker present in immature B cells, AA4.1, they evaluated transitional and mature subsets in BM and spleen. According to Allman's group studies, positive co-expression of AA4.1 and CD23 show the presence of T2 cells in BM as well as spleen. These T2-like cells can give rise to a more mature transitional subset T3 or FM. Furthermore, T1 cells have the capacity to bypass the T2 stage and differentiate into a T3 cell. FM cells can further be divided into two subsets based on IgM and IgD co-coexpression (14). Hence, transitional B cell maturation from T1 to T2 is not exclusive to the spleen. An additional transitional subset, T3, exists between the T2 and mature naïve subsets. The mature naïve subsets are characterized by lack of AA4.1 expression and CD23 and CD21 expression. They are subdivided into FO I, a population that depends on Btk-signaling and FO II cells that are long-lived and can give rise to MZP cells (26).

The greatest controversy in the transitional B cell identification lies in the identification and functional characteristics of T2 cells. Loder et al. (13) identified T2 cells as $CD21^{HI}$ and observed that these cells proliferate upon BCR engagement. On the other hand, other groups identify T2 cells based on markers CD23 and AA4.1 show that T2 cells do not proliferate upon BCR-engagement. The inconsistency between the two groups could be attributed to differences in what markers are used to isolate subsets thereby analyzing different cell populations. Recently, other groups using gating strategies similar to Carsetti's group, observed proliferation in the T2 population.

Furthermore, a difference between the schemas is the use of different levels of CD21 expression to identify transitional B cells. Carsetti's group noted that T2 cells expressed high levels of CD21 while Allman proposed T2 cells had lower levels (33). Other groups have proposed subdividing transitional and mature subsets based on levels of CD21 (34). This type of identification has been termed signal strength hypothesis where peripheral subset populations are divided into newly formed/T1 cell ($CD21^{LO}$) that gives rise to a T2-follicular precursor population ($CD21^{INT}$). The T2-FP population remains even in mice with mutations in the BCR-signaling pathway that lack FO B cells and in mice lacking MZ B cells due to mutations on Aiolos and Notch2 genes (26). Consequently, T2-FP cells are a precursor population to both follicular mature and transitional stage 2-MZ B cell precursor (T2-MZP). T2-FP cells that receive strong Btk-dependent BCR signals will differentiate into follicular mature cells. Alternately, T2-FP cells that receive weak BCR signals will be more susceptible to respond to Notch2 and NFκB1 signaling and differentiate into MZP cells that will home to the marginal zone and become MZ cells. MZ cells unlike MZP cells lack CD23 expression as well as have lower IgD levels (26).

Despite the unique precursor capacity of T2-FP cells, few studies have elucidated the functional properties of these cells. Meyer-Bahlburg et al. (4) evaluated the unique properties of this population under B cell lymphopenia and BAFF. They noted that this population had increased resistance to BCR-induced cell death, but also displayed robust proliferation to LPS via TLR signaling. CD21int-T2 population had lower levels of Notch2 compared to MZP cells. The unique properties of this population were observed under B cell lymphopenia whereby these cells proliferated and this proliferation was

dependent on antigen encounter and presence of BAFF. However, in B cell sufficient hosts CD21int-T2 cells proliferate little, although they still display bipotent differentiation into FM or MZP (4).

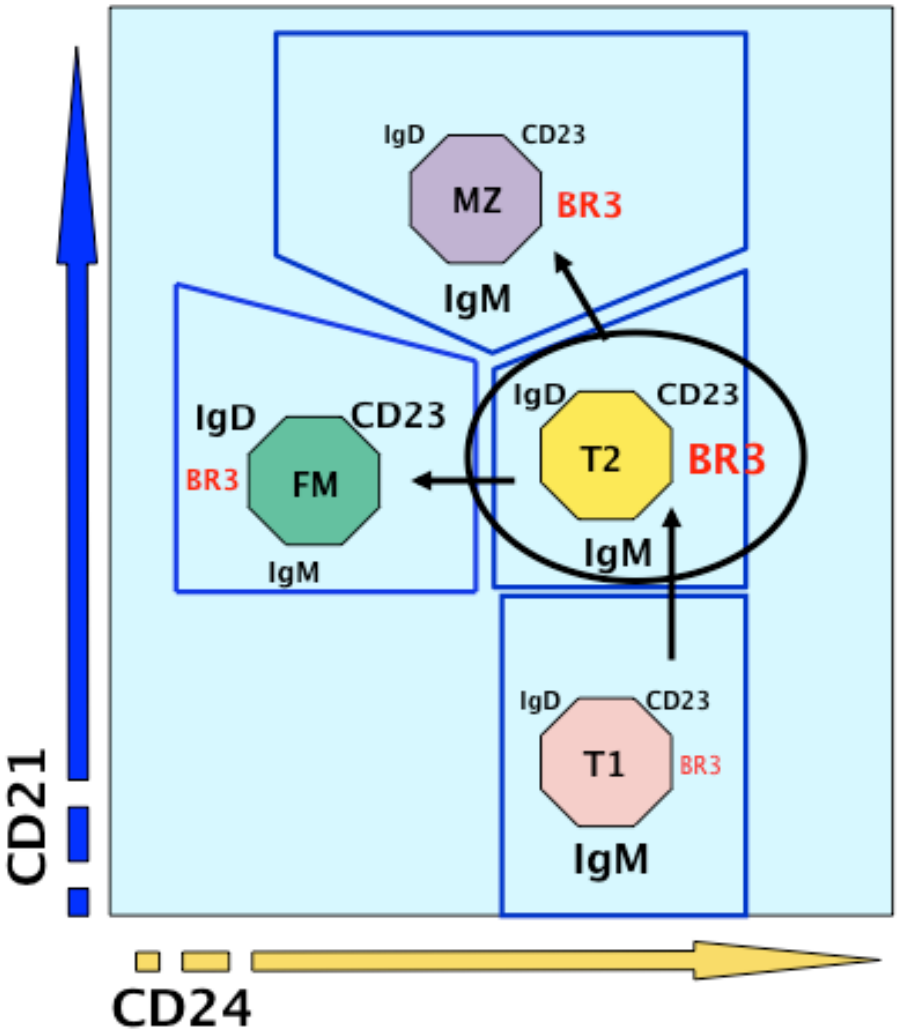

Figure 4. CD21 and CD24 co-expression in the murine spleen identifies a CD21-intermediate subset. Meyer-Balhburg et al. (4) used CD21 and CD24 co-expression to evaluate the functional characteristics of the CD21-intermediate population assigned as T2-intermediate in mouse spleen. The T2-int population proliferated in a lymphopenic host matured into the FM or MZ linage. They expressed the highest levels of BAFF-R expression compared to naïve mature cells or T1 cells. Furthermore, addition of BAFF into the lymphopenic host induced differentiation into MZ fate.

B Cell Tolerance

The presence of excess auto-reactive B cells, in B cell-mediated autoimmune disease, raise the question of when do auto-reactive B cells emerge during B cell development. Understanding B cell development under normal conditions provides clues to where potential aberrations may occur that lead to auto-reactivity. Mouse studies indicate that auto-reactive immature B cells are eliminated during the transitional stage of B cell development (12). At this stage, an immature B cell expressing surface IgM will exit the BM and transit toward the spleen where it will differentiate into a naïve mature B cell (12).

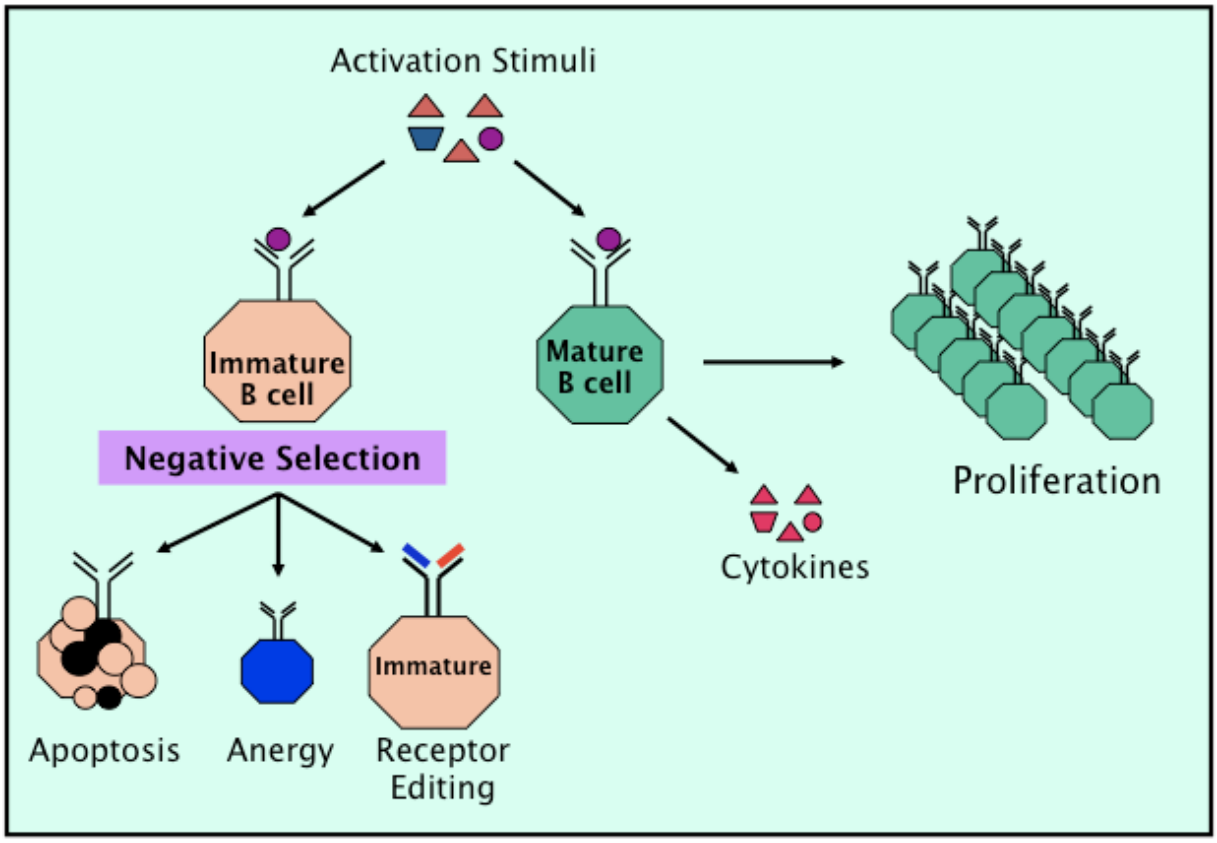

Figure 5. Differential Responses of Immature and Mature B Cells to Activation/Selection Stimuli. Unlike mature B cells, which proliferate in response to BCR engagement, immature B cells undergo negative selection following BCR interaction with ligand. These negative selection mechanisms operate to eliminate auto-reactive B cells. Depending on the strength of the antigen affinity for the BCR, an immature B cell may undergo apoptosis, anergy, or receptor editing. In receptor editing RAG-1 and RAG-2 give rise to a new light chain rearrangement resulting in a new BCR that will hopefully not be autoreactive.

Selection processes primarily function via the BCR. While mature B cells are activated and proliferate in response to BCR engagement, immature B cells will undergo negative selection processes upon BCR engagement (Figure 6). Studies in the mouse model indicate that negative selection processes primarily function at the T1 stage to remove auto-reactive B cells through three negative selection mechanisms: 1) receptor editing, 2) deletion of immature B cells (apoptosis), or 3) induction of an unresponsive state (anergy) (9, 12, 35, 36).

Regulation of auto-reactive B cells begins in the BM. Immature B cells with auto-reactive B cell receptors will undergo receptor editing (36, 37). This process is mediated by the expression of RAG genes and results in rearrangement of the light chain on the BCR. If receptor editing fails to rearrange the light chain into a non-auto-reactive one, the cell will undergo apoptosis and/or undergo anergy (36, 38, 39).

Receptor editing allows for the rearrangement of a new light chain on the BCR. When the BCR on an immature B cell is moderately activated by endogenous antigen (activation stimuli), negative selection processes will promote receptor editing to reduce the BCR's self-reactivity (39). During early B cell development, recombinatorial genes RAG1 and 2 mediate BCR arrangement. In receptor editing, re-expression or continued expression of RAG genes allows the generation a new BCR with different antigen specificity and hopefully reduced auto-reactivity (37). BCR light chain rearrangement rather than directed apoptosis upon BCR engagement in immature B cells seems to occur inherently in the bone marrow (39). Many of the BCRs display auto-reactivity during the immature stage, and receptor editing is the normal process that reduces much of this auto-reactivity among B cells (16, 40). Furthermore, in studies where Bcl-xL, a survival

protein, was over-expressed in BCR transgenic mice, auto-reactive B cells undergo receptor editing or anergy rather than deletion (39). Under normal B cell development, Bcl-xL expression is upregulated during the late transitional stages in T2 cells (17, 19).

Once receptor editing has occurred, B cells that remain self-reactive will undergo further negative selection depending on the strength of BCR stimulation. These auto-reactive cells will either be deleted by apoptosis or induced into an anergic state (35, 41). Hence, B cells expressing a BCR reactive to endogenous proteins would undergo apoptosis after leaving the protective microenvironment in the BM (16, 42). Apoptosis of negatively selected auto-reactive B cells has been attributed for the large loss of immature B cells during B cell development (43). However, immature B cells may bypass both receptor editing and apoptosis and become activated to respond to a continued immune response (36).

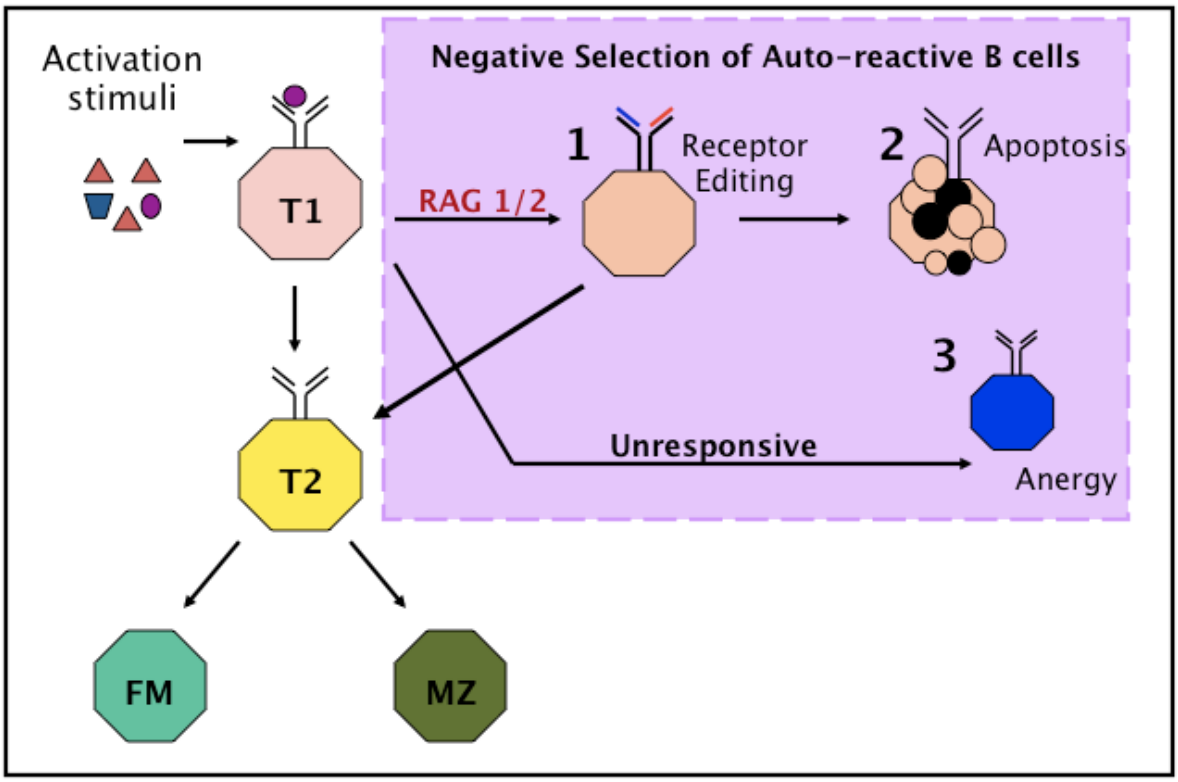

Figure 6. **Auto-reactive immature B cells undergo selection mechanisms to eliminate auto-reactive BCRs.** In the BM, immature or T1 cells may be directed to three fates in the case of self-antigen exposure. A T1 cell can rearrange its BCR due to the expression of *RAG1* and *RAG2* genes. These genes allow the rearrangement of the light chain of the BCR thereby producing a new BCR. If the rearrangement produces a more auto-reactive BCR, the cell will undergo apoptosis. A third fate is anergy. Anergy results in an unresponsive B cell. The BCR is functional, but the BCR signaling cannot activate the B cell (36, 44).

B Cell Peripheral Tolerance

In vitro and *in vivo* murine studies show that negative selection of auto-reactive B cells occurs during transitional stages of B cell development (36). Once they exit into the periphery, T1 cells that encounter antigen and become activated will undergo apoptosis (17, 45). On the other hand, T2 cells that encounter antigen may undergo apoptosis or survive depending on the availability of growth factors and T cell-produced cytokines(16, 17, 46, 47). The difference in survival between the two transitional subsets is due to the increased BCR-mediated signal transduction and expression of receptors that respond to growth factors on T2 B cells (4, 48). One of these factors is BAFF whose binding to the BAFF receptor on T2 cells propagates upregulation of survival proteins, Bcl-2 and Bcl-xL. Furthermore, T2 cells respond to IL-4, a T-cell cytokine (16, 49). While activation promotes negative selection in T1 cells, activation of T2 cells in the presence of growth factors promotes proliferation (17).

Mature splenic B cells are comprised of two major populations. The follicular (FM) naïve B cells reside in the primary follicles and primarily interact with T-dependent antigens (17, 22). The second and smaller population, marginal zone (MZ) B cells are involved with blood-borne, T-independent responses (17). Mouse studies indicate that signaling via the BCR results in selection into one or the other population. Weak signaling via BCR results in MZ fate while a strong BCR signaling results in a FM B cell fate (17, 20).

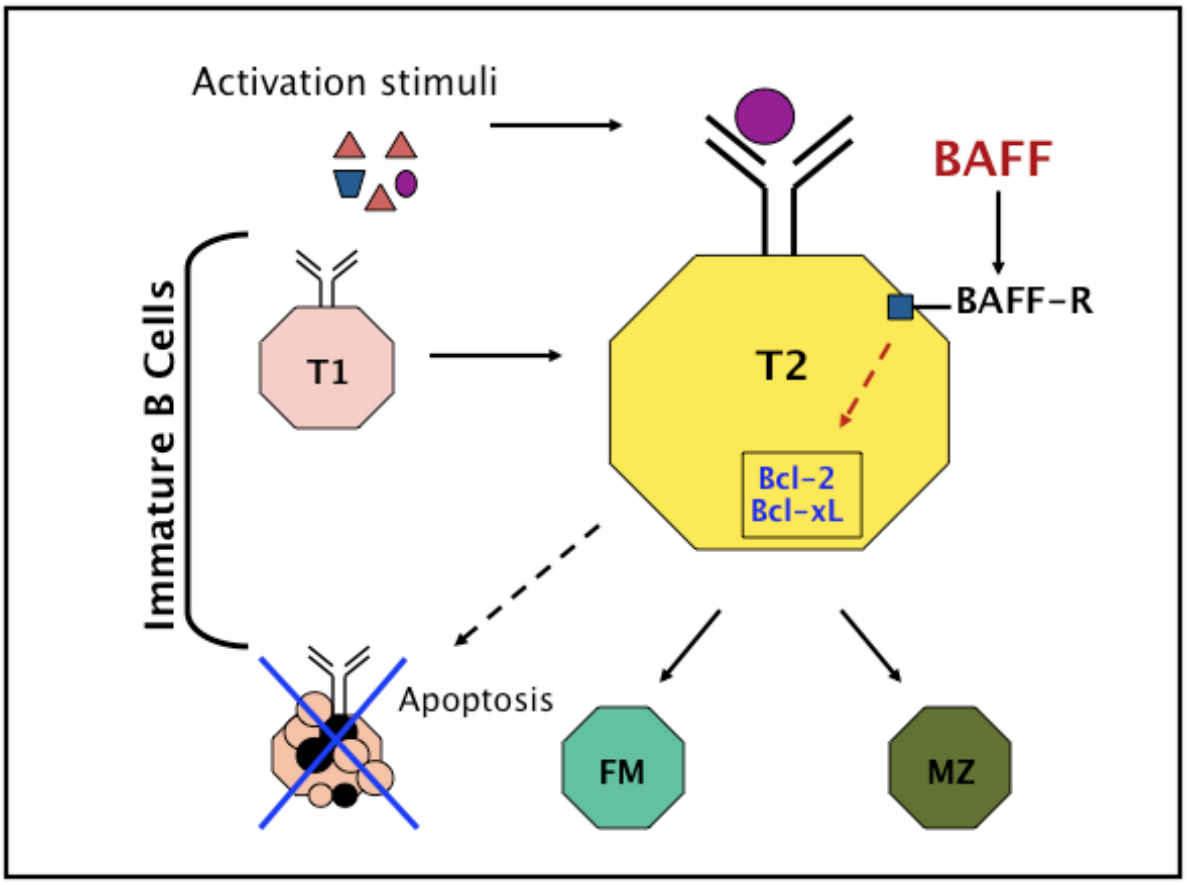

Figure 7. **T2 cells can survive under the presence of BAFF.** Unlike T1 cells, T2 cells express BAFF-R. BAFF-R expression allows them to respond to BAFF, a survival cytokine. Binding of BAFF to its receptor results in the upregulation of Bcl-2 and Bcl-xL. In the absence of BAFF, T2 cells cannot differentiate into FM or MZ. Thus, BAFF is essential for late B cell development and B cell maturation. Alternately, excess BAFF allows survival of auto-reactive T2 cells that would have been unable to compete with normal T2 cells. Consequently, under excess BAFF, auto-reactive B cells differentiate into mature naïve auto-reactive B cells (17, 50).

Auto-reactive immature B cells may arise when negative selection mechanisms fail and these cells then mature into T2 and subsequently FM cells (4, 35). Negative selection is a process that is still poorly understood and why it fails in autoimmune disease (51, 52). Selection mechanisms may be multi-factorial and evaluating each unique transitional subset under these factors is crucial for understanding how auto-reactive B cells bypass elimination (3, 53-56). However, one problem in understanding transitional B cell selection has been the discrepancy between identification schemas for transitional B cells.

Functional data from different groups have been conflicting resulting in confusion over T2 cells and how they give rise to FM cells (4, 14, 26). Consequently, to begin understanding why auto-reactive B cells emerge, we must first develop a consistent schema for identifying transitional B cells; develop a method to translate mouse studies into human; and apply this method to human studies in order to elucidate similarities and differences in mouse and human transitional B cell development.

Human Transitional B Cell Identification

Previous studies of human transitional B cells utilized markers that came from clinical studies of human disease and do not incorporate the developmental perspective that could be provided by translational data obtained in the mouse model (33, 57-59). Markers to study the development of human transitional B cells are poorly defined and a correlation with markers characteristic of transitional B cell development in the mouse has not been established.

