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MECHANISMS OF LYMPHOCYTE ACTIVATION AND IMMUNE REGULATION XI

B Cell Biology

Edited by
Sudhir Gupta

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Mechanisms of Lymphocyte Activation and Immune Regulation XI

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Preface

In recent years, major developments have been made in understanding various genetic and epigenetic regulatory processes that are critical for the generation of B cell repertoires. These include the role of chromatin regulation and nuclear organization in understating the IgH gene regulation. A role and mechanism of DNA repair proteins in somatic hypermutation has been elucidated. Genetic mutation studies have been instrumental in providing insight into some of the mechanisms involved in targeting CSR to various switch DNA regions located upstream of C region genes, especially a role of AID motifs, transcription and R-loops. Recent studies support a dominant role of receptor editing in central B cell tolerance and signaling pathways that regulate receptor editing in self-reactive and non-self-reactive immature B cells. These were some of the topics of discussion at the 11th International Conference on B cell Biology. These proceedings highlight recent developments in lymphocyte development, Ig gene rearrangements and somatic hypermutation, chromatin structure modification, B lymphocyte signaling and fate, receptor editing and autoimmunity.

First section deals with the mechanisms of B cell development and immunoglobulin gene arrangements. A contribution of E2A-protein in the induction of B cell lineage specific gene expression program and the induction of class switch recombination are discussed. Blimp-1 regulates a number of transcription factors and influences multiple gene expression programs Multiple roles of Blimp-1 in the development and differentiation of cells of B cell lineage are presented. Also, more interestingly, recent data of the role of Blimp-1 in thymocyte development, peripheral T cell homeostasis in responses of T cells to TCR are presented. Novel mechanisms of clonal selection in Th cell and B cell compartments of regulation of memory B cell responses, as well as the role of BCR-linked factors in developmental fate decisions are reviewed. Several speakers presented interesting and novel data for regulation of AID functions in SHM and CSR, including chromatin modification, cis-acting sequences and phosphorylation of AID.

Second section includes presentation regarding BCR signaling, receptor editing, immunological tolerance and autoimmunity. It has been demonstrated that CD19 associates with MHC class II signaling complex in B cells, and is required for optimal MHC class II-mediated downstream signaling. Two alternative pathways of NF-κB have emerged in normal and malignant B cell activation and development by BCR and BAFF-R. A selective expression of FcR-like proteins during different phases of B cell development makes them interesting B cell differentiation markers. Since these proteins, by virtue of cytoplasmic ITIM and/or ITAM sequences, display immunoregulatory activities, they are interesting therapeutic targets for disorders of B cells. A central role of ERK signaling pathway in integrating opposing signals to regulate the induction of Blimp and plasma cell differentiation is reviewed. Receptor editing is one of the mechanisms by which autoreactive B cells can undergo tolerance. A significant progress has been made in understanding mechanisms which regulate receptor editing, including a role of the recombining sequences (RS) and its human homologue the kappa deleting element (κDE) in BCR editing. Regulation of RAG genes in immature self-reactive and self-tolerant B cells, receptor editing of IgHC, and enforcement of allelic exclusion in the setting of receptor editing are reviewed. A new paradigm that hyporesponsiveness of B cells constitutes a critical component of a genetic diathesis to autoimmunity may represent environmentally-induced predisposition to autoimmunity.

This volume should be of interest to immunologists, cell biologists, academic clinicians, and scientists.

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1
Regulation and Function of the E2A Proteins in B Cell Development

Cornelis Murre*

1.1. Introduction

E-proteins belong to a family of transcriptional regulators, helix-loop-helix proteins (HLH), which regulate multiple developmental pathways in vertebrate organisms. Throughout B cell development, E-proteins play critical roles. Members of the E-protein family include the E2A proteins E12 and E47 as well as two closely related gene products, E2-2 and HEB. The E2A-proteins contribute to B cell commitment, to induce a B lineage specific gene expression program, to activate antigen receptor gene rearrangement, to induce class switch recombination and to promote developmental progression. Here, I present the current status on the function and regulation of E2A proteins in B cell development.

1.2. E2A proteins

The helix-loop-helix proteins can be divided into distinct categories based on expression patterns and biochemical properties[1]. Class I bHLH proteins, also named E-proteins, are DNA binding proteins that recognize specific DNA sequences (E2-box site)[2]. E-proteins are expressed in many different tissues but their levels of expression differ significantly among lineages and tissues. In lymphoid cells they bind DNA either as homo- or as heterodimers with other E-proteins. Four E-proteins are expressed in lymphoid cells, named E12, E47, E2-2 and HEB[3]. E-proteins contain an HLH dimerization domain and a basic region that mediates DNA binding[4,4a]. In addition, two conserved stretches of amino acids are present in E-proteins that function as either transactivation or repressor domains. The two domains are named the AD1 and AD2 domains[5,6,7].

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The AD1 region has been proposed to form a helical conformation upon interacting with its co-activator or co-repressor complex. A putative loop-helix structure is present in the AD2 domain that is conserved in all E-proteins[6].

The E2A gene encodes for two proteins, E12 and E47. E12 and E47 arise through differential splicing of the bHLH region[8]. E12 and E47 both bind the E2-box site but with different affinities. E12 shows low affinity whereas E47 binds with relative high affinity. In B-lineage cells homodimers of E47 and heterodimers of E47 and E2-2 are present[2]. In T-lineage cells, E47 and HEB form heterodimers[9]. Although E12 and E2-2 are expressed in B cells, their biochemical and functional properties have not been carefully studied.

The DNA binding and transactivation activity of the E2A proteins during B cell development is regulated at various levels. The DNA binding activities of the E2A proteins are regulated by another class of HLH proteins, named the Id gene products[10]. Four Id gene products are expressed in vertebrate organisms, termed Id1-4[11]. Id proteins lack a DNA binding region but contain an HLH dimerization domain. Upon interacting with the E2A proteins they block their DNA binding activity. Among the four Id members, Id2 and Id3 are the critical molecules that control the activity of E2A during B cell development. E2A proteins are also regulated by post-translational modifications[12]. Specifically, it has been proposed that E47 is dephosphorylated in developing B cells to promote homodimerization[12].

The E2A proteins have the potential to either activate or repress gene expression[13]. The transactivation abilities of E2A are mediated by the AD1 and AD2 domains. The AD1 domain contains a stretch of highly conserved residues, termed the LFDS motif[14]. In developing B cells, the LFDS motif recruits a co-activator complex containing CBP/p300[15]. The AD1 domain also functions as a repressor domain upon interacting with a family of co-repressors, designated as ETO proteins[16]. ETO members, in turn recruit the co-repressor N-COR and protein complexes that contain histone deacetylase activity[16].

1.3. The Role of E2A Proteins in Early B Cell Development

Hematopoietic stem cells (HSCs), in the fetal liver or the bone marrow, generate precursor cells of various hematopoietic cells lineages, including: B, T, natural killer, myeloid, dendritic and erythroid cells. Three distinct classes of HSCs, long term, short term and multipotent progenitors (MPP), have been identified. The long-term repopulating HSCs show extensive self-renewal properties and have the ability to mature into short-term repopulating cells with limited self-renewal ability. The short-term repopulating stem cell population has the capacity to progress into multipotent progenitor cells (MPPs), which lack detectable self-renewal activity. MPPs differentiate into either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMPs and CLPs develop into clonogenic progenitor cells, which have the ability to

differentiate into all mature hematopoietic cells. Both CLPs and CMPs undergo further separation. CMPs develop into myelonomonocytic progenitors (GMP) and megakaryocytic/erythroid progenitors (MEP) that segregate macrophage and granulocyte production from erythroid and platelet development. CLPs differentiate efficiently into pre-pro-B cells and have T lineage potential. The development of CLPs and pro-B cells is regulated, at least in part, by the E2A proteins. Specifically, E2A deficient bone marrow cells show a partially block at the CLP cell stage and a complete block at the pro-B cells stage (Hardy Fraction A[17]. Interestingly, human B cell development is blocked at a similar stage in bone marrow cells that express Id3[18]. Enforced expression of E12 and E47 in B cell progenitors indicates that both E12 and E47 are important for B lineage commitment[19].

Recently we have generated E2A deficient pre-pro-B cells[20]. E2A deficient pre-pro-B cells have the capacity to grow long term in vitro in the presence of stromal cells and IL7, SCF and FLT3-L. They express low levels of B220 and CD43 but CD19 expression cannot be detected. Unexpectedly, E2A deficient pro-B cells have the ability to undergo IgH DJ rearrangements. B-lineage specific transcripts can be detected, albeit at very low levels, in E2A deficient hematopoietic progenitor cells. Interestingly, transcription factors important for the development of alternative hematopoietic cell lineages are expressed in E2A deficient cells, including GATA-1, GATA-3 and TCF-1. Remarkably, E2A deficient pre-pro-B cells are pluripotent. Overexpression of E47 activates the transcription of B-lineage specific genes, including E□□□nd Pax5. On the other hand, GATA1, GATA-3 and TCF-1 gene expression is blocked by enforced E47 expression. Based on these observations we have proposed a model in which E2A proteins perform a dual function in hematopoietic progenitor cells[20]. They activate B-lineage specific gene expression and repress the transcription of regulators that promote the development of alternative hematopoietic cell lineages.

The data indicated that E2A gene products function in hematopoeitic progenitor cells upstream of EBF[21]. Once EBF is activated, it acts in turn to induce the expression of Pax5[22]. Subsequently, Pax5 induces a program of B-lineage specific gene expression. Recent observations have recently indicated that mb-1 expression by Pax5 requires EBF and possibly E2A activity[23].

The E2A proteins also inhibit the transcription of genes normally induced in non-B-lineage hematopoietic cell lineages. Transcription of genes involved in myeloid and erythroid development, including GATA-1, c-fms and EpoR are suppressed by E2A in pre-pro-B cells[20]. It will be of interest to determine whether E2A proteins act in concert with ETO members to inhibit the transcription of these non-B lineage specific genes.

1.4. E-proteins and B Cell Maturation

The data described above indicate that the E2A proteins act to induce and repress transcription in order to promote a B cell fate in progenitor cells.

It is unclear at this time how important the E2A proteins are to maintain a B-lineage program of gene expression at the pro-B cell stage, and a more detailed analysis will be required to address this issue. However, E2A protein levels are regulated by pre-BCR mediated signaling. Upon pre-BCR signaling E47 protein abundance transiently declines[24]. In contrast, Id3 abundance is increased upon pre-BCR signaling[25]. In expanding pre-B cells, the abundance of E47 is increased again to ultimately promote cell cycle arrest and induce Ig light chain gene rearrangement[24]. E47 abundance continues to remain high at the immature-B cell stage to allow receptor editing. E47 abundance is decreased in transitional B cell populations and declines further in the mature-B cell population. We have previously proposed that high levels of E47 enforce developmental arrest at the immature-B cell stage[24].

In the presence of auto-reactivity, E47 levels do not decrease, promoting RAG transcription and promoting IgK locus accessibility to allow continued rearrangement. In contrast, in the absence of self-antigen, BCR signaling modulates E47 activity, suppressing further rearrangement. Furthermore, it is conceivable that a decline in E47 levels would allow relief from the immature developmental checkpoint to promote maturation.

1.5. E2A Proteins and Immunoglobulin Gene Rearrangement

Our in vitro data have suggested that E2A proteins have the ability to induce IgK VJ rearrangement in non-lymphoid cells[26,27]. A role for the E2A proteins in IgK rearrangements was demonstrated in mice that carried mutations in the Ig κE1 and κE2 sites[28]. These mutations significantly perturbed IgK gene rearrangement, providing unambiguous evidence that the E2A proteins regulate IgK gene rearrangement.

It is plausible that the E2A proteins promote Ig VJ gene rearrangement by modulating chromatin structure through the recruitment of a co-activator complex containing CBP/p300. The critical question now is whether E-protein activity is suppressed once a productive IgK chain has formed. It will be interesting to see how E2A activity at the immature-B cell stage is regulated.

1.6. E2A and Class Switch Recombination

The E2A and Id gene products also play critical roles in peripheral B cell development. High abundance of E2A promotes follicular B cell development whereas high levels of Id3 favor marginal zone B cell development. In resting B cells, E2A abundance is low but detectable. However, E47 levels are readily elevated in response to mitogenic stimuli[29]. For example, E47 protein abundance is increased upon treatment with LPS, BCR and CD40 ligand. How these ligands activate E2A expression in peripheral B cells remains to be determined. In

activated B cells, E-proteins act to induce AID expression and class switch recombination[30]. Interestingly, the antagonists HLH protein, Id2, has been shown to suppress CSR[31]. It is plausible that Id2 in activated B cells inhibits E-protein activity once class switch recombination and SHM are completed.

1.7. Concluding Remarks

Studies during the past decade have demonstrated that the E-proteins play critical roles during B cell development. In pre-pro-B cells they act to induce, directly or indirectly, the expression of EBF. In pre-B and immature-B cells they act to induce IgK VJ rearrangement and to regulate receptor editing, whereas in activated mature-B lineage cells they are required to induce AID expression to promote CSR and SHM. How E-proteins regulate the expression of such a diverse set of genes at different stages remains a mystery that needs to be resolved. Likely it is the combination of transcriptional regulators that ultimately will determine lineage and stage specificity.

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2
Multiple Roles for Blimp-1 in B and T Lymphocytes

David Savitsky[†], Luisa Cimmino[‡], Tracy Kuo[*,#], Gislâine A. Martins[*] and Kathryn Calame[*,¶]

2.1. Introduction to Blimp-1

The transcriptional repressor B lymphocyte induced maturation protein-1 (Blimp-1) has been studied in our laboratory for several years. Although it was first characterized as a regulator of terminally differentiated plasma cells, it is becoming increasingly clear that Blimp-1 plays important regulatory roles in the differentiated function of several cell lineages in adult mice, in addition to critical roles during embryonic development. Here we briefly review our earlier work on Blimp-1 in the B lymphocyte lineage and describe more recent studies on B cells. We also describe our studies that demonstrate a role for Blimp-1 in the T lymphocyte lineage.

2.1.1. Mechanism of Transcriptional Repression

B lymphocyte induced maturation protein-1 (Blimp-1) is a 98 kDa protein with five-zinc finger domains encoded by the *prdm1* gene, first identified as a repressor of the human *interferon (IFN)*-β promoter[1]. Blimp-1 was subsequently identified as an induced transcript when BCL_1 B cell lymphoma cells differentiated into antibody-secreting cells and, significantly, it was shown that ectopic expression of Blimp-1 was sufficient to drive BCL_1cells to develop into

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an antibody-secreting plasma cell phenotype[2]. The transcriptional repression activity of Blimp-1 depends upon its association with proteins encoded by Groucho-related genes[3], histone deacetylases[4] and the G9a histone H3 methyltransferase[5]. Thus its repression mechanism appears to depend on covalent modifications of histones (deacetylation and methylation) that create a repressive or inactive chromatin structure. No transcriptional activation capability has been demonstrated for Blimp-1, although it remains a theoretical possibility. The Blimp-1 consensus binding site on DNA has been defined and shown to be virtually identical to that of interferon-regulatory factor (IRF)1 and IRF2. Consistent with this overlapping specificity, Blimp-1 competes *in vivo* with IRF proteins for binding to the *IFN*-β promoter[6].

2.1.2. Importance in Early Development

Blimp-1 is expressed in many embryonic cells[7], other hematopoietic lineage cells[8] and in epithelial cells[7]. *Prdm1* null mice die at day 10.5 but anterior patterning and neural crest are normal. However, absence of Blimp1 disrupts morphogenesis of the caudal branchial arches and leads to a failure to correctly elaborate the labyrinthine layer of the placenta. Furthermore, Blimp-1 is required in mouse for formation of primordial germ cells[9,10]. Blimp-1 is also necessary for early embryonic decisions in other species. In xenopus, the frog homologue of Blimp-1, X-Blimp, controls anterior mesodermal fate[11] and the drosophila homologue is required for proper tracheal system development[12]. In zebrafish, the homologue of Blimp-1 promotes the cell fate specification of both neural crest cells and Rohon-Beard sensory neurons[13] and is necessary for patterning and organogenesis[14].

2.2. Blimp-1 in Plasmacytic Differentiation

2.2.1. Blimp-1 is Sufficient to Drive Plasmacytic Differentiation

When Blimp-1 was first cloned during a subtractive screening protocol as a mRNA specifically expressed upon differentiation of BCL_1 lymphoma cells into immunoglobulin (Ig) secreting plasma cells, it was shown that *forced expression of Blimp-1 was sufficient to drive plasmacytic differentiation* of this cell line[2]. Our laboratory and others have confirmed and extended this finding to primary murine splenic B cells and human centrocytes, showing that forced expression of Blimp-1 is sufficient to trigger plasmacytic differentiation[15,16]. Blimp-1 is only sufficient to drive plasmacytic differentiation in B cells when expressed at an appropriate developmental stage (i.e. activated or germinal center B cells). When ectopically expressed in B cell lines representing earlier developmental stages, it causes apoptosis[17,18]

2.2.2. *Blimp-1 is Required for Plasma Cell Formation and Function*

We created mice in which *prdm1* exons 5-8 were flanked with LoxP sites (*prdm1*$^{F/F}$)and generated mice lacking Blimp-1 in their B cells by mating them to *CD19*$^{Cre/+}$ mice[19]. Analysis of these mice showed that Blimp-1 is required for formation of CD138^{+} Ig-secreting plasma cells (PCs) in response to either a T-independent or T-dependent antigen (Figure 2.1). There was no Ig secretion in a recall response. Splenic B cells from the CKO mice do not secrete Ig upon

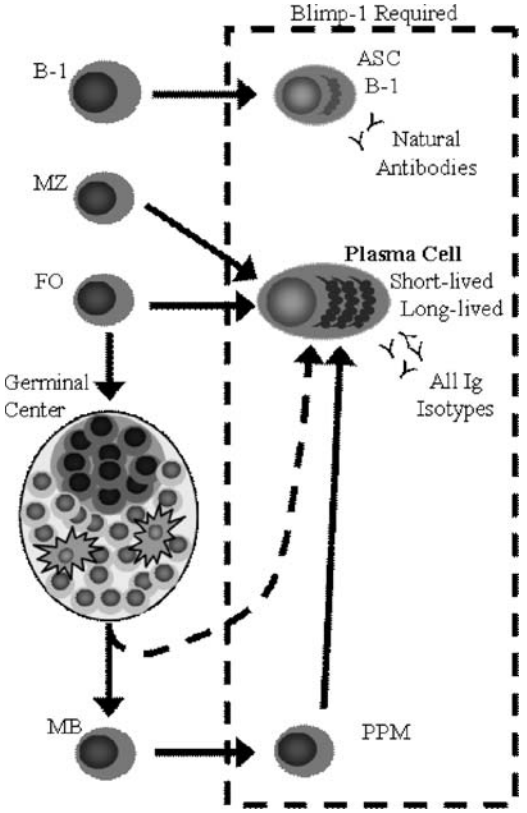

FIGURE 2.1. A Requirement for Blimp-1 at Multiple Stages in the B Lymphocyte Lineage. B-1 B cells do not need Blimp-1 for their development but require Blimp-1 for natural antibody secretion (ASC B-1). Blimp-1 is essential for the formation of both short-lived an long-lived plasma cells derived from marginal zone (MZ), follicular (FO), and post-germinal center B cells. In Blimp-1 deficient *CD19*$^{crel/+}$*prdml*$^{F/F}$ mice, all immunoglobulin isotypes are markedly reduced, germinal centers are enlarged, and pre-plasma memory (PPM) cells fail to form normally. Removal of Blimp-1 result in the loss of previously formed, long-lived plasma cells demonstrating a requirement for their mainatenance in the bone marrow.

activation with LPS. This is due in part to their failure to switch to the secreted form of μ mRNA and failure to induce XBP-1 mRNA. More detailed analyses of the failure of these cells to secrete Ig, using gene expression studies, confirmed that Blimp-1 is required for the induction of XBP-1[20], probably by relieving *Pax-5*-dependent repression of XBP-1[21,22]. XBP-1 is the proximal activator of multiple genes encoding proteins directly responsible for endoplasmic reticulum (ER) function and protein secretion. Our more recent studies on the role of Blimp-1 in the B cell lineage are summarized in more detail below.

2.2.3. Regulation of Blimp-1 Expression in B Lineage Cells

Blimp-1 mRNA and protein are expressed in Ig-secreting plasmablasts and plasma cells[23–25]. Blimp-1 is also present in a subset (~5%) of germinal center B cells[23]. Pre-plasma memory cells[26] express low levels of Blimp-1 mRNA (M. McHeyzer-Williams and Calame, unpublished).

In B cells, regulation of Blimp-1 is primarily at the level of transcriptional initiation[27]. Cytokines including IL-2, IL-5, IL-6, IL-10 and IL-21[28] induce Blimp-1 mRNA. STAT3, activated by most of these cytokines, induces Blimp-1 mRNA[29], although precise response elements on *prdm1* have not been identified. In addition, B-cell stimulation of TLR4 by LPS[15] and TLR9 by CpG (Kuo and Calame, unpublished) induces Blimp-1 mRNA and our recent studies show that NF-κB, activated in response to TLR stimulation, directly induces *prdm1* transcription (Kuo et al, submitted). AP-1 also induces *prdm1* transcription [30]. Bcl-6 represses *prdm1* both by interfering with AP-1 activation[30] and by direct binding[31]. Bcl-6-dependent repression is thought to be important for keeping Blimp-1 shut off in germinal center B cells, ensuring completion of the germinal center reactions before terminal plasmacytic differentiation ensues. Recent studies show OBF-1 is required for induction of Blimp-1 in post-germinal center B cells[32] and knock-out mice suggest that IRF4 is upstream of Blimp-1 induction in activated splenic B cells (H. Singh, pers. comm.). However, it is not known if OBF-1 or IRF4 act directly or indirectly, possibly by regulating Bcl-6. Signals that instruct germinal center B cells to overcome Bcl-6-dependent repression, exit the germinal center and become committed to plasmacytic differentiation may be revealed by further studies on Blimp-1 regulation.

2.2.4. Mechanism of Blimp-1 Action in B Cells

Identification of target genes repressed by Blimp-1 has been critical to understanding how it triggers plasmacytic differentiation. We have shown that Blimp-1 regulates three gene expression programs in B cells[20,33]. *First, Blimp-1 induces Ig secretion.* Direct repression of *Pax-5*[21] leads to de-repression of XBP-1, J chain and IgH transcription. In turn, XBP-1 is the proximal activator of numerous genes directly involved in ER biogenesis and protein processing and secretion[20]. *Second, Blimp-1 represses a proliferative program.* This includes

direct repression of *c-myc*[17,34] as well as repression of other genes such as *E2F − 1*, *PCN,* and *DNA pol*δ required for cell cycle entry, DNA replication, and mitosis. *Finally, Blimp-1 represses many B-cell functions, thereby blocking continuation of an activated B-cell phenotype*. This includes direct repression of *CIITA*, leading to decreased MHC class II expression[16], direct repression of *Pax-5*[21], required for germinal center function and expression of AID[35,36] direct repression of *Spi-B* and *Id3*[33]. Significantly, Blimp-1 also represses genes encoding Bcl-6 (required for germinal center B cells), AID (required for somatic hypermutation and class switch recombination), several proteins required for BCR signaling and CXCR5, a chemokine receptor involved in keeping B cells in the follicles. All known direct Blimp-1 targets encode transcription factors and many other targets, which may be direct or indirect, are also transcription factors, providing insight into how Blimp-1 initiates cascades of transcriptional regulation. This knowledge clarifies how this single transcriptional repressor can be sufficient to drive plasmacytic differentiation and has provided new insights into hierarchies of regulatory cascades required for terminal B cell development. Our more recent studies on Blimp-1 in the B cell lineage are described in more detail later in this article.

2.3. Blimp-1 is Required for Maintenance of Long-lived Plasma Cells

Long-lived plasma cells that reside in the bone marrow are germinal center experienced cells[37] that have usually switched isotype and have undergone somatic hypermutation and selection. The antibodies that these long-lived plasma cells secrete provide "constant vigilance" in the form of protection for future encounters with the pathogens that led to their formation. Long-lived plasma cells can survive for months to years, possibly the lifetime of the organism in some cases[38] in the absence of antigen[39] or cell division[40]. Given the fact that long-lived plasma cells continue to secrete immunoglobulin, we hypothesized that Blimp-1 might be required for their continued function.

To address this question we took advantage of genetically altered mice in which the *timing* of *prdm1* gene deletion can be controlled by activating Cre recombinase through a ubiquitously expressed, tamoxifen-inducible Cre recombinase-expressing transgenic mouse ($Rosa26^{ERCre/+}$). Equal frequencies of short-lived plasma cells were generated from wildtype and $Rosa26^{ERCre/+}prdm1^{F/F}$ B splenocytes after treatment with LPS for 3 days *in vitro* (12–20% $CD138^+$). However, $Rosa26^{ERCre/+}prdm1^{F/F}$ cultures showed a 90% reduction in PC frequency compared to only a 30% reduction in frequency of $CD138^+$ cells after treatment with 4-hydroxytamoxifen (4OHT) for an addition 4 days. Also, no Blimp-1 was detected in 4OHT-treated $Rosa26^{ERCre/+}prdm1^{F/F}$ cultures demonstrating that deletion was efficient. Splenic B cells were labeled with CFSE dye and the experiment repeated to show this was not due to the

failure to generate more plasma cells. CFSE^{Hi} (non-dividing) plasma cells disappeared more rapidly and to a greater overall extent in *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ cultures and ELISPOT analyses showed a reduction in the frequency of IgM secreting cells over control cultures.

Similar results were obtained *in vivo*. Upon administration of tamoxifen, *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ mice showed a 4-fold decrease in both previously formed (BrdU^{+}) and newly formed (BrdU^{-})plasma cells compared to control mice 3–4 weeks following tamoxifen treatment. Likewise, there was a significant loss in serum titers compared to pre-tamoxifen levels following deletion of Blimp-1 in *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ mice immunized with NP-KLH. Five weeks after tamoxifen treatment, 11 weeks post-immunization, wildtype mice had 35% starting levels while *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ mice had only 8% (four-fold lower amount). Finally, to exclude a role for bone marrow stromal cells in the maintenance of plasma cells, bone marrow containing long-lived plasma cells from wildtype and *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ mice immunized and boosted with NP-KLH were adoptively transferred to irradiated naïve mice. Recipient mice were assessed for NP-specific serum IgG_1 by ELISA 3, 5 and 10 weeks following *prdm1* gene deletion upon tamoxifen treatment. NP-specific IgG_1 fell 66-fold more in *Rosa26*$^{ERCre/+}$*prdm1*$^{F/F}$ mice compared to control mice demonstrating a cell-intrinsic requirement for Blimp-1 in maintaining immunoglobulin-secreting plasma cells.

The fascinating and important question that remains to be determined is the fate of plasma cells following removal of Blimp-1. It is unclear if when Blimp-1 is removed and CD138^{+} immunoglobulin-secreting plasma cells in the bone marrow disappear, these long-lived plasma cells die or de-differentiate. Nevertheless, our data reveal an essential for Blimp-1 in the persistence of immunoglobulin-secreting plasma cells and antibody serum titers, both important components in immunological memory (Figure 2.1).

2.4. Blimp-1 and B-1 B Cells

B-1 cells are a small subset of the B cell compartment that, unlike B-2 cells, reside primarily in the peritoneal and pleural cavities, self-renew and secrete antibodies in the absence of apparent infection[41,43]. These so-called "natural antibodies" are thought to arise from the interaction of B-1 cells with self and oxidized self antigen whose repertoire is limited and lack N region additions and somatic hypermutations[44,45]. An important function demonstrated for natural antibodies is in the pre-infection immunity against a number of bacterial and viral pathogens[46,49].

2.4.1. Blimp-1 is Required for Ig Secretion by B-1B cells

Although some B-1 cells migrate from cavities to the secondary lymphoid organs and undergo further differentiation to become antibody secreting cells[50,51],

there is good evidence that B-1 cells in the peritoneal cavity can also secrete immunoglobulin[52]. Also, although comparable fractions of plasma cells derived from LPS-activated splenic B-2 cells and *ex vivo* purified PerC B-1 cells secrete antibody, plasma cells secrete much more antibody, on a per cell basis, than B-1 cells[52].

We have recently analyzed B-1 cells in $CD19^{Cre/+}prdm1^{F/F}$ mice (Savitsky and Calame, submitted). Both B-1a and B-1b sister populations were present in normal frequencies of conditional knockout mice, as was the frequency of splenic B-1 cells, and adoptive transfer of peritoneal cavity Blimp-1-deficient B-1 cells into $Rag1^{-/-}$ recipients demonstrated normal self-renewal capabilities. Thus, Blimp-1 is not required for the formation, self-renewal or maintenance of B-1 cells.

Purified Blimp-1-deficient B-1 cells, however, did display a defect in immunoglobulin secretion (Figure 2.1). The secretion of IgM from $CD19^{Cre/+}prdm1^{F/F}$ B-1 cells *in vitro* was 14-fold lower than control cultures as was determined by ELISA. This defect was also confirmed *in vivo* by ELISA assay performed on unimmunized serum for a B-1-specific idiotype, T15. To determine if the nature of the molecular regulation of Ig secretion in B-1 cells, quantitative RT-PCR analysis for mRNA steady-state levels of Pax5, XBP-1 and μ-secreted transcripts were performed on resting and LPS-treated purified B-1 cells. These genes, previously shown to be required for immunologlobulin secretion by B-2 cells, were found to be misregulated in $CD19^{Cre/+}prdm1^{F/F}$ B-1 cells. In both the untreated and LPS-treated conditions, conditional knockout cells failed to induce μ and XBP-1 and failed to repress Pax5 transcripts normally. Pax5 was 3- and 18-fold de-repressed in purified untreated and LPS-treated conditional knockout B-1 cells, and XBP-1 and μ-secreted transcripts were 3.6- and 6.6-fold and 5.7- and 11.6-fold reduced compared to wildtype cells, respectively. Our data show that the Blimp-1/XBP-1-dependent program operates in the regulation of immunoglobulin secretion in B-1 as well as B-2 cells.

2.4.2. Blimp-1-Deficient B-1 Cells are Defective in Providing Protection to Influenza Infection

Baumgarth et al. previously showed that both B-1 and B-2 cells are required for effective immunity against influenza infection in mice. B-1 cell-derived natural antibodies, present before infection, promoted subsequent B-2 cell IgG_{2a} responses and reduced mortality[53]. This is likely the result of natural antibodies neutralizing influenza virus and fixing it to complement[54,56]. We reconstituted irradiated $Rag1^{-/-}$ mice i.v. with B-2 cells from wild type bone marrow (BM), and PerC B-1 cells i.p. from either WT or CKO mice. Three weeks post-reconstitution, mice were infected intranasally with an $<LD_{50}$ dosage of A/WSN/33 influenza virus then monitored for 2 weeks. Weight loss was used as a criterion for susceptibility to influenza infection. Two of five CKO-reconstituted mice lost greater than 25% of their initial body weight by day 8 while zero out of four WT-reconstituted mice lost more than 10% of their body weight upon

infection with influenza virus. The increased susceptibility to influenza infection of mice receiving CKO B-1 cells demonstrates the physiological relevance of the requirement for Blimp-1 in antibody secretion by B-1 cells.

2.5. Blimp-1 in Early and Pre-plasma Memory B Cells

Two subsets of antigen specific memory B cells have been described by McHeyzer-Williams and colleagues[57]. The first is characterized by surface $IgM^{-}IgD^{-}$ $B220^{+}CD138^{-}$ markers, recirculation through secondary lymphoid tissues such as spleen and lymph nodes, a high proliferative capacity, and the ability to produce limited plasma cells in a recall response. The second type, termed pre-plasma memory (PPM) B cells, is defined by $CD79b^{+}B220^{-}CD138^{-}GL7^{-}$ surface expression. These cells primarily inhabit the BM and can differentiate rapidly to plasma cells upon antigen recall. It has been suggested that PPM cells represent an intermediate developmental state between early or conventional memory B cells and plasma cells, but their identity and role are controversial[58].