The Carsetti group (33) utilized CD24 and CD38 to study immature human B cells. The co-expression pattern of CD24 and CD38 on B cells identified memory, mature, and immature transitional B cell populations in PB. Further analysis of CD27 expression distinguished between mature naïve B cells (CD27-) from memory B cells (CD27+). To confirm the uniqueness of the subsets identified in PB, bone marrow was analyzed with the same marker combinations and showed comparable memory, mature, and immature transitional subsets (33).

A notable difference between PB and BM was that the transitional B cell population (identified by high levels of CD24 and CD38) made up a greater fraction of BM B cells (33). Carsetti's group suggested that the transitional population identified by high levels of CD24 and CD38 was indeed composed of transitional B cells as further analysis of this population showed it expressed other markers ascribed to murine transitional B cells such as IgM, IgD, CD21 and CD23 (33, 60). Hence, based on surface phenotype and tissue distribution, human transitional B cells displayed similarities to murine T1 and T2 B cells (33, 61) incorporated both Carsetti's human transitional schema and evaluated ABCB1 transporter function in developing B cells. ABCB1 transporter expression is indicative of maturation status–ABCB1 transporters are expressed in mature cells that excrete xenobiotic compounds such as tracer dye from the cell while a transitional B cells show a lack of ABCB1 transporter (62). Thus high levels of CD38 expression and an absence ABCB1 transporter are useful for defining human transitional B cells (61, 63), however the correlation of these markers with the mouse model is not yet known.

Even though human transitional B cells showed similar phenotypes to murine counterparts with respect to IgM, IgD, CD21, and CD23, there is a marked difference in the expression of CD5 in human transitional B cells (58, 63). In mice, CD5 expression is used to identify a functionally distinct population of B cells (B1a cells) (64). This does not appear to be the case in humans (33, 65, 66). In humans, CD5 is expressed on the majority of B cells from cord blood and peripheral blood from young children as well as on newly formed B cells after human stem cell transplantation (58, 67). In humans, CD5 expression in adult B cells is low and transient and its presence may be a marker of antigen exposure (68) as well an identifying marker of the transitional stage rather than a murine B1a human counterpart (33, 58). CD5 expression is reported to be absent in immature cells in the BM, but is present on B cells in the periphery (57, 61).

Human Transitional B Cell Subset Phenotypes

Human transitional B cells are less functionally defined compared to mouse transitional cells. Human studies on B cell subsets rely on different sets of identification markers depending on the tissue being studied. In the past, human immature B cells were identified using developmentally regulated markers that are highly expressed in the BM. Mature, memory, and germinal center subsets have been identified using IgD and CD38 co-expression (59). A marker or even uniform set of markers was lacking to identify immature and naive mature subsets. The presence of a high percentage of memory B cells compared to mouse added another dilemma in developing an identification schema for human subsets (69). Furthermore, many of the markers that are used to identify putative transitional B cells are also upregulated upon B cell activation.

Transitional B cells in mouse tissues have utilized early B cell markers to delineate between immature and mature subsets. Carsetti's group (33) used early developmental markers CD24 and CD38. In the BM, immature B cells express high levels of CD24 and CD38. Cells with this phenotype were observed in peripheral blood along with a subset with intermediate expression of both markers. To distinguish between immature and mature, CD10 was utilized as the marker is only expressed in immature B cells. Hence, high expression of CD24 and CD38 indicated an immature/transitional subset; intermediate levels indicated naive mature subsets as they lacked CD10; and low levels of CD38 is indicative of memory B cells as some were positive for memory marker CD27 (33). Identification of transitional B cells using CD24/CD38 co-expression was further supported by the work by Jacquot's group (57). Upon evaluating mouse transitional models from Carsetti's and Allman's work, Jacquot's group assessed transitional and mature populations for expression of CD21, CD23, IgM, IgD, and BAFF-R which are used to identify transitional subsets in mice. In addition, CD5 and CD10 were included in determining marker expression profiles of human transitional and mature subsets in normal and post-hematopoietic stem cell transplantation (57). Their results showed that transitional B cells express CD5, while mature cells did not. CD10 was expressed in transitional B cells, but this expression decreased as cells lost CD24/CD38 expression. Markers BAFF-R, CD23, IgM, and IgD expression was not consistent across tissues, but CD21 displayed the most consistency (57).

Markers IgD and CD38 have traditionally been used to identify B cell subsets in spleen and tonsils. Lipsky's group (58) used the co-expression of these markers to delineate immature from mature subsets in peripheral blood. IgD expression increases

with maturation, while CD38 decreases. Cells positive for IgD expression and expressing high levels of CD38 were assigned as T1 cells. These T1 cells were high in CB and SLE PB (58). A second population assigned as "intermediate" due to its intermediate expression of CD38 was observed in CB and PB, but was mainly absent from BM. This intermediate population had higher levels of CD10 and CD24, but displayed similar levels of CD21 compared to naive mature. Intermediate cells resemble mouse T2 cells with respect to CD23 and IgD expression. A notable finding this group made was the expression of CD5 on the T1 and Intermediate populations in PB. The expression of CD5 is associated with activated B cells in humans and in mice it identifies a separate B lineage population termed B1 cells. Consequently, a major difference between mouse and human transitional subsets is the expression of CD5 in human cells (58, 61, 63).

Transitional B cell subsets display functional differences in response to BCR ligation with cognate antigen. Consequently, it's important to select the appropriate markers with the most discriminatory power for identifying human transitional B cell subsets. Lipsky's group (58) studies further elucidated on the unique functional properties of human transitional B cells. Their results showed that T1 cells survive best with IL-4 or anti-CD40 rather than BAFF. When T1 cells were activated with anti-IgM or anti-IgM/BAFF stimuli, no cell cycling occurred and cells died by apoptosis. When T1 cells were stimulated with IL4/anti-IgM or anti-CD40/anti-IgM, cells entered cell cycle, but to a lesser extent compared to mature B cells. A unique aspect to the cell death observed in T1 cells was despite positive staining for Annexin V, the cells died via necrosis rather than via a caspase-dependent pathway (58). The capacity for T1 cells to survive in the presence of T cell help may be attributed to the expression of CD5 on

human cells. IL-4 may down-regulate CD5 expression consequently rendering BCR signaling more sensitive to stimuli. Hence, transitional B cells are inhibited from responding to BCR stimulation via expression of CD5. When CD5 is down-regulated, immature cells may be induced to proliferate and differentiate into naive B cells (58).

Two groups have suggested the presence of a late transitional population or a pre-naive B cell population. Lipsky's group (63) suggested the presence of a pre-naive B cell population in PB. Hence, CD5 is expressed in human T1 cells as well as pre-naive B cells, but not in naive mature subsets. These cells display an intermediate phenotype and functional properties between transitional and mature naive B cells. Like naive B cells, pre-naive B cells have the capacity to present antigen and differentiate into plasma cells (63). Like transitional B cells, they undergo apoptosis upon sIgM and CD40L ligation. They are functionally intermediate to transitional and mature, because they do not respond as well to BAFF as naive even though BAFF-R is expressed. This lack of responsiveness to BAFF differs to mouse data on T2 cells where BAFF is absolutely necessary for progression and maintenance into the T2 stage (63). More importantly, the maturation of human T1 cells into a more mature transitional stage in PB suggests alternate sites for maturation occur in human. In contrast, transitional B cells in the mouse reside primarily in the spleen and splenic factors are necessary mediators for transitional maturation into naive B cells (12, 14, 17, 70).

The use of rhodamine123 dye (R123) has become a useful tool for discerning between late transitional and naive mature B cells. Transitional B cells are immature and don't have functional ABCB1 transporters. ABCB1 transporters are responsible for extruding fluid from the cell (62). When immature B cells are exposed to R123 dye, the

cells are unable to extrude it and will be positive for the dye. ABCB1 transporters are only found in human cells and cannot be used to assess immaturity status in mice (62). Using CD24 and CD38 co-expression rather than IgD, CD38, and CD5, Palaniachamy et al, (61) identified a T1, T2 and a late transitional population named T3. The most immature transitional B cells, T1, expressed the highest levels of CD24 and CD38. Alternately, T2 cells and late transitional B cells displayed intermediate to low levels of these markers. T1 and T2 had reduced levels of calcium signaling (61), but showed a trend toward increasing levels of CD21 expression similar to mouse transitional B cells also described in other studies (71). The late transitional population showed a similar phenotype to naive mature B cells with respect to other maturation markers such as IgM, CD23, BAFF-R, IgD, but were unable to extrude dye.

Recently, the Tangye group (71) proposed that human transitional CD24/CD38 gated subset could be divided into a CD21 low and CD21 high expressing subsets. CD10 positive cells were gated prior to delineation into transitional populations subpopulations, CD21lo and CD21hi populations. Assessment of PB from hematopoietic stem cell transplantation (HSCT) patients indicated that CD21lo cells are the first subset present in the periphery (71). Hence, CD21lo cells may be a precursor population to CD21hi cells. Furthermore, CD21lo cells are enriched for auto-reactive B cells and this suggests that they are early BM emigrants. CD21lo or CD21hi cells do not proliferate as well as mature naïve B cells, and do not seem to correspond to T3 or pre-naïve B cell populations. The CD21lo population is the most abundant population in the X-linked agammaglobulinemia (XLA) patients, suggesting that CD21lo progression into CD21hi is dependent on Btk-derived signals (71). CD21lo and CD21hi populations were found

heterogeneously in the CD24/CD38 transitional gate in normal PB. When they assessed for CD21 subsets in the CD24/CD38 transitional gate in HSCT blood, only the CD21lo subset was found. They suggested this discrepancy was due to CD24 and CD38 marker expression reflecting time of generation and export from the BM (71).

Although human studies in CB and PB have shown the presence of immature "transitional"-like cells, a generally accepted schema remains to be developed as various groups used different markers to identify these subsets. One important finding in human transitional B cells was the finding that not only BCR signaling was important in maturation, but also TLR9 (60). Normally, TLR9 signaling induces memory B cells to differentiate into plasma cells. CpG, ligand for TLR9, can prolong naive B cell survival. In humans, transitional B cell development into naive mature B cells is mediated by BCR signaling. Yet, CpG stimulation of transitional B cells as identified using CD24 and CD38 co-expression, produces IgM memory-like B cells (60). IgM memory B cells are normally generated in the spleen and are possibly generated from a different B cell lineage (72, 73). IgM memory B cells are present in developing spleen by the age of 2 years, but are absent from the lymph nodes (74-78). Furthermore, AID is unregulated in transitional B cells upon CpG stimulation. Hence, transitional B cells can be pushed to differentiate into IgM memory cells in the presence of CpG suggesting transitional B cells can mature into naive via BCR-dependent and BCR-independent signaling (60).

T Cell Help and BAFF

In the mouse, BCR signaling is important for the survival and continued developmental progression of transitional B cells, as many signal transduction proteins in B cell development are associated with the BCR (16, 17). Extrinsic factors can decrease

negative selection as T2 cells can be rescued with co-stimulatory signals from T-cell help (CD40L) and BAFF (1). Transitional B cells vary in sensitivity to T-cell signals as T2 cells respond and survive better than T1 B cells (12). In the presence of T-cell help, T1 and T2 B cells that are pro-apoptotic can survive and proliferate. Furthermore, the capacity to respond to T-cell-derived signals by immature/transitional B cells may be correlated to access these cells have to peripheral compartments containing antigen and activated T cells (16, 75).

The TNF-family protein BAFF has been implicated in promoting B cell survival. Response to BAFF binding to BAFF-R augments as B cells progress through the various splenic transitional stages (46, 48, 79, 80). BAFF promotes survival via signaling through the BAFF-R by increasing the expression of Bcl-2, Bcl-xL, and possibly up-regulating CD21 and CD23 expression (81, 82). BAFF mediates responses only in T2 and mature B cells as its binding affects BCR signaling and CD23 (81, 83). BAFF-R levels are increased upon anti-BCR stimulation of FM mature and T2 B cells (53). Impaired BAFF-R signaling causes almost complete loss of T2 and mature B cell compartments despite functional BAFF-R signaling components (48, 53).

As seen in murine T1 cells, functional studies showed that putative human T1 cells die in culture after anti-IgM stimulation (58). Mouse and putative human T1 cells express lower BAFF-R compared to more developmentally mature cells and as a consequence BAFF does not rescue mouse or human cells with a T1 phenotype from cell death after anti-IgM stimulation (17, 58).

B Cell Maturation in the Spleen

Mouse splenic microenvironment provides the necessary growth factors to facilitate the development of transitional B cells into mature subsets (17). Mouse splenic subsets include T1, T2, FM, and MZ cells. Mouse studies indicate that the spleen is the only site where MZ B cells will develop and reside. MZ B cells are involved in innate immune responses and can be activated by non-specific stimuli to kill pathogens. T1 to T2 maturation occurs in the PALS region of the spleen, but development into FM and MZ B cells occurs in different splenic sites (12, 17).

FM cells reside in the primary follicles. These cells are the conventional naïve B cells that circulate throughout the body. They respond to antigen stimulation from helper T cells making them T-dependent and have highly specific BCRs. They participate in germinal center (GC) reactions, proliferate, and undergo somatic hypermutation (SHM), thus increasing their BCR specificity (16, 17, 84). MZ B cells function as a first-line of defense against encapsulated blood-borne pathogens. They serve as connectors between the innate and the adaptive immune system. MZ B cells can become activated without T cell help and can respond to T-independent (TI) antigens such as LPS or capsular polysaccharides from encapsulated bacteria (26, 77, 85).

The splenic MZ is organized to be the first line of defense for the organism (26). Blood flow to the MZ is very reduced facilitating interaction between antigen and effector cells such as MZ B cells or macrophages. MZ B cells are normally pre-activated and have BCRs with a broad range of specificities, but with lower affinity. Thus, MZ B cells have lower BCR activation thresholds so that they may respond to lower levels of antigen (26). In order to respond better to TI-2 antigens, MZ B cells express CD21, which facilitates binding to complement fragment C3d at a much lower activation

threshold (75, 77). While mouse MZ is composed primarily of naïve B cells, the human MZ contains a large number of memory B cells as identified with CD27 and CD148 (77). Moreover, studies of human PB and tonsil, suggest that MZ-like cells circulate out of the spleen (75). A phenomenon not observed in rodents.

Several models for MZ or FM B cell commitment have been proposed. The BCR signal strength hypothesis suggests that based on the specificity of the BCR, BAFF, and constitutive Btk-independent baseline signals, the late transitional B cell will choose an FM or MZ fate. When a T2-FP cell responds well to self-Ag, it will become an FM cell. If a T2-FP cell responds poorly to self-Ag, it will become an MZ cell with the help of BAFF (16).

Marginal zone B cells have a unique phenotype that reflects their unique function within the B cell pool. A key function of marginal zone B cells is their capacity to rapidly respond to antigen and produce large amounts of IgM (86). To facilitate this process, studies suggests that MZ B cells express high levels of IgM and CD21. The high surface IgM enables high cross-linking of surface IgM (sIgM) upon activation by Type I- 2 antigen from encapsulated bacteria (73, 75, 87). TI antigens tend to be polyclonal activators where a single antigen contains several overlapping parts that can be individually recognized by low affinity BCRs (86).

CD21 Expression and Function in the Spleen

CD21 serves a complement-binding subunit after antigen binds to BCR and also directly associates with CD19. CD19 functions to lower the amount of sIgM needed to activate PLCγ to induce Ca2+ mobilization. When CD21 binds to the C3d degradation

fragment of activated C3, studies suggest it provides a recognition site for CD19. The formation of the trimolecular complex CD19/CD21/TAPA couples the capacity of CD21 to recognize antigen by complement to the signaling function of CD19 (88). Hence, CD21/CD19 complex promotes signaling through BCR and enhances response by 10-1000 fold. CD21 is thus a necessary component in MZ B cells to induce a heightened BCR signaling in response to small amount of antigen. Essentially, CD21 serves as a bridge in MZ B cells between innate and acquired immune systems.

In humans, MZ B cells are identified based on high expression of CD21, IgM, CD27, and CD1c. CD27 and CD1c are human specific markers. CD27 is up-regulated on human memory B cells. CD1c molecules are nonpolymorphic MHC class I molecules that can anchor lipid antigens (75). Co-expression of both CD27 and CD1c is considered unique to human MZ lineage cells, but has been reported in IgM+ cells in peripheral blood and tonsil. A key difference when compared to splenic MZ cells is the decreased expression of CD21 in peripheral MZ-like cells (73). MZ-like cells in the organs other than the spleen is still controversial as some propose that MZ cells migrate out of the spleen. In contrast, MZ B cells in mice are sessile staying in the spleen and are mainly composed of naïve MZ B cells (26).

Human Memory B cells

Memory B cells are an effective method for quick protection against common infections. They display unique characteristic such as prolonged lifespan, decreased activation thresholds enabling more rapid activation when compared to naïve B cells, and capacity to stimulate T cells. Traditionally, memory cells were characterized as having been derived in a T-dependent germinal center reaction (69). Thus, they became isotype-

switched and had highly mutated antibodies with even greater specificity then naïve B cells. These mutated antibodies had undergone SHM through the induction of activation-induced deaminase (AID) (87). B cells that were un-switched or had retained the IgM isotype and displayed un-mutated antibodies were excluded as true memory cells (69).

Memory B Cell Subsets are Heterogeneous

New emerging phenotypic data indicates that the memory B cell pool is far more heterogeneous than previously thought. Memory B cells can be generated via GC-dependent and GC-independent mechanisms (59). Using CD27 as a defining marker of human memory B cells, human B cell pool contains a memory B cell frequency of 40-60% when compared to mice (69, 89). This high frequency of memory B cells in human may be due to a longer lifespan in humans. Furthermore, analysis of the human CD27+ memory B cell compartment indicates that less than half of the cells have undergone isotype switch. Thus, half of the CD27+ cells express surface IgM and may express low levels of IgD (69).

Classification of human memory B cells was based on the expression of four surface markers: CD19, IgD, CD38, and CD27 (59). The co-expression of IgD/CD38 was used for the Bm1-Bm5 identification scheme used to identify B cell subsets in spleen and tonsils (59, 69). These subsets included: virgin naïve B cells, activated naïve B cells, pre-GC cells, GC cells, and memory cells. The Bm identification scheme was applied for studying PB and all subsets were recognized except GC cells (69).

A problem with using a classification system based on IgD/CD27 is that not all memory cells express CD27 (72). CD27 is useful in distinguishing memory from both naïve and transitional subsets (IgD+CD27-) (69). However, some memory cells lack

expression of CD27 (90). CD27- memory B cells were not recognized until their presence was observed in SLE patients. Studies showed that in SLE there was an expansion of cells that lacked CD27 and IgD expression (90, 91). These cells have recently been observed in PB and tonsils. Identification of these cells relied on evaluating isotype switch as well as positive staining for rhodamine123 dye. Like CD27+ cells, these CD27- cells expressed the isotypes, IgM, IgG, and IgA (91). These cells were unable to extrude the rhodamine123 dye and could not proliferate when stimulated with CpG DNA (69). Other characteristics still remain to be determined such as where these CD27- cells originate from and if they have the capacity to differentiate into plasma cells (69).

Human Marginal Zone and Memory Cells

The anatomical location of human memory cells is located in two inter-related areas: the marginal zone and the sub/intra-epithelial surfaces. Although the marginal zone is mainly considered a developmental unit, it is the site of crucial functional characteristics of marginal zone B cells (78, 92). Phenotypically, MZ B cells can be identified based on CD27 expression and high levels of CD1c and are also $CD21^{HI}$CD23- (73). These pre-activated MZ B cells are involved in T-independent responses that quickly generate short-lived IgM-secreting plasmablasts. Furthermore, MZ B cells have higher sensitivity to stimulation from TLR ligands and display a pre-selected repertoire of B cell receptors that can be self-reactive or react to bacterial pathogens (75). These characteristics allow them to act as a first line of defense against blood-borne infections. Thus, based on the pre-activated state and specific BCR repertoire, MZ B cells may represent innate immunity or a evolutionarily selected “memory” (69).

Mouse and humans have different MZ compartments. In humans, the marginal sinus is absent as well as metallophilic macrophages and has a larger perifollicular area (78). The human marginal zone develops during the early childhood and may be the reason why an infant's immune system fails to respond to T-independent antigens making them susceptible to infections by encapsulated bacteria (33, 69). In the absence of a spleen, circulating memory IgM B cells will be absent resulting in high susceptibility in these patients to infections from encapsulated bacteria such as *Streptococcus pneumoniae* (69).

Memory B cells Profile in Healthy and Autoimmune are Different

Analysis from PB of SLE patients show that B cell pool has abnormal expansion of CD27+IgD- post-switched memory B cells and these cells respond less to immunosuppressive therapy (69). Also, the BCR repertoire of memory cells shows abnormal selection, increased somatic hypermutation, and increased receptor editing (93). Besides abnormal cellular processes, an increased memory B cell pool means a higher risk for autoimmunity as these cells have lower activation thresholds and can be activated with TLR agonists and BAFF or APRIL. The CD27-IgD- subset is expanded in patients with SLE and associated with increased disease activity and active renal disease. The presence of the subset was correlated with auto-antibodies such as anti-dsDNA, anti-Smith, and the 9G4 idiotypes (90). The 9G4 idiotype expresses the VH4-34 heavy chain and that type of BCR may form anti-DNA autoantibodies (94).

B Cell Subset Homeostasis

Homeostatic Proliferation

The naïve B cell pool is maintained at a stable size suggesting there are other possible mechanisms besides immature B cell sources. One such mechanism is a unique antigen-independent proliferation observed when naïve B cells were transferred into a selectively B cell deficient or fully lymphopenic host. This characteristic of B cells was termed homeostatic proliferation (HP) and is only observed when same-lineage cells are absent. Cells undergoing HP express early activation markers, but later revert to a naïve phenotype. The signal transduction pathways that induce proliferation differ from the pathways activated by conventional antigen or mitogen induced responses (95).

Role of BAFF in Homeostatic Proliferation

Homeostatic proliferation in B cells is independent of T cells. HP is mainly limited by the presence of same-lineage B cells in a host and trophic factors. The presence of same-lineage cells provides a lineage-specific feedback to reduce competition for resources. If trophic factors are present that promote growth and survival, a lymphocyte population could be re-established to the previous "set-point" since all the factors would be used up. One trophic factor involved in homeostasis is Blys or BAFF (95, 96). Several hematopoietic cells produce BAFF such as radio-resistant stromal cells, macrophages, dendritic cells, and neutrophils. Production of BAFF can be increased under the presence of pro-inflammatory cytokines (95). The survival of mature B cell populations is dependent solely on BAFF since related cytokine, a proliferation-inducing ligand (APRIL), has no effect on mature naïve B cell populations when eliminated. Thus, BAFF via BAFF-R (BR3) binding promotes the survival signals for follicular and

marginal zone B cell populations. BAFF is necessary factor in HP (95). In the absence of BAFF, B cells failed to proliferate in a lymphopenic host, but proliferated when BAFF was added to hosts (95). In the context of autoimmune disease, patients have lymphopenic environments that would favor the expansion of transferred mature naïve B cells.

Homeostatic Proliferation and Memory B Cells

Besides mature naïve B cells, memory B cells have been studied in the context of HP. When compared to naïve B cells, memory B cells are maintained longer and have lower threshold for BCR activation. They have a reduced need for T cell help, undergo cell cycling more quickly, and can differentiate into effector populations (95). Studies indicate that increased memory B cell lifespan is antigen-independent and thus a possible explanation for their longevity could be HP. Memory B cells may migrate to splenic follicles where they come under BTK and BAFF dependent signaling that promotes HP similar to those in naïve B cells (95).

The potential for a subset to undergo HP is dependent on the competitive advantage of that subset to access and utilize of BAFF. Memory B cells may have advantage over naïve B cells due to previous BCR stimulation which up-regulates BAFF-R (49). Furthermore, memory B cells have a higher density of TLRs on their cell surface and this may allow access to a larger number of ligands providing better stimulation compared to naïve B cells. HP uses both BTK-dependent and a BTK-independent BAFF dependent signaling pathways. Both pathways are easily stimulated in memory B cells, but are not readily in naïve B cells (95).

The model of homeostatic proliferation suggests that under trophic cytokine availability, memory B cells will differentiate into effector function more easily than resting naïve B cells. Memory B cells stimulated with CpG were induced to replicate polyclonally (97). Thus, the HP model provides a mechanism by which memory B cells can be maintained in the absence of its antigen (95).

B Cell Subset Phenotypic Profiling

Recent studies have emerged highlighting the importance of evaluating PBMC B cell populations. The composition of the B cell pool can be regarded as a sentinel population that indirectly informs about the immunological activity occurring in other parts of the body. B cell-depletion resulted in clinical improvement in diseases such as SLE, RA, primary Sjogren's syndrome, and Type 1 Diabetes and showed the importance of B cells in these diseases (55, 98). We have yet to understand what antibody-independent B cell functions contribute to the development of disease. Furthermore, what B cell subsets are involved and what functions they perform in the induction and progression of disease. Identifying the proportion and collection of an individuals B cells subsets can create a "signature" profile. This profile can then be correlated with disease outcome providing a framework from which to optimize targeted therapies for the individual patient (98, 99). Human B cell subsets as described by various human studies can provide a type of diagnostic profiling that allow targeted therapies to be adjusted on a per-patient basis (98).

Human B cell functions have previously been characterized in part by comparisons of B cells from healthy individuals and those with immunological diseases. Also, more information can be acquired based on the different clinical outcomes between

groups of patients with the same disease. This heterogeneity in clinical outcomes is observed in SLE making treatment decisions complex (100). Another aspect of clinical outcomes with B cell-depletion is the differences in B cell subset proportions that differ depending on the disease. For example, B cell depletion in SLE results in early reconstitution of B transitional B cell subsets (101). The opposite occurs in transplant patients where transitional B cells are deficient and their reduced production of IL-10 is associated with reliance on immunosuppressive drugs. The number of transitional B cells can be used to predict tolerance to transplant (102).

Analysis of B cell subsets in disease showed abnormalities within the normal cellular functions. For example, SLE-patient B cells show an altered activation status particularly in the IgD+CD27- (naïve) population and also in total B cells as indicated by protein tyrosine phosphorylation patterns (69). Furthermore, B cells express activation markers and have higher Ca2+ flux, proliferative potential, antibody production, as well as bigger cell size (98). In B cell-depleted patients with SLE or RA, the presence of CD27+ (memory) B cell during post-reconstitution may be predicative of clinical relapse. These memory B cells may play some role in autoimmune pathogenesis. Taken together, defining B cell subsets and analysis of frequencies within the B cell population can provide “signatures” that can be used to correlate with different clinical outcomes (98).

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CHAPTER TWO

DIFFERENCES IN MOUSE AND HUMAN NON-MEMORY B CELL POOLS[1]

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Abstract

Identifying cross-species similarities and differences in immune development and function is critical for maximizing the translational potential of animal models. Co-expression of CD21 and CD24 distinguishes transitional and mature B cell subsets in mice. Here, we validate these markers for identifying analogous subsets in humans and use them to compare the non-memory B cell pools in mice and humans, across tissues, during fetal/neonatal and adult life. Among human CD19+IgM+ B cells, the CD21/CD24 schema identifies distinct populations that correspond to T1 (transitional 1), T2 (transitional 2), FM (follicular mature), and MZ (marginal zone) subsets identified in mice. Markers specific to human B cell development validate the identity of MZ cells and the maturation status of human CD21/CD24 non-memory B cell subsets. A comparison of the non-memory B cell pools in bone marrow (BM), blood, and spleen in mice and humans shows that transitional B cells comprise a much smaller fraction in adult humans than mice. T1 cells are a major contributor to the non-memory B cell pool in mouse BM where their frequency is more than twice that in humans. Conversely, in spleen the T1:T2 ratio shows that T2 cells are proportionally ~8 fold higher in humans than mouse. Despite the relatively small contribution of transitional B cells to the human non-memory pool, the number of naïve FM cells produced per transitional B cell is 3-6 fold higher across tissues than in mouse. These data suggest differing dynamics or mechanisms produce the non-memory B cell compartments in mice and humans.

Abbreviations

BAFF -	B CELL ACTIVATING FACTOR
BAFF-R -	B CELL ACTIVATING FACTOR RECEPTOR
BD -	BECTON DICKINSON
BM -	BONE MARROW
CB -	CORD BLOOD
CBMCs -	CORD BLOOD MONONUCLEAR CELLS
C3H/HeN-	MOUSE STRAIN
PB -	PERIPHERAL BLOOD
FM –	FOLLICULAR MATURE
MFI -	MEDIAN FLUORESCENCE INTENSITY
MZ -	MARGINAL ZONE
T1 -	TRANSITIONAL 1
T2-	TRANSITIONAL 2

Introduction

The mouse and other animal models provide important insights into human B cell development and disease (1, 2). Murine data show that B lineage committed progenitors arise from hematopoietic stem cells in the bone marrow (BM) and transit a series of developmentally sequential stages to produce immature B cells expressing surface IgM (3, 4). Immature B cells pass through the transitional 1 (T1) and transitional 2 (T2) stages and then develop into naïve follicular mature (FM) or marginal zone (MZ) B cells as they leave the BM, travel through the periphery, and move into the spleen and other secondary lymphoid tissues (5-7). Differentiation from T1 to T2 and subsequently to FM and MZ B cells in the mouse is believed to occur mostly in the spleen. Developing B cells that are autoreactive undergo negative selection following B cell receptor (BCR) stimulation in the BM or the periphery (3, 6). Survival of transitional B cells during negative selection depends on interplay between signals mediated by the BCR and the receptor for B cell activating factor (BAFF) (8-12). Mature B cells that are activated by BCR stimulation, together with appropriate co-stimulatory signals, differentiate into antibody-producing plasma cells, as well as memory B cells, that together with non-memory B cells form the B cell pool (13, 14)

Comparative studies of mouse and human B cell development have focused on B cell precursor populations and activated B cells, while cross-species comparisons of the non-memory B cell pools are lacking (15). Identifying differences in the non-memory B cell pools are important for understanding the differences in mechanisms that contribute to B cell homeostasis in the two species and in translating information obtained from mouse models to studies of human disease. Murine disease models remain our major source of mechanistic data for human disease processes that arise due to defects in

negative selection and B cell homeostasis (3, 16, 17). However, the clinical application of murine data is limited because multiple schema are used to identify transitional and mature B cells in mice (5, 8, 16, 18-20) and humans (21-26) and many of these are based on species-specific markers (Supplemental Table I). A system of common markers that can be used to identify transitional and mature B cell subsets across tissues in mice and humans has yet to be developed.

Here, we show that co-expression of CD21 and CD24 can be used to identify analogous subsets of CD19+IgM+ B cells in mice and humans. These markers allow the identification of T1, T2, and FM B cells in multiple hematopoietic tissues during fetal/neonatal and adult life in both species. Unlike other schema that are used to distinguish human transitional and FM B cells, these markers also allow MZ B cells in the human spleen to be identified. Using the CD21/CD24 schema and strict gating criteria to exclude memory B cells, we compared the contribution of transitional and naïve mature cells to the B cell pools in adult humans and mice. When compared to mice, our data show that human transitional B cells are reduced in the non-memory B cell pool across tissues. Despite the relatively small contribution of transitional B cells to the non-memory B cell pool, they give rise to a proportionally much larger naïve FM B cell compartment (3-6 fold increased across tissues) than those in the mouse. These data suggest that differences in the dynamics or mechanisms involved in B cell production are required to produce the proportionally larger FM compartment observed in humans as compared to mice.

Supplemental Table I: Cell Surface Phenotypes to Identify Mouse and Human B Cell Subsets

Mouse Schema				
Tissue	**Subset**	**Cell Surface Phenotype**	**References**	**Cross Species Utility**
Allman & Pillai				
Bone Marrow	Immature	AA4.1+ IgM^{HI} CD24^{HI} IgD^{LO} CD21^{LO}	(19)	AA4.1 mouse-only
	T2-like	AA4.1+ IgM^{HI} CD24^{HI} IgD^{HI} CD21^{LO} CD62L+ CD23+		
	Mature	AA4.1^{-} IgM^{LO} CD24^{HI} IgD^{HI} CD21^{LO} CD62L+ CD23+		
Spleen	T1	AA4.1+ IgM^{HI} CD24^{HI} IgD^{LO} CD21^{LO}		AA4.1 mouse-only CD1d mouse-only
	T2	AA4.1+ IgM^{HI} CD24^{HI} IgD^{HI} CD21^{LO} CD62L+ CD23+		
	T3	AA4.1+ IgM^{LO} CD24^{HI} IgD^{HI} CD21^{LO} CD62L+ CD23+		
	FO I	AA4.1- IgM^{LO} CD24^{LO} IgD^{HI} CD21^{INT} CD62L+ CD23+		
	FO II	AA4.1^{LO} IgM^{HI} CD24^{LO} IgD^{HI} CD21^{INT} CD62L+ CD23+		
	MZP	AA4.1- IgM^{HI} CD24+ IgD^{LO} CD21^{HI} CD23+ CD1d+		
	MZ	AA4.1- IgM^{HI} CD24+ IgD^{LO} CD21^{HI} CD1d+		
Carsetti, Pillai, and Rawlings				
Spleen	T1	AA4.1++ IgM++ CD24++ IgD+/- CD21- CD62L- CD23+/- CD1d++ LFA-1+ BAFF-R+	(5, 16, 18-20)	AA4.1 mouse-only CD1d mouse-only
	T2	AA4.1++ IgM++ CD24++ IgD++ CD21+ CD62L+ CD23+ CD1d++ LFA-1++ BAFF-R++++		
	FM	AA4.1- IgM+ CD24+ IgD++ CD21+ CD62L+ CD23+ CD1d+ LFA-1++ BAFF-R++		
	T2-MZP	AA4.1+/-IgM++ CD24++IgD++ CD21++ CD62L+ CD23+ CD1d+++ LFA-1+++ BAFF-R+++		
	MZ	AA4.1- IgM++ CD24++IgD++ CD21++ CD62L+ CD23- CD1d+++ LFA-1+++ BAFF-R+++		
Rawlings (in vivo functional studies)				
Spleen	T1	IgM++ CD24^{HI} CD21^{LO} (BAFF-R+)	(18)	
	CD21^{INT} T2	IgM++ CD24^{HI} CD21^{INT} (BAFF-R++++)		
	FM	IgM+ CD24^{INT} CD21^{INT} (BAFF-R++)		
	T2-MZP/MZ	IgM++ CD24^{INT-HI} CD21^{HI} (BAFF-R+++)		
Human Schema				
Tissue	**Subset**	**Cell Surface Phenotype**	**References**	**Cross Species Utility**
Carsetti				
Bone Marrow & Blood	Immature	CD19+ IgM+ CD27^{neg} CD24^{bright}CD38^{bright} CD10+	(22)	CD10 human-only CD38 human-only
	Transitional	CD19+ IgM^{bright} CD27^{neg} CD24^{bright}CD38^{bright} CD10^{neg} IgD+		
	Mature	CD19+ CD27^{neg} CD24^{dull} CD38+		
	Memory	CD19+ CD27+ CD24^{bright}CD38^{neg}		
Lispky				
	Immature	CD19+ IgM^{HI} CD24^{HI} CD38^{HI} CD10+ IgD+ CD21^{LO} CD23^{LO} CD20^{HI}CD44^{HI}	(23)	CD10-human only CD5-human only CD38 human-only
	T1	CD19+ CD27^{neg} CD24^{HI} CD38^{HI} CD10^{HI} CD20^{HI} CD44^{LO} CD5+		
	Intermediate	CD19+ CD24^{INT} CD38^{INT} CD10^{LO} CD20+ CD44^{HI}		
	Naive	CD19+ CD24^{LO} CD38^{LO} CD10^{NEG} CD20+ CD44^{HI}		
Anolik				
Cord Blood, Tonsil, BM, PB, & Spleen	Immature	IgM^{++} CD27^{neg} CD24^{+++} CD38^{+++} CD10+ IgD^{NEG} ABCB1^{NEG}	(25)	CD10-human only CD38 human-only
	T1	IgM^{+++} CD27^{neg} CD24^{+++} CD38^{+++} CD10+ IgD+ ABCB1^{NEG}		
	T2	IgM^{++} CD27^{neg} CD24^{++} CD38^{++} CD10+/- IgD^{++} ABCB1^{NEG}		
	T3	IgM^{+} CD27^{neg} CD24^{+} CD38^{+} CD10+/- IgD^{++} ABCB1^{NEG}		
	Naïve Mature	IgM^{+} CD27^{neg} CD24^{+} CD38^{+} CD10^{NEG} IgD^{++} ABCB1^{POS}		
	Memory	IgM+/- CD27+ CD24^{++} CD38^{+} CD10^{NEG} IgD+/- ABCB1^{NEG}		
Tangye				
Cord Blood, Tonsil, BM, PB, & Spleen	Transitional (CD21lo)	CD20+ CD27^{neg} CD10^{LO} CD5^{HI} CD21^{LO}	(21, 24, 26)	CD10-human only CD5-human only
	Transitional (CD21lhi)	CD20+ CD27^{neg} CD10+ CD5^{HI} CD21^{HI}		
	T3	CD20+ CD27^{neg} CD10^{NEG} CD5$^{LO/+}$ CD21^{HI}		
	Naive	CD20+ CD27^{neg} CD10^{NEG}		
	Memory	CD20+ CD27+ CD10^{NEG}		

To produce comprehensive cell surface phenotypes, various groups that study mouse and human B cell development have each built upon the work of previous studies to develop schema that identify B cell subsets. However, due to species differences many markers required to identify specific subsets are useful only for identifying mouse B cells or only for identifying human B cells as indicated.

Materials and Methods

Sample Procurement and Cell Preparation

Mouse Tissues

Male BALB/c mice, between 3 to 6 months of age (Charles River Laboratories, Inc., Boston, MA) and adult female C3H/HeN (Harlan, Inc) non-pregnant mice, were used for adult mouse studies. Spleens from fetuses of gestational age 18 days were isolated from pregnant female C3H/HeN mice. The use of animals was approved by the Institutional Animal Care Use Committee at Loma Linda University. BM cells were flushed from the femurs with 1 ml of sterile PBS (Cellgro, Manassas, VA). PB from BALB/c mice was obtained from the hepatic portal vein. A citrate-phosphate-dextrose solution (Sigma-Aldrich, St. Louis, MO) anticoagulant was added to blood in a 1.4 to 10 ratio. PBMCs were isolated using RBC lysis buffer method. Splenocytes from BALB/c mice were isolated by straining spleens through a 70 μM cell strainer (BD Falcon, Franklin Lakes, NJ) to create a single cell suspension in PBS. Fetal mouse spleens were processed using the same method as adult spleens.

Human Tissues

CB was collected from the umbilical cord of full-term neonates following caesarian section. Fetal spleens, pregnancy age between 17 and 23 weeks, were obtained from Advanced Bioscience Resources, Inc (Alameda, CA) or Novogenix Laboratories, LLC (Los Angeles, CA). PB from adult donors was obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL), and donated by the Blood Processing and Quality Control Lifestream in San Bernardino, CA. BM from adult male donors aged 20-35 was purchased from All Cells (Emeryville, CA) or Lonza (Walkersville, MD). Adult

spleens between the ages of 30 to 55 years of age were acquired from pathology specimens.

A citrate-phosphate-dextrose solution (Sigma-Aldrich) anticoagulant was added to blood in a 1.4 to 10 ratio. CBMCs were isolated using RBC lysis buffer density gradient centrifugation. Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) and/or RBC lysis density gradient centrifugation was used to isolate PBMCs from blood collected from filters (27). Spleens were processed using gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA) and then pressed through a 70 μM cell strainer (BD Falcon) to create a single cell suspension in PBS. Fetal spleens were processed as described above for adult spleen tissues. All human tissues were acquired and handled according to protocols approved by the Institutional Review Board at Loma Linda University.

Flow Cytometry

For flow cytometry, cells were stained with mAbs in PBS with 1% BSA for 20 minutes in the dark at 4ºC. Cells were subsequently washed and fixed with a 1% paraformaldehyde/PBS solution before analysis on BD FACSCalibur flow cytometer (BD Immunocytometry Systems, [BDIS] San Jose, CA) or MACSQuant® Analyzer (Miltenyi Biotec). The anti-human antibodies used were CD19 Pacific Blue, CD24 PE-Cy7, CD38 APC, CD27 APC-Cy7, CD21 FITC, CD1c PE (all from Biolegend, San Diego, CA), CD10 PE, CD5 PE, CD23 PE, IgD PE, IgM PE-Cy5 (all from BD Bioscience), BAFF-R PE (eBioscience Inc., San Diego, CA). The following anti-mouse antibodies were used: CD24 Pacific Blue, CD21 FITC, CD23 PE, BAFF-R PE, CD21 APC, SA-APC-Cy7 (secondary antibody for IgD biotin staining), IgM PE-Cy7 (all from Biolegend), and IgD Biotin (eBioscience). Living cells were identified by forward scatter

and side scatter gating and/or exclusion of 7-aminoactinomycin-D (eBioscience) added immediately prior to data acquisition or fixation. Flow cytometry data analysis was performed using Flowjo data analysis software (TreeStar, Ashland, OR).

Statistical Analysis

The number of patient or mouse samples assayed (biological replicates or sample size) are indicated by n values in Figure legends. Statistical significance was evaluated using a Mann-Whitney U test for comparison of the mean values taken from B cell subsets from mice and human tissues. Differences in BAFF-R expression between B cell subsets were determined by paired-t test. Values were considered significant if $p \leq .05$. Graphs and statistics of subset populations were derived using GraphPad Prism and Instat software (GraphPad Software, La Jolla, CA).

Results

CD21 and CD24 Co-expression Identifies Distinct B Cell Populations in Human Hematopoietic Tissues

Our first goal was to identify markers that define analogous populations of transitional and naïve mature B cells in mice and humans. We evaluated multiple markers used to define these subsets in mice (Supplemental Table I (5, 8, 16, 18-26), but focused our studies on CD21 and CD24 (18) because these markers are expressed on B cells in both species. In the mouse, CD21 and CD24 have been used to discriminate between transitional and mature splenic B cell subsets (Fig. 1A). These include transitional 1 (T1), transitional 2-intermediate (T2), and follicular mature (FM) B cells, as well as a subset that contains marginal zone B cells together with marginal zone precursors (MZ) B cells

(16, 18). To discriminate transitional B cells and the naïve FM B cells from other B lineage cells, we gated CD19+ B cells that were IgM+ (Supplemental Fig. 1). This excludes B cell precursors in BM and class-switched B cells in all hematopoietic tissues (3, 7). Based on the reported phenotypes of the follicular Type I and follicular Type II B cell subsets described in the mouse (28, 29), the mouse FM cells gated using this strategy would include both of these populations.

First, we assessed mouse splenic CD19+IgM+ B cells for CD21/CD24 co-expression (Fig. 1B) and observed all the transitional and mature subsets described in previous studies (16, 18). We then evaluated whether distinct subsets of human CD19+IgM+ B cells could be identified by CD21 and CD24 co-expression. A distinct population of human B cells with the FM CD21/CD24 phenotype ($CD24^{INT}CD21^{INT}$) was observed in all tissues (Fig. 1C-E). CD21/CD24 subsets with the T1 ($CD24^{HI}CD21^{LO}$) or T2 ($CD24^{HI}CD21^{INT}$) phenotypes were easily detectable in peripheral blood (PB) and BM

(Fig. 1D-E). Human CD19+IgM+ B cells with the CD21/CD24 MZ phenotype ($CD24^{HI}CD21^{HI}$) were only observed in the spleen (Fig. 1C).

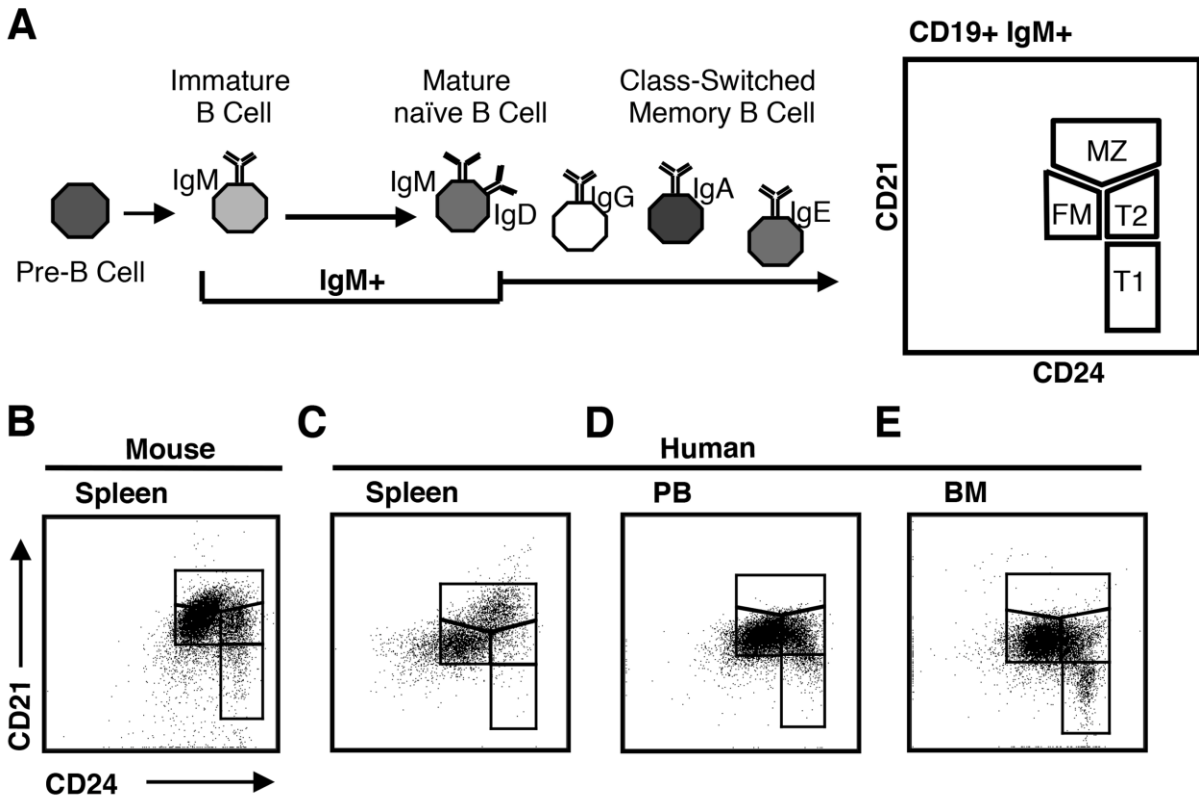
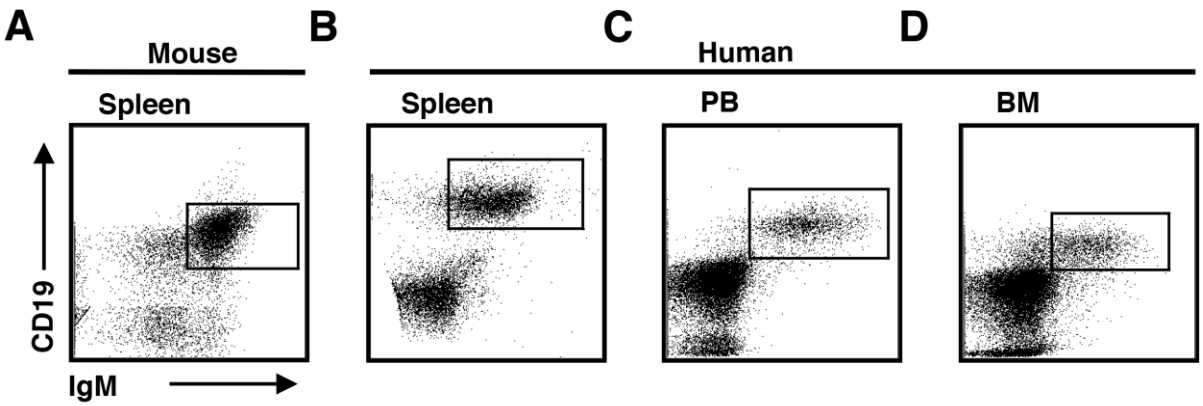


Figure 1 (*Figure 8*). CD21 and CD24 co-expression identifies distinct subsets of B cells in mouse and human tissues. *A,* Diagram of gating strategy to exclude B cell precursors and class-switched memory B cells prior to B cell subset identification using co-expression of CD21 and CD24. Identified are transitional 1 (T1), transitional 2 (T2), naive follicular mature (FM), and marginal zone plus marginal zone precursor (MZ) subsets. *B-E*, Mononuclear cells from indicated BALB/c mouse and human tissues (spleen, peripheral blood [PB], and bone marrow [BM]) were stained for flow cytometry to detect CD19, IgM, CD21, and CD24. CD19+IgM+ cells falling in lymphocyte light scatter were gated and B cell subsets as diagramed in A were gated. Data shown are representative of mouse spleen: n=4, human spleen: n=4, human PB: n=33, human BM: n=10.