Analysis of conditional knockout of Blimp-1 in the B cell lineage revealed a block in the development of PPM cells and an accumulation of late germinal center cells following a primary immune response upon immunization with NP-KLH. While total NP^{+} cells were equivalent between control and $CD19^{Cre/+}prdm1^{F/F}$ immunized mice, larger splenic germinal centers detected by PNA staining and an increase in the frequency and total numbers of $CD138^{-}B220^{+}NP^{+}GL7^{+}$ CKO B cells as compared to preimmune animals at day 7 and day 14 were observed. However, $B220^{+}$ memory cells appeared to be normal in mice lacking Blimp-1 and evidence for some aspects of functional B-cell memory was revealed in an exaggerated germinal center response upon recall in the CKO animals. However, there was a greater than 90% reduction in the frequency of $CD79b^{+}B220^{-}CD138^{-}GL7^{-}NP^{+}$ PPM in the bone marrow on days 7 and 14 post-immunization. An identical failure to develop PPM and accumulate $CD79b^{+}B220^{-}CD138^{-}GL7^{-}NP^{+}$was seen in CKO mice upon re-challenge with NP-KLH, suggesting that formation of the PPM subset requires Blimp-1 but that the formation of $B220^{+}$memory cells does not require Blimp-1.

2.6. Gene Expression Patterns in Human Memory B Cells

Germinal center B cells from human tonsils can be grown *in vitro* in short-term cultures[59,60,61]. In response to IL-4 these cells develop into surface immunoglobulin positive cells with a memory B cell phenotype ($CD20^{+}CD38^{-}$). Although exact comparisons to murine memory cells are difficult, the “memory” cells that develop in these cultures in response to IL-4 are probably most similar to the early $B220^{+}$, re-circulating memory subset in mice. The cultured

human memory cells express high levels of Bcl-2 mRNA and, with appropriate stimulation by IL-10, develop rapidly into immunoglobulin secreting cells, also consistent with a memory phenotype[59,60]. When centrocytes are grown with IL-10 they develop into immunoglobulin-secreting cells with a plasma cell phenotype ($CD20^{-}CD38^{+}$)[59,62].

We have exploited this system to study changes in transcription factors and gene expression patterns during differentiation of human tonsillar B cells from the centrocyte to the memory B cell stage (T. Kuo, Y. Choi and K. Calame, unpublished). Messenger RNA encoding Blimp-1 remained virtually undetectable throughout the culture period for memory B cells, providing strong evidence that Blimp-1 does not play a role in memory B cell formation in this setting. Levels of mRNA for two other "plasma cell" factors, IRF4 and XBP-1, began low and increased approximately 5-fold during the culture period. The significance of this is not currently understood. Messenger RNAs encoding "germinal center" factors Bcl-6, Pax5 and Bach2 all decreased rapidly to nearly undetectable levels early in the tonsillar cultures grown in IL-4. Further studies confirmed that Bcl-6 protein is absent from memory cells in these cultures and, significantly, showed that ectopic expression of Bcl-6 is sufficient to block memory cell development in this system (T. Kuo and K. Calame, unpublished). These data provide evidence that Bcl-6 does not play a role in memory B cells and shows that absence of Bcl-6 is not sufficient to allow induction of Blimp-1 in this setting.

Since our analysis of the well-defined transcription factors for germinal center B cells and plasma cells, two developmental stages thought to bracket memory B cells, did not provide evidence that any of them were expressed or important for memory B cells we performed a microarray analysis on centrocytes, memory B cells and plasma cells in the human tonsillar cultures to identify memory B cell specific transcriptional regulators (T. Kuo, A. Shaffer, Y. Choi and K. Calame, unpublished). We found that transcription factors AML-1, ATF-3, CIITA, c-JUN, NF-IL3A, STAT5a, B-ATF and XBP-1 were elevated in memory cells compared to centrocytes. The roles and targets of these factors remain to be investigated.

Memory B cells undergo activation and proliferation more rapidly than naïve B cells[63]. Consistent with this phenotype, mRNAs were found to be elevated for proteins involved in antigen presentation (MHC class II and CIITA), T-cell co-stimulation (CD80, CD86, OX40L, CD70, and CD40) and chemokines to attract T cells (IP-10, CCL17, CCL22, CX3C, and STCP) as well as for proteins involved in JNK signaling (c-jun, ATF-3, and B-ATF) and certain phosphatases. A striking increase in mRNAs encoding cytokine receptors (IL-2Rβ, IL-4Rα, IL-12Rβ1, IL-13Rα and IL-17R) was observed, consistent with a heightened response to the environment. In previous studies[64], mRNA levels were found to be elevated in memory B cells compared to centrocytes. Stimulation via IL-4[65], IL-2[65], IL-12[66], and IL-13[67] receptors on B cells are known to provide proliferation signals, but the role IL-17 in B cells is currently not clear. These studies provide new insights into gene expression patterns in memory B cells.

2.7. Blimp-1 in the T Cell Lineage

2.7.1. Regulated Expression of Blimp-1 in the T Lymphocyte Lineage

Blimp-1 expression has been observed by immunofluorescence in $CD3^+$ T cells in normal human tonsils and in reactive T cell infiltrates of human B cell lymphomas[24]. We have studied the steady-state levels of Blimp-1 mRNA within the T cell-lineage by quantitative RT-PCR from flow cell-sorted subpopulations of the thymus, lymph nodes and spleen (Figure 2.2). Blimp-1 is basally expressed in the thymus and appears to be regulated at various checkpoints during thymocyte development, with mRNA levels decreasing 3-fold during the transition from double negative (DN) to double positive (DP) differentiation then increasing in single positive (SP) thymocytes to the levels seen originally amongst the DN population. This level of Blimp-1 expression is maintained in resting, naïve ($CD62L^{Hi}CD44^{Lo}$) T cells in the periphery. However, in both the $CD4^+$ and $CD8^+$ T cell compartments the levels of Blimp-1 are increased 10-fold in memory T cells ($CD62L^{Hi}CD44^{Hi}$) and 15-fold in effector T cells ($CD62L^{Lo}CD44^{Hi}$) above the level found in the naïve population. This suggested that Blimp-1 mRNA expression was being induced upon T cell activation. Indeed, *in vitro* stimulation of naïve $CD4^+$ T cells for 6 days with plate-bound anti-CD3 and anti-CD28 caused up to a 50-fold increase in Blimp-1 mRNA, a level comparable to that found in plasma cells differentiated *in vitro* from splenic B cells stimulated with LPS for 3 days (Martins, et al. *in press*). In the case of $CD4^+$ T helper (Th) cell differentiation, the influence of cytokine-induced polarization on Blimp-1 mRNA levels was also assessed. Stimulation of naïve $CD4^+$ T cells *in vitro* either with IL-2 alone (Th0), IL-12 and anti-IL-4 (Th1) or IL-4 and anti-IFNγ (Th2) for 6 days resulted in a further 3-fold induction of Blimp-1 in Th2/Th0 cells and a 2-fold suppression in Th1/Th0 cells overall leading to a 6-fold increase in Blimp mRNA levels in Th2/Th1 $CD4^+$ T cells (unpublished). The increased level of Blimp-1 in Th2 $CD4^+$ T cells could imply that the role this repressor plays in $CD4^+$ T cell responses is supportive of its role in the B cell lineage, to promote antibody-mediated immune responses.

Blimp-1 is also expressed in regulatory $CD4^+$ T cells ($CD62L^{Hi}CD25^{Hi}$) at levels similar to those found in $CD4^+$ effectors. These cells are a specialized subset of $CD4^+$ T cells required for the suppression of immune responses and to maintain tolerance in the periphery.

2.7.2. Blimp-1 Is Involved in Thymocyte Development

Conditional deletion of Blimp-1 in the T cell lineage by $Lck^{Cre/+}$ expression causes a 70% reduction in total thymic cellularity (Figure 2.3). Development of DN cells through DN1-4 progresses normally based on CD44 and CD25 expression by FACS analysis and total numbers of DN and γ/δ T cells are unaffected by loss of Blimp-1. The reduction in total numbers is mainly due to

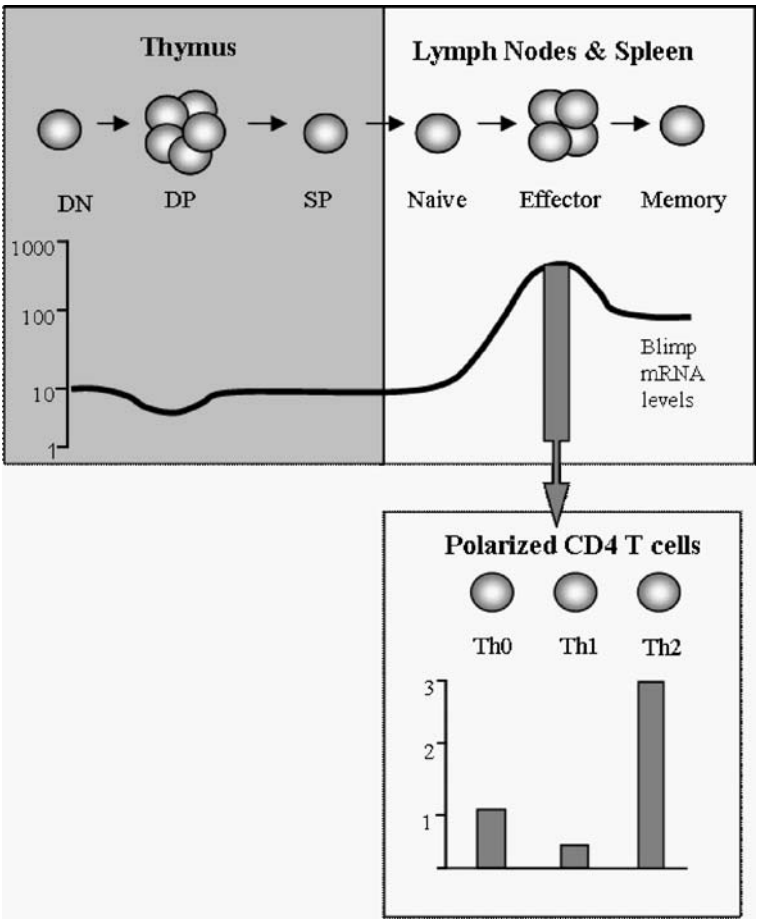

FIGURE 2.2. Expression of Blimp-1 mRNA in the T Cell Lineage. Blimp-1 steady-state mRNA, determined by quantitative RT-PCR, is basally expressed during thymocyte development and maximally expressed in antigen experienced effector and memory peripheral T cells. Differentiation of CD4 T helper (Th) cells *in vitro* under polarizing culture conditions causes Blimp-1 mRNA levels to fall in Th1 cells and rise in Th2 cells compared to neutral (Th0) conditions.

a decrease in DP and SP thymocytes populations either from decreased proliferation, survival or increased death during development. An *in vivo* BrdU incorporation assay was used to examine whether the loss of Blimp-1 caused a decrease in proliferation. The rate of BrdU incorporation was found to be the same for all thymic subsets of WT and Blimp-1 CKO mice. Therefore, the deficit could not be attributed to decreased proliferation during the DN-DP or DP-SP transitions. Differentiation does not appear to be impaired as the surface expression pattern of CD5, CD69, HSA, and TCRβ within all thymocyte subsets is normal compared to WT mice.

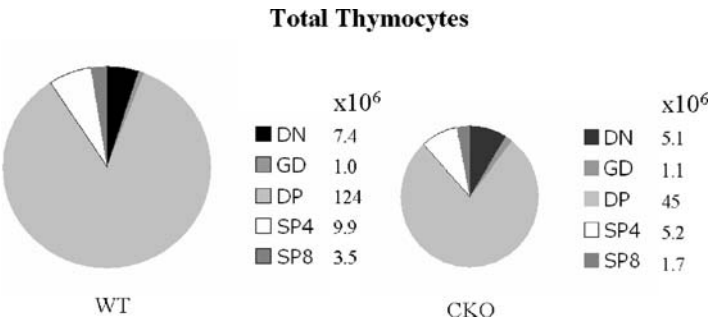

FIGURE 2.3. Decreased Thymic Cellularity in Blimp-1 CKO Mice. Total thymocyte numbers are reduced by 70% in Blimp-1 CKO mice compared to WT, as indicated by the relative sizes of the pie charts. Within each compartment of double negative (DN), gamma/delta (GD), total thymocyte numbers are approximately the same. The greatest reduction in thymocyte numbers is attributed to decreases in the double positive (DP), single positive CD4 (SP4) and single positive CD8 (SP8) compartments as indicated alongside the color key.

Identification of apoptotic (Annexin V^+) cells using flow cytometry showed that Blimp-1 KO DP thymocytes are 2-fold more susceptible to death *ex vivo* and *in vitro* when stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28. These data suggest there is an intrinsic requirement for Blimp-1 in DP thymocyte survival and suggest that selection of thymocytes may be abnormal in CKO mice. One important possibility, which we plan to address experimentally in the future, is that failure of thymocytes to survive in the CKO mice could be secondary to stress caused by abnormal T cell activation in the periphery, as described below.

DP thymocytes from $E2A^{-/-}$ mice also show increased apoptosis upon *in vitro* culture and similar to the Blimp CKO thymus have a reduction in the proportion of DP thymocytes and an increase in the proportion of SP thymocytes with a reduction in total thymic cellularity[68,69]. The *E2A* gene products E12 and E47 arise by alternative splicing and belong to a family of E proteins that include E2-2 and HEB[70,71]. The E proteins are ubiquitously expressed basic helix-loop-helix proteins whose complexes in T cells are largely composed of E2A-HEB heterodimers. The E protein complex binds to a conserved E-box motif in several T cell-specific regulatory elements, including the TCRβ and CD4 enhancers, activating transcription[71]. E proteins are regulated by Id proteins, of which Id3 is shown to play a specific role in T cell development. The Id proteins lack the DNA binding domain found in E proteins but can heterodimerize with these molecules and thus act as dominant negative inhibitors, blocking E protein DNA binding and consequently inhibiting transcriptional activation[70]. In $E2A^{-/-}Id3^{-/-}$ mice, thymocyte numbers are higher and subpopulations are restored to more normal proportions[71]. It is interesting to note that Blimp-1 directly inhibits Id3 transcription in B cells[33]. Lack of Blimp-1 in the thymus could allow for increased Id3 levels and increased

inhibition of E protein transcriptional activity in DP and SP thymocytes. Further studies using T cell transgenic mouse models will allow additional characterization of Blimp-1's role in thymocyte selection and survival. These studies, in conjunction with target gene analysis using microarray technology, may reveal the molecular mechanism of Blimp-1's role in the regulation of T cell development.

2.7.3. Mice Lacking Blimp-1 in their T Cells Develop Spontaneous Colitis

T cell CKO ($Lck^{Cre/+}$ $prdm1^{F/F}$) mice do not gain weight and survive poorly. The cause is spontaneous colitis; no other pathology has been observed in the mice. As early as 4 weeks of age, some CKO mice developed colitis, by 6-10 weeks 70% of the mice had colitis, and after 10 weeks the number increased to 83%. The mice analyzed were on a mixed C57Bl/6 and 129 background and colitis development may be different once they are back-crossed.

2.7.4. Blimp-1 is Required for Peripheral T Cell Homeostasis

Analysis of peripheral T cells in the CKO mice revealed that the total number of T cells is slightly increased as compared to the control mice. The increase in the total T cell numbers in the CKO is more obvious at the lymph nodes, especially the mesenteric ones. As the mice age and inflammation in the colon progresses the enlargement of the mesenteric lymph nodes and increase in the total numbers of T cells become more prominent. In order to avoid confusion between primary effects of Blimp-1 deletion and secondary effects from the colitis progression, we focused our analysis in young (4-5 weeks) CKO mice. At this age, the CKO mice showed similar numbers of total T cells in the spleen (Figure 2.4), but around 20% more T cells in the lymph nodes as compared to the control mice. We next evaluated the frequency and total numbers of naïve ($CD62L^{Hi}CD44^{Lo}$), effector ($CD62L^{Lo}CD44^{Hi}$), and memory ($CD62L^{Hi}CD44^{Hi}$) $CD4^+$ and $CD8^+$ cells in the control and CKO mice. We found that the frequency of naïve $CD4^+$ or $CD8^+$ is reduced in the CKO mice, especially in the spleen (Figure 2.4) but also in the lymph nodes, as compared to the control mice. CKO mice also present reduced numbers of naïve $CD4^+$ T and $CD8^+$ T cells in the spleen, although the reduction in naïve CD4 was more prominent. CKO and control mice presented similar frequency and numbers of $CD4^+$ and $CD8^+$ memory cells in the spleen (Figure 2.4), but slightly more $CD4^+$ and $CD8^+$ memory cells in the lymph nodes. The frequency and the numbers of $CD4^+$ and $CD8^+$ effector T cells are increased in spleen (Figure 2.4) and lymph nodes of CKO mice as compared to the control mice, indicating that deletion of Blimp-1 results in increased peripheral T cell activation.

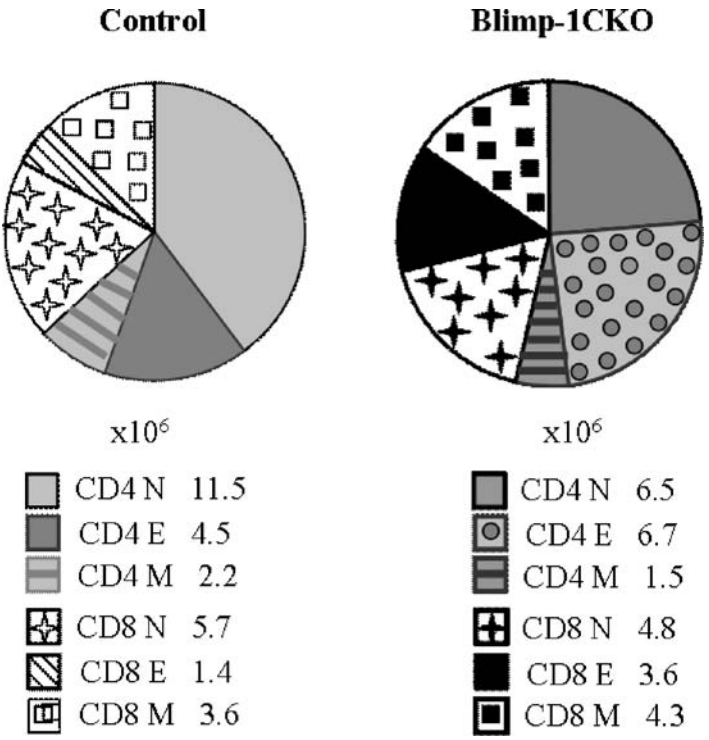

FIGURE 2.4. Increased T cell Activation in Blimp-1 CKO Mice. Representation of average of total numbers (x10^6) of naïve (N, CD62L^{Hi} CD44^{Lo}), effector (E, CD62L^{Lo} CD44^{Hi}) and memory (M, CD62L^{Hi} CD44^{Hi}) CD4$^+$ (shades of grey) and CD8$^+$ (black and white patterns) T cells in the spleen of control ($Lck^{Cre/+}prdml^{+/+}$, left) and Blimp-1 CKO ($Lck^{Cre/+}prdml^{F/F}$, right) mice. Blimp-1 CKO mice present a 2-4 fold increase in effector CD4$^+$ and CD8$^+$ T cells in the periphery.

2.7.5. Blimp-1 is Required to Control CD4$^+$ T Cell Responsiveness

We have analyzed how Blimp-1-deficient CD4$^+$ T cell respond to TCR activation. We found that purified naïve CKO CD4$^+$ T cell are more proliferative than control cells in response to sub-optimal stimulation such as anti-CD3 alone. However, stronger stimulating conditions, such as addition of anti-CD28 and IL-2 resulted, in similar proliferation in control and CKO T cells. In addition to the increased proliferative response, naïve CKO CD4$^+$ T cells also produce more IL-2 than control cells, as evaluated by intracellular cytokine staining. We found that stimulation of CKO naïve CD4$^+$T cells results in 4-fold increase in the frequency of IL-2 producers. These studies suggest that Blimp-1 is involved in establishing a threshold for response to TCR signals.

Despite the fact that CKO naïve $CD4^+$ T cells were more responsive than control cells to TCR activation, they presented equal susceptibility to activation induced cell death (AICD) as accessed *in vitro* by strong TCR re-stimulation after primary stimulation[72]. It still remains to be investigated if control and CKO cells are equally susceptible to cell death induced by lack of stimulation and/or growth factor deprivation. It is conceivable that Blimp-1 deficiency also confers an increased capacity to survive under limiting amounts of IL-2 and/or other growth factor deprivation. This, together with the hyper-responsiveness could help to explain the accumulation of the peripheral activated/effector $CD4^+$ and $CD8^+$ T cells in the CKO mice.

2.7.6. Blimp-1 Deficiency Results in Unbalanced Cytokine Production

Analysis of cytokine production in control and CKO naïve $CD4^+$ T cells revealed that $CD4^+$ T cells from CKO mice produce IL-4 in levels similar to control mice. Production of IFNγ, however, was significantly increased in CKO cells as compared to control cells. Along with the increased IFNγ production, CKO cells also had approximately a 4-fold reduction in IL-10 producing cells. This altered cytokine production was observed in effector ($CD62L^{Lo}CD44^{Hi}$) $CD4^+$ T cells sorted from control and CKO mice and re-stimulated for 4 hours *in vitro*, and also in naïve $CD4^+$ CKO cells stimulated for 6 days with plated-bound anti-CD3 anti-CD28 and IL-2 in non-polarizing conditions. These findings suggest that Blimp-1 is required to regulate cytokine production following TCR stimulation. The finding that naïve CKO $CD4^+$ T cells respond to TCR stimulation with the same skewed cytokine pattern as effector cells differentiated *in vivo*, point to a cell intrinsic defect, rather than skewed cytokine production as consequence of the inflammatory disease that CKO mice develop.

2.7.7. Blimp-1 Deficient Naïve $CD4^+$ T Cells Cause Colitis in $Rag1^{-/-}$ Mice

We used a colitis model in which naïve $CD4^+$ T cells are adoptively transferred into $Rag1^{-/-}$ hosts[73] to test if the hyper responsiveness of the CKO naïve $CD4^+$ T cells could be translated into increased colitogenic potential *in vivo*. In this model, the transfer of low numbers (10^4cells) of naïve cells to a lymphopenic environment results in homeostatic proliferation and expansion. Activation of the expanded cells by antigens in the intestinal mucosa leads to the development of colitis. We found that both, control and CKO naïve $CD4^+$ T cells caused colitis in $Rag1^{-/-}$ recipient mice, however, CKO T cells caused more weight loss than control cells. Colitis scores, determined by inflammation and histological damage in the colon, were higher in the mice receiving CKO cells. In fact, some of the mice injected with CKO cells needed to be sacrificed before the end of the experiment, due to excessive weight loss. This indicates that Blimp-1 deficient naïve $CD4^+$ T cells are more colitogenic than control cells.

2.7.8. *Blimp-1 is Required for Regulatory T Cell Function In vivo*

In previously described murine models for colitis, disease initiation and/or progression is often associated with de-regulated $CD4^+$ T cell activation. This is due to either an intrinsically enhanced T cell responsiveness[74,75] and/or defective development or function of naturally occurring regulatory T cells (Treg)[76–78]. Thus we studied how deletion of Blimp-1 affected Treg function and development.

Analysis of the Treg compartment in the CKO mice revealed that the lack of Blimp-1 does not impair Treg development: CKO mice present virtually normal numbers of $CD8^-CD4^+CD25^+$ Treg in the thymus and $CD4^+CD25^+CD62L^{Hi}$ Treg in the periphery. In fact, we found that peripheral Treg are expanded as CKO mice age and develop colitis. We next tested the function of CKO Treg by addressing their ability to suppress proliferation of naïve $CD4^+$ T cell *in vitro*. In these experiments, naïve ($CD4^+CD45RB^{Hi}CD25^-$) control or CKO T cells were stimulated with T cell-depleted mitomycin C-treated syngeneic splenic cells and soluble anti-CD3ε in the presence of increasing numbers of control or CKO $CD4^+$ $CD25^+$ Treg. Control and CKO Treg suppressed proliferation of both control and CKO naïve $CD4^+$ cells equally in this system.

We also tested if CKO Treg could function *in vivo*. This was done using a model of experimentally induced-colitis in which mice are protected from dextran sodium sulfate (DSS)-induced colitis by injection with Treg[79,80]. Sorted $CD4^+CD25^+$ ($CD62L^{Hi}$ and $CD62L^{Lo}$ in a 1:1 ratio) Treg from control, but not from CKO mice, blocked colitis development. Therefore, although CKO Treg function normally *in vitro*, they had a defective suppressor function *in vivo* in the settings we tested. These results indicate that Blimp-1 is required for proper Treg function.

IL-10 production by naïve $CD4^+$ T cells is also impaired in the absence of Blimp-1, suggesting that the defective *in vivo* activity of the CKO Treg may be caused by decreased IL-10 production. Another possibility is that responsiveness to TGFβ is compromised in the CKO Treg. Interestingly, mice expressing a truncated TGFβ receptor in the T cell lineage contained Treg that were functional *in vitro* but failed to block DSS-induced colitis[79] or naïve T cell transfer-induced colitis *in vivo*[80]. The possibility that deletion of Blimp-1 affects TGFβ responses remains to be tested.

In the CKO mice it is likely that a combination of increased T cell hyper responsiveness and partially defective Treg function contribute to colitis. Although the mechanisms by which Blimp-1 regulates developing and mature T cell homeostasis and function requires further elucidation, our data provides the first evidence for a crucial role of this transcriptional repressor in the biology of T cells. Based on the phenotype of our CKO mice and the results discussed above, we hypothesize (Figure 2.5) that Blimp-1 inhibits responses to TCR-dependent activation in at least two ways: 1) basal levels of Blimp-1 in naïve T cells raise the threshold for response and 2) higher levels of Blimp-1 induced after response to TCR regulate effector functions, including cytokine production. The molecular mechanisms involved are not known but may include repression

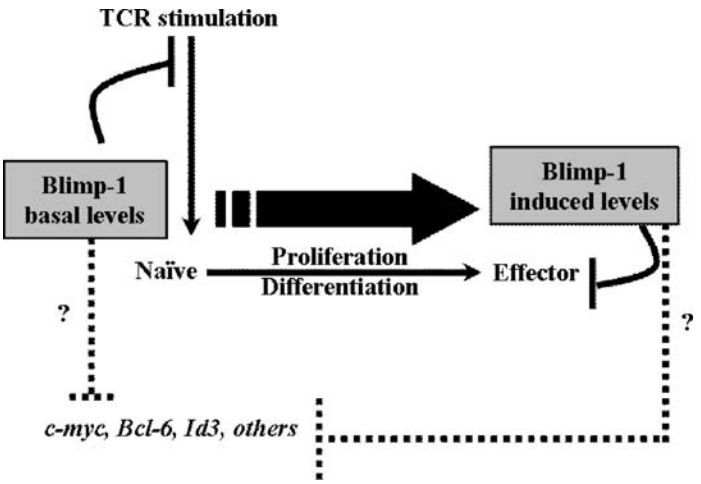

FIGURE 2.5. Hypothetical Model for Blimp-1 Regulation of T cell Activation. Naïve T cells express low levels of Blimp-1 (left box). Signals mediated through TCR activation and growth factors induce native T cell proliferation and differentiation into effector cells, and accompanied by up-regulation of Blimp-1 expression (right box). Blimp-1 in turn interferes with T cell effector function, by regulating proliferation and cytokine production. Possible molecular mechanisms for the function of Blimp-1 in T cells would include the regulation of target genes such as *c-myc*, *Bcl-6* and *Id3*, which were previously identified in B cells are Blimp-1 targets and are also expressed in T cells.

of previously identified targets such as *c-myc, Id3* and *Bcl-6*[17,33] or competition with IRF-1 and IRF-2[6].

2.8. Closing Considerations

In B cells, we understand that Blimp-1 regulates cascades of transcription factors thereby altering several important gene expression programs. Because it plays such an early and fundamental role in plasmacytic differentiation, studying Blimp-1 has revealed important aspects of this process and may help to understand other aspects of B cell regulation. Regulating the type and magnitude of response to TCR activation is clearly critical at multiple points in the life of thymocytes and T cells. Since Blimp-1 appears to modulate responses to TCR, we hope that further studies on Blimp-1 will reveal new aspects of T cell regulation.

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3
Memory B Cell Evolution: B Cell Biology

Louise J. McHeyzer-Williams and Michael G. McHeyzer-Williams

3.1. Introduction

Protein vaccination is an effective public health initiative with demonstrable utility in the prevention of infectious diseases and a safe alternative to the use of attenuated micro-organisms. Vaccine efficacy relies on Th cell regulated development of high-affinity B cell memory and its consolidation through antigen re-challenge. The rapid evolution of B cell memory occurs at the cellular level and is controlled by multiple cognate checkpoints during the development of adaptive immunity. While clonal selection is the fundamental process that underpins adaptive immunity, still surprisingly little is understood about the mechanism of its action across multiple junctures of antigen-specific memory B cell development in vivo.

Innate immune system activation initiates adaptive responses through cognate priming of naïve antigen-specific Th cells and differentiation of effector Th cells (Checkpoint I). Naïve B cells must also encounter their cognate antigen during this initial priming phase. Effector Th cells can then regulate B cell fate through cognate interactions that induce a spectrum of short-lived effector B cells (Checkpoint II). Alternately, B cell clonal expansion proceeds in secondary follicles at this developmental juncture as precursors to germinal centers (GC) that support BCR diversification and affinity maturation. In GCs, antigen-specific Th cells also impact the evolution of high affinity B cell memory (Checkpoint III).

The cellular products of the GC reaction provide precursors for the response to antigen re-challenge and a cohort of long-lived plasma cells that produce high affinity antibody. Antigen re-challenge initiates a memory Th cell controlled memory B cell response that promotes robust antibody production and the enhancement of the antigen-specific memory B cell compartment (Checkpoint IV). The memory response to antigen recall appears necessary for effective long-term protection and remains central to all vaccination programs, yet little is known of the rules that control this phase of immunity.

In this chapter, we will emphasize the open issues in this arena and present our own contributions in the context of model systems we have developed to access antigen-specific Th cells and B cells directly ex vivo.

3.2. Initiating Adaptive Immunity

Protein vaccination ideally promotes the helper T cell regulated appearance of high affinity B cell memory. Immune adjuvants drive local inflammation, inducing dendritic cell (DC) maturation and migration to draining secondary lymphoid tissue. The antigen-experienced DC that express pMHCII complexes recruit and prime naïve Th cells with TCR specificity above a threshold pMHCII binding affinity. These cognate interactions and their molecular context define the first major developmental checkpoint for B cell immunity by determining the quality and quantity of antigen-specific T cell help. At this early priming phase, naïve B cells must also encounter their cognate ligand, internalize, process and then present pMHCII complexes to procure specific effector T cell help and progress in their antigen-specific development in vivo.

3.2.1. Activating the Innate Immune System

Vaccination typically involves the co-administration of foreign protein and immune adjuvants. The immune adjuvant activates multiple facets of the innate immune system that subsequently modulate the quality and quantity of antigen-specific adaptive immunity. Adjuvants typically comprise a vehicle for antigen delivery and an inflammatory stimulant. The vehicle is often formulated to prolong the depot of antigen at the site of injection. In this manner, the protein antigen remains in the vicinity of the local innate immune system that has been directly and indirectly aggravated by the inflammatory stimuli. The discovery of Toll-like receptors (TLR) and other innate sensing mechanisms have accelerated our understanding of these inflammatory mediators and how they impact innate immunity[1,2]. These adjuvants provide a means to modify immune cell fate in vivo, however the precise influence on protective aspects of adaptive immunity remain poorly resolved.

While antigen clearance is the immune priority for survival, antigen-specific immune memory is the key to long-term immune protection. In this manner, adequate immune memory against critical protein antigens will protect against even primary infection by microorganisms expressing the antigen. Hence, the innate vaccine response must retain and faithfully convey antigen specificity to the many elements of the adaptive system. As the most efficient antigen presenting cells (APC) of the innate system, dendritic cells (DC) are central to this process of cognate regulation and are the key initiators of adaptive immunity.

DCs pre-exist as distinct cellular subtypes distributed across many potential sites of antigen entry and within draining secondary lymphoid tissue[3,4]. Upon local protein vaccination, DC sub-populations rapidly mature and migrate to the

T cell zones of these draining lymph nodes (Figure 3.1). Uptake and antigen processing is central to the capacity of antigen-experienced DCs to engage naïve Th cells expressing pMHCII-specific TCR. The level of pMHCII and the molecular context of its expression across different DC subtypes will primarily shape effector Th cell development. Interestingly, while B cells recognize unprocessed native antigen, it is possible that first contact with the BCR may occur through cell-associated antigen presentation. In this manner, the quality of APC maturation may directly impact antigen-specific B cell fate.

3.2.2. Cognate Priming of Naïve Th Cells

The central and defining outcome for naïve lymphocytes at checkpoint one is clonal selection. Hence, antigen receptor specificity is the principal guiding influence that shapes adaptive immunity. While 'bystander' activities can modify Th cell fate, cognate regulation uses the specificity, strength and context of TCR-pMHCII interactions to drive clonal expansion and effector Th cell development. Helper T cell responses using restricted TCR repertoires provide experimental access to these processes[5,6]. Earlier studies stressed the importance of the affinity of the TCR-pMHC interaction in determining T cell fate in

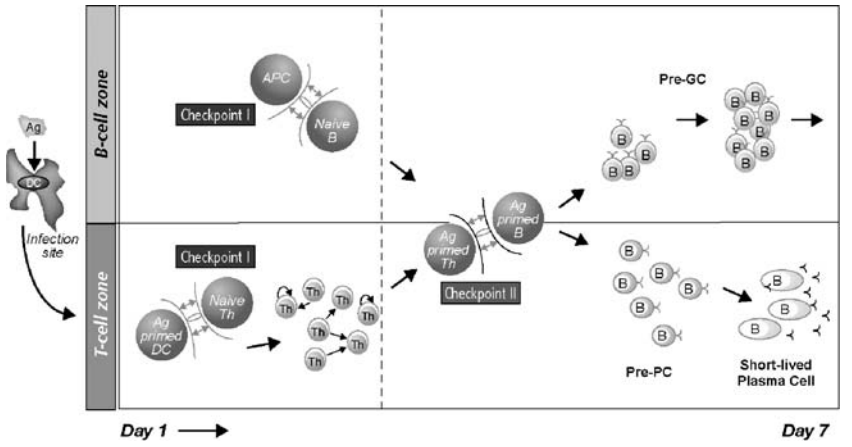

FIGURE 3.1. **Initiating Adaptive Immunity and Development of Effector Function**. Antigen experienced DC migrate from site of vaccination to the T cell zones of draining LN. Checkpoint one signifies the cognate interaction of pMHCII-expressing mature DC and pMHCII-specific naïve Th cells. Naïve B cell must also specifically recognize the protein antigen to initiate the TD B cell response and may do so in a cell-associated manner. Naive Th cells expand, differentiate into effector Th cells and migrate to the T-B borders to contact pMHCII-expressing antigen-primed B cells. Checkpoint two signifies the cognate interactions between a spectrum of effector Th cells and primed B cells that emerge after initial priming. B cell clonal expansion in the T cell zones produces pre-plasma cells as precursors to the short-lived plasma cell pathway. Expansion in the B cell zones is the pre-GC precursor to the GC reaction and production of B cell memory.

vivo[7–10]. It was possible that selective expansion of T cells expressing TCR of higher affinity in vivo rely on their capacity to compete for rare pMHC II complexes on the surface of APC[11,12]. There was also evidence in vitro[13–15] and in vivo[16,17] for the selective loss of clones expressing TCR with fast off rates for pMHCII.

In contrast, we more recently provide evidence for an affinity threshold mechanism that selects high affinity Th clones and propagates preferred clonotypes regardless of further differences in TCR-pMHCII half-lives or affinity[18]. Reducing antigen dose in these experiments does not influence the mixture of dominant clonotypes. Hence, the affinity threshold appears to be driven by intrinsic means and does not present as a mechanism involving clonal competition. Understanding the rules that drive Th cell clonal selection provides a means to attenuate adaptive immunity at a very fundamental level with impact on the subsequent B cell response.

3.2.3. Recruiting Naïve Antigen-Specific B Cells

Whether the initial B cell antigen encounter is cell associated or soluble in the context of standard protein vaccination is not yet clear. Antigen-pulsed DC can activate naïve B cells and some DC subsets have recently been shown to play a role presenting TI antigens to B cells in vivo[19,20]. Immunizing with immune complexes (IC) also clearly enhances cell associated antigen presentation[21]. Antigen presented to B cells by APC induces BCR and its signaling intermediates to cluster in ways that resemble an immune synapses[21]. This process substantially decreases B cell activation thresholds and appears to promote antigen uptake and processing. Thus, cell presentation of native antigen may be the more typical first encounter of naïve B cells with their cognate antigen providing a multitude of cellular and molecular options for regulating subsequent B cell fate.

3.3. Regulating B Cell Fate

Following clonal expansion, antigen-specific Th cells move towards the T-B borders of LNs and spleen to sample antigen-experienced B cells for pMHCII expression. Checkpoint two represents this cognate regulation of B cell fate. The range of cognate effector Th cell functions defines a broad spectrum of activities associated with the production of cyokines and expression of cell surface molecules whose combined action in vivo is still not fully appreciated. B cell expansion in the T cell zones is the prelude to short-lived plasma cell development, while expansion in the follicular regions forms secondary follicles as the precursor to germinal centers. Antibody isotype switch occurs in both arms of antigen-driven development and is most likely imprinted in a cognate manner by Th cells at checkpoint two.

3.3.1. *Effector Th Cell Function*

The fundamental nature of T cell help in vivo and the extent of particular activities required to orchestrate effective B cell immunity still remains unclear. Cytokine production is a cardinal Th cell activity that can be mimicked in vitro to drive basic aspects of B cell development such as clonal expansion, antibody isotype switch and antibody secretion. Hence, the capacity of these powerful soluble molecules to impact B cell immunity is unequivocal. Furthermore, when we measure the number of antigen-specific Th cells that express mRNA for any one cytokine directly ex vivo, the frequency is still very low[22]. What still remains difficult to discern is the actual role cytokines play in vivo and the reliance of cell surface molecules and the cognate control of cellular interactions that synergize to influence various B cell developmental processes[23].

The existence of multiple types of Th cells with sub-specialized function in adaptive immunity highlights a complex and dynamic cellular organization for T cell regulation of adaptive immunity. The original Th1/Th2 paradigm represented extreme ends of immune responsiveness that are now more reasonably viewed as a spectrum of outcomes controlled by the nature of the immune stimuli. Regulatory Th cell subsets have received extensive attention in the context of homeostasis and autoimmunity, but have also been found within the context of adaptive responses with proposed impact on the contraction of effector Th cells. We have also uncovered a pre-existing division in naïve Th cell development based on Ly6C expression with separable pre-immune TCR repertoires[24]. The antigen-experienced progeny of the Ly6Chi subset have a substantially higher differential capacity to induce plasma cells in the context of protein vaccination. The basis of these effector Th cell differences and the mechanism of their action in vivo remain unclear.

Effector Th cell physiology also changes in fundamental ways upon antigen-experience. We recently demonstrated the change in TCR signaling events as a consequence of antigen experience[25]. By day 7 after antigen challenge, TCR cross-linking no longer induced the elevation of intracellular calcium. Furthermore, polyclonal stimulation of effector Th cells in vitro using anti-CD3, anti-CD28 and IL-2 could not induce proliferation. Curiously, responsiveness returned to many cells by day 11 after antigen challenge. We interpret these trends as evidence for an antigen-specific memory Th cell compartment at sufficient frequencies to detect in vivo that can now respond to antigen re-challenge.

3.3.2. *Short-Lived Plasma Cells and Clonal Selection*

Checkpoint two shapes development in the non-GC pathway to short-lived PC production. Kelsoe and colleagues[26–28] demonstrated the broad BCR repertoire of short-lived PCs, however the comparative affinities of this compartment and the early GC B cells have not been directly measured. In our early studies[29,30], using the same polyclonal TD response to the hapten NP (4-hydroxy-3-nitrophenyl-acetyl), we presented evidence for sub-selection of the pre-diversified BCR of early GC B cells based on the restricted length of the

V_H186.2 CDR3 junctional region. More recently, Nussenzweig and colleagues[31] demonstrated the preferential selection of high affinity BCR transgenic B naïve cells into the GC pathway providing strong evidence for an early affinity-based selection mechanism in the primary B cell response.

Divergent development of specific B cells may be ultimately orchestrated by pMHCII-specific Th cells at all stages of development. However, based on evidence for clonal assortment of the BCR, specificity appears to play a primary role in cell fate determination. Whether, there is a similar intrinsic affinity threshold for the initial B cell recognition events as seen for the Th cell compartment, or an early episode of clonal competition before BCR diversification that initiates this division, is yet to be resolved. We propose an affinity threshold mechanism for initial B cell clonal selection prior to BCR diversification and affinity maturation. These earliest selection events may be controlled by a different set of principles than found in the GC cycle of activities for memory B cell development.

3.3.3. Commitment to Antibody Isotype Switch

The switch to antibody isotypes downstream of IgM/D occurs in both the non-GC and GC pathway of B cell development. Hence, it is clear that the GC is not required for isotype switch and that perhaps the Th cell regulated commitment to isotype switch occurs at an earlier stage of the response. We propose that this functionally critical cell fate decision may be another important attribute controlled by checkpoint two interactions. Thus, it is likely that sub-specialized effector Th cells control differential isotype switch. These effector functions would be acquired during the regulated induction of a propensity to secrete the appropriate cytokine profile and express the appropriate constellation of cell surface co-stimulation. It will be important to identify these sub-specialized effector Th cells to evaluate the impact of the initial immune stimuli and predict the isotype profile of the early antibody response. The mix of effector Th cells at checkpoint two may also potentially impact the spectrum of isotype in the long-lived memory compartment post-GC development.

3.4. Evolution of B Cell Memory

In Th cell regulated B cell response to protein antigens, memory B cell development proceeds through the GC reaction (Figure 3.2). Secondary follicles of antigen-experienced pre-GC B cells polarize into the dark/light zone organization of the typical GC reaction. These zones broadly support distinct phases of the GC cycle of activity. Clonal expansion and somatic hypermutation (SHM) of the BCR occurs in the centroblasts of the dark zone. Expression of variant BCR is selected in the centrocytes of the light zone with positive selection of high affinity variants on antigen somehow consolidated by cognate signals from GC Th cells. This last phase of the cycle defines checkpoint three as a critical

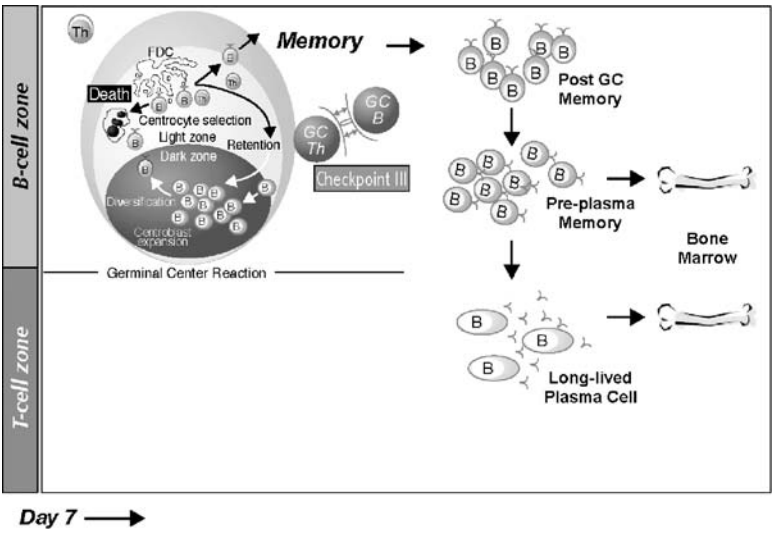

FIGURE 3.2. **Evolution of B cell memory**. Pre-GC expansion results in the formation of secondary follicles that subsequently polarize into the light and dark zones of the germinal center reaction. Clonal expansion is accompanied by somatic diversification of antibody variable region genes in the dark zone. Exit from cell cycle and expression of variant BCR allows for antigen-specific positive selection in the light zone. Regulation of GC B cell fate involves pMHCII-specific GC Th cells in ways that constitute checkpoint three of this developmental pathway. As depicted, multiple subsets of memory B cells emerge from the GC reaction of the primary immune response to constitute the high affinity memory B cell compartment.

developmental event in the commitment to the memory B cell compartment. Exit from the GC cycle signifies entry into the memory B cell compartment of either memory B cells as precursors for the response to antigen recall or long-lived plasma cells that continually produce high affinity antibody for the life of the animal.

3.4.1. Clonal Evolution in the GC Cycle

Over time, secondary follicles polarize into the light/dark zone organization of the GC reaction[32,33]. Expansion of antigen-specific B cells and BCR diversification by SHM initiates accelerated evolution in this antigen-specific B cell response[34]. Activation-induced cytidine deaminase (AID) is expressed by GC B cells and is required for both SHM and class switch recombination (CSR)[35]. The majority of centrocytes expressing variant BCR will die due to a decrease or loss of antigen binding after SHM[36–38]. However, the GC B cells that express higher affinity variant BCR are positively selected. Follicular dendritic cells (FDC)[39,40] and GC Th cells[41] are involved in this process in ways that remain poorly understood. Most models suggest immune complex (IC) trapping on follicular dendritic cells

(FDC) is the most likely means for variant BCR to receive a rescuing signal from native antigen and then GC Th cells provide cognate control of cell fate. Mapping the immediate and long-term outcome of positive selection and the assortment of the BCR repertoire at this critical developmental checkpoint has been difficult to access experimentally and remains an important challenge for the field.

The control of proliferation is another important variable that may directly or indirectly control the efficacy of clonal evolution in the GC. Large GCs that form in the absence of AID[34] are thought to indicate the lack of a negative feedback mechanism that follows SHM and/or CSR and allows dysregulated clonal expansion. The large GC in the absence of the CDK inhibitor p18INK4c more directly supports this contention[42]. Interestingly, blocking cell cycle arrest also impedes plasma cell formation connecting cell cycle and cell fate determination in this latter model. Large GC in the absence of Blimp-1[43] may also be related to the lack of cell cycle control together with a block in plasma cell formation. Interestingly, in this model there was also evidence for a defect in memory B cell subset generation. Ablation of the regulatory sub-unit of calcineurin, CnB1 in B cells[44] blocks NFAT transcriptional activity and also results in a large GC phenotype.

3.4.2. pMHCII-Specific GC Th Cells

During anti-protein immune responses, the Th cells within the light zone of the GC express pMHCII-specific TCR. There appears to be sequential movement of antigen-specific Th cells from the T cell zones into the GC microenvironment[45,46] with evidence that suggests GC Th cells can also move between different GC[47]. Interestingly, all antigen-specific Th cells responding to a protein antigen are not represented within the GC reaction, indicating pre-GC functional differentiation for responders Th cells[48]. Furthermore, non-GC Th cells can re-emerge in a memory response indicating that the GC is not required for memory Th cell development[48]. The function of the GC Th cells in the regulation of memory B cell development has been more elusive to define.

3.4.3. Multiple Memory B Cell Subtypes Exit the GC Cycle

The GC reaction produces at least two broad categories of affinity-matured memory B cells[23]. The most typical memory B cells are the precursors for a memory response to antigen recall. These memory B cells are easily distinguished functionally from the second cellular compartment of memory, the long-lived plasma cells[49]. The long-lived plasma cells are terminally-differentiated cells continually produce high affinity antibody and will not be drawn into a secondary response. In both categories of memory B cells the expression of antibody isotype distinguishes separable memory B cell compartments. By definition, these subsets differ in the antibody they can produce, but may also

differ in migration patterns, survival needs and the requirements for re-activation at the time of antigen recall. Hence, it is prudent to separate B cell memory by antibody isotype before evaluation of development programming and regulation of cell fate.

Early studies indicated populations of long-lived cells with slow or no turnover in vivo[50] that have more recently been shown to survive independent of the expressed BCR specificity[51]. Loss of sIgM/sIgD and expression of downstream isotype are indicative of antigen experience, but are not required for memory cell development[30,52,53]. Expression of mutated BCR with evidence for affinity increasing changes is the most useful molecular marker for the memory compartment[28–30]. However, there are abundant examples of germline-encoded BCR expressed by memory response precursors. Location has also proved useful with the appearance of antigen binding cells with downstream isotypes and mutated BCR in the blood very soon after intentional priming. The combination of location, phenotype, genotype and time after intentional priming has many elements of a comprehensive definition for memory B cells[54].

3.4.4. Pre-Plasma Memory B Cells

The more typical memory response precursors retain a high level of expression for a glycosylation variant of the B cell isoform of CD45R, B220 (seen by mAb 6B2)[29,55]. There is now evidence of a second subset of memory B cells that down-regulates this glycoform displaying reduced 6B2 levels[23]. Upon adoptive transfer of the different memory cell subsets into $RAG2^{def}$ recipients, antigen-specific clonal expansion and differentiation into PCs indicated that both compartments contained memory response precursors (104). The cellular and humoral immune response pattern on transfer and challenge and the broad parent to progeny relationship displayed allowed us to proposed a model of linear development for B cell memory. In this model, the $6B2^{hi}$ memory compartment lies developmentally upstream from the $6B2^{lo}$ pre-plasma memory compartment that itself appears to be a more immediate cellular precursors for long-lived PCs. The dynamics and details of the primary response to this antigen were similar (105) suggesting that this heterogeneous memory cell development was established as a consequence of the primary response GC reaction.

3.5. Consolidating B Cell Memory

The boost with antigen re-challenge seems necessary to establish adequate long-term protection following protein vaccination. The cellular and molecular control of the memory response resides largely at checkpoint four as an interface between memory B cells and memory Th cells (Figure 3.3). Both $6B2^{hi}$ and $6B2^{lo}$ sub-types of memory response precursor can respond to re-challenge and both sub-types re-establish higher levels in vivo after the acute recall phase of the response. Short-lived PCs producing high affinity antibodies are the dominant

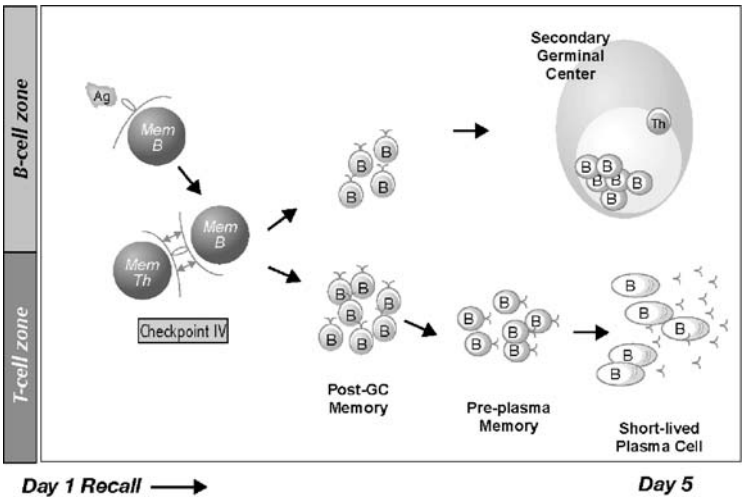

FIGURE 3.3. **Consolidating B cell memory**. Antigen-specific memory B cells are the most likely APC to initiate memory Th cell responses required at checkpoint four to initiate the response to antigen recall. Memory cell expansion produces large numbers of high affinity memory response plasma cells that appear short-lived. Secondary GC reactions are also part of the memory response. All cellular facets of the memory compartment, $6B2^{hi}$ post-GC memory B cells, $6B2^{lo}$ pre-plasma memory B cells and $CD138^{+}$ long-lived plasma cells are boosted to higher levels even after the secondary response wanes.

cellular outcome of secondary challenge with evidence for a minor secondary GC reaction. The long-lived memory PC compartment is also enhanced after recall with higher numbers of cells and serum levels of specific antibody that persist after the acute response.

3.5.1. Antigen Presentation and Re-Shaping B Cell Memory

While vaccination strategies use adjuvant in the boost response, memory B cell responses to protein antigen can occur in their absence. Hence, it is likely that memory B cells act as the principal antigen presenting cells in the memory response to re-challenge. However, in the presence of adjuvant at the boost, the innate immune system may re-shape the memory B cell compartment. It will be important to evaluate the mechanism of regulation at this memory phase of the response to promote the most protective long-term effector B cell immunity.

Antibody isotype controls the type of antigen clearance effector mechanism. What controls the re-expansion of isotype-switched memory B cells is not understood. To alter isotype profiles would change the quality of long-term B cell immunity. This spectrum of memory B cell isotypes may be under the control of differential memory Th cell function, that exerts its impact at checkpoint four. Alternatively, memory B cell recruitment may be influenced by innate immune

stimuli present at re-challenge. The impact of antigen re-challenge on continued long-term protection may be measured by the change in the isotype of serum antibody levels. However, it would also be important to measure the change in the non-secreting memory compartment as a predictor of the response to future antigen exposure.

Continued maturation of affinity can be demonstrated in many model antigen systems. While the results of increasing affinity among the memory responder population can be observed at the level of SHM and BCR affinities, the underlying mechanisms are still not clear. It is possible that limiting antigen dose at re-challenge exerts a selective pressure for the recruitment of the highest affinity memory B cells. This process of clonal selection would represent a more classical cellular drive without BCR diversification. Alternatively, the secondary response GC reaction may recruit memory B cells and re-initiate the GC cycle of expansion, diversification and positive selection. These issues have not been experimentally addressed in recent times with the cellular resolution currently available.

3.5.2. Memory Effector Th Cells

Memory B cell responses require Th cell control, at least during the first few days after antigen re-challenge. Under typical protein vaccination conditions, memory Th cells specific for pMHCII complexes would be the most abundant Th cell population available for this process. In the B10.BR (I-Ek restricted) response to pigeon cytochrome c (PCC), we can measure the rapid emergence of the memory Th cell response that reaches similar peak levels to the primary response, but 3-4 days earlier[6,46]. The frequency of PCC-specific Th cells producing any one cytokine at the peak of this response was surprisingly similar to what was observed after the initial priming[22]. Hence, using the same adjuvant and antigen dose as the initial priming induced a similar spectrum of memory effector Th cells. How these memory Th cells differ in the deployment of effector function, or the impact of these functions in the context of B cell memory is still poorly appreciated. Furthermore, the influence of adjuvant on this phase of vaccination needs to be resolved for its practical utility in the induction of adequate and appropriate B cell memory.

3.5.3. Memory B Cell Fate

Upon adoptive transfer, both $6B2^{hi}$ and $6B2^{lo}$ sub-types of memory B cells responded to antigen re-challenge. As discussed, the propensity for clonal expansion and plasma cell production differed between the two populations allowing us to propose a linear progression of development. Thus, the $6B2^{hi}$ subset was developmentally upstream from the $6B2^{lo}$ subset that was immediately upstream from plasma cells. Our analysis of the Blimp-1 conditional knockout animals supported this model of linear progression[43]. In the absence of Blimp-1, there was a block in the development of the $6B2^{lo}$ pre-plasma memory B cells that

accompanied the loss of PCs at all stages of the primary and memory response to antigen. Similarly, following secondary challenge there is a demonstrable increase in both memory sub-compartments as well as a rapid and substantial production of plasma cells[55]. The memory response PCs are largely affinity-matured products from the primary response and based on their rapid appearance and disappearance in the spleen, also appear to be short-lived. Thus, the cellular subsets developed in the GC reaction during the primary response re-emerge upon secondary challenge. Importantly, after the acute secondary response subsides, all memory B cell subsets can be found at substantially high frequencies in vivo.

3.6. Concluding Remarks

Vaccination is an important public health initiative with substantial impact on the prevention of infectious diseases. Understanding the mechanisms that shape antigen-specific B cell immunity remains a substantial challenge for this important undertaking. In this chapter, we have outlined the major developmental checkpoints that control the quantity and quality of effector B cell responses and the resultant B cell memory that is the major mechanism of vaccine action. At each stage of the developing immune response there are multiple cellular and molecular variables that impact cell fate. We have tried to emphasize what we currently understand to highlight what remains unclear at this point in time.

Our strategy has been to develop animal models, specialized new reagents and experimental strategies to provide direct access to the antigen-experienced lymphocyte compartment. Our priority has been to work in non-transgenic animals, as we believe that precursor frequency governs timing in the developing response and remains the most fundamental variable of immune-regulation in the primary response to antigen. This focus has led us to investigate the mechanisms that underlie clonal selection in the Th cell and B cell compartment and how these cell fate decisions potentially impact immune function. Finally, the regulation of the memory response to antigen recall has received little attention in recent years as a specialized and potentially unique means to modify initial vaccination response. We have begun analysis of this later phase of immunity and the role of adjuvant may provide access to important new facets of regulation.

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4
BCR-linked Factors in Developmental Fate Decisions

Randall J. Brezski and John G. Monroe*

4.1. Introduction

The initial diversity of the B cell antigen receptor (BCR) results in a pool of B cells that can recognize both self and foreign antigen. The formation of the B cell antigen receptor takes place in the bone marrow. Upon successful heavy chain gene rearrangement, the pre-BCR is first expressed on the surface of pre-B cells. Upon successful rearrangement of the light chain locus, the clonotypic antigen receptor is expressed on the surface of immature stage B cells. The immature stage B cell marks an important checkpoint in B cell development since this is the first stage where self reactive clonotypic antigen receptors can be sensed and eliminated by "negative selection." The B cells that emigrate from the bone marrow and first enter the spleen are known as transitional B cells. The transitional B cell stage also represents a developmental checkpoint in the spleen where self reactive antigen receptors can be sensed and eliminated. B cells then transit from the transitional stage to mature B cells that are immuno-competent and differentiate into effector cells after antigen receptor engagement.

After successful gene rearrangement of the heavy and light chains of the B cell receptor (BCR), these BCR components do not change during development unless the BCR encounters antigen. There are multiple outcomes for developing B cells after antigen encounter. In the immature/transitional B cell stages, upon antigen encounter the BCR can undergo receptor editing where there is once again rearrangement of the BCR locus. Self antigen stimulated immature/transitional and mature B cells can also enter a state of unresponsiveness, termed anergy. We and other have shown that in vitro stimulation of immature/transitional B cells undergo apoptosis while mature stage B cells undergo proliferation. It is therefore of interest as to why BCR stimulated immature/transitional stage B cells undergo

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apoptosis, while antigen receptor stimulated mature B cells undergo proliferation and differentiation[1].

Antigen receptor induced apoptosis of immature/transitional B cells is an intrinsic response of this stage of B cell development, although there are multiple extrinsic factors that can redirect cell fate. Costimulation of immature/transitional stage B cells with PMA, CD40, or IL4 can rescue the apoptosis response but not proliferation[2]. The molecular mechanisms that govern signal strength and the particular signaling pathways induced also appear to be governed by the microenvironment that the BCR resides in after receptor stimulation. Much attention has recently been given to cholesterol-enriched membrane microdomains, termed lipid rafts[3]. The lipid rafts are enriched in positive signaling molecules such as the Src family tyrosine kinases, and exclude tyrosine phosphatases such as CD22 and CD45[4]. Interestingly, BCR stimulation of mature B cells results in inducible translocation of the BCR into lipid rafts, while BCR stimulation of immature/transitional B cells does not result in BCR association with the lipid raft[5,6,7]. We have addressed the role of cholesterol in the development of B cells and how cholesterol-enriched lipid raft microdomains affect BCR signaling.

4.2. Proximal Signaling in B Cells

Stimulation of the BCR by crosslinking results in series of proximal signaling events that leads to altered gene expression and unique functional outcomes, such as apoptosis vs. proliferation[5]. The first events in genereation of BCR induced signals are the phosphorylation of the Immunoreceptor Tyrosine Based Activation Motifs (ITAM) of Igα and Igβ by the Src family kinase Lyn. This phosphorylation event results in the recruitment of the tyrosine kinase Syk, the adapter protein SLP-65 (also known as BLNK), and the formation of a macromolecule signaling complex. This complex is characterized by the recruitment and activation of PLCγ2, Btk, PI3K, Vav as well as other proteins (Figure 4.1). Our lab has particularly been interested in the activation of the PLCγ2 → NFkB → c-myc pathway, since the ability to activate and sustain this pathway differs between the immature/transitional and the mature B cell stages[5,8].

The phosphotidyl inositol phosphates are important lipids in proximal signaling. They serve as both anchors for plekstren homology (PH) domain containing proteins and can be cleaved into second messengers. PLCγ2 both contains a PH domain and can cleave phosphotidyl inositol 4,5 phosphate into diacyl glycerol (DAG) and inositol triphosphate (IP3)[9]. DAG activates multiple signaling pathways such as PKCβ leading to activation of the NFkB → c-myc pathway, and RasGRP3 which activates the MAPK pathway. The importance of the PLCγ2 pathway in B cells is exemplified by the fact that mice deficient in this protein have severely reduced peripheral mature B cells[10]. B cells deficient in PKCβ have relatively normal B cell development. However, PKCβ defiant B cells have no response to T1 independent antigens or anti-BCR, slight impairment of T dependent antigens and CpG and normal secondary responses to

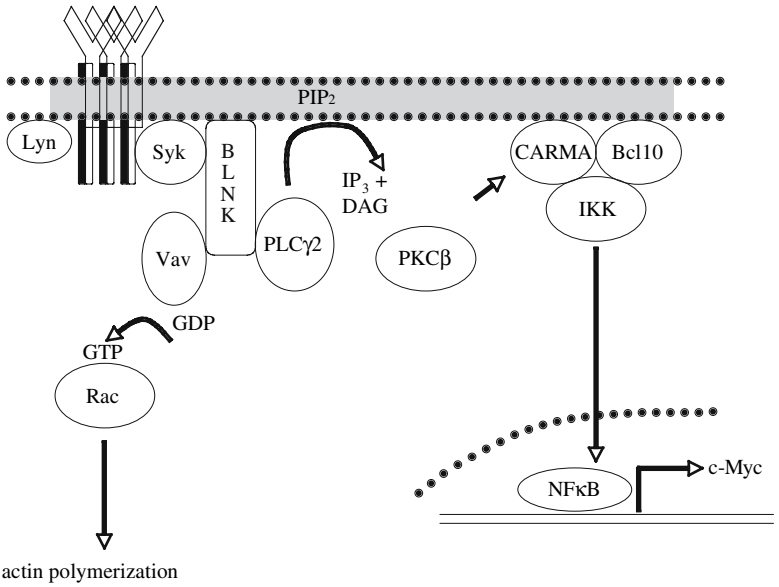

FIGURE 4.1. Proximal signaling pathways generated upon stimulation of the BCR.

T dependent antigens[11]. Mice deficient in the NFkB proteins c-Rel and RelA do not upregulate expression of c-myc, and the mature B cells have impaired survival when treated with anti-BCR in vitro[12]. Taken together, these genetic studies indicate that signals generated through PLCγ2 activation and subsequent propagation of these downstream B cell signals are critical for B cell activation and survival.