Supplemental Figure 1 (*Figure 9)*. CD19+ and IgM+ gates for subset identification. *A- D,* Representative CD19+ IgM+ gating to identify B cells prior to subset identification using co-expression of CD21 and CD24 in indicated mouse and human tissues.

CD21/CD24 Gating Identifies MZ Cells in Human Spleen

Our assessment of human hematopoietic tissues showed the varying levels of CD21 expression (low, intermediate, and high) that have been used to distinguish B cells subsets in the mouse. Since previous studies indicate that high levels of CD21 are characteristic of murine MZ B cells (16, 19, 30), we wanted to confirm the identity of the splenic IgM+ human B cells in the CD21/CD24 MZ gate.

Human MZ B cells have been identified as $CD1c^{HI}CD27^{HI}$ (21, 31-34). As shown in Fig. 2A, IgM+ splenic B cells that are $CD1c^{HI}CD27^{HI}$ fall within the CD21/CD24 MZ gate while the CD1c–CD27– cells display a phenotype consistent with FM B cells. Converse gating in Fig. 2B shows that human splenic B cells in the CD21/CD24 MZ gate are predominantly $CD1c^{HI}CD27^{HI}$, while cells in the FM gate show reduced co-expression of these molecules. Thus, the expression of CD1c in combination with CD27 on IgM+ B cells in the CD21/CD24 MZ gate provides evidence that CD21 and CD24 can be used to discriminate MZ lineage B cells in the human spleen. As an additional means of verifying the identity of human B cells in the CD21/CD24 MZ and FM gates, we evaluated expression of IgM and IgD. Human MZ B cells are $IgM^{HI}IgD^{L0}$, while FM B cells show lower levels of IgM and higher levels of IgD (31, 34). As shown in Fig. 2C, CD19+IgM+ human splenic B cells in the MZ gate express higher levels of IgM and lower levels of IgD, as compared to those in the FM gate.

The co-expression of CD24 and CD38 has been used to distinguish mature B cells from transitional and memory B cells in human studies (22, 23, 25, 26, 35, 36). We compared the ability of CD24/CD38 and the CD21/CD24 schema to discriminate human FM and MZ cells in combination with CD27. First, to eliminate the majority of memory B cells (including MZ B cells), we gated on CD19+IgM+CD27– human splenic B cells.

The remaining B cells in the spleen form a homogeneous population that falls within the mature CD24/CD38 B cell gate (Fig. 2D, left panel). When the CD24/CD38 "mature" population was gated and evaluated for CD21/CD24 co-expression, a distinct FM population was observed, as well as B cells expressing the high level of CD21 that is characteristic of MZ cells. Thus, co-expression of CD21 and CD24, as compared to CD24 and CD38, allows splenic MZ B cells to be more stringently distinguished from FM B cells. Taken together, the above data demonstrate that markers commonly used to distinguish human MZ and FM B cells support the use of CD21/CD24 co-expression to identify FM and MZ cells in human spleen.

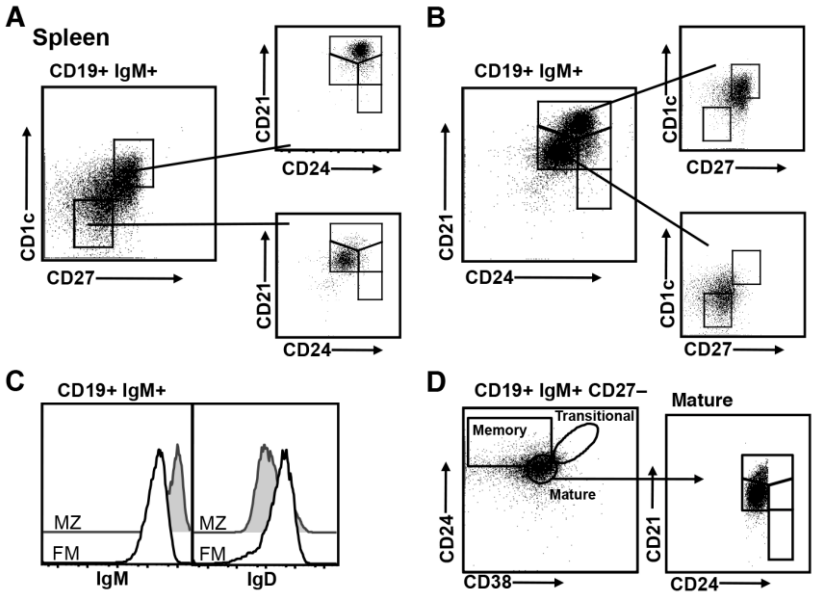


Figure 2 (*Figure 10)*. CD1c and CD27 co-expression validate the identity of the CD21/CD24 MZ subset in human spleen. Human splenic cells were co-stained for flow cytometry to detect CD19, IgM, CD24, CD21, and indicated markers. CD19+IgM+ cells falling in lymphocyte light scatter were gated. *A,* Co-expression of CD1c and CD27 was plotted (left panel). CD1c–CD27– and CD1c+CD27+ populations were gated and their distribution with respect to CD21/CD24 subsets was plotted (right panels). *B,* CD19+IgM+ cells were gated into CD21/CD24 subsets (left panel). Co-expression of CD1c and CD27 in the CD21CD24 MZ (top right panel) and FM (bottom right panel) subsets is shown. *C*, CD19+IgM+ cells were gated into CD21/CD24 subsets and expression of IgM and IgD in the MZ and FM subsets is shown in histograms. *D*, CD24 and CD38 co-expression in gated CD19+IgM+CD27– cells was plotted. Gates for memory, transitional and mature B cell subsets based on the CD24/CD38 identification schema are shown (left panel). Cells in the mature subset were gated and their distribution with respect to CD21/CD24 subsets is plotted (right panels). Data shown are representative of n=4 adult human spleens.

Human-Specific Developmental Markers Confirm the Maturation Status of CD21/CD24 Subsets

Memory B cells make up approximately 40% of the B cell pool in adult humans, but less than 5% in adult mice (14, 37). Given the large percentage of memory B cells in humans, as compared to mice (14, 37), we developed a stringent gating strategy to exclude human memory B cells prior to assessing the developmental status of immature and naïve B cells identified using the CD21/24 schema (14, 38, 39). In PB and BM, human memory B cells have classically been identified based on their expression of CD27. However, recent reports suggest that not all IgM+ memory B cells express CD27 (38, 39). Therefore, we used expression of CD27 together with strategies based on CD24/CD38 co-expression to exclude memory B cells. PB and BM cells were co-stained with CD27, CD38, CD24, CD21, IgM and CD19. To exclude CD27+ memory B cells, IgM+CD27– cells were gated (Fig. 3A, top panel). Then, co-expression of CD24 and CD38 was plotted and gates were drawn to exclude the CD24/CD38 "memory" B cells (Fig. 3A bottom panel). The application of this gating strategy allowed us to identify non-memory human B cells so that we could evaluate the maturation status of CD21/CD24 subsets using a panel of human-specific developmental markers.

In human B cells, CD24 and CD38 are developmentally regulated with increased levels of these markers correlating with immaturity (22, 25, 26, 35). To verify the maturation status of human CD21/CD24 B cell subsets we evaluated CD24/CD38 co-expression in human hematopoietic tissues. Human PB and BM were co-stained for flow cytometry and gated as shown in Fig. 3A. To determine whether B cell subsets identified based on co-expression of CD24 and CD38 supported the developmental status predicted by the mouse markers (CD21/CD24), each of the gated CD24/CD38 transitional and

mature subsets (Fig. 3B-C, left panel) were evaluated for CD21/CD24 co-expression (Fig. 3B-C, right panels). Cells in the CD24/CD38 "transitional" B cell gate are distributed within the CD21/CD24 T1 and T2 gates (Fig. 3B-C, top right panels) while cells in the CD24/CD38 "mature" gate fall exclusively in the CD21/CD24 FM gate (Fig. 3B-C, bottom right panel). This distribution supports the developmentally immature status of T1 and T2 phenotypes based on co-expression of CD21 and CD24.

Human transitional cells identified by CD24 and CD38 co-expression fall within both the CD21/CD24 T1 and T2 gates. Further analysis was required to determine whether cells in the CD21/CD24 T2 gate represent a more mature B cell population than those in the T1 gate. Expression of the human-specific developmental markers, CD10 and CD5, as well CD38 has been used to distinguish between transitional and mature subsets in human CD19+ B cells (22-26, 35, 40, 41). Each of these markers is expressed at higher levels in the most immature IgM+ cells and their expression progressively declines during maturation of B cells in adults (36, 41). We evaluated expression levels of these markers in CD21/CD24 subsets to confirm maturation status.

PB and BM cells were stained for co-expression of CD21, CD24, CD38, CD27, CD10, CD5, IgM and CD19 and gated into CD21/CD24 subsets, excluding memory B cells as shown in Fig. 3A. Histograms of developmental marker expression in CD21/CD24 subsets are shown in Fig. 3D. The marked decrease in CD10 and CD38 expression between T1 and T2 subsets provides evidence that the T1 subset is more immature than the T2 subset. This is also supported by a similar decrease in CD5 expression that is most clearly observed in PB. The lack of CD10 and CD5 expression in cells within the CD21/CD24 FM gate in human PB and BM (Fig. 3D) as well as spleen

(data not shown) provides additional evidence that CD21/CD24 co-staining can be used to distinguish transitional and mature naïve human B cells. Taken together, these data indicate that in humans, as in mice, CD21/CD24 co-staining can be used to identify developmentally sequential populations of transitional and mature naïve B cells, as well as a population that contains MZ cells in the spleen.

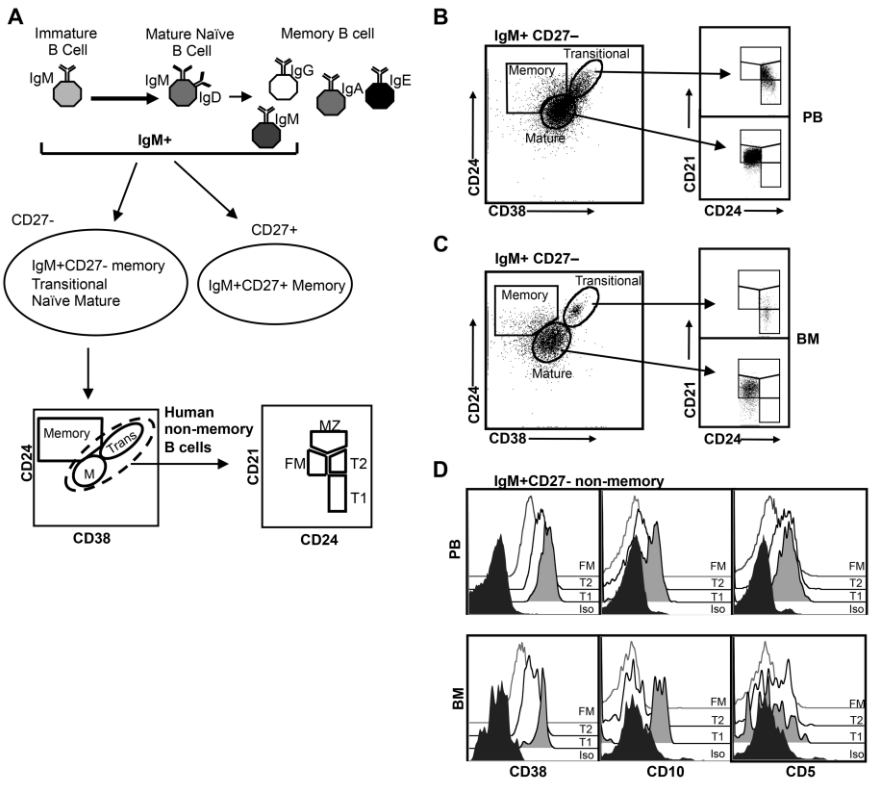


Figure 3 (*Figure 11)*. Human markers validate the developmental status of B cell subsets identified by CD21 and CD24 co-expression. *A,* Diagram of gating strategy to exclude both CD27+ and CD27– IgM+ memory B cells in human tissues. *B-C,* PB and BM cells were co-stained for flow cytometry to detect IgM, CD27, CD38, CD24, and CD21. IgM+CD27– cells in lymphocyte light scatter were gated as shown to identify memory, transitional and mature B cell subsets based CD24/CD38 expression as shown (left panel). Cells in the mature and transitional subsets were gated and their distribution with respect to CD21/CD24 subsets is shown (right panels). *D*, IgM+CD27– cells in the CD24/CD38 non-memory gate (dashed oval in panel A) were gated into CD21/CD24 subsets and evaluated for CD38, CD10 and CD5 expression. Data shown are representative of n=27 adult human PB and n=10 adult human BM.

Patterns of Differential BAFF-R Expression are Similar in Mouse and Human CD21/CD24 Subsets

BAFF provides survival, differentiation, and growth signals that play a major role in the maintenance of the normal B cell pool (42-44). Differences in the levels of BAFF-R expression in different B cell subsets are believed to provide one mechanism for differential BAFF responses during the negative selection process (44). Studies in the mouse have shown that BAFF-R is reduced in T1 cells as compared to later stages of B cell development (12, 45). Data obtained using human-specific markers to identify developmental subsets suggests that this is also the case in humans (23, 25, 26, 36). Here we compared BAFF-R expression in analogous B cell subsets in mouse and human tissues identified using the CD21/CD24 schema.

Non-memory B cells from mouse and human spleen, PB, and BM were gated into CD21/CD24 subsets as described in Fig. 3A. For the evaluation of MZ cells in human spleen, IgM+CD27+ cells were included. Median fluorescence intensity (MFI) of BAFF-R staining is shown in Fig. 4. These data show that the pattern of BAFF-R expression is similar in mouse and human B cell subsets, identified based on CD21/CD24 co-expression Consistent with previous reports in mouse and human (12, 23, 25, 26, 36, 45), we found that BAFF-R expression is lower in T1 cells and increases with maturation. These data establish that conclusions drawn from the previously reported patterns of differential levels of BAFF-R expression are applicable to B cell populations compared across species in mouse and humans when markers common to both species are used to identify developmental subsets of B cells.

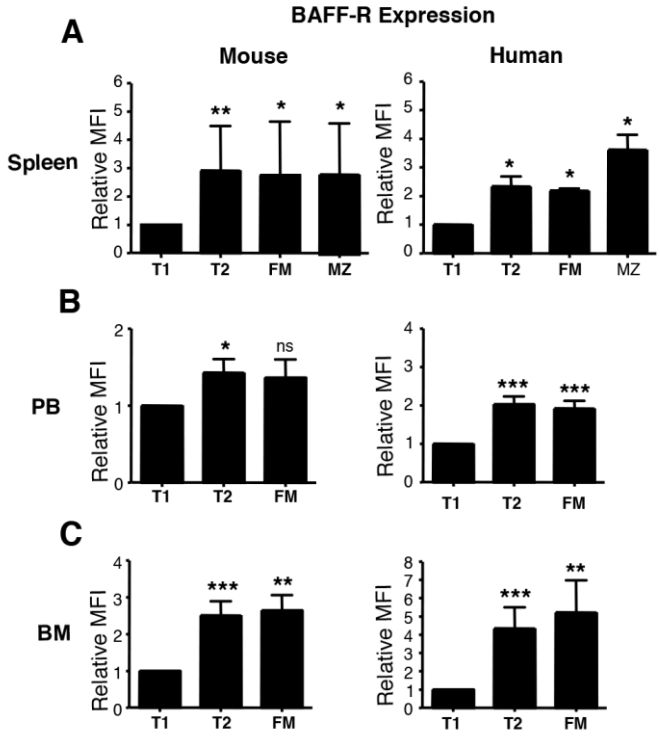


Figure 4 (*Figure 12)*. Patterns of differential BAFF-R expression are similar in mouse and human CD21/CD24 B cell subsets. *A-C*, Mononuclear cells isolated from adult mouse and human spleen, PB, and BM were stained for IgM, CD21, CD24, BAFF-R and in human samples for CD27, CD38 and CD19 as well. IgM+ B cells falling in lymphocyte light scatter in mouse tissues were gated into CD21/CD24 subsets. Human PB, BM, and splenic T1, T2, and FM cells were gated as described in Fig. 3. For MZ cells in human spleen, CD19+IgM+ cells were assessed for CD21/CD24 MZ phenotype and this included both CD27+ and CD27– cells. For each tissue, graphed are the means + SEM of relative BAFF-R expression in each CD21/CD24 subset (normalized to BAFF-R levels in the T1 subset in that tissue). Data are from n=9 mouse spleen, n=7 mouse PB; n=9 mouse BM, n=4 human spleen, n= 15 human PB and n=9 human BM. Statistical differences are shown as *p < .05; **p< .01; *** and p < .001.

CD21/CD24 Co-staining Identifies Developmental Subsets of Human B Cells in Fetal/Neonatal Tissues

In the experiments described above, we established the validity of CD21 and CD24 co-staining for the identification of developmental subsets of immature B cells and for distinguishing FM and MZ B cells in adult human tissues. Next, we wanted to determine whether CD21/CD24 co-staining can be used to identify developmental subsets at early points in life and thus provides a tool for comparing the timeline for emergence of mature B cells in mice and humans.

We first determined whether CD21/CD24 could be used to identify distinct B cell subsets present in human neonatal blood and fetal spleen. Populations corresponding to T1, T2, and FM were observable in both neonatal blood (umbilical cord blood, CB) (Fig. 5A) and fetal spleen (Fig. 5B).

To verify the maturation status of these subsets, we evaluated the expression of the human-specific developmental markers CD38, CD10 and CD5. B cells in CD21/CD24 subsets present in CB (Fig. 5D) and fetal spleen (Fig. 5E) show a progressive decline in the expression of CD38 and CD10 in the T1, T2 and FM subsets, respectively, similar to that observed in adult PB and BM. All the CD21/CD24 B cell subsets in human CB and spleen show at least low levels of CD5, regardless of maturation status (Fig. 5D, E). This is consistent with previous reports of CD5 expression on human fetal/neonatal B cells (23, 24, 46, 47). These results provide evidence that the CD21/CD24 schema can be used to identify developmental subsets of human transitional and mature B cells in fetal/neonatal tissue, as well as adult tissue.

By Mid Gestation, the B Cell Pool in Humans, But Not Mouse, Includes Mature B Cell Subsets

The period of fetal development is considerably longer in humans than in mice (~266 days versus 20 days) (48, 49). To determine whether this disparity impacts the composition of the B cell pool prior to birth we used the CD21/24 schema to identify the B cell subsets present in human and mouse fetal spleens (Fig. 5B-C). The IgM+ cells in human fetal spleens obtained at approximately mid-gestation (18-23 weeks, pregnancy age) included substantial populations of T1, T2, and FM B cells (Fig. 5G). In contrast, virtually all of the IgM+ cells in mouse fetal spleen (harvested 2 days prior to end of gestation which occurs at 20 days) have a T1 phenotype (Fig. 5C, H). Consistent with the emergence of mature B cells in human fetal spleen prior to birth, the B cell pool in the circulating blood of human newborns (umbilical cord blood) shows a B cell pool comprised of primarily of T2 and FM (Fig. 5F). In contrast, in the mouse at a point ~90% of the way through gestation T1 cells make up ~97% of splenic B cells (Fig. 5 D, H). This is consistent with reports of murine neonatal B cell development which show that the splenic B cell pool consists primarily of T1 cells as late as one week after birth (5). Thus, using the CD21/CD24 schema to identify analogous developmental subsets we show that the human non-memory B cell pool is comprised of T1, T2 and FM B cells well before birth, while the mouse B cell pool includes only T1 cells late in gestation.