4.3. Characterization of the PLCγ2 → NFκB → C-myc Signaling Pathway in Immature/Transitional VS Mature B Cells

There are many similarities in the early signaling profiles of immature/transitional B cells and mature B cells. After receptor stimulation, both developmental cell types have the ability to form BCR aggregates on the surface of the cell. Both developmental stages inducibly phosphorylate the tyrosines in proteins involved in proximal signaling as well as generate an early wave of calcium flux after receptor stimulation. However, since the in vitro stimulation of these two developmental stages results in two separate fate decisions, we decided to explore downstream signaling pathways for differences. One obvious pathway to investigate is the NFkB pathway, which has been implicated in generating survival signals[13].

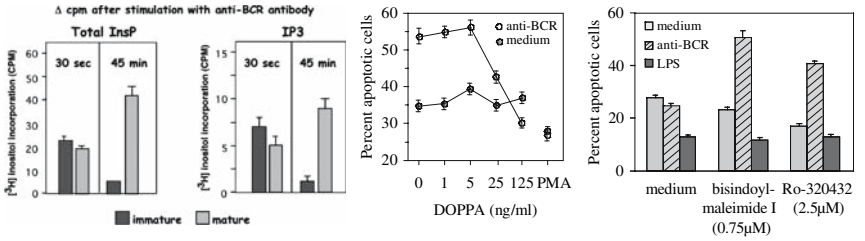

FIGURE 4.2. Isolated immature and mature B cells were stimulated with anti-BCR for the indicated times and total inositol phosphates (InsP) and IP_3 were isolated by ion exchange chromatography and quantitated by liquid scintillation. Shown are BCR-induced changes in cpm with unstimulated background controls subtracted (left panel). Adding a pharmacological agent that activates PKCβ rescues apoptosis of immature/transitional B cells (middle panel). Blocking PKCβ activation in mature B cells results in apoptosis after anti-BCR stimulation (right panel).

Our lab and others have shown that immature/transitional stage B cells have an inability to sustain signals generated through the PLCγ2 → NFkB → c-myc pathway[5]. At early time points, immature/transitional cells flux calcium and produce similar levels of IP3. However, the production of IP3 is reduced at 45 minutes compared to mature B cells (Figure 4.2). Furthermore, production of c-myc protein is transient in immature/transitional B cells compared to mature B cells. In order to determine if the survival aspects of NFkB signaling could be rescued in immature/transitional B cells, this developmental population was treated with a pharmacological agent that activates PKCβ specifically. Pharmacological activation of PKCβ in immature/transitional B cells does in fact rescue apoptosis but not proliferation. If the PKCβ pathway is blocked in mature B cells, after anti-BCR stimulation, these cells take on an immature/transitional phenotype and undergo apoptosis[8]. Taken together, these data show that this pathway is integral in the fate decisions that developing B cells make after anti-BCR stimulation.

4.4. Influence of Cholesterol-enriched Domains on the PLCγ2 → NFκB → C-myc Signaling Pathway in Immature/transitional Vs Mature B cells

The concept of membrane heterogeneity has recently played an important role in understanding how antigen receptor signals are initiated and propagated. The cholesterol-enriched membrane microdomain, termed the lipid raft, has gained much attention[14]. The lipid raft has a high concentration of sphingolipids, which contain longer, saturated acyl chains than glycerophospholipids. This results in a highly hydrophobic environment that allows tight packing of cholesterol within the saturated acyl chains of sphingolipids. Because of this the lipid raft environment favors some proteins that have undergone particular fatty acid

modifications such as myristoylation and palmitoylation. The Src family kinase member Lyn contains both of these fatty acid modifications and is constitutively localized to the lipid raft. This results in an optimum microenviroment for BCR signal initiation. Lipid rafts also contain members of the phosphotidyl inositol lipids. It has also been shown that NFkB pathway proteins inducibly localize to rafts, so this microdomain has been implicated in propagating NFkB signaling[15].

We and others have shown that immature/transitional stage B cells do not inducibly colocalize the BCR with lipid rafts after anti-BCR stimulation[5–7]. Mature B cells do inducibly colocalize the BCR with lipid rafts. Since cholesterol is a key component in the formation of lipid rafts, we wanted to determine if transitional/immature B cells had different plasma membrane levels of cholesterol compared to mature B cells (Figure 4.3). We found that transitional B cells contained 3–4 times less cholesterol than mature B cells. These results were confirmed with gas chromatography analysis. We also determined that the membrane levels of immature/transitional B cells can be manipulated to contain

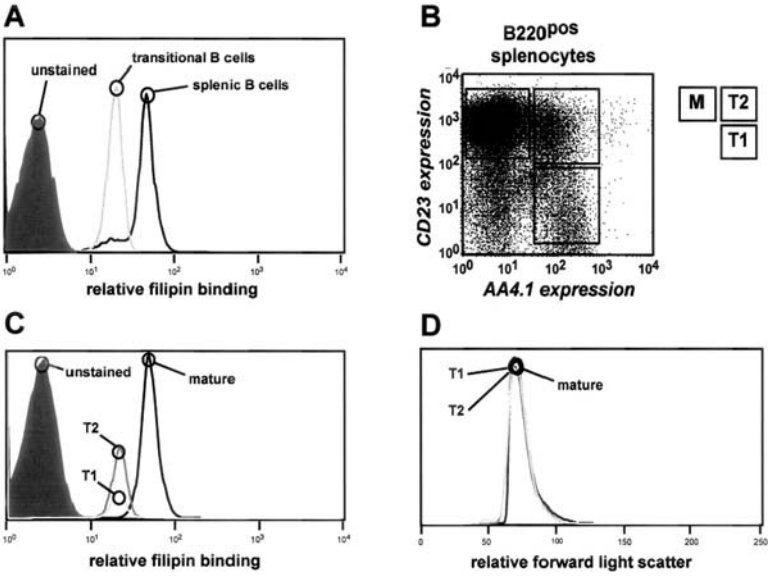

FIGURE 4.3. Transitional immature B cells contain less cholesterol than mature B cells. A, B cells were prepared from the spleens of BALB/c mice 14 days after irradiation (transitional B cells) or non-irradiated BALB/c mice (mature B cells). The cells were then fixed with paraformaldehyde and allowed to bind to filipin. Unstained mature B cells were used as a staining control. Cholesterol levels in transitional immature B cells were two to three times lower than mature splenic B cells. B and C, B cells were purified from the spleens of non-irradiated BALB/c mice. B220pos cells were then examined for their expression of CD23 and AA4.1. Three populations were then gated based on these markers, M (CD23pos and AA4.1lo), T2 (CD23pos and AA4.1hi), and T1 (CD23neg and AA4.1hi). Each of these populations was then examined for filipin binding as shown in C. D, forward scatter of the three populations.

relatively the same level of cholesterol as mature B cells. This provided an experimental model to determine how cholesterol levels affect particular signaling pathways in developing B cells[5].

As stated previously, anti-BCR stimulation of immature/transitional B cells results in BCR aggregates on the cell surface, but these aggregates do not co-polarize with lipid rafts. Cholesterol-enrichment of immature/transitional B cells rescues anti-BCR induced co-polarization of BCR aggregates with lipid rafts. Therefore, it seems likely that cholesterol-enriched immature/transitional B cells signal from a lipid raft environment. We first tested if anti-BCR induced signals in cholesterol-enriched immature/transitional B cells affected the NFkB signaling pathway (Figure 4.4). Induction of phospho-PLCγ2 was rescued by the cholesterol-enrichment, such that the treated cells now mirrored the mature B cell phenotype. In order to test if this had an affect on downstream NFkB signaling, we determined message levels of c-myc. Immature/transitional B cells induce c-myc message at one hour after anti-BCR stimulation, but are unable to maintain those levels through 4 hours. Mature B cells induce c-myc message at one hour after ant-BCR stimulation and are able to maintain production of

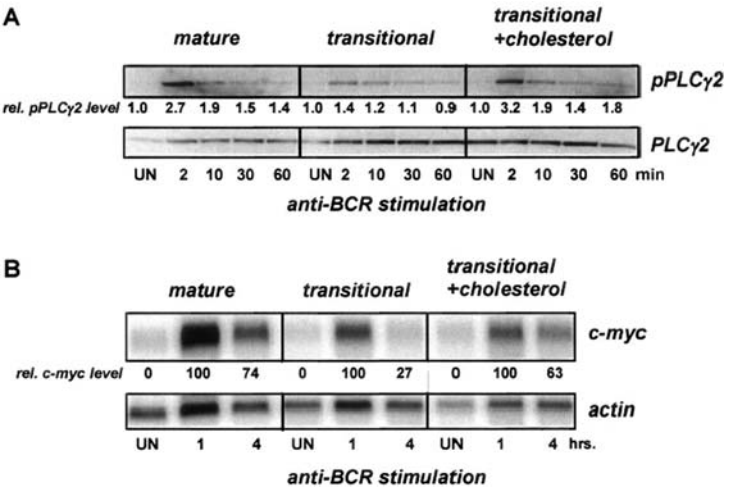

FIGURE 4.4. Cholesterol addition to transitional immature B cells results in a signaling phenotype similar to mature B cells. *A*, purified splenic B cells from wt BALB/c mice (mature) and day 14 sublethally irradiated mice (transitional immature) were unstimulated or stimulated for 2, 10, 30, and 60 min, lysed, and proteins were separated on SDS-PAGE. PLC_2 and phospho-PLCγ2 were detected by Western blot. The relative phospho-PLCγ2 levels indicate the relative increase in phospho-PLCγ2 compared with background. *B*, purified splenic B cells from wt BALB/c mice (mature) and day 14 sublethally irradiated mice (transitional immature) were unstimulated or anti-BCR stimulated for 1 and 4 h. c-*myc* transcripts were identified by Northern blot analysis. β-Actin transcripts are shown as a control. Relative c-*myc* levels represent the percentage of signal remaining relative to peak induction at 1 h.

c-myc message through 4 hours. Cholesterol-enrichment of immature/transitional B cells rescued the ability to sustain message through 4 hours post stimulation. These data suggest that BCR signals generated in a lipid raft environment influence sustained signaling of the PLCγ2 → NFkB → c-myc pathway[5].

4.5. Links Between Cholesterol-enriched Domains, Directed Actin Polymerization, and Sustained Signaling

It has become increasingly clear that there is a connection between cytoskeletal reorganization and the ability to sustain surface receptor induced signaling[16]. For instance, in several cell types, blocking cytoskeleton rearrangement results in transient signaling, particularly with MAPK signaling[17]. One signaling pathway leading to actin polymerization involves the Guanine Nucleotide Exchange Factor (GEF) Vav and the small Rho GTPases such as Rac1, cdc42, and RhoA. RhoGTPases are either active when bound to GTP or inactive when bound to GDP. Vav facilitates the association of Rho GTPases with GTP, which regulates downstream effectors, including actin polymerization and cytoskeleton rearrangements[18].

In T cells, upon antigen-receptor stimulation, the cytoskeleton polarizes toward peptide pulsed APCs, antigen-coated beads, and anti-CD3 coated plates. Defects in these signaling pathways result in blockage of directed actin polymerization toward APCs, beads, and plates as well as blocking of membrane ruffling. Specifically, the activation of a Vav1-Rac1 pathway leads to cytoskeleton reorganization and promotes T-cell/APC conjugate formation[19]. Interestingly, defects in polarization of the actin cytoskeleton in T cells leads to transient signaling. In particular, T cells that have deficiencies in cytoskeleton polarization do not sustain phosphorylation of ERK or produce IL2[17,20].

In B cells, Vav1/Vav2 null mice and Rac1/Rac2 null mice have similar phenotypes. Both null mice have a block in B cell development, specifically in the ability for transitional immature B cells to differentiate into mature B cells. In addition, anti-BCR induced signals leading to proliferation and survival are compromised, indicating that signaling through the GEF Vav and Rho GTPases are integral in B cell development and antigen receptor signaling[21,22,23]. The adaptor proteins Grb2 and BLNK are required for the recruitment of Vav to the lipid raft[24]. Recruitment of Vav to rafts results in GTP-loading of Rac1. Grb2 null and BLNK null mice and cell lines are defective in Vav raft localization and Rac1 GTP-loading. It has also been shown that the adaptor protein BAM32 is required for efficient GTP-loading of Rac1. B cells lacking Bam32 and subsequent Rac1-GTP do not undergo membrane ruffling and have a transient signaling phenotype[25]. Since these phenotypes are similar to immature/transitional B cells, we tested if there are deficiencies in Vav/Rac1 signaling in immature/transitional B cells.

We have seen that immature/transitional B cells are defective in directed actin polymerization. Cholesterol-enrichment of transitional immature/transitional

B cells rescues directed actin polymerization. In fact, the ability to undergo directed actin polymerization correlates with the ability to sustain anti-BCR induced signals. We hypothesize that directed actin polymerization can influence sustained signaling through two models. First, cytoskeletal reorganization directed towards antigen engaged BCR can facilitate a greater concentration of signaling proteins to form a long lasting macromolecular signaling complex. Second, the small Rho GTPases, which are responsible for directed actin polymerization, have been shown to recruit PI5K[26]. PI5K can help maintain phosphotidyl inositol levels, leading to sustained activation of the PLCγ2 $\rightarrow$ NFkB $\rightarrow$ c-myc pathway.

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5
Gene Regulatory Networks that Orchestrate the Development of B Lymphocyte Precursors

Harinder Singh[1], Jagan M. R. Pongubala[2] and Kay L. Medina[3]

Abstract: The B cell developmental pathway represents a leading model within the hematopoietc system for the analysis of gene regulatory networks, which orchestrate cell fate specification and commitment. Considerable progress is being made in the characterization of regulatory components that comprise such networks and examining their connectivity. These components include the cytokine receptors Flk2 and IL-7R as well as the transcription factors PU.1, Ikaros, E2A, EBF and Pax-5. We review recent experimental evidence concerning the molecular functions of these regulatory components and attempt to connect them in sequentially acting and inter-dependent regulatory modules.

5.1. Transcriptional Control of B Cell Fate Specification and Commitment

The B lineage represents a leading system for analyzing transcription factors that orchestrate cell fate specification and commitment within the hematopoietic system[2,20]. The transcription factors E2A, EBF and Pax-5 are intimately involved in the generation of B cell precursors[1,10,23,26]. Both E2A and EBF are required for specification of the B cell fate that involves activation of early B lineage genes, mb-1, B29, □5, VpreB (components of pre-B cell receptor) and initiation of DNA rearrangements at the IgH locus. The E2A gene encodes two basic helix-loop-helix proteins, E12 and E47, generated by differential splicing[14]. EBF is

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an atypical helix-loop-helix zinc finger protein that is expressed exclusively in the B lineage within the hematopoietic system[7]. Targeted inactivation of the E2A or EBF gene results in a block in B cell development at the stage of onset of early B lineage gene expression and the initiation of D-J rearrangements at the IgH locus[1,10,26]. E2A/EBF compound-mutant heterozygotes exhibit a more severe defect in B lymphopoiesis than the single-heterozygotes[16]. E2A has been proposed to induce expression of the EBF gene and the two factors appear to function synergistically to activate transcription of several early B cell genes[6,16].

Unlike with E2A and EBF, targeted disruption of Pax-5 results in a block to B cell development after the onset of early B lineage gene expression[23]. Pax-5-/- pro-B cells properly express most early B lineage genes and undergo D_H-J_H and proximal V_H-DJ_H gene rearrangements[23,8]. However, these Pax-5-/- pro-B cells are not committed to the B cell lineage. They display multilineage capacity and generate a wide range of hematopoietic cell types upon transplantation in vivo or culture in vitro[15,17]. Thus, Pax-5 is required for commitment to the B lineage and suppression of alternative cell fates. Importantly, Pax-5 is also needed for maintenance of B cell identity via the active repression of lineage inappropriate genes[12]. The conditional deletion of Pax-5 in B lineage cells results in the mis-expression of myeloid genes including M-CSFR (macrophage-colony stimulating factor receptor). It should be noted that Pax-5 represses the expression of myeloid genes while inducing the expression of B lineage genes that include mb-1, CD19, and BLNK Based on these analyses, we propose that E2A and EBF can be regarded as primary B cell fate determinants (required for cell fate specification) and Pax-5 as a secondary B cell fate determinant (required for cell fate commitment).

Recently, E2A-deficient cells that resemble B lineage progenitors (cKit+ B220+CD43+CD19−) have been shown to retain multilineage developmental potential suggesting that E2A is necessary not only for B cell fate specification, but also important in restricting alternate developmental fates[9]. Importantly, EBF and Pax-5 expression is compromised in E2A-/- progenitors. Since EBF is required for the developmental activation of the Pax-5 gene[11,16] and these two transcription factors are expressed specifically at the onset of B cell development, these results raise the possibility that restriction to the B cell fate involves the sequential or concerted action of EBF and Pax-5. Consistent with this possibility, a functional bypass experiment has shown that EBF can induce the generation of properly specified B cell precursors from E2A-/- progenitors[19].

5.2. Role of IL-7 Signaling in B Cell Fate Specification

The cytokine receptors Flk2 and IL-7R are essential for fetal as well as adult B lymphopoiesis[21,24]. Furthermore, signaling through the IL-7R appears to regulate the earliest events in B lineage development[13]. Thus the IL-7R receptor is an attractive candidate for generating signals that could be used in specification of the B cell fate. We have demonstrated that the transcription factor PU.1

is required for the generation of hematopoietic progenitors expressing Flk2 and IL-7R[11]. PU.1 appears to directly regulate the expression of the IL-7Rα gene[4] and may similarly control expression of Flk2. We have therefore suggested that a major function of PU.1 in B lymphopoiesis is to generate progenitors that are competent for the induction of the B cell developmental program. Cumano and colleagues have analyzed the role of IL-7R signaling in early B cell development and its pathway of action using IL-7-/- mice. There is a profound block to B cell generation in the bone marrow of adult IL-7-/- mice (8-12 weeks of age)[3]. There is a modest reduction of CLPs in the bone marrow of IL-7-/- mice. Therefore, it is possible to isolate IL-7-/- CLPs and quantitatively assess their ability to give rise to B, T and NK cells using stromal cell or fetal thymic organ cultures[5]. IL-7-/- CLPs have greatly diminished B cell developmental potential. Importantly, IL-7-/- CLPs can give rise to T and NK progeny with similar frequencies as wild-type CLPs. Thus, IL-7 signaling in the bone marrow appears necessary to induce or sustain B cell fate determinants in CLPs. Intriguingly, IL-7-/- CLPs express considerably lower levels of EBF and Pax-5 transcripts when compared with wild-type CLPs whereas GATA-3 transcripts are equally abundant. As GATA-3 is required for T cell development but dispensable for B cell generation[18], these results reinforce the specific developmental defect manifested by IL-7-/- CLPs. Finally, expression of EBF in the IL-7-/- CLP's restores B cell development. Since EBF regulates expression of the Pax-5 gene, IL-7 signaling likely induces the EBF gene in CLP's. Collectively these results strengthen the possibility that, IL-7R signaling via EBF promotes specification of the B cell fate.

5.3. EBF as a Primary B Cell Fate Determinant

Using PU.1-/- fetal liver hematopoietic progenitors that are blocked in B cell development and functional bypass experiments, we have proposed a gene regulatory network that mediates B cell generation from multipotent progenitors, MPPs[11]. However, in this network the identity of the primary B cell fate determinant remains to be elucidated. Such a determinant is expected to promote B cell fate choice at the expense of other developmental options by antagonizing the expression or activity of alternate lineage determinants. Paradoxically, E2A, EBF and Pax-5 have each in turn been suggested to regulate B cell fate choice[6,22,25]. The induction of E2A activity via downregulation of *Id* gene expression has been widely considered to promote B cell fate choice, however, such a function has not been experimentally tested[6]. Precocious expression of EBF or Pax-5 in HSCs has been shown to efficiently promote B lymphopoiesis *in vivo*[22,25]. However, these analyses did not quantitatively evaluate the developmental potencies of purified MPPs expressing EBF or Pax-5. Therefore, these studies failed to distinguish between the roles of EBF or Pax-5 in regulating B cell fate choice *versus* promoting the expansion of B lineage progenitors.

To address this unresolved issue, we have expressed each of the key B lineage transcription factors (E2A, EBF or Pax-5) directly in purified MPPs and quantitatively assessed their ability to generate B lymphoid and myeloid progeny (Pongubala and Singh, unpublished results). Our experimental strategy and accompanying molecular analyses have led to the identification of the primary B cell fate determinant and provided insight into the mechanisms by which it dictates cell fate choice. Our results strongly suggest that EBF is the limiting factor for B cell fate determination in MPPs. Precocious expression of EBF in MPPs results in induction of Pax-5 and the expression of early B lineage genes. Importantly, EBF promotes B cell development from MPP's at the expense of myeloid cell fate options. EBF can also re-direct myeloid progenitors to adopt the B cell fate. In doing so, EBF downregulates the myeloid cell fate determinants, PU.1 and C/EBP□. Finally, using EBF-/- progenitors that retain multilineage potential we demonstrate that EBF can antagonize myeloid differentiation not simply as a consequence of inducing Pax-5. Based on these results, we propose that EBF functions as the pivotal node of a gene regulatory network, which integrates multiple developmental inputs to dictate B cell fate choice in MPPs.

5.4. Perspective

The B cell developmental pathway represents a leading system for the analysis of regulatory circuits that orchestrate cell fate specification and commitment. Considerable progress has been achieved, within the past decade, in the identification and characterization of various regulatory components. These include, transcription factors and signaling molecules. It is now possible to initiate the assembly of molecular circuits that underlie cell fate choice and specific developmental transitions. We have attempted to assemble such control circuits, which operate in multipotential progenitors (MPPs), lymphoid progenitors (CLPs), pro-B and pre-B cells, respectively. Detailed knowledge of regulatory networks that specify distinct immune cell fates should facilitate the directed and efficient generation of lineage specific progenitors from embryonic stem cells for therapeutic purposes.

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6
Rules for the Rearrangement Events at the L Chain Gene Loci of the Mouse

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6.1. Introduction

One B-lymphocyte produces – and displays on its surface – one, and only one antibody, one immunoglobulin (Ig) structure in 10^4 to 10^5 copies with one heavy (H) and one light (L) chain amino acid sequence. This central dogma of immunology, the basis of the clonal selection hypothesis (Jerne, 1955, Burnet, 1957) is observed in mouse B cell development by the H chain locus for 98 % to 99 %, and with the L chains loci for 95 % to 97,5 % of all peripheral splenic and lymph node B cells. (For a review see[9]) Hence, in the vast majority of these B cells – but not necessarily in other B cell lineages such as the BI lineage – only one of two IgH-, and only one of six IgL (one κL and three λL chain loci) alleles are translated into proteins which assemble to Ig molecules and are deposited as antigen-specific receptors (BcR's) on the cell surface. This choice of one allele of the H-chain and L chain loci is termed "allelic exclusion".

The molecular mechanisms and cellular developmental pathways, by which B-lymphocyte lineage cells achieve this allelic exclusion, appear to be different for the H- and the L chain loci. Ig gene loci have to undergo V(D)J-recombination before they can be expressed as mature mRNA molecules and translated into protein. (For reviews see[8,9])

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6.2. Allelic Exclusion at the H Chain Locus

Rearrangements at the Ig-loci occur in stepwise fashion at different stages of B cell development. First D_H segments are rearranged on both IgH-chain alleles to J_H-segments. D_HJ_H/D_HJ_H rearranged pre BI cells develop from pluripotent hematopoietic stem cells, myeloid/lymphoid – and lymphoid progenitors, in which the rearrangement machinery, i.e. the RAG1 and RAG2 enzymes, are expressed, and in which the D_H and J_H-regions of the IgH chain loci become accessible to this rearrangement machinery first. Second, pre BI cells enter V_H- to D_HJ_H-rearrangments and become pre BII cells. (Figure 6.1) Successful, i.e. productive, in-frame-rearrangements generating μH chain proteins are probed by surrogate light (SL) chain. Surrogate L chain is already expressed in pre BI cells. As soon as μH chains are expressed they are probed for their ability to form a pre B cell receptor (preBcR) with SL chains, so that they can be displayed on the surface of pre BII cells. (For a review see [10]) A large part, i.e. between 50 % and 80 % of all pre BII cells with newly generated μH chains apparently cannot form a functional preBcR, and are likely to be excluded from further B cell development, unless they can enter a V_H to D_HJ_H-rearrangement on the second allele, or a V_H-replacement on the $V_HD_HJ_H$-rearranged allele.

Pre BII cells expressing a fitting μH chain, hence displaying a preBcR on their surface, are stimulated to proliferation by preBcR crosslinking induced in a cell-autonomous, ligand-independent fashion. Functional preBcR

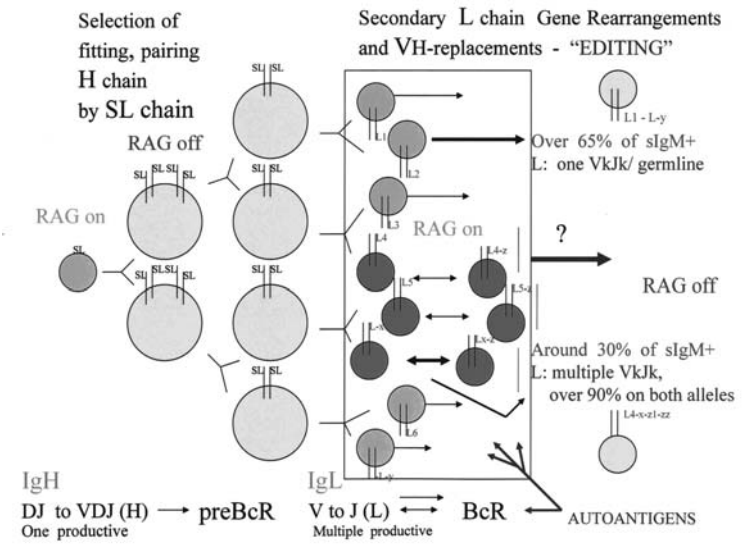

FIGURE 6.1. The scenario of B cell repertoire development through the stages of V_H to J_H-rearrangements on the H chain locus, followed by Vκ to Jκ-rearrangements on the κL chain locus of the mouse. For details see the text.

expression also signals the downregulation of SL chain expression, thereby limiting the proliferative expansion of pre BII cells. Expression of μH chain also signals downregulation of the rearrangement machinery, i.e. RAG1 and RAG2 expression, and closes the D_HJ_H-rearranged H chain alleles for further V_H to D_HJ_H-rearrangements. However, since triple deficient $V_{preB1}^{-/-}$, $V_{preB2}^{-/-}$, $\lambda5^{-/-}$ B-lineage cells still show allelic exclusion of their IgH chain locus expression, the signalling for this allelic exclusion cannot be mediated by the classical preBcR, but s likely to be effected by an alternate form of a preBcR not involving SL chain. Since double productively $V_HD_HJ_H$-rearranged pre BII cells and mature B cells have been found in numbers expected from a random process of in-and out-of-frame rearrangements at the H chain alleles, and since in all these cases only one of the two productively rearranged alleles could form a functional preBcR, allelic exclusion mediated by successful μH chain expression should be achieved by a pairing mechanism of these μH chains with a SL-chain-analogous partner.

6.3. Allelic Exclusion at the L Chain Gene Loci – Rules of V_L to J_L-Rearrangements Derived from Single Cell Pcr Analyses

When large, proliferating pre BII cells with a functional preBcR cease to proliferate and become resting, small pre BII cells, because SL chains become limiting, or when μH chain-expressing pre BII cells develop to a comparable small, resting pre BII cell stage without proliferation, they open the κL-and λL-chain gene loci for access, and reactivate the rearrangement machinery, i.e. RAG1 and RAG2. (Figure 6.1)

Single cell PcR analyses have been developed with primers which detect non-rearranged κL chain loci (i.e. in germ-line configuration), Vκ to $Jκ_1$-, Vκ to $Jκ_2$-, *V*κ to $Jκ_4$, *V*κ to $Jκ_5$ and *V*κ to RS rearrangements, all with efficiencies between 85 % and 95%. Furthermore, a universal $V_{λ1}/V_{λ2}$ – to $J_{λ1}/J_{λ2}/J_{λ3}$ – primar set was designed to detect V_L to J_L-rearrangements at the 8L chain loci with similar efficiencies.[1a] Hence, we could expect to detect simultaneously in a single cell the maximum of all the possible six rearrangements in approximately half of all the single cells assayed. Multiple rearrangements at the κL chain locus are detectable since all L chain-rearranging cells are non-dividing, hence do not loose excised DNA after deletion-type V_L to J_L-rearrangements. The use of a general primer for the Vκ-segments in the κL chain locus allowed the detection of all Vκ-segments of the locus.[4] Sequencing of the Vκ-Jκ-joints, furthermore, enabled the detection and quantification of in- and out-of-frame V_L-J_L rearrangement joints.[1,2]

The analyses of Vκ to Jκ-rearrangements were first done at both alleles of the κL chain locus in wildtype B-lineage cells. This made a clear assignment of an individual VκJκ-joint to one of the two rearrangements – competent alleles

impossible and, therefore, did not allow to follow multiple rearrangement events on a single allele. However, they allowed a number of interesting conclusions:

1. The first Jκ-segments, $J\kappa_1$, is most often used – three times as frequently as Jk_2 and six times as frequently as Jk_5.
2. Fifteen to 25 % of all immature and mature B cells carry a single Vκ to Jκ rearrangement. It appears that the fastest cells entering the sIg^+ B cell pools, first immature, then mature are the ones with only one VκJκ-rearrangement. These cells continue to predominate the peripheral mature B cell pools. Since mature B cells no longer are rearrangement-competent, i.e. do no longer express RAG1 and RAG2, they appear to be selected preferentially into the peripheral mature pools. The deposition of Ig in the surface membrane appears to be one mandatory step in this selection, as induced destruction of the capacity of mature B cells to produce sIg induces their apoptosis.[11] It remains to be seen whether relative fitness of H/L-pairing and/or ligand antigen binding to Ig play additional rules in the selection of B cells.
3. While 12 % of all pre BII cells, 25 % of all immature B cells and 50 % of all mature B cells carry only one VκJκ -rearrangement and have the second κL chain allele in germline configuration, 14 % of all pre BII cells, but only 5 % of all immature B cells and 6 % of all mature B cells have two VκJκ rearrangements, and no germline κL chain allele. This indicates, first, that both alleles are rearranged once – and only once. Hence, if the first allele is either non-productively rearranged, or produces an κL which is incompatible with a rapid transfer of the cell into the immature and mature B cell compartments, then the second allele is opened for rearrangements – rather than continuing rearrangements with secondary rearrangements only at the first allele. This is in contrast to the prevailing notion that rearrangements begin, and are continued, at one allele while the second allele remains inaccessible.[12,13,14] If the first allele produces an autoreactive L chain, which induces secondary L chain rearrangements not at the same, but at the other allele, it might lead to B cells producing two L chains, thereby defying the dogma of allelic exclusion – with the added danger of a rescue of autoreactive BcR-expressing B cells into the peripheral mature B cell pools.
4. Multiple Vκ to Jκ- and to RS-rearrangements, as well as λL chain gene rearrangements can be detected in 30 % of all pre BII cells and of all immature B cells and in 25 % of all mature B cells. However, these rearrangements could not be allocated to a given allele, necessitating the need for analyses on a single κL chain allele.

Therefore, single cell PcR analyses were also done with pre BII cells, immature and mature B cells of wildtype /$JC^{-}\kappa - F_1$, mice, i.e. in the potential presence of κL chain proteins in single B-lineage cells, or in the absence of κL chain proteins, i.e. in Cκ-/$JC^{-}\kappa - F_1$, cells. Again, a number of conclusions were drawn from these analyses.

1. As in cells with two rearrangements-competent alleles single rearrangements prefer the most 5'-located $J\kappa_1$-segments.
2. The frequencies of B-lineage cells with a single VκJκ-rearrangement increase, as in cells with two rearrangement-competent alleles, from pre BII (10 %, two competent alleles – 25 %, one competent allele), over immature B cells (20 %, two competent alleles – 40 %, one competent allele) to mature B cells (35 %, two competent alleles – 48 %, one competent allele). By contrast, the same subsequent stages of B cell development of $C^{-}\kappa/J\kappa^{-}$-κL chain deficient mice remain constant at around 25 %. This indicates that successful VκJκ-rearrangements, i.e. VκJκL chain^{+} sIg^{+} B cells are positively selected during B cell development. This positive selection must be done by $V_\lambda J_\lambda^{+}$ L chain sIg^{+}B cells in the $C\kappa^{-}/JC\kappa^{-}$ mice. In the B-lineage cells of these $C\kappa^{-}/JC^{-}\kappa$ - mice single Vκ to Jκ-rearrangements are found at the expected 2:1 ratio of non-productive over productive rearrangements.
3. On the other side, small pre BII cells are the major cellular sites for secondary L chain gene rearrangements. (Figure 6.1) Thus, multiple rearrangements, with a peak of 2 to 3 per cell, are more frequently detected in pre BII cells (63 %), than in immature B cells (42 %) than in mature B cells (34 %). No major differences in these percentage values are seen between the different mouse strains. Rearrangements to more 3'-located J6-segments are more frequent in pre BII cells than in immature or mature B cells. Rearrangements on the λL chain gene loci are more frequent in pre BII cells than in immature B cells or in mature B cells.

   These results suggest that those pre BII cells which do not rearrange productively and those immature B cells, in which a productive V_L to J_L-rearrangement has led to an incompatible or autoreactive BcR are kept in the pre BII compartment, respectively returned from the immature B back to the pre BII compartment to enter secondary rearrangements.

   These conclusions can also be reached from the precise in and out-of-frame analyses of subsequent Vκ to Jκ-rearrangements at the one rearrangement – competent allele. Thus, a productive rearrangement in a cell with multiple rearrangements is often followed by secondary unproductive and/or productive rearrangements. It indicates that productive rearrangements to not necessarily terminate subsequent rearrangements. While in pre BII cells the last 'secondary' rearrangement can often be non-productive, only in the sIgM^{+} immature and mature B cells is the last 'secondary' rearrangement also a productive one.
4. Lambda L-chain-expressing sIgM^{+}B cells from mice with two rearrangement-competent κL chain alleles carry increased numbers of $V\kappa J\kappa_5$- and Vκ RS-rearrangements and have decreased numbers of non-rearranged, κL chain germline alleles. This is likely to be a consequence of the five-fold higher rate of $V_L H_L$-rearrangements at the κL-chain, compared to the λL chain loci – and this is so even in κL chain protein-deficient B lineage cells of $C\kappa^{-}/JC\kappa^{-}$ mice.
5. In pre BII cells of 6Lchain-defective ($JC\kappa^{-/-}$) or – deficient ($C\kappa^{-/-}$) mice only 15 % - 25 % of the normally sized cell compartment (i.e. $\sim 4 \times 10^7$

cells /bone marrow /mouse) carry a λL chain rearrangement. The rest have all L chain gene loci in their non-rearranged germline configurations. Hence, κL- and λL-chain gene loci rearrange independently of each other. Since the κL chain locus rearranges at higher rates than the λL chain loci do, pre BII cells that have no L chain gene rearrangements. That shows that these pre BII cells can develop in a cellular differentiation program that can become disconnected from the status of Ig gene rearrangements, indicating that at least the development of ckit$^-$ CD25$^+$ CD19$^+$ pre BII cells is Ig rearrangement-independent.[1]

6. Usage of potentially functional Vκ-segments: The 141 V6-segments of the UPkappa L chain locus are not used at equal frequencies for rearrangements[3]. Of the 94 potentially functional Vκ-segments 84 have been found used in over 1000 sequenced joints. Half of all rearrangements use only 10 or 11 of the 94 potentially functional Vκ-segments. The position of a Vκ -segment within the 5' to 3' orientation of the locus is not important for its usage in rearrangements. Multiple rearrangements often occur in closer proximity within the locus. (Andersson, J., Yamagami, T., Rolink, A. and Melchers, F. in preparation).
7. Usage of Pseudo-Vκ-segments: Of the 21 RSS-mutated pseudo-Vκ-segments only one was found used in one of over 1000 sequenced joints, indicating that these pseudogenes are practically never used. Of the 26 pseudogene Vκ-segments with deletions and stop codons 9 were found used in 29 joints of the over 1000 analysed in total, one of them in more than two thirds of all rearrangements.
8. Rearrangements by deletions and by inversions: Rearrangements by inversion or by deletion occur at a ratio of 2 to 1 i.e. as expected from an equal usage of the 32 potentially productive Vκ-segments with the same orientation as Jκ and Cκ in the locus, and of the 62 segments with opposite orientation.

In conclusion, the following picture of rearrangements at the L chain gene loci during B cell development emerges (Figure 6.1): Large pre BII cells, which have expanded by proliferation through stimulation by the preBcR and have closed their Ig H chain loci for further V_H to D_HJ_H-rearrangement, come to rest and open their κL and λL chain loci, so that the reactivated rearrangement machinery with RAG1 and RAG2 can begin V_L to J_L rearrangements. Rates of rearrangements at the κL chain locus are five times higher than those at the λLchain loci. Rearrangements are random, i.e. occur at a 2:1 ratio out-of-frame to in-frame. Some 10 % of all potentially usable Vκ-segments are preferentially used in the first rearrangements, and they use the most proximal 5' located $Jκ_1$ most frequently. Rearrangements by deletions or by inversions of the intervening DNA segments are equally probable. Two thirds of the emerging sIgM$^+$B cells are immediately selected with one VκJκ-rearrangement on one allele leaving the other allele non-rearranged in germline configuration to become sIgM$^+$ mature B cells of spleen and lymph nodes, in which no further rearrangements occur. In the other part of the developing pre BII and immature B cells secondary rearrangements, most frequently at the κL chain locus, occur, probably because of two

reasons: non-productive rearrangement events leading to no L chain expression, or productive events leading to L chain productions which, combined with the pre-expressed μH chain, generates an auto-reactive BcR-expressing immature B cell (a process termed "editing"). The autoantigens are expressed in the primary lymphoid organ, i.e. in bone marrow and in the immature compartments of the spleen. B cells with subsequent productive rearrangements expressing non-autoreactive L chains are allowed to enter the mature, peripheral B cell pools of spleen and lymph nodes, where they constitute one third of the emerging B cells. As pre BII and immature B cells continue to be involved in secondary rearrangements at the 6L chain loci, and as they go forth and back between these two stages of development, the slower rearrangements at the 8L chain loci become more frequent, increasing their chances to fix a developing B cell as a fitting, mature 8L chain$^+$ sIgM$^+$ in the peripheral pools.

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7
Regulation of AID Function *In Vivo*

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and Tasuku Honjo*

7.1. Introduction

Extensive proliferation of antigen–stimulated B lymphocytes is accompanied by two distinct genetic alterations, namely somatic hypermutation (SHM) and class switch recombination (CSR).[1] SHM introduces point mutations at high frequency in DNA encoding variable (V) regions of immunoglobulin (Ig) and their immediate downstream flanking region, followed by selection in germinal centers for high-affinity antibody-producing cells against a given antigen. On the other hand, CSR takes place between repetitive DNA sequences (S regions) located 5' to Ig heavy chain constant-region genes. CSR is a region-specific recombination to diversify effector functions of Ig by changing Ig isotypes from IgM to IgG, IgE, or IgA.

Activation-induced cytidine deaminase (AID) is required for both CSR and SHM because AID deficiency in mouse and human completely abolishes these two genetic alterations.[2,3] In addition to AID, CSR and SHM require transcription of target DNAs, S regions and V regions, respectively.[4–10] All of the other factors required for CSR and SHM appear to be expressed ubiquitously because the two reactions can be induced by AID overexpression in non-B cells such as fibroblasts if the target DNA is actively transcribed.[11–13] However, studies on transgenic (Tg) mouse lines ubiquitously expressing AID suffered from T cell lymphomas or lung microadenomas but not B cell lymphomas,[14] indicating a possibility that transgenic AID is negatively regulated in B cells. In addition, we have recently shown that the transgenic AID expressed constitutively only in B cells, albeit its abundance, is much less efficient for CSR and SHM than the endogenous AID, suggesting that AID is negatively regulated in B cells or unknown modification is necessary for full activation of AID.[15]

Recently Basu et al. reported that phosphorylation of AID by protein kinase A (PKA) is critical to class switch recombination (CSR) in B cells.[16] They found

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that serine 38 (S38) of B cell-derived AID is phosphorylated by mass spectrum analysis and suggested that threonine 27 (T27) may be another phosphorylation site of AID by *in vitro* experiments using PKA. Most importantly they found that mouse (m) S38A and mT27A mutants did not efficiently rescue CSR to IgG1 when introduced retrovirally into AID deficient splenic B-cells. They concluded that phosphorylation of both S38 and T27 is indispensable for CSR. We therefore examined whether phosphorylation of AID is required for AID full activation and found that phosphorylation of AID is not essential to CSR and SHM by AID. AID requires unknown modifications to set physiological range of activity in B cells.

7.2. Analysis of B Cell Specific AID Transgenic Mice

7.2.1. Generation of B Cell Specific AID Tg Mice

To study how AID is regulated to induce CSR and SHM in B cells, we generated AID conditional Tg mice that express AID specifically in B cells. Expression of the conditional AID transgene construct is designed in such a way that the AID protein synthesis is blocked by insertion of GFP flanked by two lox*P* sites although its transcription is constitutive and ubiquitous by the regulation of the promoter, pCAG (Figure 7.1A).[15,17] AID can be expressed only after Cre-mediated deletion of the GFP cDNA from the transgene. This lox*P* GFP-AID Tg (single Tg, Tg/---) mice were crossed with the mice carrying the Cre gene knocked into the downstream of the CD19 promoter (CD19-cre mice)[18] so that GFP is deleted at the pro B cell stage to express AID only in B lineage cells[19,20]

We performed PCR analysis of genomic DNA from several tissues of the double Tg mice. PCR primers were designed to generate 1718 bp and 361 bp products from the intact and recombined transgenes, respectively (Figure 7.1A). CD19-cre dependent recombination of the AID transgene was found in bone marrow (BM), spleen (SPL), mesenteric lymph nodes (MLN), and Peyer's patches (PP) (Figure 7.1B). As shown in Figure 7.1C, GFP is expressed constitutively from the transgene in all the $B220^+$ or $CD19^+$ cells in the single Tg mice whereas GFP expression is lost efficiently in the $B220^+$ or $CD19^+$ cells in the double Tg mice. Northern blot hybridization detected two bands for each of endogenous and transgenic AID transcripts (Figure 7.1D). Endogenous AID transcripts are known to have two variants due to differential polyadenylation site usage[21] while transgenic AID transcripts are derived from the intact and recombined AID transgene. In consistence with loss of GFP, recombined Tg-derived AID mRNA was detected in SPL of the double Tg mice together with endogenous AID mRNA. The expression level of endogenous AID mRNA in the LPS-stimulated B cells was augmented but still much less than Tg-derived AID (stimulated SPL cells in Figure 7.1D). Furthermore, the protein amount of endogenous and transgenic AID in LPS-stimulated splenocytes was measured by immunoprecipitation (IP) using an anti-AID monoclonal antibody (clone #243),

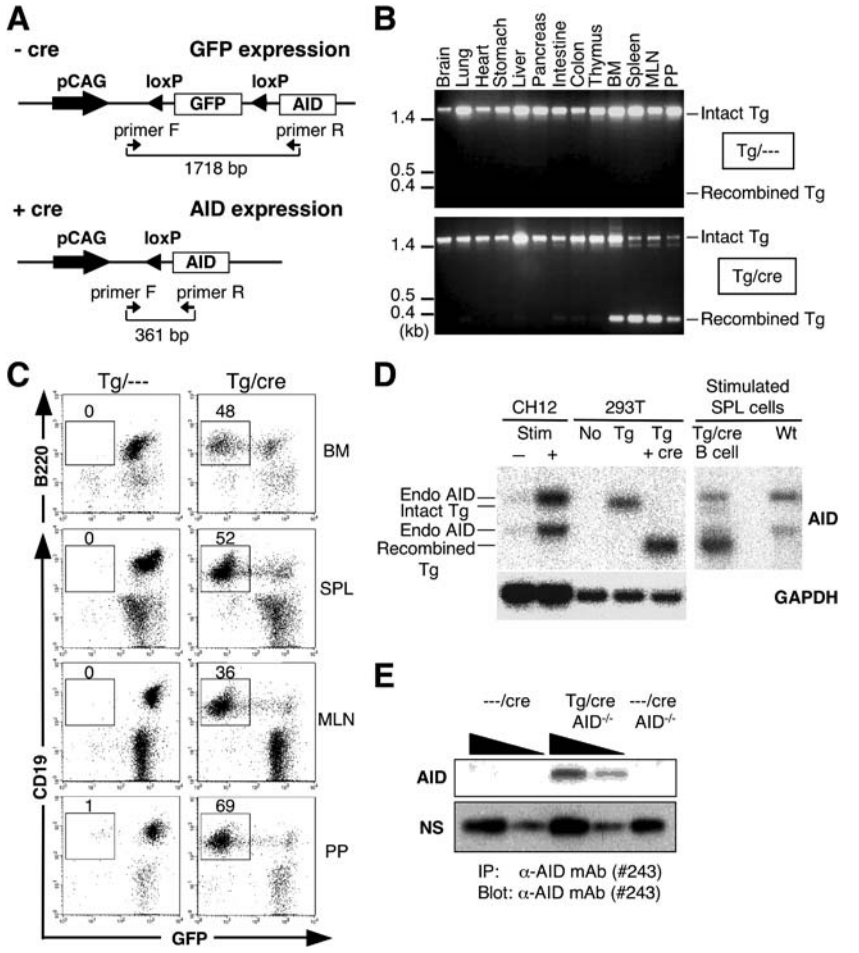

FIGURE 7.1. B cell specific expression of AID in the double Tg mice. (Modified from Ref. 15) (A) The conditional AID transgene construct that expresses AID after Cre dependent recombination. (B) PCR analysis of the recombination of the transgene DNA in various tissues of the Tg mice with or without CD19-cre. (C) Flow cytometric profiles of B lymphocytes from several lymphoid organs of the double Tg mice, after staining with antibodies for B cell markers (B220 or CD19). Numbers indicate percentages of B cell marker^{+} GFP^{-} cells in lymphocytes. (D) Northern blot analysis of endogenous and transgenic AID expression in the lymphoid tissues of wild-type and Tg mice. Total RNA from unstimulated SPL was extracted and electrophoresed. Blots were probed for AID mRNA and re-hybridized with a GAPDH probe after stripping the AID signal. RNA samples from stimulated and unstimulated CH12F3-2A (CH12) cells were prepared for endogenous AID control. RNAs from the HEK293T (293T) cells transfected with the transgene vector with or without Cre-expressing vector are used for controls of transgene AID before or after recombination, respectively. To compare the amounts of endogenous and transgenic AID mRNA, RNA was prepared from purified GFP^{-} B220^{+} SPL B cells of the double Tg mice after stimulation by LPS for 3 days (stimulated SPL cells).

followed by immunoblot. Transgenic AID was clearly detectable in the double Tg mice of AID knockout background (Tg/cre AID$^{-/-}$), whereas endogenous AID (---/cre) was undetectable in activated splenocytes by IP-Western blot (Figure 7.1E). The transgenic AID protein is expressed far more than the endogenous AID.

7.2.2. *Transgenic AID is Inefficient in CSR*

To examine whether or not CSR is augmented by constitutive expression of AID, we measured *in vitro* CSR efficiency of splenocytes from the double Tg mice (Figure 7.2A). The double Tg splenocytes showed a slightly higher CSR efficiency than the single Tg and non-Tg mice splenocytes after stimulation with LPS and/or IL4 (Figure 7.2A), suggesting that transgenic AID has some activity to induce CSR. It is noted that the CSR efficiency of splenocytes from the single Tg mice was similar to those from non-Tg mice (Figure 7.2), excluding the possibility that the integration of the transgene itself affected CSR in B cells. To distinguish the transgenic AID activity from the endogenous one, we analyzed *in vitro* CSR efficiency of the double Tg-AID$^{-/-}$ splenocytes. Naïve B cells of the double Tg-AID$^{-/-}$ mice were negative for surface IgG. Only when subjected to stimulation by LPS and/or IL4 to induce CSR *in vitro*, they switched to IgG1 or IgG3-expressing cells (Figure 7.2B), indicating that the transgenic AID has CSR acitivty. However, the CSR efficiency of the double Tg-AID$^{-/-}$ splenocytes was only less than 30% of wild-type control. To excrude a possibility that other factors required for CSR are downmodulated in B cells in double Tg-AID$^{-/-}$ mice, we isolated GFP^{-} splenocytes of the double Tg-AID$^{-/-}$ mice, infected them with AID-expressing retrovirus and measured CSR activity by flow cytometry (Figure 7.2C). AID expression by retrovirus infection enhanced the CSR efficiency of the B cells from the double Tg-AID$^{-/-}$ mice to the level similar to or more than wild-type (Figure 7.2C), indicating that other factors required for CSR were not limiting. These results indicate that constitutively expressed AID is somehow modified to downmodulate its activity of CSR in B cells, in spite of its abundance.

7.2.3. *Transgenic AID is Inefficient in SHM*

We analyzed the frequency and pattern of SHM in the 3' flanking region of the rearranged heavy-chain V exon of B cells in the double Tg mice.[22] First, we

FIGURE 7.1. *(Continued)* (E) AID protein production from the double Tg mice with AID$^{-/-}$ background. AID protein in splenocytes of the double Tg x AID$^{-/-}$ mice and their controls after stimulation with LPS for 4 days was immunoprecipitated by an anti-AID monoclonal antibody (#243), and subjected to immunoblot by the same antibody after SDS-PAGE. Three fold dilutions of input amounts are shown. Non-specific signal (NS) from the blot is shown as an internal control to indicate input protein amounts.

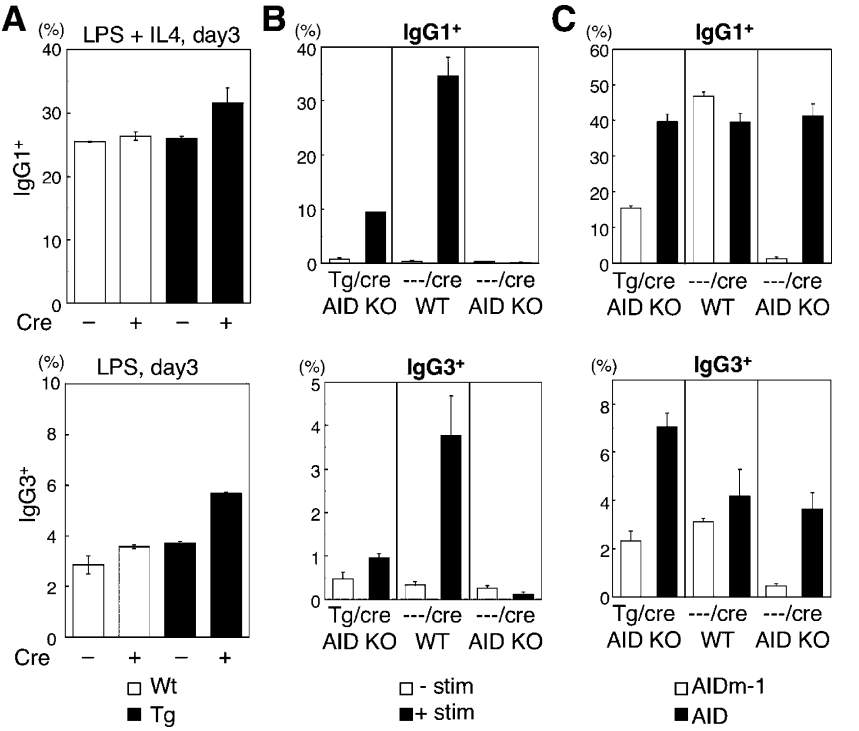

FIGURE 7.2. Analyses of CSR induced by Tg AID. (Modified from Ref. 15) (A) *In vitro* class switching of splenocytes from the AID Tg mice in the presence of endogenous AID. Splenocytes after 3-day culture with LPS and/or IL4 were stained with anti-B220, and anti-IgG1 or IgG3 antibodies and analyzed by flow cytometry. The percentages of $IgG1^+$ or $IgG3^+$ B cells in the $B220^+$ fractions are indicated in each panel. Results obtained from 2 individual mice aged 13–14 weeks were summarized as column graphs with mean ± SD values. (B) *In vitro* class switching of splenocytes from the AID Tg mice in the absence of endogenous AID. Splenocytes were stimulates with LPS and/or IL4 for 4 days. The percentages of IgG^+ or $IgG3^+$ B cells in the GFP^- $B220^+$ fractions are indicated in each panel. Results obtained from 3 individual mice aged 21–30 weeks were summarized as column graphs with mean ± SD values. (C) GFP^- splenocytes from the AID Tg mice were stimulated with LPS and/or IL4, and infected with AID-expressing retrovirus one day after stimulation. Splenocytes with 4-day culture were analyzed as described in (B). AIDm-1, a loss-of-function mutant of AID.[13]

analyzed SHM in PNA^+ B cells from PP of the double Tg mice, in which SHM are introduced abundantly by spontaneous antigen stimulation.[23] The mutation frequency of B cells in the double Tg mice was not significantly higher than that in the control single Tg mice (Figure 7.3A). We analyzed SHM in the PNA^- B cells to check if the constitutively expressed transgenic AID has SHM activity or not. There was a low but significant level of SHM in PNA^- PP B cells from the double Tg B cells. To exclude a possibility that observed SHM was derived

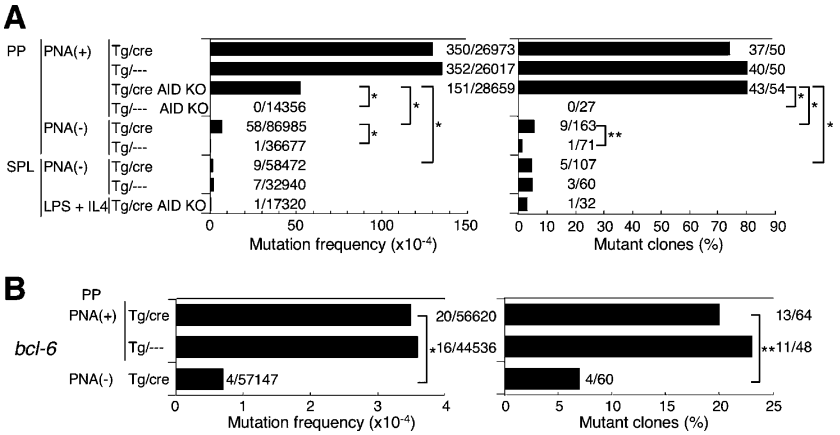

FIGURE 7.3. Analyses of SHM in B cells of the AID Tg mice. (Modified from Ref. 15) (A) Mutation frequency at the 3'– flanking region of the JH4 exon of the Ig heavy chain V region. Numbers of mutation/nucleotides analyzed (left panel), and the numbers of mutated clones/Ig clones analyzed (right panel) are indicated. Statistical significance was evaluated by Fisher's exact tests for indicated sets of data. * $P < 0.01$, ** $P < 0.05$. For each analysis, 1–4 individual mice aged 9–27 weeks were used. (B) Mutation frequency at the *bcl-6* gene. Numbers of mutation/nucleotides analyzed (left panel), and the numbers of mutated clones/clones analyzed (right panel) are indicated. Statistical significance was evaluated as described in (A).

from contaminated PNA^+ PP B cells, we then analyzed SHM in PNA^- spleen B cells, which accumulate little SHM under the normal condition.[24] We detected no more than background levels of SHM in PNA^- spleen B cells from the double Tg mice (Figure 7.3A). *In vitro* stimulation of spleen cells by LPS and IL4, which activates transcription of the Ig locus and induces CSR to IgG1 and IgE, did not induce SHM (Figure 7.3A). SHM was introduced to PNA^+ B cells in the double Tg-$AID^{-/-}$ mice (Figure 7.3A), indicating that Tg AID is functional for SHM. However, the efficiency of SHM in the double Tg-$AID^{-/-}$ B cells was no more than 40% of the single Tg B cells, which express only endogenous AID, again suggesting that constitutively expressed AID in B cells seems inactivated for the SHM activity as well as CSR activity.

To test whether the target specificity of SHM by transgenic AID is regulated similarly to the endogenous AID, we analyzed SHM on non Ig genes; *bcl-6* which is known to be subjected to SHM in normal germinal center B cells and GC-derived B lymphoma cells.[25] Since overexpressed AID is known to mutate itself,[11] we sequenced the AID transgene and found that no accumulation of SHM was found in the genes for the transgenic AID (0 out of 16560 bp, $<0.6 \times 10^{-4}$/bp) even in the PNA^+ B cells of the double Tg mice. On the other hand, SHM was accumulated on the bcl-6 gene in the PNA^+ B cells of the double Tg mice with the frequency similar to the single Tg mice (Figure 7.3B). In addition, the base specificity of SHM in the double Tg mice was similar to

the wild-type or the single Tg mice without strong bias to dG/dC bases (data not shown). These results indicate that the transgenic AID in B cells is regulated in a manner similar to the endogenous AID in terms of the base specificity and locus specificity, although transgenic AID is downregulated for SHM frequency with unknown mechanism.

7.3. B-Cell Specific Regulations Against Side Effects of AID

In the present study, we have shown that constitutively expressed AID is downmodulated for both CSR and SHM in B cells by either inactivation or lacking a modification for full activation. Constitutive expression of AID is potentially deleterious to B cells because AID may introduce double-strand breaks not only in the Ig V region genes, but also in other genes including oncogenes, which further induces chromosomal translocations and mutations. To avoid such negative effects of AID, AID in B cells should be regulated tightly in such a way that limits the function of AID within a short time range after stimulation by antigen. These regulations appear to be unique to B cells because AID expression in non-B cells including T cells, fibroblasts and hybridomas can induce CSR and SHM in artificial assay constructs,[11–13,26] although the same SHM construct was very inefficient in CH12 B cell line expressing AID.[27] In addition, T lymphoma cells generated in AID Tg mice continue to accumulate mutations.[14,28] These results suggest that the variation of SHM efficiency may reflect the absence and presence of SHM-specific cofactors (either stimulatory or inhibitory) in CH12 and T lymphoma cells, respectively. Also, we speculate there are CSR-specific cofactors to regulate the AID function in B cells.

7.4. Is AID Phosphorylation Essential to Class Switch Recombination?

Recently phosphorylation of AID at T27 and S38 residue was reported to be critical for CSR activity in stimulated B cells, suggesting that phosphorylation might be a modification required for full activation of AID.[16] However, we report here that the same mutations of mouse and human AID retain CSR activity equivalent to 50% or more of respective wild-type AID in the identical assay[29] using AID deficient splenocytes (Figure 7.4A,B). Even the double mutations at T27 and S38 showed the detectable activity of CSR; especially hT27AS38A had about 25% of CSR activity compared to wild-type AID. In addition, as shown in the previous sections, constitutively overexpressed AID in B cells is less efficient in both CSR and SHM, although transgenic AID is far more than endogenous AID and should be phosphorylated since it is expressed in B cells,[16] indicating that phosphorylation at T27 and S38 may not be essential to CSR

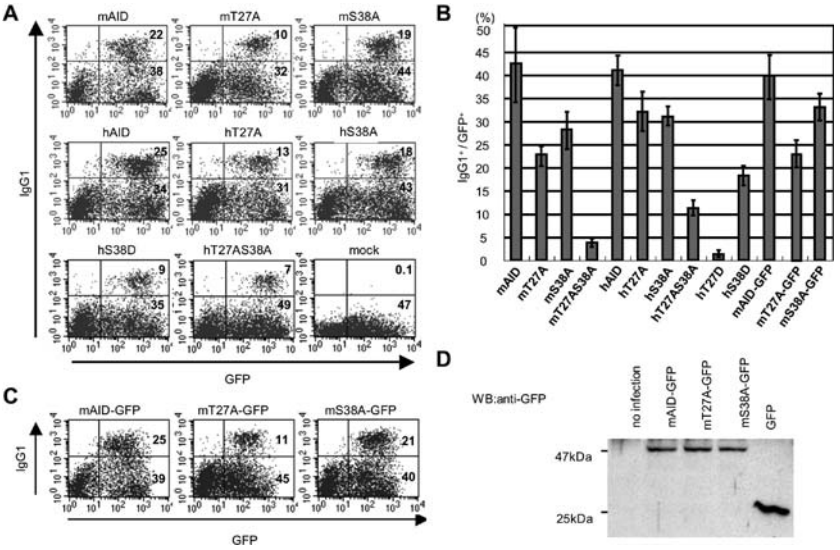

FIGURE 7.4. T27A and S38A AID mutants rescued CSR activity in *vivo*. (A)Flow cytometry assessing CSR in AID deficient spleen cells stimulated with IL-4 and LPS and infected with retroviruses expressing wild-type or mutant AID as described previously.[29] CSR was measured by IgG1 cell surface expression and infected cells are GFP^+. Data are representative of 3–6 individual experiments. Numbers indicate percentage of $IgG1^+GFP^+$ cells (top right) and $IgG1^-GFP^+$ cells (bottom right). (B) CSR activity is presented as percentage of $IgG1^+$ cells normalized to GFP^+ populations (mean $\pm$ s.d.) from 3–6 individual experiments. (C) Flow cytometry assessing CSR in AID deficient spleen cells stimulated with IL-4 and LPS and infected with retroviruses expressing wild-type or mutant AID-GFP fusion proteins. Analysis were done as shown in (A). (D) Wild-type or mutant AID-GFP fusion proteins expressed in infected splenocytes were assessed by Western blot analysis using anti-GFP antibody. The equal amount (50μg) of cell lysate was loaded on each lane.

activity in B cells. Since S38 is not conserved in fish AIDs that have CSR activity,[30,31] Basu et al. raised the possibility that the aspartate residue at position 40 could be a constitutive mimicry of S38 phosphorylation.[32] However, we found that hT27D and hS38D were less active than hT27A and hS38A, respectively (Figure 7.4A, B) although their protein expression levels were comparable (data not shown). We monitored the protein expression level of mutants more directly by using the AID-GFP fusion protein by both flow cytometry and Western blot (Figure 7.4C, D). mS38A-GFP and mT27A-GFP had CSR activity of 80% and 50%, respectively, compared to mAID-GFP. Furthermore, mS38A induced SHM in GFP transgene expressed in NIH3T3 cells with the frequency (47 out of 18417 bp, 2.6×10^{-3}/bp) comparable to mAID (19 out of 7016 bp, 2.7×10^{-3}/bp).[13,29]

Although phosphorylation of AID was shown to take place,[16] it is not known what fraction of AID is phosphorylated. We assume that only a minor population

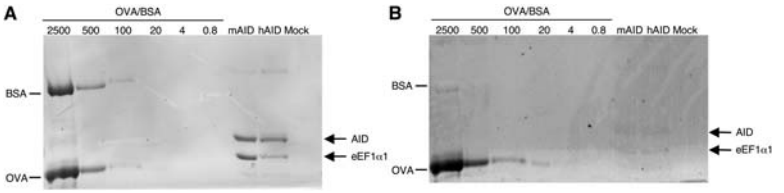

FIGURE 7.5. Phosphorylation of AID in CH12F3-2 cells. (A) Coomassie Brilliant Blue staining of affinity-purified AID protein. An epitope-tagged AID protein was expressed in CH12F3-2 cells and purified from cytoplasmic extracts by using anti-FLAG M2 agarose beads. Indicated amounts (ng) of OVA and BSA are loaded as phosphorylated and nonphosphorylated protein standards, respectively. eEFα1 was co-purified with AID as evidenced by peptide mass fingerprinting. mAID: mouse AID, hAID: human AID, Mock: tag alone. (B) Pro-Q®Diamond staining of affinity-purified AID protein. The number of known phosphate groups on OVA protein is 2.

of AID protein may be phosphorylated in B cells because we could not detect significant phosphorylation of AID using the ProQ method (Figure 7.5).[33] AID phosphorylation was reported to be specific to B cells,[16] whereas AID is active for CSR and SHM in non-lymphoid cells.[12,13] Relative activities of AID mutants were similar in non-lymphoid and B cells. A slight reduction of CSR activities in T27A and S38A may be due to non-conserved replacements. In fact, mutants such as T27L, T27N, T27G and T27R showed more drastic reduction (less than 5% of wild type) in CSR activities (data not shown). The apparent difference of analyses with the same mutants could be due to lower expression levels of the mutants in experiments reported by Basu et al.[16] A lower AID expression by a less efficient viral infection provides a narrower dynamic range of CSR assay. Taken together we conclude that phosphorylation of T27 and S38 is not essential to the function of AID *in vivo*. It still remains to be seen whether there are unknown mechanisms for full activation of AID or inactivation of AID.