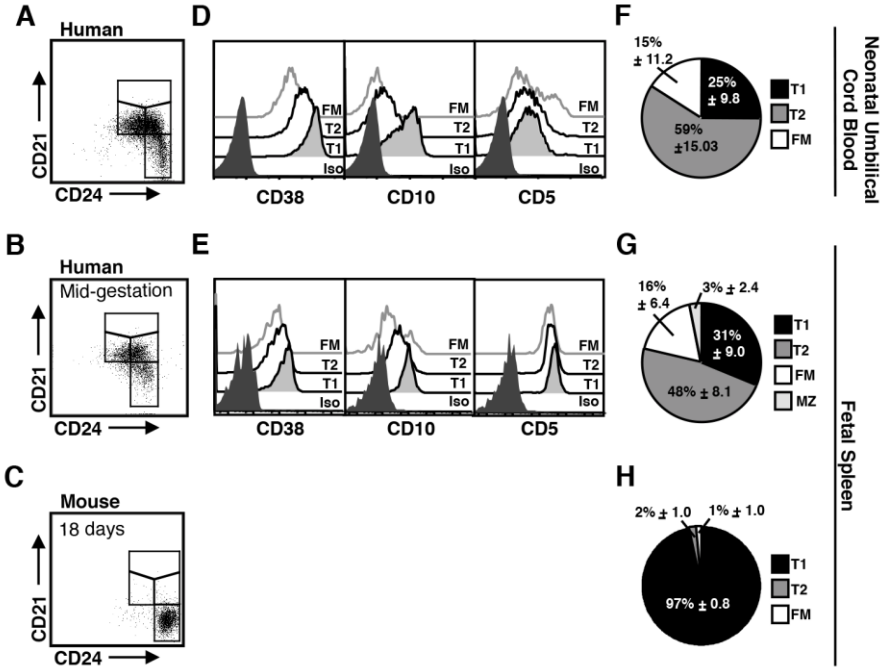


Figure 5 (*Figure 13)*. Use of CD21/CD24 to compare emerging B cell pools during fetal/neonatal development in mice and humans. Mononuclear cells were isolated from human cord blood (CB), human fetal spleen and pooled mouse fetal spleens. Cells were stained for flow cytometry to detect CD19, IgM, CD21, CD24, and in human samples for CD38, CD10, and CD5 as well. *A-C*, Cells falling in lymphocyte light scatter and that were CD19+IgM+ or IgM+ were gated into CD21/CD24 subsets. *D-E*, Subsets in human CB and human fetal spleens were evaluated for CD38, CD10 and CD5 expression. *F-H,* Graphed are the frequencies ±SD that each of the CD21/CD24 subsets contribute to the composition of the B cell pool. The mean ±SD for subsets in each tissue was derived from: n=10 human CB; n=6 human fetal spleens obtained at 18–23 weeks, (pregnancy date); and pooled mouse fetal spleen from C3H/HeN mice, day 18 of 20 day gestation, assayed in 3 independent experiments. Flow cytometry histogram and dot plots are representative data from indicated tissues.

Transitional B cells Are Reduced in the Non-Memory B cell Pool in Humans as Compared to Mice

Comparisons of the non-memory B cell pools in mice and human have been limited, in part because obtaining splenic tissue from human patients is challenging and PB has rarely been evaluated in the mouse. Here, we compare the contribution of B cell subsets to the IgM+ non-memory B cell pool in multiple tissues across species, using the CD21/CD24 schema to identify B cell subsets in spleen, PB, and BM from mice and humans (Fig. 6).

First, we compared B cell pools in adult spleen in mice and humans. To include MZ cells in our census of the splenic B cell pool in humans, CD21/CD24 gates were set on CD19+IgM+ cells gated without regard to CD27 expression (Fig. 6A). For the identification of human transitional and FM cells, memory B cells were excluded using the strategy diagramed in Fig. 3. Data from the analyses of human MZ and non-memory subsets were combined for comparison to CD21/CD24 subsets in the IgM+ splenic B cell pool in the mouse (Fig. 6D, G).

In both mice and humans, the splenic IgM+ B cell pool is comprised primarily of FM and MZ cells (Fig. 6D, G). Mouse spleen shows clear populations of T1 and T2 cells, although 5% of total IgM+ cells are T1 cells. In contrast, T1 cells are barely detectable in human spleen, while the contribution of T2 cells is less than half that observed in mouse (Fig. 6D, G). Thus, the major difference in the splenic non-memory B cell pool in mice and humans is the reduced contribution of transitional B cells, primarily T1 cells, in humans.

Next, we used the CD21/CD24 schema to compare B cell subsets in the PB and BM from adult mice and humans (Fig. 6B-C). In contrast to the spleen where mature B

cells comprise most of the non-memory pool, in mouse PB and BM, transitional B cells predominate (Fig. 6E, F, H, I). This was strikingly different from what we observed in human tissues where transitional B cells comprised 30% or less of the non-memory B cell pool in PB and BM (Fig. 6E, F, H, I). Thus, while transitional B cells formed the majority of cells in the non-memory B cell pool in mouse PB and BM, in humans, transitional B cells comprised a much smaller fraction of the non-memory B cell pool in these tissues, as they did in the spleen.

Production of FM B Cells, Per Transitional B Cell, Is Greater in Humans Than Mice

To gain insights into differences in the dynamics of B cell production in mice and humans, we examined the relative proportions of T1, T2 and FM subsets in the non-memory B cell pool across tissues in mice and humans (Table I). To facilitate comparisons across tissues, the MZ compartment was excluded from the IgM+ B cell pool in the spleen.

We used the ratio of FM to transitional B cells (T1+T2 cells) as an indicator of the capacity for transitional B cells to give rise to naïve FM B cells (Table I). In the mouse spleen, the ratio of FM B cells to transitional B cells was 3.7 to 1 while in human spleen it was 21.9 to 1. In mouse PB and BM, the ratio of FM to transitional B cells was $\leq$0.6. to 1. In human PB and BM, this ratio is much higher, 2.2 to 1, and 2.1 to 1, respectively. Taken together, these data show that across tissues the ratio of FM to transitional B cells is ~3-6 fold higher in humans than mouse (Table I). Our human data is consistent with previous reports that used human-specific markers to evaluate transitional B cell frequencies. Work by Cuss et al. showed that the frequency of transitional B cells is

much lower than that of naive mature cells and is reduced in the periphery and secondary lymphoid tissues as compared to BM (24). Thus, if the FM to transitional B cell ratio is used as an indicator of the capacity for transitional B cells to give rise to FM B cells, our data suggest that this capacity is much higher for human than mouse transitional B cells.

Differences in Transitional Subset Predominance in Mice and Humans Show Tissue Specificity

To gain further insights into the dynamics of naïve B cell production in mice and humans, we used data obtained with the CD21/CD24 schema to compare the relative contribution of T1 and T2 cells to the non-memory B cell pool and to the transitional B cell compartment across tissues in mice and humans (Fig. 6D-I). A comparison of T1 and T2 cell frequencies in the non-memory B cell pool shows that in the mouse, the contribution of T1 cells was the greatest in the BM. Here they comprised ~50% of the non-memory B cell pool with progressively smaller contributions in PB and spleen (Fig. 6G-I). While the frequency of human T1 cells was greater in BM than PB or spleen, T1 cells made up only ~25% of the BM non-memory B cell pool (Fig. 6F, I) in humans. Murine T2 cells contribute most to the non-memory B cell pool in PB where they comprise a majority of the cells (Fig. 6E, H). In human tissues, T2 cells are also most abundant in the PB non-memory B cell pool, however, their frequency is about one third of that observed in mouse. Our human data is consistent with a previous study which found that T2 cells, identified using human-specific markers, are most abundant in PB (26).

A comparison of the T1 to T2 ratios among transitional B cells in BM shows that T1 cells outnumbered T2 cells by 5 to 1 in the mouse, but only by 3 to 1 in humans

(Table I). In PB, the ratio of T1 to T2 cells is similar in both species (~1:7). However, when mouse and human spleen are compared we find that the ratio of T1 to T2 cells is 1:3.3 in mice versus 1:25.4 in humans (Table I). Thus, PB has the highest frequency of T2 cells in both mice and humans, but the relative proportion of T2 cells, as compared to T1 cells, is highest in the spleen, particularly in humans where this ratio is ~8 fold greater than in the mouse. Taken together these data show a disparity between T1 to T2 cell ratios in mice and humans. While ratios in the periphery are similar, the proportion of T1 cells is larger in mouse BM while the proportion of T2 cells in the transitional B cell compartment is larger in human spleen.

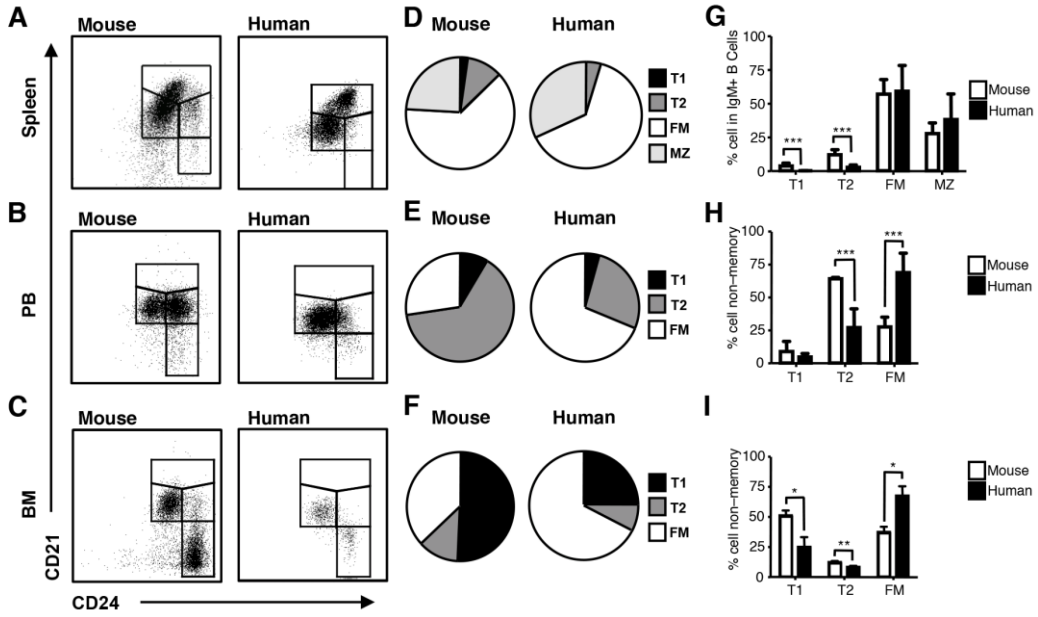


Figure 6 (*Figure 14)*. Transitional B cells are reduced in the non-memory B cell pool in humans as compared to mice. Mononuclear cells were isolated from adult mouse and human spleen, PB, and BM. Cells were stained for IgM, CD21, CD24, and in human samples for CD27, CD38 and CD19 as well. *A-C,* IgM+ B cells falling in lymphocyte light scatter were gated in mouse tissues. For human spleen, CD19+IgM+ cells falling in lymphocyte light scatter were gated. Human PB and BM were gated as described in Fig. 3. CD21/CD24 gates are as shown. *D*, Pie graphs showing the composition of the IgM+ B cell pool with respect CD21/CD24 subsets gated as described in results to include CD27+ and CD27– MZ cells. *E-F.* Pie graphs showing the composition of the non-memory B cell pool with respect CD21/CD24 subsets. *G-I,* Graphed are the mean $\pm$ SD of the percentages that each CD21/CD24 subset contributes to the IgM+ B cell pool in spleen and the non-memory B cell pool in PB and BM. Graphed are data from n=16 adult mouse spleen; n=6 adult mouse PB; n=9 adult mouse BM; n=4 adult human spleen; n= 33 adult human PB; and n= 11 adult human BM; Mice were adult male BALB/c and non-pregnant female C3H/HeN mice. Statistical differences between analogous subsets are shown as *p < .05; **p< .01; *** p < .001; and **** p< .0001.

Table I: Ratios of FM to transitional B cells in the non-memory B cell pool across tissues in mice and humans.

	Mouse	Human
Subset Ratio	FM : T2 : T1	FM : T2 : T1
Spleen	15.9 : 3.3 : 1	577 : 25.4 : 1
PB	3.2 : 7.4 : 1	15.3: 6.0 : 1
BM	0.7: 0.2 : 1	2.7 : 0.3 : 1
Subset Ratio	FM:Transitional	FM:Transitional
Spleen	3.7 : 1	21.9 : 1
PB	0.4 : 1	2.2 : 1
BM	0.6 : 1	2.1 : 1
Mouse:Human Ratio	Mouse (FM:Transitional) :	Human (FM:Transitional)
Spleen	1 : 6.0	
PB	1 : 5.8	
BM	1 : 3.5	

Discussion

To facilitate translational studies of human B cell maturation, our first goal was to identify, from among the multiple murine and human schema, a set of common markers that could be used to identify analogous populations of immature and mature B cells across tissues and at different points in life in mice and humans. Such a tool could be used by basic science and clinical researchers to translate data from animal models to studies of human B cells and for developing and testing new hypotheses. To provide a foundation for such studies, our second goal was to use the CD21/CD24 schema to compare the composition of the non-memory B cell pool across tissues in fetal/neonatal and adult B cell production in mice and humans.

We evaluated several candidate markers by flow cytometry for their conserved ability to identify analogous B cell subsets in mice and humans. CD21 emerged as a promising candidate based on its ability to identify functionally distinct subsets in both species. In human studies, CD21 expression was used by Suriyani et al. (26) to subdivide immature B cells into two transitional populations. During B cell reconstitution in patients receiving hematopoietic stem cell transplants, they noted that B cells expressing lower levels of CD21 precede those expressing higher levels (26). In the mouse, CD21 together with CD24 had been used to identify T1 B cells, as well as a T2-intermediate population (designated T2 here) that gives rise to both FM and MZ precursors/MZ B cells–all identified based on CD21/CD24 co-expression (16, 18). In addition, rabbit T1 and T2 subsets had recently been identified based on CD21/CD24 co-expression patterns similar to those observed in mice (1) suggesting that CD21 expression in context of CD24 might be broadly conserved across species in B cell development. The above data

suggested that CD21 had the potential to distinguish between developmentally sequential subsets of immature B cells, as well as FM and MZ B cells in humans. However, CD21 expression had not been evaluated in context of CD24 co-expression in humans, and its ability to identify analogous B cell subsets in mice and humans was unknown. Here, we validate the use of C21/CD24 co-expression to identify analogous B cell subsets in human hematopoietic tissues. Among human CD19+IgM+ B cells, we show that co-expression of CD21 and CD24 identifies distinct populations that correspond to the T1, T2, FM, and MZ subsets identified in the mouse (18).

Human B cells within the CD21/CD24 MZ gate were found exclusively in the spleen and their identity was validated by co-expression of the human MZ lineage markers, CD1c and CD27. The ability to distinguish FM cells from cells that are MZ or MZ precursors in the human spleen (Fig. 2) is an advantage of the CD21/CD24 schema over other systems currently used in human studies (23, 50). While mouse MZ B cells are restricted to the spleen, in humans MZ-like B cells (circulating IgM+ memory B cells that have similarities to MZ B cells) have been associated with other secondary lymphoid tissue including tonsils, lymph nodes and mucosal-associated lymphoid tissues (31, 34, 51). In addition, circulating CD1c+CD27+ cells have been observed in PB and are considered MZ-like cells (31, 34, 51). We found that the CD21/CD24 schema identifies a distinct population of $CD21^{HI}$ MZ cells present in the human spleen, but absent from PB (Fig. 1C-D). Non-splenic MZ-like B cells have been described as CD21+ (34). However, consistent with data shown in previous reports (21, 24, 31, 34, 52), our studies indicate that these cells express the intermediate levels of CD21 that we observed for FM B cells in the spleen and all mature IgM+ B cells in the PB. It is possible that the splenic

microenvironment is required for expression of the high levels of CD21 found on splenic MZ lineage B cells (52-54). Alternatively, it has been suggested that the diverse functional and phenotypic characteristics ascribed to MZ and MZ-like cells may be due to multiple MZ and MZ-llike B cell lineages (51). The ability of the CD21/CD24 schema to identify immature transitional B cells as well as to distinguish FM and MZ B cells in mouse and human spleen will facilitate studies to define different pathways/lineages of human MZ and MZ-like B cell differentiation and the role of the spleen and other secondary lymphoid tissues in this process.

The maturation status of human CD21/CD24 non-memory B cell subsets in multiple adult and fetal/neonatal tissues was verified using markers of maturation specific to human B cell development (22, 23). Our data provide evidence that the CD21/CD24 schema can be used to identify B cell subsets in multiple tissues and at different points in ontogeny across species (Fig. 3-4). Data from clinical studies demonstrate that CD10 and CD38 are expressed at high levels on transitional B cells that first emerge in the periphery during B cell re-constitution following stem cell transplant (24, 26, 36) or B cell depletion therapy (25) and that expression of these markers decreases with differentiation to the mature B cell stage. Changes in expression of CD38 and CD10 verified the maturation status of human T1, T2, and FM cells identified based on CD21/CD24 in both adult (Fig. 3) and fetal/neonatal (Fig. 5) tissues.

Mouse studies indicate that BAFF functions as a growth stimulus, promoting the survival and proliferation of T2 but not T1 cells (6, 43). The differential effects of BAFF on murine T1 and T2 cells is due in part to differential BAFF-R expression during differentiation: T1 cells show reduced BAFF-R as compared to T2 and mature B cells

(7). Here, we evaluated levels of BAFF-R expression in analogous subsets of human B cells identified using the CD21/CD24 schema. Consistent with previous mouse and human reports (12, 23, 25, 26, 36, 45), we found that BAFF-R is reduced on human T1 cells as compared to later stages in B cell development. However, unlike previous studies that used markers whose expression was mouse-specific or human specific to identify B cell subsets, we assessed BAFF-R expression using common markers between the species.

The period of fetal development and adult life span are dramatically different in mice and humans (48, 49). To gain insights into the impact of life span on human versus mouse B lymphopoiesis, we used the CD21/CD24 schema to identify analogous populations of mouse and human B cell subsets at early points in life. We compared the composition of the non-memory B cell pools during fetal/neonatal development in mice and humans. Our fetal data (Fig. 5) are consistent with previous studies showing the murine splenic B cell compartment is comprised primarily of T1 B cells as late as one week post partum (5) and that substantial populations of mature B cells are present in the human spleen midway through fetal development (33). Our data from human CB and adult mouse PB show that when humans first encounter pathogens at birth their non-memory peripheral B cell pool is populated with T1, T2 and mature B cells (Fig. 5H) and that the distribution of B cell subsets within this pool is similar to that in adult mice (Fig. 6E). An interesting question is whether the mechanisms at work in the production and maintenance of the adult mouse B cell pool most closely model those at work in humans during adult life or during the fetal/neonatal period.

A comparison of the non-memory B cell pool in adult mice and humans showed dramatic differences. In contrast to adult mice, where transitional B cells predominate in the BM and PB, humans show a non-memory B cell pool comprised of ~70% FM B cells, across tissues in BM, PB and spleen. A comparison of T1 to T2 ratios in the transitional B cell compartment in mice and humans shows that the proportion of T1 cells in BM is greater in the mouse than in humans. Conversely, the proportion of T2 cells, within the transitional B cell compartment, is larger in spleen in humans than in mice. Taken together, these data provide evidence that transitional B cells comprise a much smaller fraction of the overall human non-memory B cell pool than in the mouse. Despite their low frequency, human transitional B cells, give rise to a proportionally much larger naïve B cell compartment than those in the mouse. These data suggest that the dynamics, and potentially the mechanisms, of new B cell production, negative selection, and homeostasis that act in concert to give rise to the non-memory B cell pool present in mice and humans, are different. The disparity in T1 to T2 ratios in mice and humans suggest potential points in B cell development where differing dynamics and/or mechanisms may be at work: 1) reduced *de novo* B cell production and/or increased negative selection among human T1 cells in the BM and/or spleen and 2) increased expansion at the human T2 stage in the spleen and/or periphery.

The production of new B cells declines during life (55), and given the differing life spans in mice and humans, the extent of this decline may not be the same in both species (56). Given the similarity between B cell pools in fetal/neonatal humans and adult mice, it seems plausible that the progressive declines in lymphopoiesis that occur during life and the increased life span of humans compared to mice might be a contributing

factor to the reduction in T1 cells that we observed in human BM as compared to mice (Fig 6R, I).

The above findings raise the question of whether similar mechanisms are responsible for the survival and proliferation of transitional B cells to produce the FM and MZ of the non-memory B cell pool. While BAFF plays a critical role in mouse B cell homeostasis (6, 43), its role in human B cell development is less clear. BAFF levels are elevated in patients with auto-reactive B cells (12), but biologics that target BAFF have been less effective in clinical trials than expected (12, 44), raising the question of whether additional mechanisms may be key players in the production of normal and autoimmune B cells in humans. It is interesting to note that normal serum BAFF levels in mouse are 10 fold higher (57) than those in humans (58), raising the question of whether factors other than BAFF may be more important in the production and maintenance of human B cells. In mouse, recent studies suggest that factors other than BAFF may contribute to these processes (59-61) raising the possibility that they play a role in human B cell development as well. It is also possible that differences in other types of cells that interact with B cells, such as follicular dendritic cells (62), influence B cell differentiation and growth leading to increased efficiency in the production of mature B cells from T1 cells in humans as compared to mouse.

The implications of our data for the differences in mouse and human B cell production and homeostasis are striking if we consider that the reduced transitional B cell compartment in humans is able to give rise to a FM B cell compartment that is 3-6 fold expanded across tissues in humans as compared to mice. Even if the fundamental mechanisms responsible for B cell production and homeostasis are similar in mouse and

humans, at minimum a difference in the dynamics of these processes is needed to account for the difference in composition of the non-memory B cell pool between mice and humans.

The data presented here provide a foundation for understanding how the production and maintenance of the B cell pool in humans is different from that in mice and the CD21/CD24 schema provides an important new tool to address this question. This will be important for understanding B cell-mediated autoimmunity (63) and the reconstitution of B cells following stem cell transplant and radiation or chemotherapy. It will also be important for understanding mechanisms that regulate human B lymphopoiesis, an area of increasing importance since the use of B cell depletion therapies is becoming more prevalent in the treatment of B-cell mediated autoimmune disease and malignancy. The CD21/CD24 schema will facilitate the two-way flow of information from the bench to the clinic allowing data obtained from murine disease models, as well as transgenic and knockout mice to be directly related to analogous human B cell populations identified in patient samples. The ability to isolate B cell subsets from patient samples based on CD21/CD24 co-expression opens new opportunities for functional comparisons of human hematopoietic processes with those in mouse, rabbit and other animals. Information obtained from patient samples can now be used to refine animal models to address the clinical questions that are key to impacting patient care and improving health.

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CHAPTER THREE

USE OF CD21/CD24 MODEL TO ADDRESS HUMAN SPECIFIC QUESTIONS: UNPUBLISHED RESULTS

Abstract

Understanding how human B cell subsets function and are regulated is critical for evaluating their specific role in B cell development and maintenance of tolerance. The translational model can provide us with a framework in order to address human specific questions. The heterogeneity of memory B cell phenotypes in humans could mean specialized function by specific memory subsets. We focused on IgM+CD27+ and IgM+CD27- memory B cells and their co-expression of CD21 and CD24. For further characterization of the IgM+ memory cells, we assessed CD1c, a molecule involved in lipid presentation to γδ T cells. The function of this subset of T cell is still not completely understood. Lastly, we evaluated CD5 transcripts. Alternate splicing of the *CD5 gene* results in the transcription of alternate CD5 isoforms that are truncated and retained in the cytosol. The expression of these alternate isoforms is exclusive to human B cells and is the result of HERV-E insertion into the *CD5 gene*. To our knowledge, our data is the first to assess the alternate CD5 (E1B) transcripts in pre-B cell lines, Nalm-6 and SupB15. The presence of these isoforms has not been evaluated in human transitional B cells.