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8
Targeting of AID to Immunoglobulin Genes

Ursula Storb, Hong Ming Shen, Simonne Longerich, Sarayu Ratnam, Atsushi Tanaka, Grazyna Bozek and Serhiy Pylawka*

8.1. Introduction

The activation induced cytosine deaminase AID is required for and initiates somatic hypermutation (SHM) of Ig genes. The question addressed in this paper is how AID gets targeted to the sequences in which it deaminates cytosines to uracils. There are three levels of targeting by AID that must be understood.[1] 1) How is AID targeted to Ig genes and not most other genes that are also expressed in B cells undergoing SHM? 2) Why in the targeted genes are the 5' 100–200 nucleotides and the C region and other 3' regions not targeted? 3) How does AID target both strands in double-stranded DNA? In this paper I will address questions 2) and 3).

8.2. Why is the Very 5' End of Ig Genes Not Mutated?

It had been shown several times, that the 5' 100 to 200 nts of Ig genes are spared from SHM.[2–6] We asked the question whether this meant that AID did not access or act in the very 5' end, or whether it did create uracils in this region but they were repaired without error, just as in other regions of the genome not undergoing SHM. We reasoned that if AID acted in the very 5' end, in the absence of the uracil glycosylase Ung, most uracils would be retained and copied during DNA replication, resulting in CG to TA transitions (Figure. 8.1). Analyzing SHM in Ung-/- mice clearly showed the same distribution as in wild type mice of mutations in the 5' end of an endogenous lambda1 gene (not shown) as well as in an Ig-kappa transgene (Figure. 8.2).[7] It was concluded that 1) A footprint of AID (transitions from C and G in Ung-/- mice) is not seen in the

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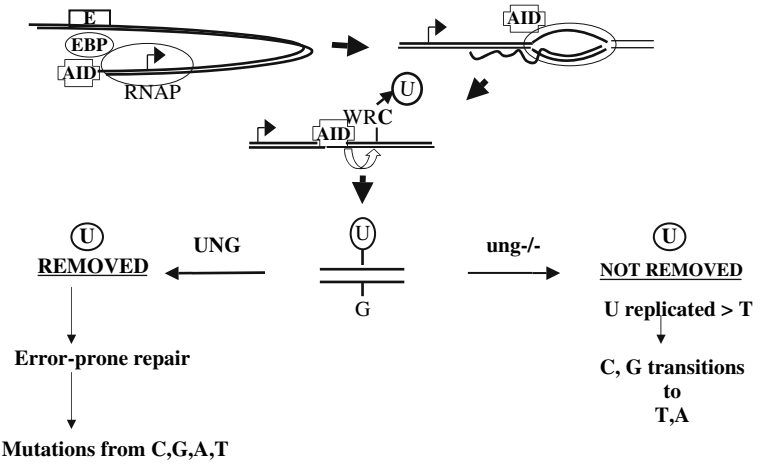
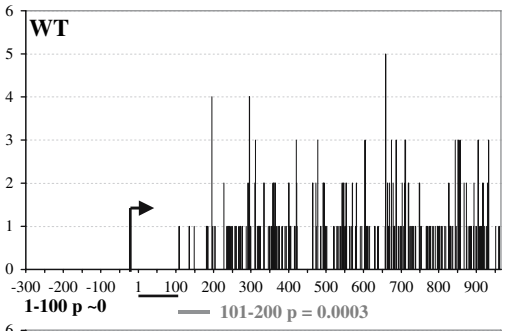
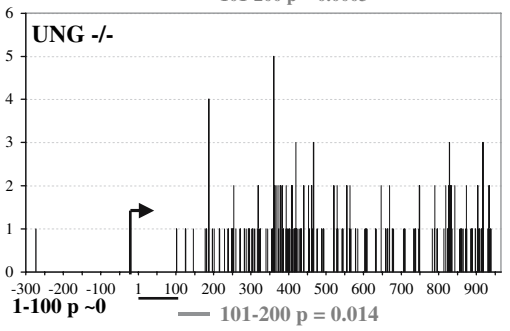


FIGURE 8.1. Model of initiation of SHM.
E, Ig enhancer; EBP, enhancer binding proteins; RNAP, RNA polymerase II; WRC, AID hotspot (W = A or T; R = purine); Ung, uracil glycosylase; C, cytosine; U, uracil; G, guanine; T. thymine; A, adenine



from	to	mutations	hotspots
-300	-201	0	11
-200	-101	0	10
-100	-1	0	15
1	100	0	11
101	200	10	12
201	300	25	11
301	400	37	7
401	500	28	7
501	600	28	8
601	700	41	11
701	800	33	10
801	900	40	12
901	964	26	12



from	to	mutations	hotspots
-300	-201	1	11
-200	-101	0	10
-100	-1	0	15
1	100	0	11
101	200	13	12
201	300	16	11
301	400	37	7
401	500	37	7
501	600	16	8
601	700	15	11
701	800	13	10
801	900	27	12
901	964	27	12

FIGURE 8.2. Mutations at the 5' end of a kappa transgene, RS, in wildtype and Ung-/- mice. Bent arrow, transcription start. p values show the likelihood that the low level of mutations in the 5' end are due to chance. (Modified from Ref. 7)

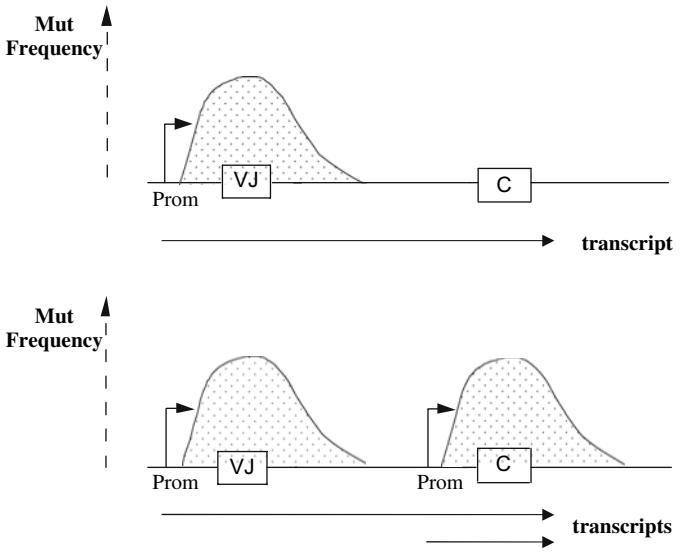


FIGURE 8.3. Somatic mutation is linked to transcription initiation. This figure summarizes data in[9].

5' end of Ig genes. 2) It is likely that AID does not deaminate cytosines in the first 100 transcribed nucleotides. 3) AID begins to act between +100 and +200 nts from the start of transcription. 4) At about 200 nts a plateau in mutation frequency is reached that lasts at least until 964 nts (the most 3' nt analyzed in this study).

What is the reason for the absence of AID function at the very 5' end? We entertain the following possibilities partly based on our finding that SHM is apparently dependent on transcription initiation (Figure. 8.1 and Figure. 8.3). 1) RNA polymerase must be in the processive mode to interact with AID. 2) Transcription-induced negative supercoils must have reached a threshold level before enough single-stranded cytosines are available for AID action (see below). 3) AID co-factors, such as RPA,[8] are not recruited. 4) A nucleosome is stably positioned over the first ~ 146 nts and AID cannot access DNA within nucleosomes. These possibilities are under further investigation.

8.3. Why are the C Region and 3' End of Ig Genes Not Mutated?

It has been found that Ig gene mutations decrease beyond about 1 kb from the promoter and are essentially absent after about 2 kb and certainly in the C region of kappa light chain and heavy chain genes.[5,10] There are a few mutations in the C region of lambda light chain genes[11], but the JC intron in lambda genes is especially short (1.1 kb) and the C region begins about 1.6 kb kb from the start

of transcription.[12] We have again used Ung-/- mice to ask whether transitions from C and G occur in the normally spared 3' end of Ig genes.[7]

Again, we found that the distribution of mutations is the same in Ung-/- and wildtype mice in both heavy and light chain genes. For example, in heavy chain genes with VD rearrangement to JH2, the sequence just 3' of JH2 is very highly mutated in both Ung-/- and wildtpe mice, but the region further 3', namely 3' of JH4 has hardly any mutations in both types of mice (Figure. 8.4). We concluded that there is no "footprint" of AID in the 3' end of Ig genes and that therefore AID does not gain access or act in this region.

What are the reasons for lack of AID activity in the 3' end of Ig genes? We entertain the following possibilities: 1) AID travels with RNA polymerase and dissociates from the transcription complex before ~ 2 kb from the promoter to become engaged with the DNA. 2) AID is present throughout the Ig gene but cannot act at the 3' end. The lack of activity 3' may exist if AID cannot access DNA within nucleosomes. This would allow access only between nucleosomes, however, if that space were too narrow, AID may be excluded. Interestingly,

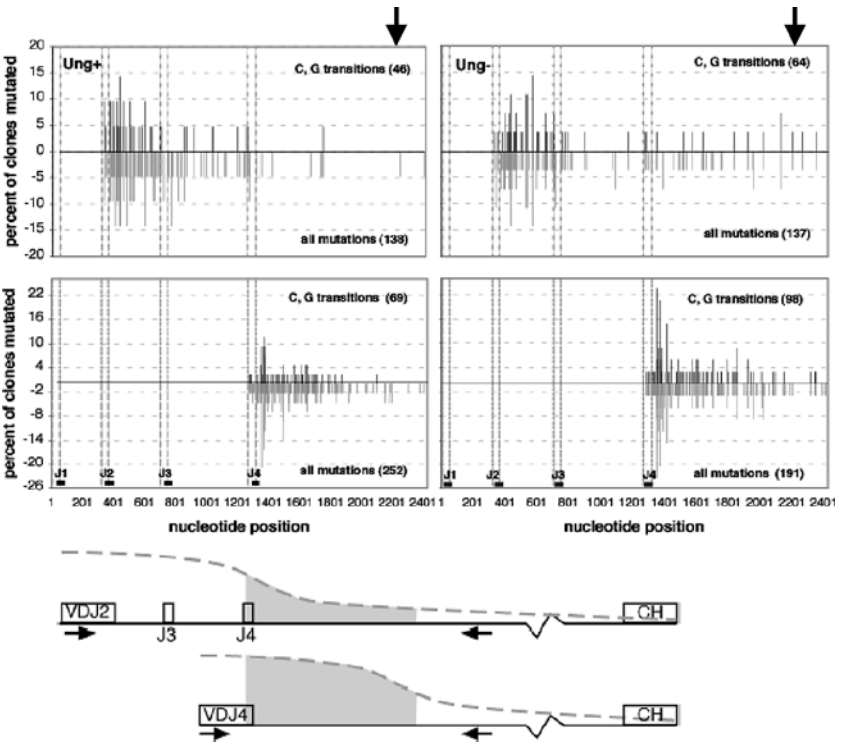


FIGURE 8.4. Mutations 3' of JH2 in VDJ2 rearranged genes (top) and 3' of JH4 in VDJ4 rearranged genes (bottom). Each panel shows C, G transition above, and the total mutations below the central line. The vertical arrows indicate that there are very few mutations 3' of JH4 in both wt and Ung-/- mice. (Modified from Ref. 17)

recent evidence has shown for the genome that nucleosomes are spaced increasingly tightly starting just 3' of the promoter and reaching maximal tightness at about 2 kb from the promoter (J. Widom, personal communication). These possibilities are currently under investigation.

8.4. How Can AID Target Both DNA Strands Equally?

In vitro assays have shown that AID efficiently targets single-stranded DNA, but cannot target double-stranded linear DNA.[13–15] When transcribed, double-stranded DNA is targeted by AID, but mainly on the non-transcribed strand.[8,16–18] *In vivo*, however, both DNA strands undergo SHM equally. This discrepancy suggested that the cell free assays do not represent the *in vivo* situation. However, we have found that supercoiled DNA is an excellent target of AID *in vitro* on both DNA strands, and have postulated that *in vivo* AID may access negative supercoils arising during transcription.[19]

Recently, we have tested the interaction of AID and transcription *in vitro*.[20] We used two circular plasmids that contain two different antibiotic resistance genes, one for ampicillin and one for kanamycin (Figure. 8.5). In pKMT7, the initiator ATG of the Amp gene has been changed to ACG so that the gene can only be expressed into protein when ACG has been deaminated to AUG in a cell free assay with AID. The Amp^r gene is transcribed by T7 polymerase. Plasmid pKMT7 confers kanamycin resistance to *E.coli*, where it is transcribed from the lac promoter. When pKMT7 is treated with AID without transcription, no mutations are found in the Amp^r gene and the plasmid does not confer ampicillin resistance when transformed into uracil glycosylase-deficient *E.coli* (not shown). However, when transcribed, the plasmid shows a high frequency of mutations and confers ampicillin resistance due to mutation of the ACG to AUG

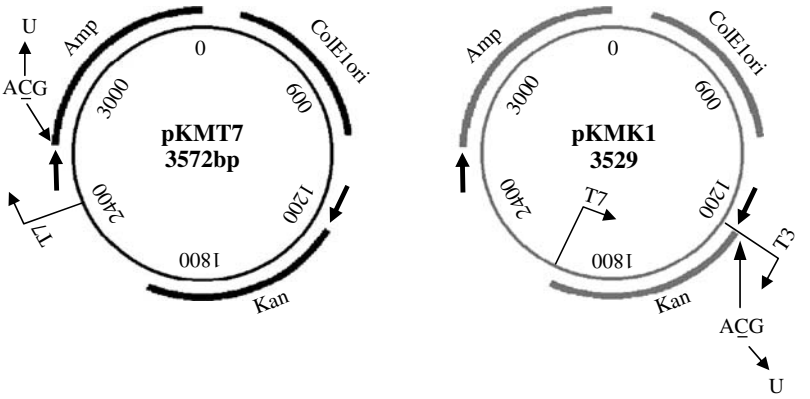


FIGURE 8.5. Maps of the plasmids pKMT7 and pKMK1. (Modified from Ref. 20)

(ATG retained in uracil glycosylase-deficient *E.coli*; not shown). The majority of the mutations are on the non-transcribed strand because only when this strand is mutated will an ATG initiation codon be created for selection in ampicillin. When that AID-treated pKMT7 plasmid transcribed by T7 RNA polymerase is selected in kanamycin, mutations are seen on both strands in the Amp^r gene, with some preference actually for the transcribed strand (Figure. 8.6). These findings suggest that transcription increases AID access and that the transcribed DNA strand has at least an equal chance to the non-transcribed strand to be targeted.

The influence of transcription was further tested with the other plasmid, pKMK1 (Figure. 8.5). pKMK1 confers ampicillin resistance, but has the ATG initiation codon of the Kan^r gene changed to ACG, requiring AID deamination to AUG for expression of kanamycin resistance. The Kan^r gene can be transcribed in a cell free AID assay with either T3 polymerase, producing sense mRNA, or from a T7 promoter in the other direction, producing antisense RNA. Surprisingly, the Kan^r gene was almost exclusively mutated by AID on the sense strand regardless of the direction of transcription. Figure. 8.7 shows that with transcription from the T7 promoter most AID-induced uracils occur on the transcribed strand. This strand is also mainly mutated when the gene is transcribed in the opposite direction (from the T3 promoter, Figure 8.5) which in that case is the non-transcribed strand (not shown). Thus, the Kan^r sequence has an overwhelming strand bias in AID targeting that is unrelated to the direction of transcription, but enhanced by transcription, especially when transcribed from the T7 promoter.[20]

In the hope of gaining some insight into the rules of targeting AID we treated the pKMT7 and pKMK1 plasmids with bisulfite (Figure. 8.8). Bisulfite deaminates single-stranded cytosines to uracil, thus revealing where in supercoiled

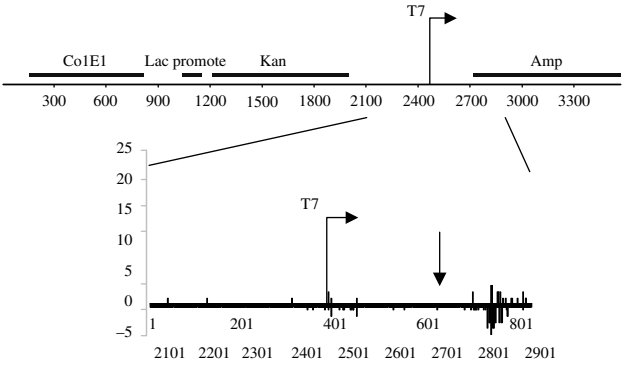


FIGURE 8.6. Mutations in the Amp^r gene in pKMT7 treated with AID during transcription with T7 polymerase (kanamycin selection). Bars above the horizontal axis are C > T, below G > A transitions. The vertical arrow indicates the location of the ACG which for selection in kanamycin does not need to be deaminated by AID. Hypermutable regions see Figure. 8.5.

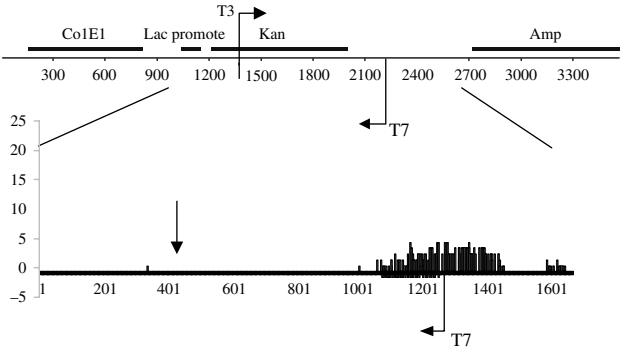


FIGURE 8.7. Mutations in the Kan^r gene in pKMK1 treated with AID during transcription with T7 polymerase (ampicillin selection). Bars above the horizontal axis are C > T, below G > A transitions. Hypermutable regions see Figure. 8.5.

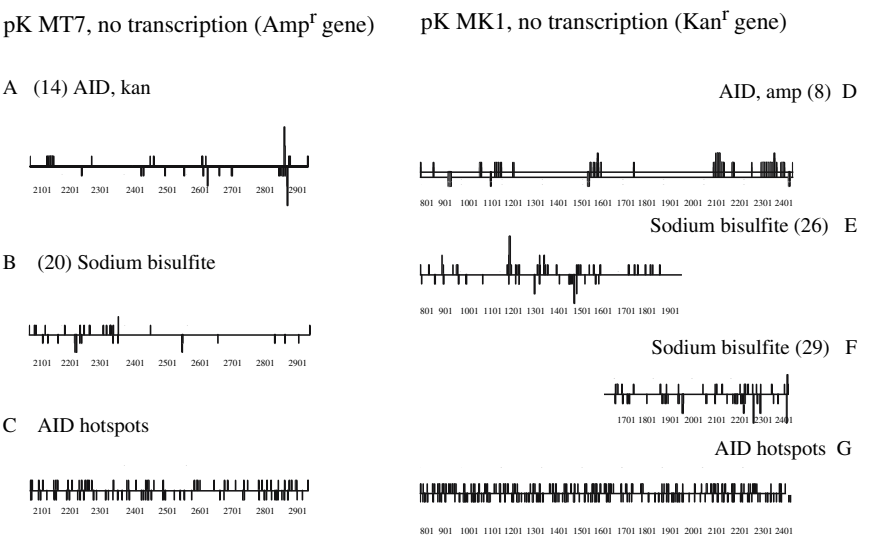


FIGURE 8.8. AID accesses different cytosines compared with bisulfite.
A-C, pKMT7. A AID treated; B bisulfite treated; C AID hotspots.
D-F, pKMK1. D AID treated; E, F bisulfite treated; G AID hotspots.
In () nos. of clones sequenced. Bars above (below) lines indicate C to T conversions (or WRC for the hotspots) on the top (bottom) strand.
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DNA cytosines may be flipped out to allow AID access. Surprisingly, the cytosines accessible to bisulfite are in general different to the ones accessible to AID for both plsmids. Their distribution also does not relate to AID hotspots (WRC/GYW) (Figure 8.8).

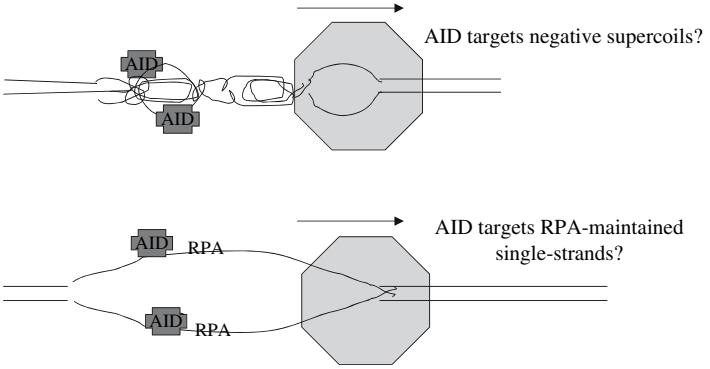

FIGURE 8.9. Two models of AID access to double-stranded DNA. The octagon represents pol II.

Thus, the rules for targeting of AID are not fully understood. While cytosines in AID hotspots in SHM and *in vitro* generally have a greater chance of being targeted than non-hotspots, less than 50% of mutations are in hotspots, and a large proportion of hotspots are not mutated (especially *in vivo*). Thus, some as yet unknown structural/conformational properties of the primary sequence appear to play a major role.

Concerning targeting by AID in SHM *in vivo*, it is possible that indeed negative supercoils arising in the wake of the transcribing RNA polymerase create flipped out cytosines that AID can access (Figure 8.9, top). Alternatively, AID alone or when associated with RPA may prevent the reannealing of the two DNA strands behind the RNA polymerase so that the transcription bubble becomes extended (Figure 8.9, bottom). Either of these models would explain how *in vivo* AID can equally target both DNA strands. These questions are under further investgation.

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9
Targeting AID to the Ig Genes

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9.1. Introduction

Antibody diversity is essential to protect us from pathogenic organisms and their products, as well as from other toxic substances in our environment. Prior to the exposure to antigens, B cells differentiate in the bone marrow and rearrange their heavy chain variable (V), diversity (D) and joining (J) germ line genes, as well as their light chain V and J genes. This generates a diverse repertoire of B cells expressing IgM antibodies that will react through their rearranged V regions with all possible antigens. However, IgM antibodies are usually of low affinity and do not leave the circulation to penetrate tissues throughout the body or enter the mucosal secretions. Higher affinity antibodies of IgG, IgA and IgE isotypes can neutralize viruses and toxins and inactivate potentially toxic substances. These antibodies are generated when B cells differentiate further in response to antigens. Activated B cells become centroblasts and centrocytes in the germinal centers of secondary lymphoid organs where they express activation induced cytidine deaminase (AID).[1] AID triggers somatic hypermutation (SHM) of V regions to produce higher affinity antibodies and class switch recombination (CSR) to allow the higher affinity V regions to be expressed with all possible constant (C) regions. SHM occurs at a rate of $\sim 1 \times 10^{-3}$ mutations per base per generation[2,3] and is preferentially targeted to the underlined base in D<u>G</u>YW (D=A/G/T, Y=C/T, W=A/T) and complementary WR<u>C</u>H (R=A/G, H=A/C/T) hotspot motifs.[4] In the V region, these mutations begin ~100-200 bp downstream from the site of initiation of transcription, are most frequent in the coding exon of the V region, end ~1.5-2 kb downstream of the promoter, and spare the intronic enhancer and C region[5] (Figure 9.1).

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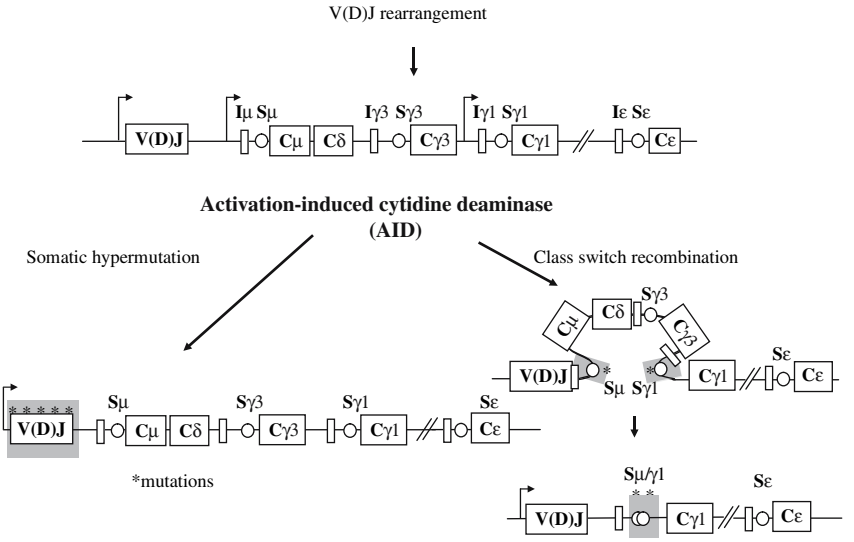

FIGURE 9.1. **The generation of antibody diversity by SHM and CSR.** After successful V(D)J rearrangement, B cells express IgM (μ) or IgD (δ) heavy chains encoded by the VDJ and the Cμ or Cδ exons, respectively (top diagram). Following stimulation with antigen, the V(D)J region undergoes AID-induced mutations (***) to change the affinity of the antibody variable region (left diagram). If stimulated by specific lymphokine(s), the B cells express germline transcripts from the promoters that are located just 5' of Iμ and of the downstream I regions, making the switch regions accessible to AID (right diagram). In this example, Sμ and Sγ1 switch regions recombine to produce a new, chimeric, Sμ/Sγ1 switch region to bring the V(D)J region in close proximity to Cγ1 that encodes an IgG1 heavy chain (right diagram).

With the discovery of AID, these characteristics of SHM led us to suggest that AID converted dC to dU on DNA, and that a uracil DNA glycosylase could convert the dU to an abasic site and recruit base excision repair and error-prone DNA polymerases.[6] Subsequent studies confirmed this general idea and led to a more detailed model[7] in which the dG:dU mismatches created by AID in the V region for SHM or in switch regions (SR) for CSR (Figure 9.1) are resolved differently. In SHM, dG:dU can be replicated to generate C-to-T or G-to-A transition mutations, removed by uracil DNA glycosylase (UNG) to create an abasic site that can then be bypassed by error-prone polymerases[7] or potentially processed by the MRE11 complex.[8] Alternatively, the dG:dU can be targeted by mismatch repair (MMR) to initiate an error-prone polymerase mediated mutation process that introduces mutations at A:T and G:C bases that are not in hot spots.[9] In CSR, dG:dU mismatches are converted into double stranded DNA breaks that trigger recombination between the donor μ SR and recipient γ, α, or ε SRs.[10]

It had been known for many years that transcription of the V region and SRs is required for SHM and CSR, but the reasons for this were obscure.[11,12] However, once AID was semi-purified in a form that was enzymatically active,

it became clear that the substrate of AID *in vitro* was dC on single-stranded DNA.[13] When double-stranded DNA was transcribed *in vitro*, dCs on the non-transcribed strand of transcription bubbles were converted to dUs. In addition, there was a preference for the same hot spot motifs that were targeted *in vivo*.[14,15] Similarly, R-loop structures in SRs, in which the nascent mRNA forms RNA-DNA hybrids with the G-rich transcribed strand of DNA, leave the untranscribed strand available as a single stranded substrate for AID.[16] While these studies provide an explanation for the need for transcription, they do not explain why both the transcribed and non-transcribed strands are mutated with approximately equal frequencies *in vivo*.[17] Biochemical studies showed that dCs in supercoiled regions upstream of the transcription bubble could be deaminated by semi-purified AID, perhaps explaining why both strands are mutated *in vivo*.[18] However, none of these findings explain why the C regions and other highly transcribed genes[19] either do not undergo AID induced mutations or accumulate mutations at much lower rate than the V region.

Given the high mutation rate that is generated by AID in antibody V regions, it seems likely that numerous mechanisms cooperate to recruit AID to V but not to C regions, and to protect other genes from its mutagenic affect. The need for such rigorous control was dramatically illustrated when AID was ubiquitously overexpressed in mice which died primarily of T cell lymphomas and lung adenomas.[20] These T cell lymphomas harbored point mutations in the T cell receptor (TCR) and c-*myc* genes. One mechanism for limiting potential damage by AID is that its expression is restricted to germinal center B cells.[21] Most of the AID protein in centroblast B cells is located in the cytoplasm, so the activity of AID may also be restricted by factors that limit its residence in the nucleus.[22,23] Phosphorylation of AID appears to be important for its nuclear localization and activation.[24] In addition, mutational analyses suggest that there are proteins that associate with AID and facilitate its transport into the nucleus and its differential targeting to the V or SRs.[25–27] However, so far, the only AID-associated protein that seems to affect its activity and targeting to hot spot motifs is replication protein A (RPA), a single-stranded DNA binding protein that may increase accessibility of AID to its DNA substrate.[28]

While transcriptional control of the expression of AID, nuclear localization, posttranscriptional modifications[24], and association with co-factors must all contribute to the targeting of AID to hot spots in the V or SRs, these mechanisms do not explain why the C regions of Ig genes and other non-Ig genes that are highly transcribed in centroblast B cells are not mutated at the same high rate as V regions.[19] This suggests that there are additional factors required for the preferential targeting of AID to V but not to C regions, and to Ig but not other genes. It is important to identify such factors not only to gain a more complete understanding of how CSR and SHM are regulated, but also because mistakes that occur during CSR and SHM lead to mutations and chromosomal rearrangements that appear to be responsible for many of the B-cell lymphomas in humans.[29,30]

In the following sections we will summarize our attempts to examine the roles of increased accessibility mediated by changes in chromatin structure, *cis*-acting sequences, and MMR in the targeting of AID.

9.2. The Generation of Increased Accessibility through Changes in Chromatin Structure

Even before AID was described, it was suggested that V(D)J rearrangement, SHM and CSR were probably restricted and targeted to different regions of the Ig gene by processes that make those regions accessible to the enzymes required to mediate these processes.[31] In recent years, accessibility to factors that regulate the transcription of particular genes has been associated with the modification of histones that leads to local changes in chromatin structure, especially in promoter regions, so it was logical to think that the coding regions of the V region might also be made accessible to AID through the acetylation of histones. To examine this possibility, we studied the BL2 human Burkitt's lymphoma centroblast-like B cell line that can be induced to undergo SHM by co-stimulating with anti-IgM, as a surrogate for antigen, and helper T cells.[32,33] We showed that following co-stimulation, but not with anti-IgM or T cells alone, there was hyperacetylation of H4 and H3 in histones associated with V, but not in the histones associated with C region. Furthermore, we found that treatment of the cells with Trichostatin A (TSA), an inhibitor of histone deacetylation that causes global hyperacetylation of histones, resulted in the mutation of the C region and increased the mutation of the V region.[33] TSA also caused mutation of the V region within 200 bp of the transcription start site (Figure 9.2).

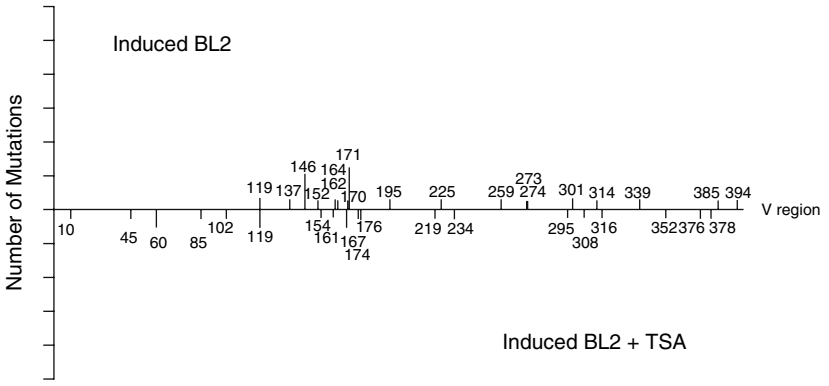

FIGURE 9.2. **Hyperacetylation results in promoter proximal mutations.** BL2 Burkitt's lymphoma cells were stimulated to undergo V region mutation and the heavy chain V region was sequenced.[33] The upper panel shows the distribution of mutations that arise normally in this cell line. If the BL2 cells are pretreated with Trichostatin A (TSA) that causes global hyperacetylation of the histones, mutations now appear in the V region proximal to the promoter (lower panel). The numbers indicate nucleotide position.

This was the first time that mutations had been routinely observed in the region of V that is proximal to the promoter, even though thousands of V regions have been sequenced over the years. In subsequent studies others using transgenic mice also observed greater increases in the acetylation of histones associated with the heavy chain the V region compared to the C region, though this hyperacetylation was found in both germinal center and naïve spleen B cells.[34] This could suggest that the histone modifications precede the expression of AID, or that changes in chromatin structure are not required for SHM. These same *in vivo* studies did not find differential acetylation between V and C in the light chain, but this particular C region is only 1.6 kb away from the promoter and still within the domain that is targeted for SHM. Thus, there is a discrepancy between *in vivo* and *in vitro* studies, and between the heavy and light chains *in vivo*. Since all of these observations are at best correlations, it remains to be seen whether these changes in chromatin structure are required for the correct targeting of AID.

Although centroblast B cells undergo both SHM and CSR (Figure 9.1), it is possible to carry out each of these processes independently, suggesting that they are regulated by different signals and factors. In addition, when B cells are stimulated with different combinations of mitogens and cytokines, particular SRs are targeted for mutation, double-stranded breaks, and recombination.[12] We have used primary mouse B cells in short-term culture to determine whether this selective targeting of AID to different SRs is also associated with changes in chromatin structure. The process of CSR begins with transcription of sterile, or germ line, transcripts from promoters just upstream of the donor Sμ region and of the relevant downstream SR. AID then initiates a process of mutation, double stranded DNA break formation, and recombination in that particular SR.[10]

To study the role of chromatin structure in this process, naïve mouse spleen B cells were treated in short-term culture with LPS to induce switching to IgG3. We found that there was constitutive transcription and hyperacetylation of the donor μ SR. LPS stimulated the hyperacetylation of histone H3 associated with the recipient Sγ3, but not of the histones associated with Sγ1 (Figure 9.3).[35] This was consistent with switching to IgG3. However, when both LPS and IL-4 were used to stimulate switching to IgG1 rather than IgG3, there was transcription of both the γ3 and γ1 SRs but there was more acetylation of the histones associated with Sγ1 than of Sγ3 (Figure 9.3). The correlation between hyperacetylation and AID targeting was further highlighted when we sequenced the DNA co-immunoprecipitated from recipient SRs by antibodies to hyperacetylated histones. We found hyperacetylated recipient SRs harbored more mutations than the input DNA. Acetylation of H4 occurred in both the γ3 and γ1 SRs with both stimuli, revealing a dissociation of the degree of H3 and H4 acetylation and of H4 acetylation with the ultimate targeting of switching.[35] While these findings, and those reported by others[36,37], suggest that SRs may be made selectively accessible to AID through the modifications of chromatin, they are correlations and additional experiments will be needed to prove that modifications of histones are required for targeting AID to particular SRs.

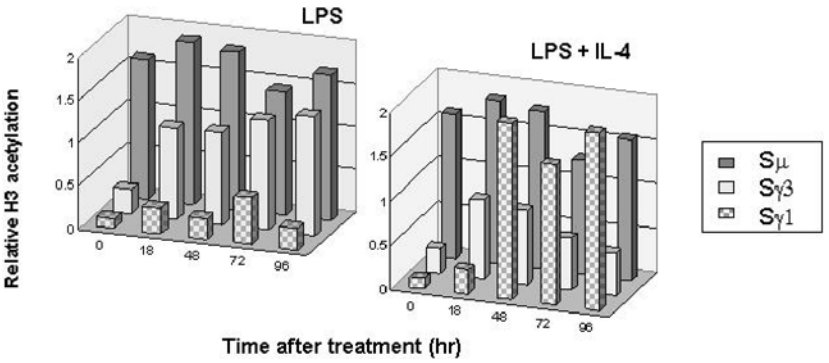

FIGURE 9.3. **Hyperacetylation of histone H3 is associated with switching to the downstream C regions.** Spleen B cells were stimulated with LPS to switch to IgG3 (left hand panel) or with LPS plus IL4 to switch to IgG1 (right hand panel). At various times after stimulation, chromatin immunoprecipitation of the donor Sμ and recipient Sγ3 and Sγ1 regions (see legend) were carried out with antibodies to acetylated histone H3 to determine the degree of acetylation of the histones associated with each of the SRs.[35] The relative amount of acetylation is depicted with bar graphs.

9.3. The Role of *Cis*-Acting Sequences in Targeting SHM

Because transcription is required for SHM and CSR, and *cis*-acting elements target transcription factors to specific genes, it seemed plausible that some of the combinations of *cis*-acting elements that regulate transcription might also target AID to V regions. This led a number of investigators to generate mice with ectopically located, often multicopy, Ig heavy and light chain Ig transgenes, with heterologous or modified promoters or enhancers. Such transgenes often undergo SHM in a manner similar to the endogenous V region *in vivo*, suggesting that all of the information required for targeting SHM is present in the Ig gene and its immediate flanking sequences.[11,38] In the endogenous genes, a number of different promoters allowed normal control and targeting of SHM.[39,40] While the 3' enhancer of the κ light chain is required for SHM in ectopically located light chain genes,[41] gene targeting indicates that this is not the case for the endogenous gene.[42] In the endogenous Ig heavy-chain gene, the hs3b and hs4 elements of the 3' enhancers were not required for SHM even though they were for CSR.[43] Many different sequences have been inserted in place of the V region itself in an attempt to identify DNA motifs that have a role in targeting SHM *in vivo*, but it appears that almost any sequence situated between the V region promoter and an intronic enhancer will be targeted by AID for SHM *in vivo*.[39,44] These studies confirmed that transcription is required for SHM but raised a question as to whether there are any particular motifs or combinations of transcriptional factors that are required to target AID. In addition, some highly expressed non-Ig genes seem to be routinely targeted for mutation in normal centroblast B cells

and in cultured cells, but the rates of mutation of these non-Ig genes are often orders of magnitude lower than the rate of mutation of the heavy and light chain V regions.[45–49]

The role of the heavy chain intronic enhancer has also been examined in both mouse and human B cell lines that undergo SHM in culture. As in the *in vivo* studies, transcription is required but other enhancers such as those from viruses still target AID preferentially to hot spots in the V region and its immediate flanking sequences.[50] In fact, when AID is overexpressed in B or non-B cells, it will targets highly expressed non-Ig genes, including AID itself, for mutation, even though there are no Ig transcriptional regulatory elements associated with those genes.[46,47,51]

These and other studies raise the question of whether *cis*-acting sequences are required to target AID to the V and SRs of Ig genes.[52] In both *in vivo* and *in vitro* models, the fact that the rate of mutation is roughly proportional to the rate of transcription has made it difficult to dissect out the potential role of the individual motifs within promoters and enhancers, because any mutation or deletion that leads to a decrease in the rate of transcription will also lead to a decrease in the rate of SHM. We were able to overcome this "technical" problem by using variants of the Sp6 hybridoma cell lines developed in Marc Shulman's laboratory.[53,54] In these cell lines, homologous recombination had been used to replace the intronic regulatory elements in the endogenous μ heavy chain gene with intronic regions that lacked the core Eμ enhancer, its flanking matrix attachment regions (MARs) or both Eμ and the MARs. The μ switch region was also deleted and the potential influence of the 3' enhancer which, as noted above, does not play a role in SHM in the endogenous gene, was blocked by inserting *gpt* as an insulator upstream of the δ constant region gene[55] (Figure 9.4). The loss of both MARs did not affect the levels of μ mRNA or protein. As expected, the loss of the core Eμ and the loss of both the core Eμ and the MARs resulted in the loss of μ heavy chain expression (Figure 9.4).[56] However, in recombinants lacking either the Eμ and Eμ plus MAR, rare subclones could be identified that had variegated expression of the μ heavy chain, and expressed steady state mRNA and the μ heavy chain at wild type levels .[57] This provided us with stable cell lines that highly expressed the μ heavy chain in the absence of the core Eμ, or both the core Eμ and MARs, and allowed us to separate the role of these elements in regulating transcription from their role targeting SHM.

Each of the genotypes was stably transfected with AID, and the V regions and C regions of multiple independently transfected clones were sequenced to assay for SHM.[56] The parental cells (Figure 9.4-gpt-) and cells in which the endogenous intronic region had been replaced with all of the parental elements (gpt+) had the same frequency of V region mutation, showing that homologous recombination did not affect SHM. The presence of the GPT gene did not significantly affect the frequency of V region mutation and there were few mutations in the C region of any of the clones examined. The cells lacking both MARs had an $\sim 50\%$ decrease in SHM while the cells lacking the core Eμ had an even lower rate of V region mutation, similar to the PCR error rate and to the rate in subclones that

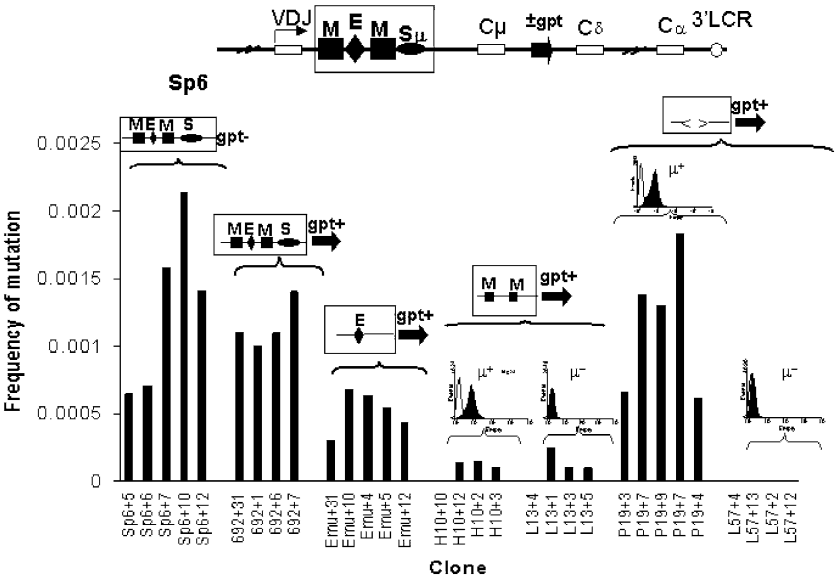

FIGURE 9.4. **The role of the intronic enhancer and its flanking MARs in targeting SHM in the Sp6 hybridoma cell line.** The intronic region in the endogenous μ heavy chain gene of the Sp6 hybridoma cell line (top diagram) has been replaced with constructs that lack either the core Eμ intronic enhancer, the two flanking matrix attachment regions (M), or both. The Sμ region was also removed and the function of the 3' enhancer was insulated with a gpt gene. Cells that expressed wild type levels of the μ heavy chain were isolated (see inserted FACS patterns) and were compared with cells that had lost expression of the μ heavy chains after removal of the core Eμ or both the core Eμ and the M. Cells of each of the genotypes were transfected with AID and independently transfected subclones were examined for their frequency of V region mutation by sequencing (vertical bars).[56] All of the cells mutated the transfected AID gene at approximately the same frequency.

no longer transcribed the heavy chain gene (Figure 9.4). We confirmed that AID was active and could induce SHM in the cells that were not mutating their V regions by sequencing the transfected and highly expressed AID transgene, which we had shown previously mutates itself, and that its frequency of mutation was approximately the same in all of the genotypes.[56] While this suggested that the core Eμ and perhaps the MARs contained *cis*-acting sequences involved in targeting AID to the V region, we were surprised to find that deletion of both the MARs and Eμ restored the rate of AID induced mutation of the transcribed V region to wild type levels. The restriction of SHM to V and not to C and the targeting to hot spots were the same as in the wild type cells.[56]

These results indicate that *cis*-acting sequences in Eμ can play a role in the selective targeting of AID to the V region. However, the unexpected finding that the rate of V region mutation in the cells lacking both Eμ and MARs was the same as the parental cell line suggests that these elements exert both negative

and positive regulation. The finding that the loss of both Eμ and the MARs permitted parental levels of SHM is consistent with the observation that highly expressed non-Ig genes that do not have any *cis*-acting Ig regulatory elements can be targeted by AID. For example, some of the proteins that bind these elements could act in concert in B cells to target AID to V regions but not to C regions, or other highly expressed genes. However, factors that bind to one or the other regulatory element, when acting alone, are not sufficient to target AID and could even protect non-Ig genes from the action of AID. In this context, it is important to note that Eμ shares motifs with other enhancers that can recruit specific transcription factors.[58]

While these findings, like others in the literature cited above, suggest that *cis*-acting elements may play a complex role in targeting AID to V region and in protecting non-Ig genes, it is important to remember that the experiments described above have been done in hybridomas that represent a late stage of B cell development where AID is not usually expressed and SHM and CSR do not occur. Recent studies using the Ramos human centroblast-like Burkitt's lymphoma cell line show that when the hs3b and hs4 elements of the 3' enhancer are substituted for the core Eμ, mutation is still targeted to the V region but the mutations occur in A:T bases in hot spot motifs more often than in G:C bases,[59] which is opposite of what happens in cultured B cells containing Eμ where 80% of the mutations are in G:C bases.[52] This again suggests a complex role of *cis*-acting elements in SHM process.

Recent gene replacement studies in mice found that core Eμ is not required for V region SHM *in vivo*.[60] While these Eμ lacking mice also have a mild deficiency in B cell development, this does not seem sufficiently to explain the difference between the *in vitro* and *in vivo* results. However, these mice retain their 3' enhancer elements while in the hybridomas we attempted to insulate the role of the 3' enhancer using a *gpt* gene. In addition, it is possible that in the *in vivo* experiments, rare B cell clones that had undergone low levels of mutation were positively selected because they were the only ones making higher affinity antibodies and this could have masked even a significant defect in SHM. Thus, the role of *cis*-acting sequences still needs more investigation both *in vivo* and *in vitro.*

9.4. Role of Mismatch Repair in Targeting SHM and CSR

Before the discovery of AID, mice and humans with defects in many repair processes were screened for defects in SHM and CSR. These studies revealed that deficiencies in some of the MMR proteins resulted in decreases in SHM and CSR.[9] This was a surprising and paradoxical finding since MMR normally maintains genomic stability by removing mismatched DNA bases that arise during DNA replication, repair, and recombination. However, MMR can also inhibit homeologous recombination between slightly divergent DNA sequences and can trigger apoptosis after extensive DNA damage by activating cell-cycle

checkpoints.[61,62] The general process of MMR is initiated by the recognition of mismatched bases. The Msh2-Msh6 dimer recruits exonuclease 1 (Exo1), the Mlh1-Pms2 heterodimer and PCNA, as well as other downstream factors, to excise the DNA containing the mismatch and replace the excised strand (Figure 9.5). Deficiency in MMR proteins Msh2 or Mlh1 plays a causal role in hereditary non-polyposis colorectal cancer (HNPCC).[63] Msh2 knock-out mice die early, predominantly from T-cell lymphomas and some intestinal tumors, whereas Msh6 knock-out mice die later predominantly from B-cell lymphomas.[64]

Because MMR is such a ubiquitous and essential process, initially there was concern that the decrease in SHM and CSR observed in Msh2, Mlh1 and Pms2 deficient mice was the result of some indirect influence of the loss of MMR on B cell differentiation.[65] However, deficiency in Msh2, Msh6 or Exo1 all led to a loss of mutations in A:T bases in SHM, and to changes in the how the double stranded breaks at the sites of recombination in SRs were resolved. This suggests that MMR is directly involved in the biochemistry of SHM and CSR. This was supported by other findings including the demonstration of Exo1 at the sites of SHM in a cell line undergoing somatic mutation.[66] Furthermore, Msh6 and Exo1 are associated with active switch regions when primary B cells are stimulate to undergo CSR, confirming that MMR plays a direct role in CSR.[67] MMR recognizes AID-generated dG:dU mismatches to produce half of the V region mutations *in vivo*, including most of the mutations in A:T, and it appears to also play a role in CSR in generating and processing the double-stranded breaks in the switch regions.[68]

Once it was clear that MMR was contributing to SHM, CSR and B cell lymphomagenesis,[9,64] it became important to determine the relative importance

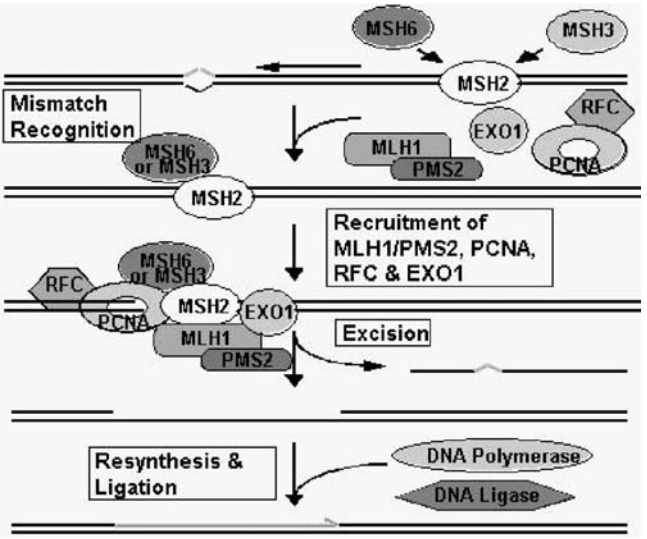

FIGURE 9.5. **Factors involved in mismatch repair.**

of the different roles of MMR in these processes. The examination of Msh6-null revealed that deficiency in Msh6 resulted in the focusing of AID-induced somatic mutations on fewer G:C containing motifs in the V region,[69,70] suggesting that there could be some sort of interaction between the targeting of AID and of MMR. To examine both the increased focusing of SHM and the relative roles of the different functions of MMR, we carried out a very extensive comparison of wild type, Msh6 null, and mice that carry a separation-of-function Msh6 mutant with a single amino acid substitution of threonine at 1217 to aspartic acid ($Msh6^{TD/TD}$).[67,71] This mutation is located near the sites of interaction of Msh2 and Msh6 and results in loss of ATPase activity in the heterodimer. A comparable mutation to this in yeast also causes a loss of the ATPase activity of the Msh2-Msh6 dimer and in man leads to early onset HNPCC. *In vitro* this mutation allows the Msh2-Msh6 dimer to bind the mismatch but there is no repair of the mismatch. However, signaling for apoptosis is retained.[71] $Msh6^{TD/TD}$ mice have a similar increase in B cell lymphomas as the null mice, even though they can trigger apoptosis in the face of excessive DNA damage, suggesting that the loss of signaling for apoptosis is not the reason that Msh6 deficient mice get B-cell lymphomas.[71] Like the $Msh6^{-/-}$ mice, the $Msh6^{TD/TD}$ mice have a decrease in SHM largely attributable to a loss of mutations in A:T.[67] However, the actual frequency of mutations is intermediate between the wild type and the null mice (Figure 9.6), suggesting that the Msh6 protein has an ATP-independent function in SHM that does not require the repair of the mismatched bases. Because both $Msh6^{TD/TD}$ mice and $Msh6^{-/-}$ mice lack the second phase of SHM, V region mutations in these mice should reflect the first phase, i.e., AID activity alone.

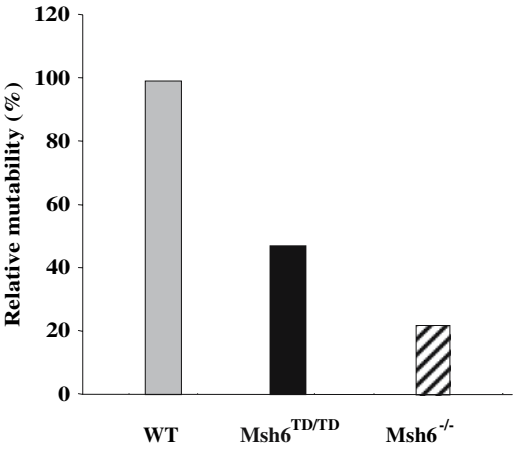

FIGURE 9.6. **Relative frequency of SHM in wild type, $Msh6^{TD/TD}$, and $Msh6^{-/-}$ mice.** Germinal center cells from Peyer's patches of wild type, mutant, and null mice were examined for the frequency of mutation in the Jh2-Jh4 intronic region. The frequency of mutation in the wild type mice is made 100% and the relative frequency of mutation of the other genotypes are depicted as bar graphs.[67]

We postulate that the physical presence of the Msh6 protein plays a role in the targeting of AID and thus modulates the first phase of SHM. In addition, the *Msh6*$^{TD/TD}$ mice have a decrease in CSR that is comparable to the knock out but the sites of recombination in the SRs, which were abnormal in the knockout, are similar to the wild type mice, again suggesting a role for the Msh6 protein itself. We used the chromatin immunoprecipitation assay to show that the mutant Msh6 protein was associated with SRs that were undergoing mutation and recombination.[67]

The notion that Msh6 plays a role in targeting AID is further strengthened by the observation that there are hot spot and non-hot spot motifs targeted by AID in the *Msh6*$^{TD/TD}$ mice but not in *Msh6*$^{-/-}$ mice[67] (Figure 9.7). Collectively, our results suggest that Msh6 provides some sort of scaffolding function that does not require enzymatic activity but does contribute directly or indirectly to the targeting of AID. These studies also highlight the complexity of the regulation and ordering of the queue of enzymes involved in mutation, translesional replication, base excision repair, transcription and MMR that play a role in both SHM and CSR.

Jh4 flanking region (5 ' to 3 ')	***Msh6*** $^{TD/TD}$ **Mutated %**	***Msh6*** $^{-/-}$ **Mutated %**	***P Value***
WGW/WCW			
85 TGG*A**G**T**T*TT	**7.2**	0.6	**<0.01**
159 TA*AA**C**T*GTC	**12.3**	0	**<0.0001**
242 TGG*A**G**T*CCC	**7.2**	0	**<0.001**
525 CTT*A**G**T*GAT	**7.3**	0	**<0.001**
102 TGC*A**G**A*CTA	**19.6**	0	**<0.0001**
514 AGG*A**G**A*GCT	**7.2**	0.6	**<0.01**
203 ACA*T**C**T*TTG	**5.1**	0	**<0.01**
77 CTT*T**G**T*TAT	**12.3**	2.8	**<0.01**
223 ATC*T**G**T*GTG	**12.3**	0	**<0.0001**
221 GAA*T**C**T*GTG	0	**5.6**	**<0.01**
366 ATT*A**G**T**T*GT	0	**11.2**	**<0.0001**
450 GGC*A**C**T*TTC	0	**9.0**	**0.0001**
413 TAC*A**G**T*ATC	5.1	10.7	0.107

FIGURE 9.7. **Targeting of mutation to some representative residues in Msh6$^{TD/TD}$ and Msh6$^{-/-}$ mice.** Each of the residues in the Jh4 flanking region was analyzed in 5-6 different mice for the frequency of mutation. Here we illustrate that some residues targeted by AID for mutation in the mutant mice, that expressed an enzymatically inactive protein, were not targeted in the null mice and vice versa.[67] The underlined base is the site of mutation (number on the left). The italicized triplets are hot spots for AID mutation. Bold and italicized numbers indicate statistical significance. Gs or Cs in RGYW or WRCY hotspot motifs are in bold.

9.5. Conclusions

AID is a highly mutagenic enzyme that is required for the affinity maturation of the antibody response and for class switching. Errors in the regulation and targeting of AID can lead to B cell lymphomas and it is likely that it is regulated at many different levels including its expression, interactions with associated proteins, enzymatic activation and nuclear localization. In addition, once AID enters the nucleus, its targeting to antibody V and switch regions and not to other highly expressed genes or regions must also be tightly controlled. Here we have summarized some of our own evidence, along with those of others, that chromatin modifications, *cis*-acting sequences, and the regulation of the large queue of enzymes that coordinate with AID, are also involved in the targeting of AID to Ig genes.

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10
DNA Replication to Aid Somatic Hypermutation

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10.1. Introduction

Immunoglobulin (Ig) heavy and light chain loci undergo V, (D) and J gene recombination in bone marrow, giving rise to the diverse pre-immune repertoire of V(D)J segments of B cell receptors (BCR). After encountering antigen, naïve B cells divide and differentiate in germinal centers of secondary lymphoid organs. Here, Ig V(D)J gene segments are the targets of point-mutations at a rate of 10^{-3} change per base per cell division. This rate is a million-fold higher than that of mutations occurring spontaneously in the genome at large; hence, the term somatic hypermutation (SHM). Mutated Ig V(D)J regions provide the structural substrate for positive selection by antigen of high affinity mutants, which are characteristic of a mature antibody response. The Ig locus of germinal center B cells also undergoes class switch DNA recombination (CSR), which replaces the constant μ (Cμ) region of the heavy chain with a downstream Cγ, Cα, Cε region, thereby endowing antibodies with new biological effector functions.

Activation-induced cytidine deaminase (AID) initiates SHM and CSR[1,2] by directly deaminating cytidine in DNA (dC), thereby yielding premutagenic uracil DNA lesions (dU)[3–5]. SHM preferentially targets the RGYW/WRCY (R = A or G, Y = C or T, W = A or T) mutational hotspot, which contains the preferred AID deamination motif WRC[6–8]. dU is a non-bulky DNA lesion that, if not repaired, could pair with dA without blocking the DNA replication fork, thereby giving rise to dG → dA and dC → dT transitions. Consistent with this prediction, mice with a double deficiency of Ung, a uracil-DNA glycosylase (UDG), and MutantS homolog 2 (Msh2), a mismatch sensor and initiator of the mismatch repair (MMR) cascade, display only dG → dA and dC → dT transitions, likely due to ablation of both the Ung-mediated base excision repair (BER) and the

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Msh2-mediated MMR pathways[4]. Accordingly, in contrast to other DNA lesions, such as abasic sites or pyrimidine dimers, dU in the DNA template would not block transcriptional elongation by human RNA polymerase II, leading to incorporation of either G or A in nascent transcripts[9]. In B cells, this would lead to the introduction of same mutations in antibodies as those that would be generated from mutated DNA templates carrying dG → dA and dC → dT transitions. Thus, dU repair in Ig V(D)J DNA is not under the feedback pressure from faulty transcription; instead, it is dealt with by Ung and Msh2 in an actively mutagenic fashion during DNA synthesis, as effected by error-prone lesion-bypass or translesion DNA synthesis (TLS) polymerases. Here, we will discuss DNA repair factors and their assembly into a putative "mutasome", which is centered on the proliferating cell nuclear antigen (PCNA), and their role in the SHM process.

10.2. Abasic Sites Generated by Ung Stall the Replication Fork

During T cell-dependent immune responses, B cells are activated by cognate antigen and CD40 ligand (CD154) expressed on T cell surface to blast with an average cell division time of eight hours. In mice immunized with carrier-conjugated hapten or sheep red blood cells (SRBC), germinal centers appear within four days[10,11] and expression of AID, which is induced by CD154 and/or cytokines such as IL-4 and TGF-β, begins within hours and peaks at day five[12]. In addition, expression of TLS polymerases critical to SHM, such as DNA polymerase (pol) θ, pol η and pol ζ, is significantly upregulated in B cells by the same stimuli that induce AID expression and SHM[13–15], suggesting that SHM is a tightly regulated active process (Figure 10.1). Mutations in V(D)J DNA are detected thereafter[10], supporting the contention that after generation of initial dU lesions by AID, B cell division and accompanying DNA replication are necessary for mutations to be fixed in the genome. Ung, but not other UDGs, such as SMUG1, contributes most, if not all, of the dU deglycosylation activity in SHM, as suggested by the greatly diminished expression of SMUG1 in germinal center B cells[16,17]. Ung can deglycosylate dU generated by AID in three different contexts: i) as dU in single-stranded DNA (ssDNA); ii) as dU:dG in double-stranded DNA (dsDNA); or iii) as dU:dA in dsDNA after one round of DNA replication that places a dA opposite a dU which escaped repairing. The deglycosylation activity of Ung on dU in ssDNA or dU:dG is 6- and 4-fold higher than that on dU:dA, respectively[18], suggesting that the first two events generate major mutagenic intermediates in SHM. As such, they will be the main focus of our discussion.

AID effectively deaminates dC in ssDNA[7], which transiently exists in the transcription bubble or immediately upstream of the replication fork. A coupling of deamination and deglycosylation of dU in such a context would explain the ready induction of mutations in BL2 Burkitt's lymphoma B cells induced by

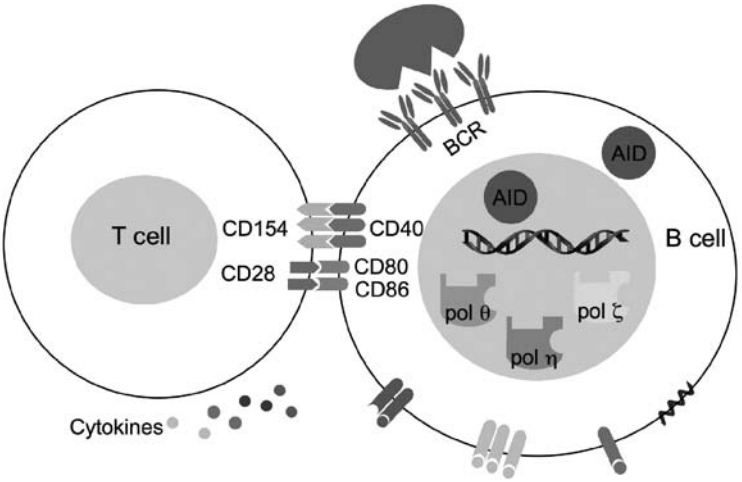

FIGURE 10.1. Induction of AID and error-prone TLS polymerases by the same stimuli that are required for SHM induction. BCR crosslinking and T:B cell interaction through CD154:CD40, CD28:CD80 and/or CD28:CD86 coengagement are required for SHM[21,22]. CD154:CD40 engagement induces AID for expression, which is further augmented by T cell-secreted cytokines[12]. Both BCR crosslinking and CD154:CD40 engagement upregulate error-prone pol θ and the Rev3 catalytic subunit of pol ζ in both human and mice. Pol η is upregulated in germinal center B cells in mice, but not in humans[13–15].

antibodies to CD19, CD21 and BCR[19]. Interestingly, in such B cells, about 50% of the mutations were dC/dG transitions, likely resulting from either "replicating over" of dU or the insertion of dA across the abasic sites generated by Ung (see below), two events that require at least one round of DNA replication.* DNA strands unwound by a helicase during replication may serve as the substrate for both AID and Ung localized at replication foci[20]. The coupling of AID-mediated deamination with Ung-mediated deglycosylation of dU is also implied by the failure to remove dU from ssDNA in B cells from a patient with hyper-IgM syndrome expressing a mutated and non-functional Ung[16]. One possible mechanism for such a coupling to occur is through replication protein A (RPA), which has been shown to bind both phosphorylated AID[23] and Ung[20]. In an *in vitro* experiment, half of AGCA in a single-stranded oligonucleotide was deaminated by AID and deglycosylated by Ung within 5 minutes[8], suggesting that Ung, if at a high local concentration, as effected by either a high protein level or a high k_{cat}/K_m ratio, would be able to process most of dU in ssDNA.