Abbreviations

HERV -	HUMAN ENDOGENOUS RETROVIRUS
E1A –	EXON 1A TRANSCRIPT
E1B -	EXON 1B TRANSCRIPT
TR-CD5 –	TRUNCATED CD5 PROTEIN
FL-CD5 –	FULL LENGTH CD5 PROTEIN
LTR –	LONG TERMINAL REPEATS

Section One

Introduction

Evaluating key differences in B cell development and function in mice and humans requires further investigation. Some of these differences are memory B cells and B cells subsets only expressed in humans. For example, one of the difficulties in evaluating memory B cells is the phenotypic heterogeneity within this subset (1). Human memory B cells were traditionally identified based on positive CD27 expression. Further studies of human subsets noted the presence of IgG+CD27- population of cells that exhibited the functional characteristics of memory B cells. Thus, CD27 identifies memory B cells, but not all memory B cells express the marker (2). Later characterizations of memory subsets indicated the presence of IgM+CD27- memory subset that is primarily expanded in SLE blood. To date, memory B cells are still being characterized as some have observed under disease state (3, 4).

Another memory B cell subset of interest is the IgM+CD27+ cells. In the spleen, the expression of these markers concomitantly with CD1c expression delineates MZ B cells in humans (5). In contrast to mice where MZ cells remain in the spleen, human studies show the presence of the MZ phenotype in human PB. Thus, these cells have been described as circulating MZ cells (5, 6). CD1c expression has also been evaluated in SLE patients PB. Within the unswitched memory subset (IgD+CD27+) the CD1c-expressing cells were decreased in SLE compared to normal PB (1). The reasons why CD1c-expressing cells would be reduced in SLE have not been elucidated.

CD1 molecules are involved in presenting microbial lipids to T cells (7). Within the CD1 family, only CD1c is expressed in B cells. Presentation by CD1c molecules is restricted to γδ T cells (7). Studies in γδ T cells from mouse and humans show there are

functional differences between the species. For example, human γδ T cells recognize all five CD1 molecules, while mice only express CD1d (8).

The CD21/CD24 model provides a starting point for evaluating human B cell subsets and human specific markers expression such as CD1c. Furthermore, CD21 and BAFF-R expression are closely associated as indicated in mouse studies (9). Evaluating where IgM+ memory cells fall within the CD21/CD24 schema could provide insight into cells that may respond similarly to BAFF as the T2 subset. In the subsequent studies, we evaluated IgM+27+ and IgM+CD27- cells for CD21/CD24 expression. We also evaluated the expression of CD1c in non-memory B cell subsets and we show that T2 and MZ subsets show the highest expression of CD1c.

Materials and Methods

Sample Procurement and Cell Preparation

Human Tissues

CB was collected from the umbilical cord of full-term neonates following caesarian section. PB from adult donors was obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL), and donated by the Blood Processing and Quality Control Lifestream in San Bernardino, CA. Adult spleens between the ages of 30 to 55 years of age were acquired from pathology specimens.

A citrate-phosphate-dextrose solution (Sigma-Aldrich) anticoagulant was added to blood in a 1.4 to 10 ratio. CBMCs were isolated using RBC lysis buffer density gradient centrifugation. Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) and/or RBC lysis density gradient centrifugation was used to isolate PBMCs from blood collected

from filters (10). Spleens were processed using gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA) and then pressed through a 70 μM cell strainer (BD Falcon) to create a single cell suspension in PBS. All human tissues were acquired and handled according to protocols approved by the Institutional Review Board at Loma Linda University.

Flow Cytometry

For flow cytometry, cells were stained with mAbs in PBS with 1% BSA for 20 minutes in the dark at 4ºC. Cells were subsequently washed and fixed with a 1% paraformaldehyde/PBS solution before analysis on BD FACSCalibur flow cytometer (BD Immunocytometry Systems, [BDIS] San Jose, CA) or MACSQuant® Analyzer (Miltenyi Biotec). The anti-human antibodies used were CD19 Pacific Blue, CD24 PE-Cy7, CD38 APC, CD27 APC-Cy7, CD21 FITC, CD1c PE (all from Biolegend, San Diego, CA), CD23 PE, IgD PE, IgM PE-Cy5 (all from BD Bioscience). Living cells were identified by forward scatter and side scatter gating and/or exclusion of 7-aminoactinomycin-D (eBioscience) added immediately prior to data acquisition or fixation. Flow cytometry data analysis was performed using Flowjo data analysis software (TreeStar, Ashland, OR).

Results

Reports on human memory cells indicate that IgM+CD27- memory cells are present in normal PB. To eliminate any of these cells from our evaluation of CD21/CD24 subsets, we developed the non-memory gating strategy. However, we wanted to further evaluate the co-expression of CD21/CD24 in IgM+ cells that were either CD27 positive

or negative. Using multicolor flow cytometry, we stained PB, non-autoimmune and autoimmune spleen with IgM, CD27, CD21, and CD24. In PB, the cells gated IgM+CD27+ fell in the T2 gate while the IgM+CD27- cells primarily expressed an FM phenotype (Figure 14 A). In normal spleen, the majority of the IgM+CD27- cells fell in the FM fate while the CD27+ cells were found in the MZ gate. A few of the IgM+CD27+ cells were observed in the T2 gate. In contrast, autoimmune spleen showed an increased number of cells in the T2 gate that is clearly not observed in normal spleen (Figure 14 B).

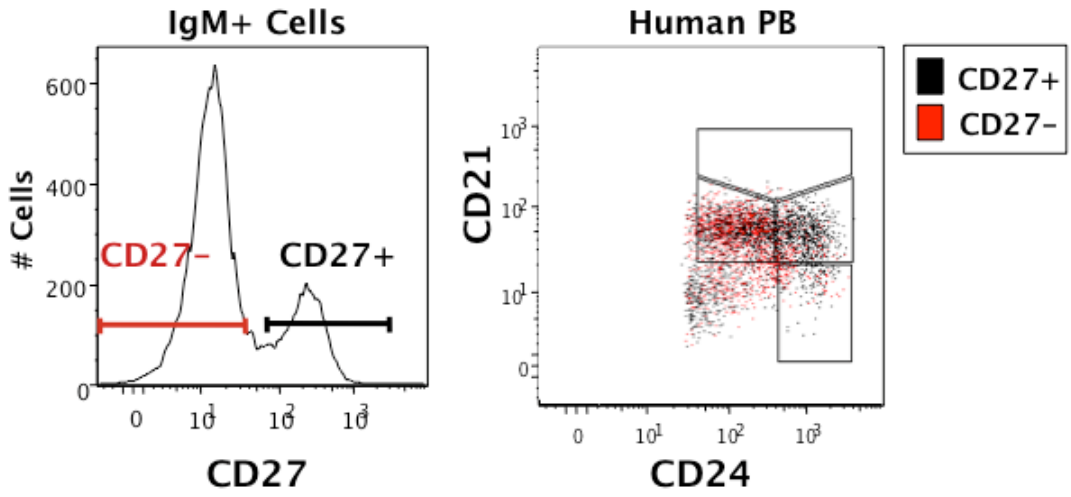
We then evaluated two autoimmune spleens, R3 and R4. We stained these cells with IgM, CD27, CD21, and CD24. First, we gated IgM+ cells and subsequently gated CD21 and CD24. We evaluated CD27 expression in the subsets as seen in Figure 15 (*left panels*). Both R3 and R4 spleen MZ cells have higher levels of CD27, while only the T2 cells in R4 showed higher levels of CD27. Both spleens showed that the CD27+ cells were mainly distributed in the MZ and T2 gates. While the CD27- population fell mainly expressed the FM phenotype (Figure 15 A and B).

We wanted to further evaluate the IgM+CD27+ cells since these cells were observed in both PB and MZ as well as expanded in autoimmune spleen. We evaluated human spleen and PB for CD19+IgM+ cells for CD1c and CD27 co-expression. CD1c is highly expressed in MZ cells along with CD27 (5). We observed that the CD1c+CD27+ cells expressed an MZ phenotype in spleen. However, in PB, these cells expressed a T2 phenotype with some cells falling in the T1 gate (Figure 16 A and B). Thus, the circulating MZ cells (CD1c+CD27+), as described by others fall in the T2 gate not in the MZ gate.

We wanted to further evaluate the IgM+CD27- memory population (Figure 17 A-C). Previous studies have shown that they express varying levels of CD24 (1). We gated IgM+CD27- cells in human spleen, PB and BM. Then, we delineated transitional, mature and memory subsets based on CD24/CD38 co-expression as described (11). Within all the tissues examine, the memory gate included a small number of cells. When we demarcated these cells for CD21/CD24 co-expression, the cells had a T2 phenotype. In the spleen (Figure 17 A), we observed some cells in the MZ gate and these could be precursors of MZ cells as described in mouse studies (12).

We wanted to evaluate the IgM+CD27- cells for markers that although expressed in mouse, studies indicate they may be regulated differently in humans (8). We evaluated expression of CD23, IgD, and CD1c in IgM+CD27- cells from spleen, PB, and CB. We assessed expression of these markers by the CD21/CD24 subsets. In all the tissues, CD23 was expressed highest in FM cells (Figure 18 *left panel)*. In spleen, the T2 population showed heterogeneous expression of CD23 with a high and low population. IgD was highly expressed in all T1, T2, and FM cells. A decrease in expression was observed in MZ cells in the spleen (Figure 18 *middle panel)*. Assessment of CD1c expression showed that consistently in all tissues with exception of spleen, T2 cells show the highest level of CD1c expression (Figure 18 *right panel)*. In CB, the difference in CD1c expression within the subsets is less pronounced. This could be attributed to CB being fetal tissue (13).

A



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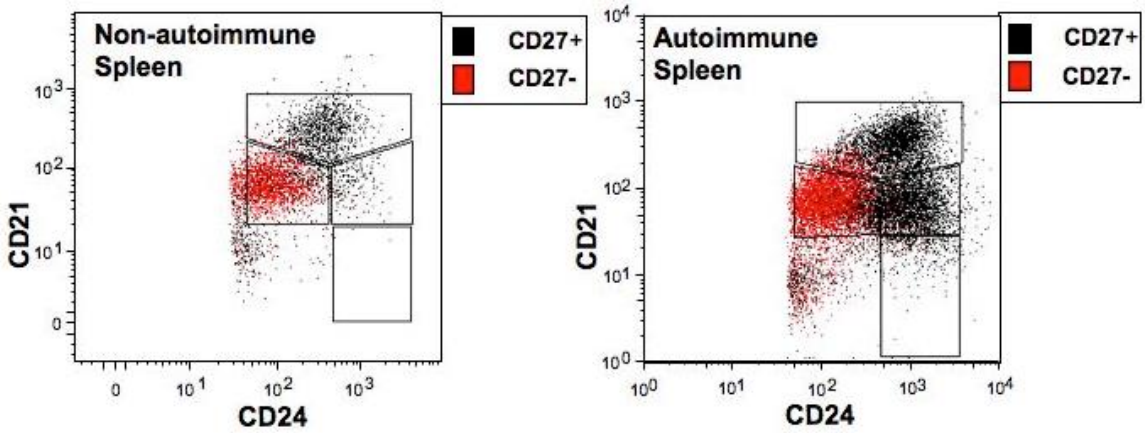

Figure 15. IgM+CD27+ cells can display a CD21/CD24 T2 phenotype as observed in both human PB and adult spleen. PB, normal spleen, and autoimmune spleen were stained with fluorescent antibodies against IgM, CD27, CD21, and CD24. The cells were subsequently run with flow cytometry. *A,* Histogram shows CD27 gating of IgM+ cells and (*B)* dot plot of overlays of CD21 and CD24 expression of CD27+ (red) and CD27- cells (black) cells.

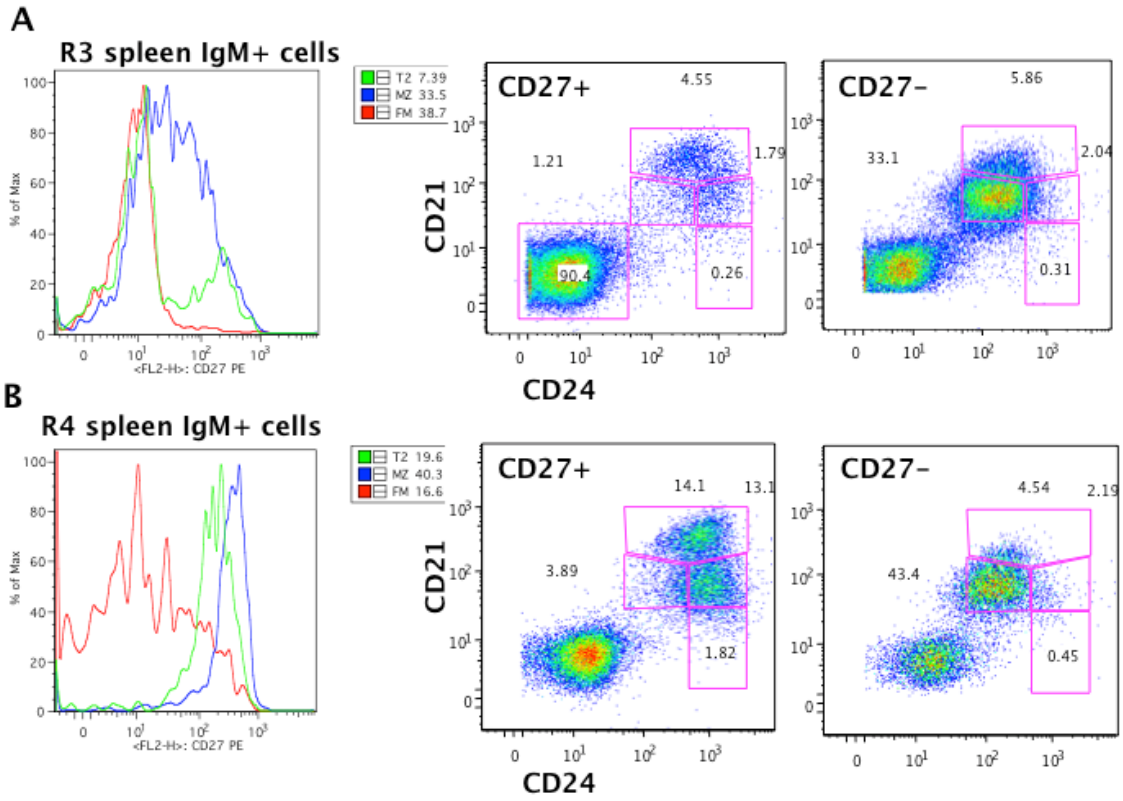

Figure 16. Comparisons of two autoimmune spleens show that IgM+CD27+ cells fall in both MZ and T2 gates. Two autoimmune spleens (R3) and (R4) were stained with fluorescent antibodies against IgM, CD27, CD21, and CD24. The cells were subsequently run with flow cytometry. *A-B left panels,* Histograms show overlays of IgM+ CD21/CD24 subsets cells and their CD27 expression. (*A-B right panels),* Dot plot display CD21/CD24 co-expression from CD27+ or CD27- cells.

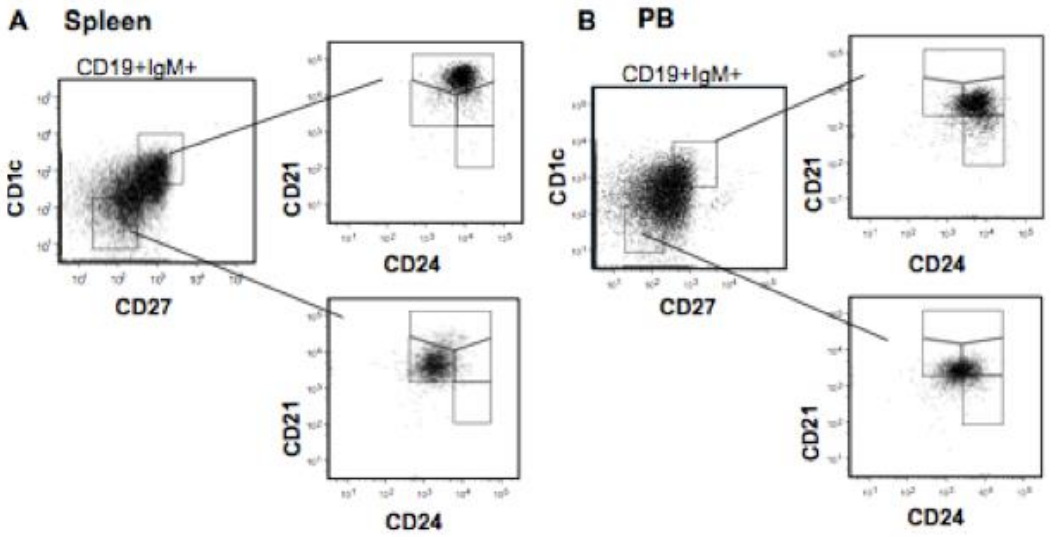

Figure 17. CD1c+CD27+ cells in spleen fall in the MZ gate, but in PB they show a T2 phenotype. PB and normal spleen were stained with fluorescent antibodies against CD19, IgM, CD27, CD21, CD24 and CD1c. The cells were subsequently run with flow cytometry. *A-B* CD19+IgM+ cells were first evaluated for CD1c/CD27 co-expression and positive and negative gates were drawn. Gates were assessed for CD21/CD24 co-expression.

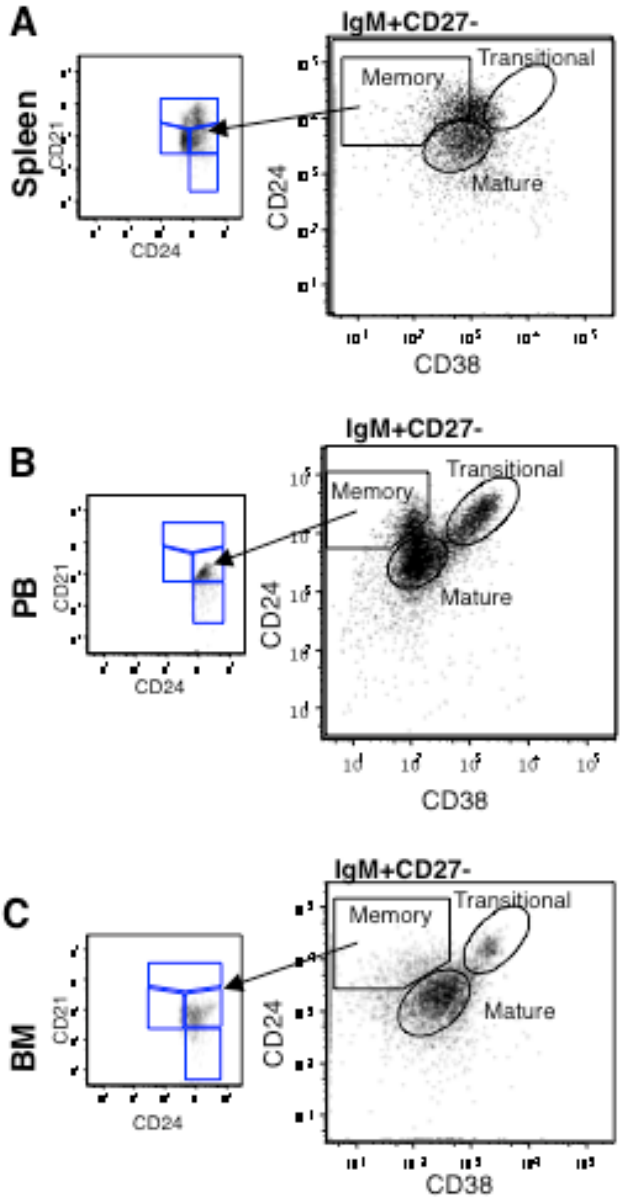

Figure 18. The IgM+CD27- cells with a memory phenotype fall in the T2 gate. Spleen, PB, and BM were stained with fluorescent antibodies against IgM, CD27, CD21, CD24, and CD38. *A-C right panels*, IgM+CD27- cells were demarcated into memory, mature, and transitional based on CD24 and CD38 co-expression. *A-C left panels*, memory cells were then evaluated for CD21/CD24 co-expression and subsets were delineated.

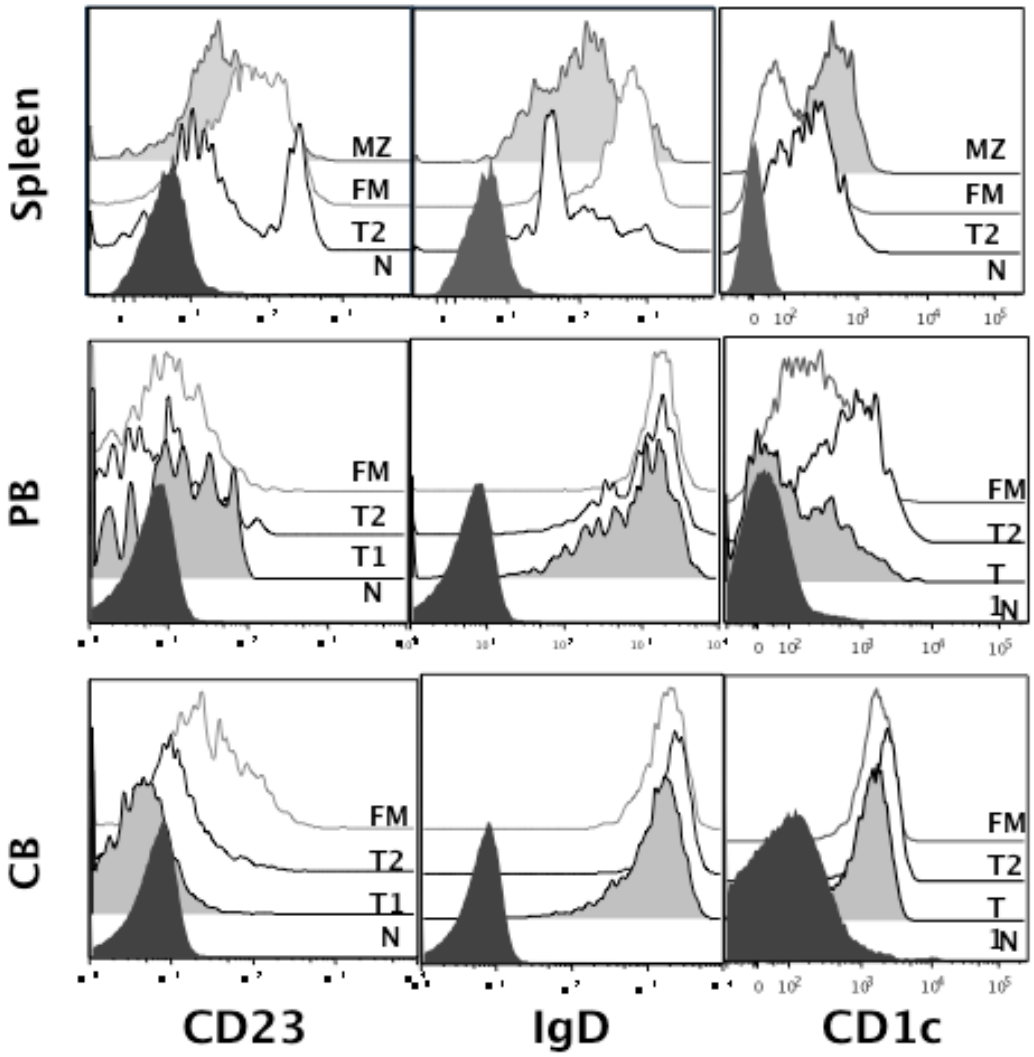

Figure 19. Expression of human specific markers by CD21/CD24 subsets. Spleen, PB, and CB were stained with fluorescent antibodies against CD19, IgM, CD27, CD21, CD24, and CD23, IgD, and CD1c. CD19+IgM+CD27- cells were delineated into CD21/CD24 subsets. Shown are histograms with CD23, IgD, and CD1c expression overlays of isotype (N), T1, T2, FM, and MZ cells.

Discussion

Our assessment of IgM+CD27+ cells and their expression of CD21/CD24 showed that that these cells fell in the T2 gate. Unlike the naïve mature (FM) cells, which lose CD24 expression with maturation. IgM+CD27+ cells retain their CD24 expression. A possible reason why these cells retain their CD24 expression may be due to activation of T2 cells prior to their full maturation. In mice, T2 cells under activation stimuli mature as indicated by decrease in CD21 and CD24 expression (14). Studies in humans show that patients unable to carry GC reactions can generate IgM+CD27+ cells with mutations on their BCRs (2). Thus, IgM+ naïve mature cells may be generated and activated as indicated with CD27 expression without the spleen. Furthermore, IgM+CD27+ cells have been considered circulating MZ cells as they participate in T-independent reactions and recognize capsulated bacteria (6).

Our use of CD27 and CD1c co-expression showed that the MZ phenotype when assessed in the spleen, the cells fell in the CD21/CD24 MZ gate. Cells in the same CD1c+CD27+ gate fall in the CD21/CD24 T2 gate. Interestingly, autoimmune spleen shows that the IgM+CD27+ population falls in both MZ and T2 gates. A possible explanation for the prevalence of IgM+CD27+ cells expressing the T2 phenotype could be that these cells are immature cells that may have encountered antigen and become activated as described in mouse studies. Furthermore, these cells express CD1c and may express a lineage of B cell dedicated to presenting to γδ cells. In humans, CD24 expression decreases upon maturation and is further decreased in activated B cells (15). Yet, IgM+CD27+ cells retain their CD24 expression regardless of activation. Further studies are needed to evaluate these IgM+CD27+ cells in both normal and autoimmune

tissues. CD24 expression is low in activated B cells that would be CD27+ (15). The high expression of both CD24 and CD27 creates a paradox in order to understand these cells. It is possible that CD24 could demarcate between functionally different IgM+CD27+ cells. Furthermore, the high expression of CD24 on these cells supports the notion that further in-depth analysis of B cell phenotypes is necessary.

Most studies primarily rely on a few markers to identify specific subsets (16, 17). Yet, multi-color flow cytometry of more than 4 colors show that these specific subsets display heterogeneity. Identification of novel B cell subsets is important for evaluating the B cell pool in both healthy and diseased individuals. Abnormal expansion or absence of specific B cell subsets within the B cell pool would be indicative of abnormal B cell homeostasis. We have recently begun to discover the heterogeneity of B cell subsets (18).

Section Two

Introduction

Human transitional B cells express surface CD5 and this expression decreases upon maturation (19). Surface CD5 expression on B cells can affect B cell responses to growth or activation stimuli via the B cell receptor (BCR) (20, 21). CD5 directly ligates the BCR and thereby modulate BCR signaling. CD5 positive B cells display hyporesponsiveness to BCR ligation when compared to CD5-negative B cells indicating CD5 increases the activation threshold for a naïve mature B cell to become activated (22). Aberrant CD5 expression may promote the presence of naïve mature auto-reactive B cells has observed in B cell-mediated autoimmune diseases (22). BCR hyporesponsiveness may allow auto-reactive B cells to evade mechanisms that eliminate self-reactive B cells. Hence, CD5 has the potential to contribute to auto-reactivity by influencing how a developing B cell responds to self-antigen.

CD5 Expression and BCR Response

Membrane CD5 expression is closely regulated as CD5 decreases BCR activation in response to stimuli. CD5 expression in murine B cells display hyporesponsivess to BCR ligation when compared to CD5-negative B cells. CD5+ B cells have low intracellular Ca^{2+} mobilization, decreased proliferation, and increased apoptotic susceptibility after BCR cross-linking (23). Lower intracellular Ca^{2+} mobilization promotes the induction of RAG expression causing receptor editing. Survival of CD5+ B cells is linked to BCR affinity. B cells with low BCR affinity do not receive growth signals resulting in deletion while B cells with intermediate BCR affinity upregulate CD5 expression and with CD40 engagement survive. T-independent stimulation can also

induce CD5 expression. This process would positively select those B cells that have the higher affinity BCRs to mature and differentiate. Essentially any B cell can express CD5, as CD5+ B cells generate CD5- B cells, but survival is dependent on BCR affinity (23).

CD5 expression in self-reactive B cells raises the activation threshold for the BCR. Studies in CD5-deficient mice indicate that CD5 cross-linking has negative effects on BCR-mediated signaling due to its association with the SH2-containing phosphatase 1 (SHP-1) (21, 24). SHP-1 plays a critical role in modulating BCR signal transduction since B cells from moth-eaten mice that have a mutation in SHP-1 show hyperresponsiveness (21, 24). CD5 via SHP-1 affects proximal signaling in B cells (24). CD5 expression is associated with activation of RAG1/2 transcription (21, 23). Interestingly, despite lack of CD5 surface expression, in CD5-negative B1b cells contain CD5 mRNA. CD5 expression completely disappears upon EBV transformation of B cells (25).

CD5 expression is tightly regulated in B cells as its presence decreases BCR responsiveness to stimuli thereby increasing activation threshold (22). In other words, only antigens with very strong affinity would induce BCR activation. Endogenous expression of CD5 is not present in normal B cells except in fetal-derived B cells (B1a) and according to some studies in transitional B cells (19, 26). Moreover, CD5 expression in transitional B cells seems to be present in humans and not in their mouse counterparts (11, 16, 17, 19, 27). CD5 presence in transitional B cells and B1a cells must have biological consequences, which have not been fully explored.

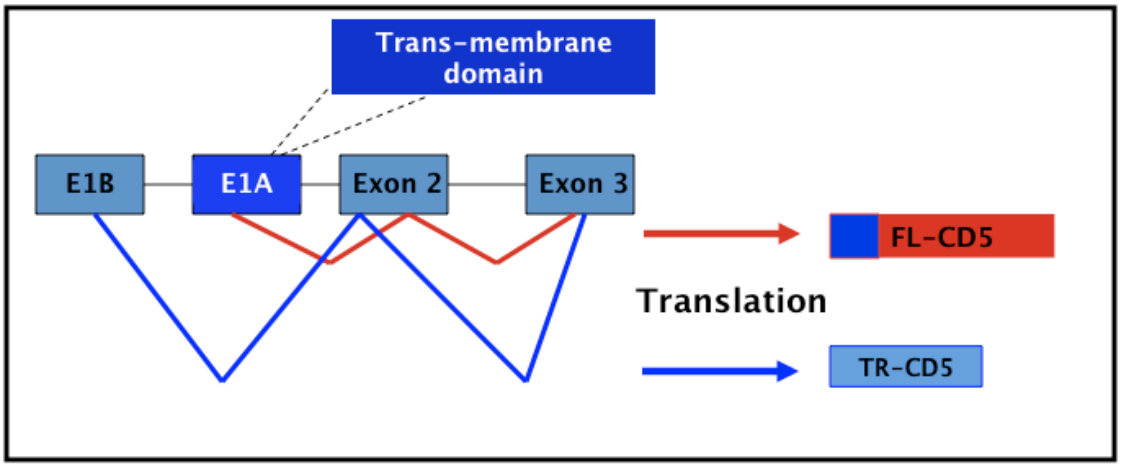

Figure 20. Alternative splicing of the *CD5 gene*. Splicing of exon E1A results in the translation of full length (FL-CD5) protein that contains a transmembrane domain. When exon E1B is splice, the resulting protein is truncated (TR-CD5) due to lack of the transmembrane domain. Figure adapted from Renaudineau et al. (21).

Human CD5 Isoforms

Human B cells are unique in that they express CD5 in two isoforms where one produces a full length CD5 (FL-CD5) on the cell surface and the other a truncated CD5 (TR-CD5) protein that is retained in the cytosol (Figure 1) (21). The human CD5 locus contains a human endogenous retrovirus (HERV) gene insertion upstream of the CD5 gene resulting in the exclusive transcription of an alternative exon 1 in B cells (21, 28, 29). Transcription from the retroviral sequence results in the transcription of an alternative exon 1 in B cells (21, 25). This alternative CD5 exon 1 is assigned E1B to distinguish it from conventional exon 1 (E1A) (Figure 1). E1B is located 8.2 kb upstream of E1A within the *CD5* gene (21). E1A transcription contains the translation initiation site that gives rise to FL-CD5 protein that is expressed on the membrane. E1B transcripts produce truncated TR-CD5 that lacks the leader peptide hence its retention in the cytosol. E1A transcripts are found in both B and T cells, while E1B have not been identified in T cells (21, 25).

Studies suggest CD5 expression in humans is regulated at the protein level. Surface CD5 expression in human B cells may be controlled by the interaction between the two alternative exons (21). The decision to synthesize E1A or E1B isoforms influences CD5 membrane expression. Since they are not translocated to the membrane, E1B products downregulate membrane CD5 expression. E1B transcription splices out E1A giving rise to CD5 molecules that lack the leader peptide and are therefore sequestered in the cytoplasm. Consequently, SHP-1 is not recruited and this results in increased antibody production (25).

A possible relationship may exist between density of membrane CD5 and the E1A transcription, as cells that have a high expression of CD5 such as in CLL strictly transcribe E1A transcripts (21). B cell activation supports the transcription of E1A and raises the membrane density of CD5. CD5 expression is observed after BCR cross-linking or engagement by T-independent type 2 antigens (21, 22). By increasing CD5 expression, BCR signaling is attenuated and may prevent cells from BCR engagement by self-antigen or apoptosis (22, 25). Full-length CD5 protein takes SHP-1 to the membrane causing an increase of phosphatases to surround the BCR and promoting tolerance of self-reactive B cells. The truncated CD5 protein does not carry SHP-1 to the membrane and increases the strength of BCR-mediated signaling and facilitates B cell expansion (24, 25). Experiments with Jurkat T cells transfected with E1B-cDNA showed that the truncated CD5 protein decreases the surface expression of full-length CD5 (25).

TR-CD5 results from alternative splicing of the transcribed *CD5 gene*. The *CD5 gene* contains a human endogenous retroviral (HERV) insertion that provides an alternative exon 1 (E1B) (22). A HERV insertion into the *CD5 gene* provides alternative exon E1B that is only expressed in human B cells and not in T cells. The biased expression of E1B in B cells may be associated with the nature of the E1B exon (21). E1B exon contains HERV-E elements that may alter how this exon is regulated (Figure 2). Alternative splicing of E1B results in the translation of a TR-CD5 protein.

Studies indicate that the expression of E1B is suppressed in healthy controls via DNA methylation. However, SLE patients display hypomethylation of E1B after BCR-mediated B cell activation and show higher levels of E1B. This outcome was also

observed in experiments where DNMT1s were blocked, E1B transcript levels increased and E1A levels decreased (22, 25).

The two CD5 isoforms can interact in the cytoplasm where TR-CD5 can sequester FL-CD5 preventing its translocation to the cell membrane. Overall, the expression of E1B suggests another mechanisms by which FL-CD5 expression can be regulated. Surface CD5 expression in human B cells may be controlled by the interaction between the two alternative isoforms (21). TR-CD5 can bind to FL- CD5 and sequester it inside the cytoplasm. Consequently, SHP-1 is not recruited and this results in BCR hyperresponsiveness (Figure 3) (21). Surface CD5 raises the threshold for the BCR response triggering the expression of RAG genes that would allow for receptor editing (23). This revision may play a role in preventing autoimmune disease. Aberrations in CD5 expression have been observed in autoimmune disease (17).

The presence of E1B decreases CD5 surface expression and prevents signaling functions of CD5 such as the translocation of SHP-1. Failure to translocate SHP-1 would prevent the production of antibodies against infectious agents as well as autoantibodies. CD5 raises the threshold for the BCR response triggering the expression of RAG genes and inducing revision of immunoglobulin variable genes. This revision may play a role in preventing autoimmune disease. Recent findings suggest that the modulating effects of CD5 signaling on the BCR may prevent autoimmunity rather than promoting auto-antibody-related diseases. Aberrations of the transduction through CD5 have been observed.

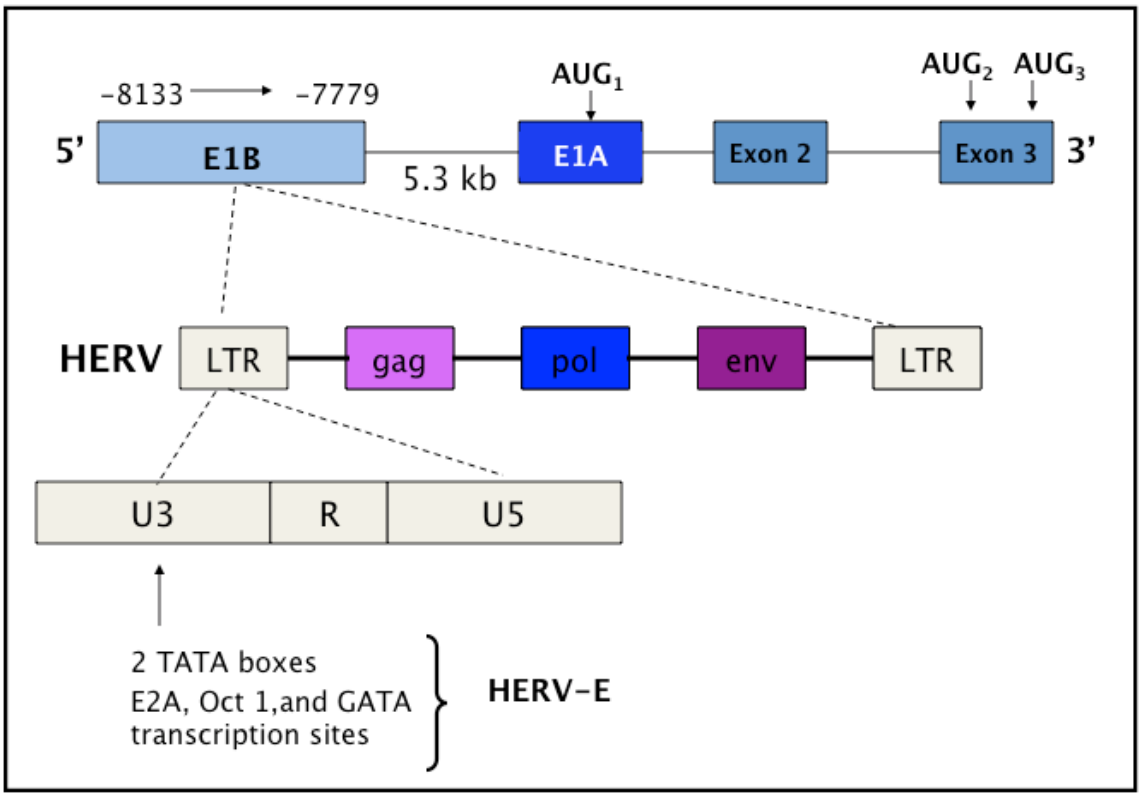

Figure 21. *CD5 gene* E1B exon is a result of a HERV insertion. Full-length HERVs contain an LTR, *gag* (secretion), *pol* (reverse transcriptase), and *env* (envelope) genes (30). All ERVs have long terminal repeats (LTRs) that border the coding sequences. LTRs contain regulatory sequences such as a promoter, enhancer, polyadenylation signal, and factor-binding sites (31). The LTR is composed of a U3 (3' unique region), R (repeat), and U5 (5' unique region). The U3 on the 5' LTR functions as a viral promoter. Host cellular transcription regulatory elements can utilize the U3 promoters to control ERV expression (32). HERV families display differences in transcription, as LTR and ERV elements may innately possess enhancer, promoter, and polyadenylation functions (33, 34). HERV-E insertions into the promoter regions of some genes induce locus rearrangement and alteration of tissue-specific expression (35).

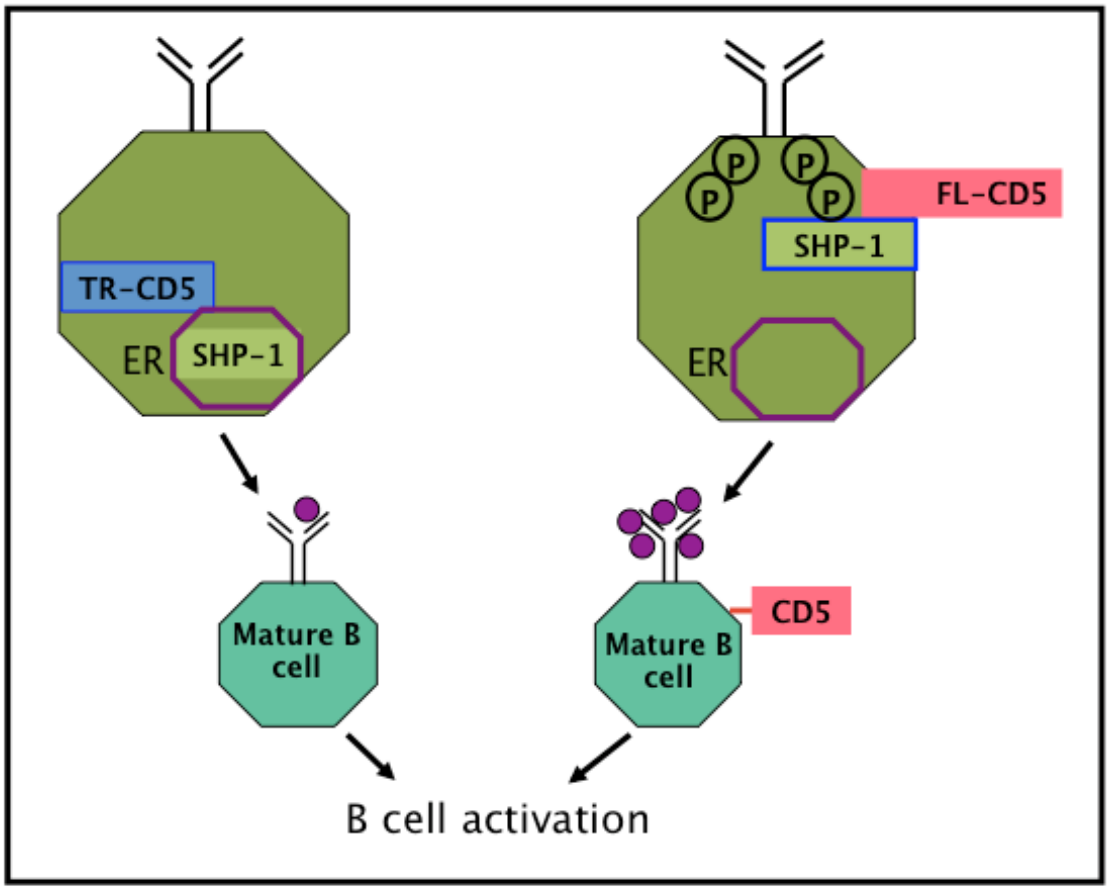

Figure 22. CD5 isoforms either promote sequestration of SHP-1 or transport to the cell surface. TR-CD5 lacks the transmebrane domain and does not have the leader peptide. TR-CD5 remains in the cytosol. FL-CD5 contains a leader peptide that homes it to the cells surface. In the ER, SHP-1 binds to the FL-CD5 thus getting transported close to the cell surface and BCR transmembrane domain. SHP-1 binds to phosphatases that will be used in BCR signal transduction. Thus, SHP-1 binding to phosphatases results in BCR hyporesponsiveness where more activation stimuli is needed to commence signal transduction. Consequently, CD5 attenuates BCR response to activation stimulus.

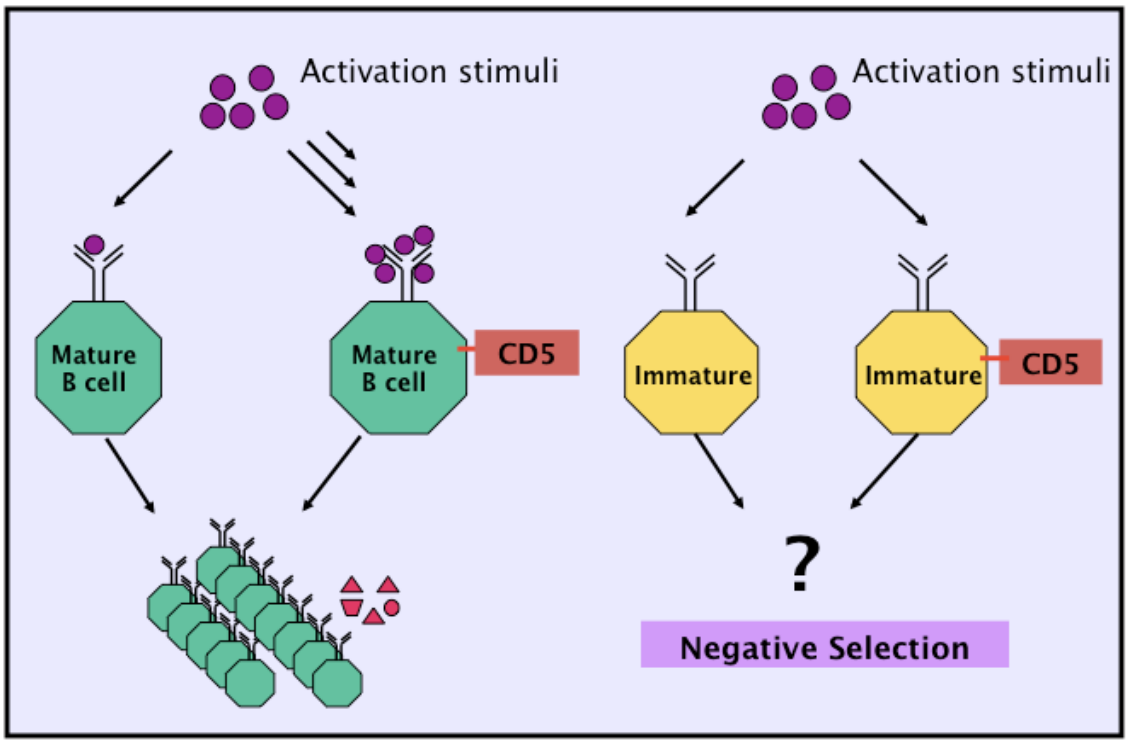

Figure 23. Human immature B cells express CD5. CD5 expression on mature B cells renders them hyporesponsive to activation stimuli. In immature B cells, negative selection of auto-reactive B cells is mediated through strenth of BCR response to self-antigen. Thus, CD5 expression on immature cells could play a role in negative selection process and may promote more auto-reactive B cell maturation.

FL-CD5 is expressed in transitional B cells and may play a role in the negative selection of auto-reactive immature B cells via association with the BCR. Studies suggest CD5 is transiently expressed in transitional B cells in adults (19, 27). A high proportion of fetal-derived B cells in CB exhibit surface CD5 expression and our preliminary data show that a large proportion CB B cells express markers of mouse T1 B cells. Studies indicate that CD5 is upregulated in some B cell subsets in autoimmune diseases (36). The presence of TR-CD5 isoform down-regulates membrane CD5 levels.

In the case of transitional B cells, membrane CD5 may allow auto-reactive B cells to bypass peripheral tolerance mechanisms that are heavily dependent on BCR activation by reducing the threshold for BCR activation (25). CD5 mRNA transcripts are known to be present in CD5 negative B cells (21). Expression of FL-CD5 is down-regulated by the presence of (TR-CD5) (25). The expression of CD5 isoforms in human transitional B cells has not been reported.

Materials and Methods

Sample Procurement and Cell Preparation

Human Tissues

CB was collected from the umbilical cord of full-term neonates following caesarian section. PB from adult donors was obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL), and donated by the Blood Processing and Quality Control Lifestream in San Bernardino, CA. A citrate-phosphate-dextrose solution (Sigma-Aldrich) anticoagulant was added to blood in a 1.4 to 10 ratio. CBMCs were isolated using RBC lysis buffer density gradient centrifugation. Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) and/or RBC lysis density gradient centrifugation was used to

isolate PBMCs from blood collected from filters (10). Nalm6 and SupB15 pre- B cell lines were donated. All human tissues were acquired and handled according to protocols approved by the Institutional Review Board at Loma Linda University.

Polymerase Chain Reaction

PBMCs, CBMCs, and Pre-B ALL cell lines were re-suspended in RNA STAT60 (Tel-Test Inc, Gainesville, FL). Total mRNA was subsequently extracted using chloroform/ethanol precipitation method. cDNA was synthesized using Qiagen Omniscript kit (Qiagen, Alameda, CA). Reverse-Transcriptase was carried out using Hotstart TaqDNA Polymerase (Qiagen, Alameda, CA). Primers for CD5 isoform amplification were: CD5 Reverse 5' –AGG CCC AGA GAG TGA CAC AT-3, CD5 Forward (full length) 5'-ATG CCC ATG GGG TCT CTG CAA C-3', CD5 Forward (truncated) 5'-GGA CTC TGG CCA GCT TGA-3'. Touchdown PCR was performed with the following thermocyler conditions: 15 min at 95 C, 5 cycles of 30 s at 94 C, 40 s at 62.2 C, 1 min at 72 C, 25 cycles of 30 s at 94 C, 40 s at 54 C, 1 minute at 72 C, and final extension of 10 min at 72 C.

Results

CD5 isoforms have been evaluated in PB and PB from SLE patients. SLE patients express higher levels of FL-CD5 transcripts (21). The purpose of these studies was to evaluate the expression of CD5 isoforms and optimize PCR conditions for studying isoforms in human transitional B cells. We evaluated CD5 isoforms in human PB (Figure 23). Due to the HERV-E insertion in the *CD5 gene*, the translation initiation sites for the truncated protein are found within Exon 3. Next, we evaluated CD5 isoform transcripts in

two different (Figure 24). The truncated CD5 transcripts were not as readily detectable compared to surface CD5. Lastly, we evaluated CD5 transcripts in CB, PB, SLE PB, RA PB, and pre-B cell lines, Nalm-6 and SupB15 (Figure 25). We did not observe surface CD5 transcripts in both Nalm-6 and SupB15, while they were present in SLE and RA PB. When we assessed expression of the truncated CD5 transcripts, Nalm-6 and SupB15 expressed the transcripts. RA did not express the truncated transcripts. Finally, these studies are the first to assess for alternate truncated CD5 isoforms in RA PB, Nalm-6, and SupB15, which is characterized as being CD5 negative.

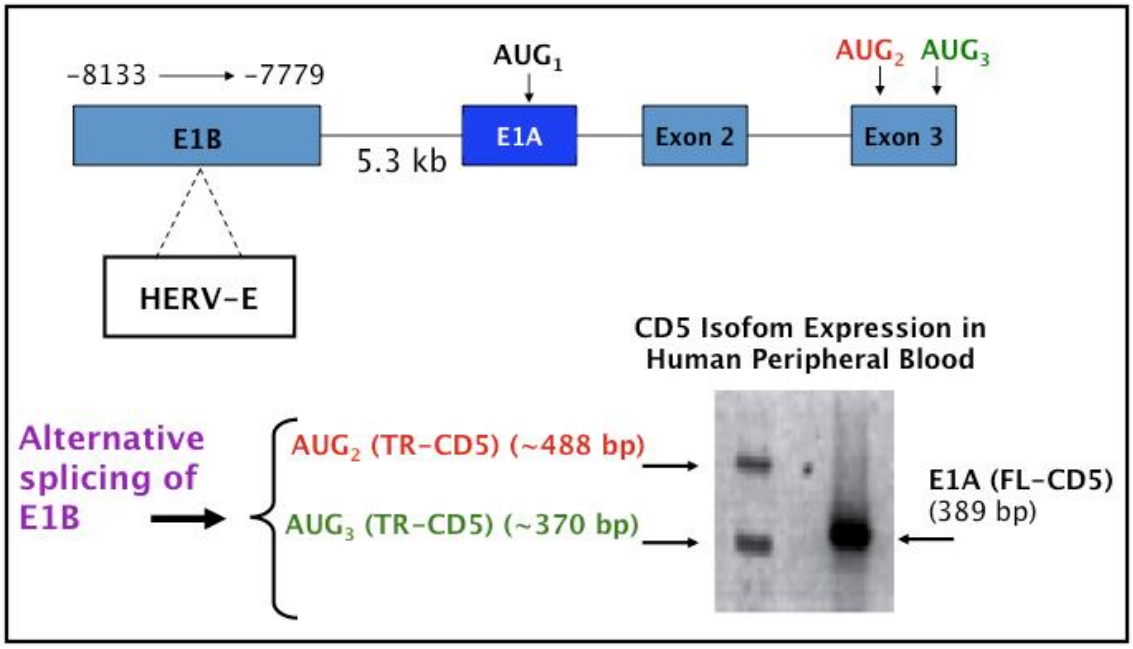

Figure 24. HERV-E insertion in the *CD5 gene* and subsequent splicing favoring E1B exon results in alternate CD5 transcripts. Splicing produces two transcripts that yield the truncated CD5 protein. Touchdown RT-PCR of PBMCs to assess CD5 transcripts yielded E1A transcript (FL-CD5) and two E1B transcripts (TR-CD5).

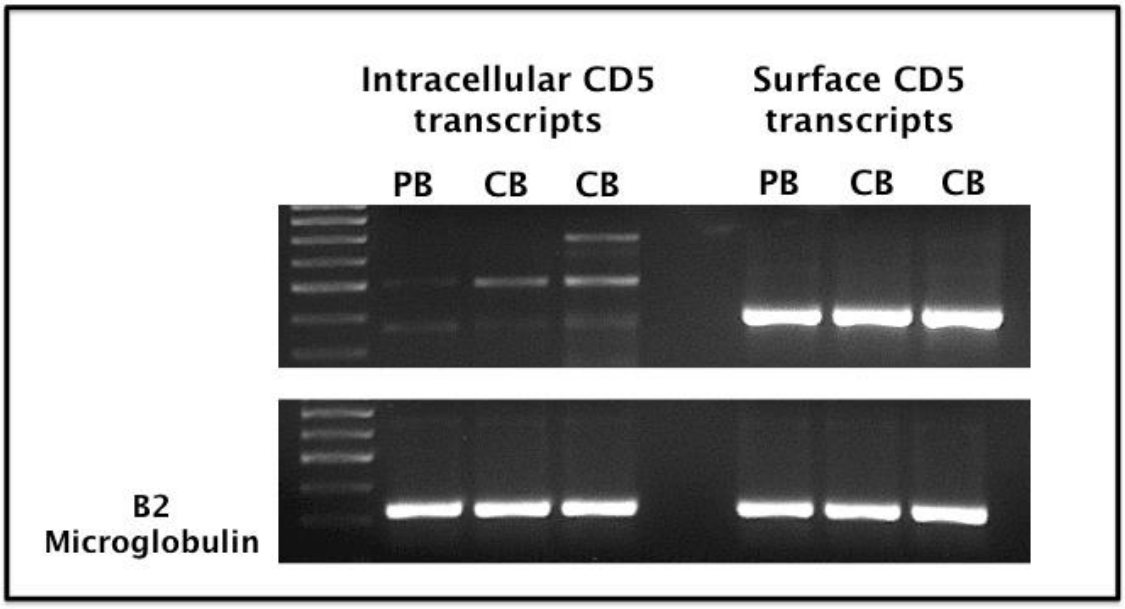

Figure 25. RT-PCR of CD5 transcripts shows they are present in PB and CB. PBMCs and CBMCs were evaluated for CD5 transcript expression E1B (intracellular CD5) and E1A (surface CD5) using touchdown RT-PCR.

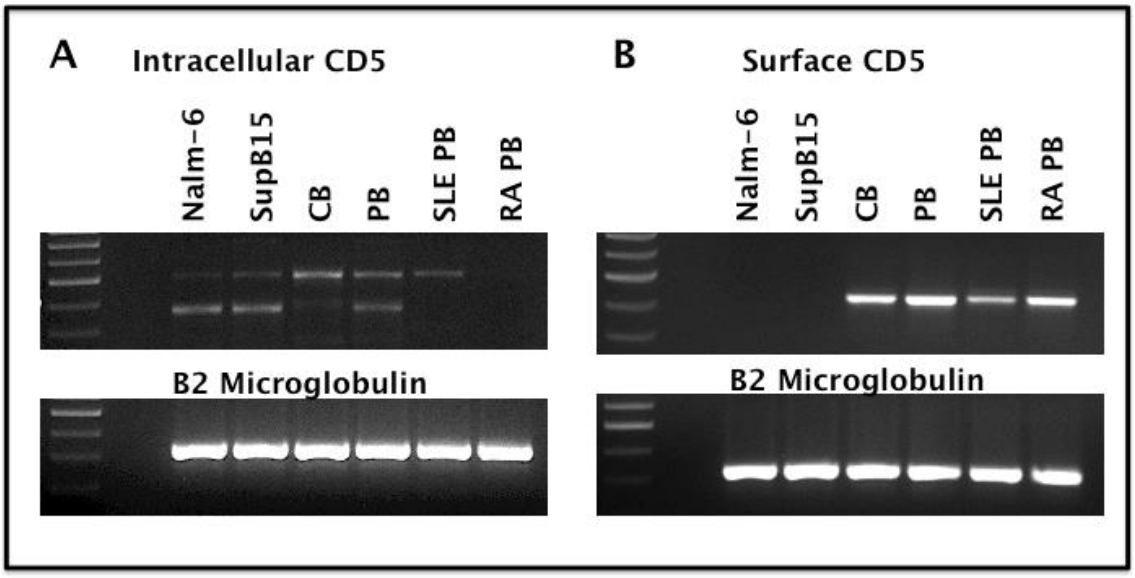

Figure 26. CD5 E1B transcripts were observed in pre-B cell lines and RA PB. CB, PB, RA and SLE PB, and Nalm-6 and SupB-15 were assessed for CD5 transcripts using touchdown RT-PCR.

Discussion

Previous studies showed that B cells uniquely express the truncated form of CD5 (21, 25). The selective expression of the TR-CD5 could be attributed to the HERV-E insertion in the *CD5 gene*. HERV-E insertions can result in tissue-specific expression of that gene. We assessed for CD5 transcripts in PB and CB as well as autoimmune blood. SLE PB has previously been assessed for transcripts and the B cells show increased levels of CD5 both at transcript and protein level. This increase in expression may be due to demethylation status observed in SLE. Demethylation results in expression of HERV elements in human genes (22). Our studies were the first to evaluate RA and pre-B cell lines. RA showed only E1A transcripts and this might be due to lack of B cells. We must enrich for B cells in RA blood and evaluate these transcripts. The most interesting part of our data was the expression of alternate transcripts in the pre-B cell lines. SupB-15 is considered CD5 negative in its panel of identifying markers. The potential reasons for expressing these alternate transcripts in these cell lines need to be further explored. From other CD5 studies, we know that SHP-1 is transported near the cell surface and there attenuates BCR signaling. TR-CD5 isoforms keep SHP-1 sequestered in the ER. The role TR-CD5 proteins could play in pre-B cell lines could provide understanding of aberrant mechanisms that occur in pre-B ALL cells.

Overall, TR-CD5 expression is a key difference between mouse and human B cells. While mouse B1 cells express CD5 and also those naïve B cells that have been activated, human studies have yet to build a case for the presence of B1 cells (37). In humans, fetally-derived B cells express CD5 as well as activated B cells. Interestingly, human transitional B cells express CD5 and expression is lost upon reaching maturation status(19). The differences in CD5 expression between the species raise the question of

whether mouse B1 cell studies can be translated to human. A possible explanation could be the difference in lifespan between the two species. CD5+ B1 cells in the mouse arise at birth and are present in large numbers throughout mice lifespan since in human CD5+ cells tend to be fetally-derived and seem to decrease as humans age (11, 20).

If the identifying marker for B1 cells is CD5 expression in mouse, human CD5 expression on a B cell does not necessarily identify it as a B1 cell. Mouse B1 cells tend express poly-reactive BCRs that are not very specific.

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CHAPTER FOUR

DISCUSSION

The success of B cell targeted therapies in treating autoimmune diseases such as SLE and RA indicate that B cells can be viewed as the primary instigators in initiating massive inflammatory responses via T cell interactions (1). Previously, B cell function was mainly considered under the regulation of T cells (2). Recent studies have shown that B cells can mediate effector functions without T cell help (1, 3). Thus, the presence of mature naïve auto-reactive B cells has the potential to contribute to initiating an immune response or exacerbating using both T cell-dependent and independent functions (4).

Mouse studies have made important discoveries elucidating the potential mechanisms that permit auto-reactivity during B cell development (5-7). While in humans, clinical studies have provided insights into aberrations in B cell population dynamics under autoimmunity (6, 8, 9), which indicate abnormal B cell population homeostasis. Despite the wealth of information in mouse and human B cell studies, the application of knowledge gained from the studies was difficult due to lack of a translational system (10-12). Specifically, through mouse models various schemas for transitional B cell development have been proposed (13, 14). Studies on transitional B cell development is important for understanding how auto-reactive B cells evolve or bypass selection mechanisms that get rid of them (4, 15). Moreover, transgenic models where B cell development displays abnormal regulation show extensive autoimmune dysregulation (5, 7). Mouse models then provide understanding of how B cell tolerance

works in maintaining overall immune health (5, 7). They provide insight into the role B cells play in adaptive and innate immune responses. These studies highlighted how B cells can have dual roles by displaying both damaging and protective traits (16, 17). The protective characteristics of B cells is important to further explore as B cell-depletion or targeted therapy is increasingly being used to treat autoimmune diseases as well as transplant patients (3, 6, 18).

Our studies using CD21/CD24 co-expression to develop a translational model between mouse and human have provided insights into differences in the composition of the B cell pools between the species. The model provided a consistent method to study analogous populations of transitional and mature B cell subsets in mice and human. Furthermore, it provided a method to assess human MZ B cells in combination with transitional and follicular mature naïve B cells. From a systematic analysis of B cell subset populations in mouse and human hematopoietic tissues, we concluded that human B transitional production is much lower compared to mouse. However, the number of naïve B cell produced per transitional B cells is about 3- to 6- fold higher compared to mouse. The discrepancy in naïve B cell production indicates that humans may utilize different homeostatic mechanisms to maintain the B cell pool numbers.

More importantly, this model provided a method for identifying human transitional B cell subsets whose function can be correlated to its mouse counterpart. For example, the human T2 subset can functionally be assessed for its response to BAFF in the context of homeostatic proliferation. Studies by Meyer-Balhburg et al. (19) showed that CD21/CD24 T2-intermediate population under lymphopenia and in the presence of BAFF undergoes proliferation and maintain more auto-reactive B cells. Mouse splenic T2

cells undergo homeostatic proliferation and this population contains a high number of auto-reactive B cells(19). This property of mouse T2 cells should be explored in human T2 cells in all hematopoietic tissues.

Although human studies are generating a wealth of human B cell development data from both B cell depletion therapy (1, 3) and hematopoietic stem cell transplantation (HSCT) (20), human studies are limiting and must rely on mouse studies. For example, transgenic mouse models provided insights into genes and signaling pathways involved in the differentiation of a B cell subset into one or two lineages as described for T2-follicular precursor cells in mouse spleen (13). Mouse models of disease also provide the initial trial for therapies that will be used for humans (5, 21). Thus, human studies will remain interconnected to mouse studies, but the parameters for the extrapolation of data between the species must be systematic and efficient (10, 12, 22).

Human-Specific Characteristics of B Cells

Once human B cell studies develop parameters for extrapolating mouse data, human studies can begin to address human-specific questions relating to B cell development. One example of differences in human B cells is the presence of unique surface markers that are expressed only in human B cells or expression of markers whose function differs from mouse (10, 23). One of the challenges in developing a translational model was evaluating which markers were commonly expressed between the species and retained both phenotypic and functional properties. Another example of human variation from mouse is the insertion of endogenous retroviral elements into human genes or HERVs (24). HERVs are normally under methylation and their affect on gene expression is observed under disease status, which promotes hypomethylation such as SLE (24).

Once demethylated, HERVs can promote the expression of alternate isoforms for certain genes such as *CD5*. Some of these genes are involved in B cell development and could contribute to differences in B cell pool regulation under disease state (25).

These human specific differences must first be addressed by identifying what is similar in analogous subsets between mouse and humans. Using the CD21/CD24 model we can evaluate analogous populations under growth and activation stimuli. The differences in response can then be correlated to human-specific characteristics of the B cell subset. For example, CD5 is expressed in human transitional B cells and not in mouse transitional B cells (26-28). CD5 modulates BCR response to activation making the BCR hypo-responsive (29). Thus, a question that emerges from differences observed is whether the presence of certain markers changes the function of the B cell and what stimuli they respond to.

An example how expression of a specific marker is associated with a specific B cell subset is the expression of CD1c on B cells (30). CD1c is only expressed in human antigen presenting cells such as B cells and dendritic cells and functions in lipid presentation to T cells (30). CD1c+ B cells recognize a specialized subset of T cells known as γδ cells (31, 32). This subset of T cells makes up a much smaller fraction of T cell population and displays unique characteristics (33). Their T cell receptor is made of different a γ chain and δ chain. Unlike other T cells (αβ type), γδ cells don't need antigen processing nor MHC presentation and function mainly in recognizing lipid antigens (33). CD1c expression is necessary for γδ T cell activation and highlights the dual role a surface marker can have in phenotypic identification and function in a cell (34).

Understanding Human B Cell Tolerance

The varied results from B cell depletion therapy in patients with SLE highlights the effect of genetic variability on patient response (15). Genetic variability promotes differences in gene expression, which can be translated to abnormal expansion of a particular B cell subset or aberrant expression of a survival cytokine (1, 5, 15, 35). Several altered genes in an individual can result in lack of efficacy from B cell targeted therapies (3, 6). Furthermore, B cell mediated autoimmune diseases are multi-factorial and genes involved in altered immune responses have not been completely elucidated (2, 3). The varied immune response in patients indicates there are differences in B cell tolerance maintenance as well. Evaluating what causes a patient to respond well to therapy would be beneficial for improving patient care, but also understanding regulation of B cell tolerance.

Most of the studies that provide insights into understanding B cell tolerance have been done in genetically similar mice (5). The effect of B cell-targeted therapies on B cell homeostasis is first evaluated in mice. Hence, evaluating the parameters in mice that correlate with successful treatment and what are those parameters in humans would provide further understanding on why some of the therapies work well in some patients, but not in others. The CD21/CD24 model can provide a framework for establishing parameters in mouse and humans. Evaluating B cell subset composition within the B cell pool can provide insight into abnormal B cell homeostasis (9). An abnormal proportion of a B cell subset, either enlarged or decreased, can offer insights into either excess levels of a growth cytokine or absence of one (36, 37). A potential scenario could be two patients receive B cell depletion therapy and both show successful depletion of B cells. After two months, one patient shows a higher number of T2 and FM B cells compared to

the other patient, while T1 cells are the same proportion with other patient. After evaluating serum BAFF levels from both patients, the patient with enlarged T2 and FM cells had higher levels of BAFF. Thus, a conclusion that could be drawn is that a patient will express varying levels of BAFF depending on the individual's genetic makeup and immune health. From mouse studies, excess BAFF can promote the expansion of auto-reactive immature and naive B cells (19, 38, 39). Thus, in the patient scenario, analysis of the expanded T2 and FM B cells in the patient shows that those B cells have auto-reactive B cells. Consequently, this patient could be given Belimumab therapy to control the levels of BAFF after B cell depletion. B cell tolerance in the emerging B cells is re-adjusted for that particular patient.

Based on our knowledge of B cell subset populations from mouse and human studies, we can potentially target that subset to readjust B cell tolerance (5, 35). Analogous B cell subset identification between mouse and human is vital as are the cytokines involved in regulating that subset in the mouse. For example, mouse studies have shown that BAFF is vital for progression from T2 into FM and MZ differentiation (38, 40). Based on these mouse studies, Belimumab was developed to target BAFF-responding cells (5, 35). More studies need to be done to characterize how human B cell subsets respond to BAFF and whether BAFF plays a similar role in human B cells (41).

B cell Reconstitution and Aging

As humans age, their immune system produces less lymphocytes (42). Mouse and human studies show there is a marked decrease of B cell production, as the BM begins producing more myeloid cells (43). Thus, B cell homeostasis must maintain proportions

of subsets to keep the B cell repertoire functioning properly to generate a B cell repertoire to protect the individual (44).

Due to the importance of memory B cells in protecting the individual as they age, this population contributes a large proportion of cells to the B cell population (45). From studies done in mouse, we know that memory B cells become activated more quickly and use survival factors such as BAFF more readily than do naïve B cells (46). Thus, depletion of B cells in aged individuals may be problematic in maintaining B cell tolerance since a decrease in B cell progenitors and genes regulating B cell development occurs with aging. Altered B cell pools have been observed in aged mice (47). The B cell repertoire in aged mice showed decreased B cell progenitors and increased lifespan of immature B cells (48). Furthermore, aging causes a dysregulation of the immune system, in particular, the BM microenvironment and an increased production of autoantibodies is observed (49, 50). An example of decreased immune system function is the response of elderly humans to vaccinations with tetanus toxoid, which results in the development of more autoantibodies (50).

To date, we do not know the progenitors of all B cell subsets or how they are derived or even their function (9, 51). For example, CB B cells mature without the need of a functional spleen. Differences in function between B cell subsets derived from CB, BM precursors, or from the peritoneal cavity have not been fully addressed. One key difference in fetal-derived mature B cells is the persistent expression of CD5 (52, 53).

B cell depletion equally eliminates fetal-derived, BM-derived B cells, and peritoneal cavity-derived cells. The question remains if fetal-derived B cells can be replenished after B cell depletion. To date, the implications of losing fetal-derived B

cells and the impact it may have on B cell subset dynamics in the context of maintaining tolerance has not been explored.

The heterogeneity of the memory B cell subsets adds another layer of complexity to understanding human B cell tolerance. The variety of memory B cells categorized based on phenotype or observed under disease such as SLE requires studies to determine what functional role the different subsets within the B cell pool and during immune response (45). Furthermore, as memory B cells accumulate with aging, the impact of depleting these cells must also be considered as the memory B cell pool provides protection for a host of organisms. For example, memory B cell repertoire may have to be re-established with vaccinations. Also, T cell diversity decreases with age and this could impact T cell and B cell interactions (54). Differences in tolerance and how it's reestablished in the absence of one B cell subset requires more study.

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