* PCR amplification of dU generated from dC deamination would also give rise to apparent dC → dT and dG → dA transitions. However, this seems unlikely in this study because under PCR conditions, incorporation of dA across dU by high fidelity DNA polymerases, such as Pfu® (Invitrogen Corp.) used in this study, is inefficient (our unpublished data).

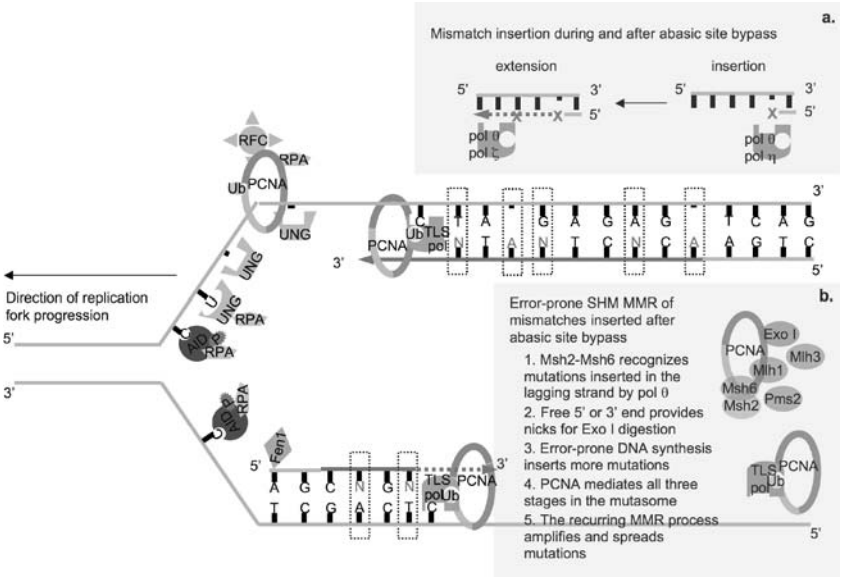

FIGURE 10.2. An integrated model of SHM during DNA replication. The depicted model is based on the assumption that AID mediates SHM through deamination on both DNA strands. Coupling of dC deamination and dU deglycosylation generates abasic sites that stall replication fork progression in DNA replication foci. During DNA replication, template strands are transient unwound by the DNA helicase, yielding ssDNA stabilized by RPA, which would recruit phosphorylated AID to deaminate dC. dU:dG generated by AID during V gene transcription would be unwound and result in dU in ssDNA as well (not depicted here). Coupling of dC deamination and dU deglycosylation generates abasic sites, depicted as shorter bars without a base, that stall replication fork progression in DNA replication foci. Loading of the monoubiquitinated PCNA trimeric ring, possibly facilitated by Ung and dependent on RFC and RPA, initiates the assembly of a mutasome that introduces mutations during repair of abasic sites. **a.** Abasic site bypass is effected through polymerase switch in both the leading and lagging strands. Monoubiquitinated PCNA plays a central role in the switch from the high fidelity pol δ and pol ε to TLS polymerases. These TLS polymerases are able to incorporate nucleotides across abasic sites and continue synthesizing DNA. Two different TLS enzymatic activities are necessary to bypass abasic sites, first, insertion, as performed by pol θ, pol η, Rev1 and, perhaps, pol ι, and, then, extension, as performed by pol θ and pol ζ. Further, because of the unique dual functions of pol θ, i.e., inserting mismatches and extending past inserted mismatches, pol θ would generate additional mutations, depicted as "N", when copying undamaged template after abasic sites bypass. **b.** Error-prone MMR of mismatches generated during the extension stage after abasic site bypass. Mismatches are recognized by Msh2-Msh6, possibly aided by PCNA, and MutL dimers are recruited to initiate the DNA excision stage. Strand nicks necessary for the entry and activity of the exonuclease I (Exo I) are provided by 3'-end of the leading strand and both 5'-end and 3'-end of the Okazaki fragments in the lagging strand. Subsequent patch DNA re-synthesis by error-prone TLS polymerases will generate additional mutations which would initiate another round of MMR. Recurring SHM MMR would spreads the mutations along the V(D)J DNA. A similar SHM MMR process initiated by dU:dG in the template DNA strand would also, possibly aided by the Mre11-Rad50-Nbs1 (MRN) complex, amplify mutations. dU:dG that is not repaired will be replicated over to generate dC → dT and dG → dA transition mutations.

Recognition and deglycosylation of dU:dG by Ung have been hypothesized to occur mainly in the nucleoplasm[24]. Here, the resulting abasic sites would be primarily repaired in an error-free fashion through a short patch BER process, which is independent of DNA replication and underpinned by nicking of DNA by apurinic/apyrimidinic endonucleases (APE) and insertion of a single nucleotide by the high fidelity DNA pol β[24]. Such abasic sites could also be processed through a long-patch BER process, as effected through cleavage by the structure-specific flap endonuclease 1 (Fen1) and gap-filling of 2-8 nucleotides by the high fidelity pol β or pol δ[25]. Alternatively, dU:dG deglycosylation by Ung localized in DNA replication foci would generate abasic sites[20], which, if not processed by APE, would effectively block nucleotide incorporation by the high fidelity replicative pol δ and pol ε and halt the DNA replication fork. Similarly, deglycosylation of dU in an ssDNA template during DNA synthesis, as discussed above, would also block the replication fork (Figure 10.2). The processive nature of AID, as shown by its deamination of a series of dC in the same ssDNA[7], suggests that AID and Ung can generate a high number of abasic sites in the template strands. These abasic sites need to be bypassed or repaired efficiently in dividing germinal center B cells. Interestingly, in the absence of APE-mediated cleavage, Ung remains tightly bound to abasic sites with an affinity even higher than to the dU in dsDNA[26], thereby likely playing an additional role in recruiting DNA repair factors to bypass abasic sites.

The current knowledge of DNA replication and repair has stemmed from studies in *E. coli* and yeast cells as well as *in vitro* systems reconstituted with defined DNA templates and purified protein factors. In both *E. coli*. and yeast cells, stalling of the replication fork by unrepaired abasic site can be overcome through either homologous recombination (HR) or TLS[27], both of which are functional in mammalian cells. Existing genetic and biochemical evidence strongly suggests that error-prone TLS polymerases bypass abasic sites and insert mismatches in SHM. This is reflected in the upregulation of TLS polymerases, which are needed to cope with the pace of generation of abasic sites by processive AID and Ung in germinal center B cells.

10.3. Abasic Site Bypass by TLS and Polymerase Switch

At the replication fork stalled by lesions in the template strand, the high fidelity pol δ and pol ε are substituted by TLS polymerases that are able to incorporate nucleotides across and/or past damaged bases, including abasic sites ("polymerase switch")[28]. The high number of AID-generated abasic sites that need to be bypassed suggests that TLS polymerases bind to template strands during SHM for a much longer time than that during general DNA repair processes, before the reversion to pol δ and pol ε occurs. Two enzymatic activities are essential to carry out the abasic site bypass, one that incorporates nucleotides across from abasic sites (insertion), and another that elongates past the nascent primer end (extension)[29] (Figure 10.2a). DNA pol η, Rev1, pol ι or even the high fidelity pol δ possess various degrees of the inserting ability, but cannot extend past the inserted nucleotide. Pol ζ cannot insert,

rather it efficiently extends nucleotides incorporated across abasic sites. Pol θ is the only TLS polymerase that possesses both enzymatic activities[30] (Table 10.1). The significant upregulation of TLS polymerases by stimuli that also induce SHM suggests that SHM co-opts the otherwise high-fidelity abasic site bypass machinery to actively introduce mismatches. As pol θ could function as both an "inserter" and an "extender", thereby bypassing abasic sites with a much higher efficiency than other TLS polymerases[30], it plays a major role in this process and overall SHM. This has been shown by the more than 70% reduction of the overall mutation frequency in a knockout mouse strain in which pol θ expression is ablated[15]. In another knockout mouse strain, in which 99 of the 532 amino acids of the polymerase domain of pol θ were deleted and the polymerase activity was inactivated, the mutation frequency at dC/dG decreased by 40% and most of the decrease was focused on the AID deamination motif WRC, strongly suggesting that at least in SHM, bypass of abasic sites

TABLE 10.1. Properties of eukaryotic DNA polymerases and their role in SHM

DNA polymerase	Class	Proposed main function[1]	Fidelity[1]	Processivity[2]	Role in SHM[3]
pol α	B	primase in DNA replication	$10^{-4} - 10^{-5}$	moderate	no
pol β	X	BER	$10^{-4} - 10^{-5}$	low	no
pol γ	A	mitochondrial DNA replication	$10^{-5} - 10^{-6}$	high	ND[4]
pol δ	B	DNA replication	$10^{-5} - 10^{-6}$	high	no
pol ε	B	DNA replication	$10^{-5} - 10^{-6}$	high	ND[4]
pol ζ	B	translesion DNA synthesis	$10^{-4} - 10^{-5}$	moderate	mispair extension
pol η	Y	translesion DNA synthesis	$10^{-2} - 10^{-3}$	low	insertion of mutations at dA/dT
pol ι	Y	translesion DNA synthesis	$10^{-1} - 10^{-5}$	low	no or marginal
pol κ	Y	translesion DNA synthesis	$10^{-3} - 10^{-4}$	low	no
pol λ	X	NHEJ	$10^{-4} - 10^{-5}$	low	no
pol μ	X	NHEJ	$10^{-4} - 10^{-5}$	low	no
Rev1	Y	translesion DNA synthesis	ND[1]	low	dC insertion across abasic sites
pol θ	A	translesion DNA synthesis	$10^{-2} - 10^{-3}$	ND[4]	insertion across abasic sites; mispair insertion and extension

[1] Defined as base substitution error frequency during nucleotide incorporation on intact single strand template; Reference[39].
[2] Reference[29].
[3] Reference[40–42].
[4] ND, not determined.

depends on pol θ[31]. An abasic site is non-instructional and all four nucleotides can be incorporated across it. Nevertheless, dA is preferentially incorporated across abasic sites by pol θ ("A rule")[30,32,33], thereby yielding dC → dT and dG → dA transitions. This would partially explain why among dC/dG mutations, transitions are more prevalent than transversions, being 50% *in vivo* and in hypermutating Ramos B cells[34] instead of 33% if all four nucleotides are equally incorporated (dG insertion does not generate a mutation). In contrast, Rev1 efficiently inserts dC regardless of the template sequence[35], possibly through a mechanism entailed by the high affinity interaction between incoming dCTP and an arginine residue in the active site of Rev1[36]. The virtual absence of dC → dG transversions in the non-transcribed strand of the Vλ1 segment DNA in Rev1 deficient mice is consistent with the role of Rev1 in inserting dC across abasic sites during SHM[37].

Pol ζ and pol θ are the only TLS polymerases known to efficiently extend the nascent DNA strand past an abasic site by copying undamaged portion of DNA template, consistent with their major role in SHM[13,15,31,38]. Pol ζ is relatively error-free when copying undamaged DNA template. In contrast, pol θ can insert mismatches opposite an undamaged DNA template and extend past inserted mismatches, consistent with its error-prone function in the extension stage of the abasic site bypass (Figure 10.2a). Mismatches inserted by pol θ can also be extended by pol ζ. Such mismatches could further trigger an error-prone MMR entailing pol θ, pol ζ, pol η and, perhaps, pol ι, instead of the canonical high fidelity pol δ and pol ε, to spread the mutations (see below). The dominant role of pol θ in SHM likely reflects the involvement of this TLS polymerase in the following crucial stages of the process: mismatch insertion across from abasic sites, mismatch introduction during extension past abasic sites and gap-filling during error-prone MMR.

Evidence in both human and mice indicates that pol η plays a role in SHM. Pol η preferentially introduces mutations at dA/dT[43]. Indeed, in *Xeroderma pigmentosum* variant (XP-V) patients, who are congenitally deficient in pol η, the mutation spectrum shows a strong bias towards dC/dG[14], and in pol η-deficient mice, the overall frequency of mutations is normal but mutations at dA/dT are decreased by two thirds[44,45]. Similar decreased mutations at dA/dT in mice deficient in the MMR proteins Msh2, Msh6 or Exo I suggest that pol η is involved in the error-prone MMR of SHM. As in abasic site bypass, the function of pol η would be restricted to inserting a mismatch that is then extended by either pol θ or pol ζ. Finally, pol ι seems to play only a marginal role in SHM, as mice with pol ι deficiency display a decreased mutation frequency only when crossed with pol η-deficient mice[44].

10.4. The Mutasome: PCNA, MMR and TLS

The modalities and mechanisms of the polymerase switch to bypass abasic sites in SHM remain to be determined. In *E. coli*, the β-sliding clamp simultaneously binds both the high fidelity pol III and the low fidelity pol IV in a tool-belt fashion. Pol IV is activated only when pol III stalls[46]. Interestingly, while the

moving pol III suppresses pol IV, reflecting the copying of an intact DNA template, the active and moving pol IV, as occurring after a bypassed DNA lesion, is quickly taken over by pol III[46]. The mammalian β-sliding clamp, PCNA, likely plays an analogous role in polymerase switch. PCNA is the pivotal factor for almost all DNA transactions, including DNA replication and repair[47]. PCNA has a unique six-fold head-to-tail trimeric ring structure with an inner side rich in α-helices that allows binding to and sliding along DNA (Figure 10.3)[48–50]. Each PCNA monomer has two functional domains connected by a coiled loop from Leu121 to Asp132. Such a coiled loop mediates the interaction with pol δ, Ung, Fen1 and DNA ligase I through the QxxI/LxxFF motif in those ligands. The C-terminus of PCNA is important for its binding to pol ε and PCNA loading protein replication factor C (RFC) through the similar QxxI/LxxFF motif[47,51].

The polymerase switch process is possibly co-opted and subverted by SHM. The PCNA-binding motif QxxI/LxxFF of Ung would recruit PCNA to the replication fork stalled by the Ung-abasic site complex, which in turn recruits TLS pol η, pol ζ and pol θ. The role of Ung in recruiting PCNA in SHM polymerase switch could be analyzed by generating knockin mice that carry a mutated QxxI/LxxFF motif in Ung. The interaction of TLS polymerases with PCNA likely depends on the posttranslational modification of PCNA, as PCNA is ubiquitinated upon UV irradiation, and interaction between pol η and monoubiquitinated PCNA is critical to confer UV resistance in mammalian cells[52]. Monoubiquitinated PCNA Lys164 constitutes another binding motif that is recognized by the ubiquitin-binding domain (UBD) in pol η, pol ι[53] and possibly other TLS polymerases. Further, PCNA, together with RFC and RPA, strongly

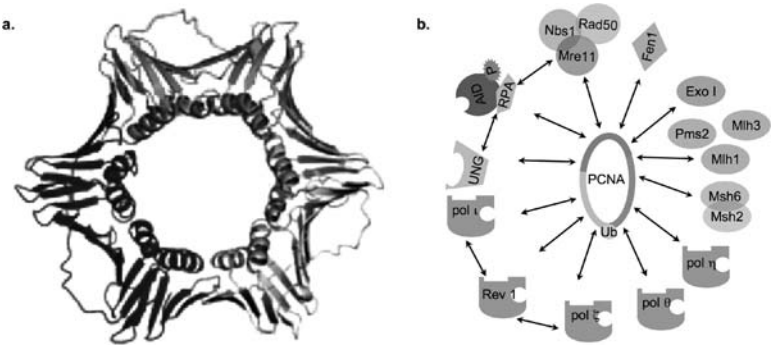

FIGURE 10.3. Structure of PCNA and its interacting proteins in the mutasome. **a.** Ribbon representations of human PCNA.* **b.** The mutasome contains PCNA and different DNA replication/repair factors that interact with PCNA and are involved in SHM.

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enhances the insertion activity of human pol η[32,33] and PCNA binds to pol ζ and strongly stimulates its TLS activity to copy UV-damaged DNA template *in vitro*[54], suggesting that PCNA functions not only as an "adaptor" but also as an "inducer" of TLS polymerases during SHM. PCNA might also interact with pol θ and augment its abasic site bypassing, mismatch insertion and extension functions. The role of monoubiquitinated PCNA in the SHM polymerase switch can be addressed in knockin mice with a mutation in Lys164 in PCNA. This mutated PCNA cannot be ubiquitinated[52] and thereby would be unable to activate TLS polymerases[53]. PCNA also undergoes polyubiquitination and sumoylation in yeast[55,56], suggesting the existence of more posttranslational modifications of the mammalian PCNA that could play a role in the SHM polymerase switch. Negative regulators of the polymerase switch would include the deubiquitinase USP1, whose degradation correlates with the accumulation monoubiquitinated PCNA in mammalian cells[57]. The recent report that ubiquitination of PCNA neither decreases the affinity of this adaptor/inducer for the high fidelity pol δ and pol ε nor does it increase the affinity of PCNA for TLS polymerases, suggests the possible existence of an inhibitory intermediate factor[58].

The homotrimeric nature and different interaction motif/domains of PCNA suggest that multiple proteins could bind to PCNA simultaneously. However, the plethora of PCNA-binding proteins would be tightly regulated for their interaction with PCNA and there may exist a dynamic change in the protein factors bound to PCNA. Indeed, PCNA could function as a "docking bay" for DNA replication/repair factors, such as RPA, Ung, TLS polymerases, Msh2-Msh6 heterodimer, Exo I and the MRN complex. Therefore, a term mutasome has been proposed to describe a protein complex localized in the DNA replication foci, in which PCNA plays a central role in coordinating the functions of different protein factors in the polymerase switch during abasic site bypass and subsequent error-prone MMR (see below), thereby effecting SHM[42].

The docking bay function of PCNA would depend on its mobility between the nucleoplasm and replication/repair foci. Whereas during the G1/G2 phase, most PCNA is diffused and mobile, PCNA in the S phase is transiently enriched and immobilized to the replication foci, effectively raising its local concentration that is critical for DNA transactions[59]. DNA damage would further increase the residence time of PCNA, allowing it to be engaged in more time-consuming DNA repair processes, such as abasic sites bypass or MMR in SHM. UV-induced DNA damage results in PCNA accumulation within replication/repair foci, suggesting a DNA replication-dependent lesion repair mediated by PCNA [59]. Such a PCNA-based DNA replication/repair process would likely occur in mutasome during SHM. Depending on different types of DNA damage, PCNA carrying the Lys164 mutation and therby deficient in ubiquitination displays different kinetic distribution patterns, as compared to the wildtype PCNA[59,60], suggesting that monoubiquitinated PCNA plays specific roles in the repair of different DNA lesions, including abasic sites and mismatches around the stalled replication fork in Ig V(D)J DNA. Based on analysis of the cell cycle-dependence of SHM-specific dsDNA breaks (DSB), it is suggested that SHM primarily

occurs in the S/G2 phase when DNA replication is about to end or has ended[61,62]. While the exact role of DSB in SHM awaits definition, monoubiquitinated PCNA specifically recruited to the V(D)J DNA may be a good indicator for the crucial DNA lesions and cell cycle-dependency of SHM.

10.5. Error-prone MMR Amplifies Mutations in the Mutasome

In the mutasome, mismatches inserted by pol θ during the extension stage following abasic site bypass would be subjected to the surveillance of the Msh2-Msh6 heterodimer, the sensor for single-nucleotide mismatches in MMR. In general, MMR corrects misincorporated nucleotides in the newly synthesized strand during DNA replication[63]. However, in the mutasome, an environment with a high local concentration of error-prone TLS polymerases and monoubiquitinated PCNA, the MMR process would be co-opted to become highly mutagenic. The late phylogenic emergence of SHM, appearing first in sharks[64], suggests that "SHM MMR" (Figure 10.2b) likely evolved from the error-free MMR process that appeared earlier in *E. coli.* and yeast cells.

PCNA in the mutasome would play a role in all three stages of SHM MMR; i) mismatch recognition; ii) mismatch excision; and iii) re-synthesis of DNA patch. In mammals, MMR is mediated by proteins homologous to prokaryotic MMR factors, such as Msh2, Msh3 and Msh6 and MutL homologues (Mlh) 1, Mlh3, postmeiotic segregation increased (Pms)1 and Pms2. Consistent with the role of Msh2-Msh6 as the sensor for single-nucleotide mismatches[63,65], mice deficient in Msh2 and/or Msh6 exhibit an overall decreased frequency of mutations. In these mice, residual mutations show a significantly altered spectrum, with decreased mutations at dA/dT and concurrent increased mutations at dC/dG (Table 10.2)[4,66–69]. SHM MMR, as occurring in the mutasome, could also access AID-generated dU:dG mispairs that have escaped Ung-mediated deglycosylation in DNA template strands in proximity of a stalled replication fork. Msh2-Msh6 binds to dU:dG with an affinity comparable to that to dT:dG *in vitro*[70]. PCNA plays a role in the mismatch recognition stage by binding to Msh6 and increasing its affinity to the mismatches[63]. PCNA also interacts with Mlh1, which heterodimerizes with one of its three partners, Pms2, Pms1 or Mlh3. Mlh1, Pms2 and Mlh3 are involved in SHM, albeit in different ways, as mice deficient in Mlh1, Pms2 or Mlh3 display different spectra of mutations[71–73]. The Mlh dimer, as recruited by the Msh2-Msh6, would coordinate the mismatch recognition stage and the subsequent strand excision stage, although a MutL-independent pathway possibly exists in *mlh1*$^{-/-}$ mice, as suggested by the moderate increase in dC/dG mutations as compared to *msh2*$^{-/-}$ and *msh6*$^{-/-}$ mice[72].

A hallmark of MMR is the exclusive targeting of the nascent DNA strand during the DNA excision stage. In *E. coli*, only the nascent and transiently unmethylated strand is nicked by the MutH endonuclease[63]. The resulting nick

TABLE 10.2. MMR proteins and the impact of their deficiencies on SHM[1]

	Frequency of mutations[2,3]	Spectrum of mutations	
		dC/dG:dA/dT[3]	ts:tv at dC/dG[3]
MutS homologs			
Msh2	↓	↑	↑
Msh3	=	=	=
Msh6	↓	↑	↑
MutL homologs			
Mlh1	↓[4]	↑ =	=
Mlh3	=	↑	=
Pms2	↓[4] =	↑ =	=
Exonuclease			
Exo I	↓	ND[5]	ND[5]

1. Data are from the references quoted in the Table 1 of Wu et al. (2006)[73].
2. Mutations in the J_H4 intronic DNA were analyzed unless noted otherwise.
3. ↑ increase; ↓ decrease; = no change; ts, transition; tv, transversion.
4. Mutations in Ig V(D)J DNA were analyzed.
5. Not determined.

provides the entry point for Exo I to excise the mismatch-containing DNA segment[63,74]. Consistent with the role of Exo I in mammalian MMR, this exonuclease contributes to SHM, as suggested by the altered mutation spectrum in Exo I-deficient mice[75]. In eukaryotes, Mlh1-Pms2 heterodimer (MutLα) is a latent endonuclease that is activated in a mismatch-, MutSα-, PCNA- and RFC-dependent manner and generates nicks preferentially on the nascent discontinuous strand (lagging strand), but also notably on the nascent continuous strand (leading strand), thereby providing entry points for Exo I[76]. However, the identity of the endonuclease that generates nicks in the template DNA strand containing dU that escaped deglycosylation remains unclear. Interestingly, Mre11-Rad50, which are part of the MRN complex, possesses an evolutionarily conserved lyase activity[77], which could nick a nearby abasic site generated by Ung in the template strand, thereby providing the entry point for Exo I. Consistent with its role in nicking abasic sites, Mre11, but not the ubiquitous APE, is enriched on V(D)J DNA of hypermutating B cells and the MRN complex promotes SHM[77,78], suggesting that at least some SHM MMR is initiated by this complex. Both Exo I and MRN bind to PCNA[79,80], indicating that PCNA is also involved in DNA strand excision.

DNA digestion by Exo I would yield ssDNA which would be "coated" and protected by RPA before re-synthesis of the DNA patch. The TLS polymerases would be recruited in the mutasome by the monoubiquitinated PCNA to effect DNA synthesis in an error-prone fashion. Indeed, the interaction between pol η

and Msh2-Msh6 *in vivo* is possibly facilitated by PCNA[70]. Pol θ, pol η, Rev1 and, perhaps, pol ι could insert even more mismatches, which are extended by either pol θ or pol ζ, thereby spreading the mutations along the whole V(D)J DNA. The newly introduced mutations at dA/dT would be subject to a new round of Msh2-Msh6-initiated MMR, thereby giving rise to an amplification loop that would lead to further introduction of mutations. Such an amplification loop could also explain the ready emergence of mutations that are not transitions at dC/dG in BL2 cells induced by BCR/CD19/CD21 crosslinking[19]. Finally, consistent with the mutation spectrum in Msh2-deficient mice, in which mutations are more focused on dC/dG in the mutation hotspot RGYW/WRCY and the putative AID deamination motif WRC[66], multiple rounds of error-prone synthesis by TLS polymerases would be instrumental in spreading mutations farther away from the original dU lesion site.

10.6. Conclusions

It is clear that the AID-generated dU lesion is the initiator of a cascade of DNA repair events which are mediated by either Ung or Msh2 and eventually introduce mutations in the Ig locus[5]. However, the relationship between these events and DNA replication is not clearly defined. The involvement of DNA repair factors, such as PCNA, RPA and RFC, which are also crucial DNA replication factors, and Ung or Msh2 in the correction of nucleotides misincorporated during DNA synthesis strongly suggests a tight correlation between SHM and DNA replication. Based on existing genetic and biochemical data, we propose a mechanistic model explaining how mutations would be introduced with a high frequency in the Ig locus (Figure 10.2). Central to this model are the multiple functions of PCNA and the intervention of TLS polymerases, whose levels are greatly upregulated in germinal center B cells. In the mutasome, abasic site bypass and error-prone MMR are the main processes responsible for the introduction of mutations. Further, the highly regulated, both spatially and temporally, functions of Ung and Msh2 would also explain why SHM is mainly confined to the fast dividing germinal center B cell, a differentiation stage entailing a high DNA synthesis rate. Finally, the amplification of mutations by the error-prone SHM MMR would further upregulate the rate of mutations in Ig V(D)J DNA to the high levels that are the hallmark of SHM.

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11
Regulation of Activation Induced Deaminase via Phosphorylation

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11.1. Summary

Immunoglobulin gene diversification by somatic hypermutation (SHM), class switch recombination (CSR), and gene conversion is dependent upon activation-induced cytidine deaminase (AID). AID is a single-stranded DNA specific cytidine deaminase that is expressed primarily in activated mature B lymphocytes. AID appears to catalyze DNA cytidine deamination of immunoglobulin heavy (IgH) and light chain (IgL) variable region (V) exons and IgH switch (S) region sequences to initiate, respectively, IgH and IgL somatic hypermutation (SHM) and IgH class switch recombination (CSR). Here, we will discuss the implications of recent studies that demonstrate the role of AID phosphorylation in augmenting AID activity with respect to these two processes.

11.2. AID Functions by Deaminating Cytidines in DNA

The mechanism by which AID modulates CSR and SHM of S regions and/or V genes is an area of intense study. Both RNA-editing and DNA deamination models have been proposed[1,2,3,4]. While there has been no direct evidence in support of an RNA editing mechanism in CSR and SHM, there has been abundant evidence that these processes are mediated by the deamination of cytidines in DNA by AID. First, we and others have shown via *in vitro* biochemical assays that AID can efficiently deaminate cytidines in DNA. Second, AID mutates

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DNA when expressed in bacteria, where the possibility of an unedited B cell recombinase mRNA is very remote[2,5–10]. Third, chromatin immunoprecipitation assays have shown that AID specifically associates with IgH S regions in a fashion that is specific for their CSR activity[7,11]. Finally, there is a large body of genetic data based on mutant mice and a mutant chicken B cell line that argues for the presence of uridine in DNA of regions undergoing CSR and SHM, providing strong evidence that AID functions in SHM and CSR via cytidine deamination of DNA, followed by further processing via Base Excision Repair (BER) or Mismatch Repair (MMR) pathways to yield mutations or double strand breaks (DSBs)[12,13,14,15,16].

11.3. Generation of DNA Substrates for AID *in vivo*.

We have shown that linear single-strand (ss) DNA, but not linear double-strand (ds) DNA, serves as an effective AID substrate *in vitro*[6]. As most DNA in cells is in a duplex form, the mechanism by which AID can gain efficient access to dsDNA *in vivo* has also been a topic of considerable focus. IgH CSR requires S regions, transcription through S regions, and AID[17–19]. As outlined below, recent work has elucidated the relationship among these requirements, which likely also applies, at least in part, to SHM.

Detailed analysis of mammalian S region sequences revealed that their non-template strand is G-rich[20] and that, correspondingly, when such S regions were transcribed *in vitro* in their physiological orientation, the transcribed RNA hybridizes with template strand DNA to form a stable RNA-DNA hybrid structure termed an R-loop[21–24], in which the non-template strand is looped out as ssDNA. *In vitro* studies showed that AID can efficiently deaminate transcribed dsDNA that can form R loops when transcribed in the sense (R-loop forming) direction but much less so when they are transcribed in the anti-sense (non-R-loop forming) direction[6]. In addition, AID preferentially deaminated the non-transcribed (looped out) DNA strand. As mammalian S regions have the capacity to form R-loops both *in vitro* and *in vivo*[25], R-loop formation can provide one mechanism of AID targeting to S regions, which are specifically transcribed during CSR[25,26]. However, the issue of how AID gains access to the other strand is still an open question, although several possible mechanisms have been considered[17,27].

The R-loop mechanism does not provide an explanation as to how AID could get access to transcribed variable regions during SHM, which are not G-rich on their non-transcribed strand and which do not, based on in vitro studies, have a clear-cut propensity to form R-loops[25]. Notably, variable regions and, even more so, S regions are particularly rich in RGYW (R = purine, Y = pyrimidine, W = A or T nucleotide) motifs, which are known hotspots for SHM (and AID activity) suggesting they may have some role in AID targeting[27]. To elucidate the mechanism by which AID accesses sequences that do not form R loops, we employed biochemical approaches to identify the ubiquitously expressed

Replication Protein A (RPA) as a co-factor that could allow AID to deaminate transcribed variable region sequences in an *in vitro* transcription reaction[7]. This work further revealed that phosphorylated AID from activated B cells interacts with RPA, a single strand DNA binding protein, and that this complex can deaminate the non-template strand of RGYW-containing substrates, such as V genes when transcribed in *in vitro* with T7 DNA polymerase[7]. We also found that the AID/RPA complex appeared to bind preferentially to transcribed DNA sequences that were rich in RGYW motifs. These findings led us to propose that RPA may function to promote AID access to transcribed DNA by somehow stabilizing ssDNA loops in the context of transcription bubbles on V gene substrates. The higher density of RGYW sequences in S and V regions (compared to random regions of the genome) might help facilitate access of AID to these sequences[7]. The fact that S regions are so rich in RGYW motifs further suggested that, in addition to a potential R-loop mechanism, AID also may be targeted to S regions via the RPA/transcription dependent mechanism[27].

Xenopus S regions are AT-rich and do not form R loops, but do have regions rich in the palindromic AGCT sequences, a subset of the RGYW motif[28]. To test whether such sequences could mediate CSR in mammalian cells, we replaced an endogenous mouse Sγ1 region with a Xenopus S region. Strikingly, we found that the *Xenopus* S region could functionally substitute for this endogenous mouse S region to effect *CSR in vivo*[28]. Moreover, the high-density AGCT patches in the Xenopus S region served as recombination hot-spots for CSR, both in Xenopus and in mice. *In vitro* transcription studies similarly showed that these sequences are a preferred target of the AID/RPA complex[28]. Based on these studies, we proposed that AID also might access mammalian S regions in an R-loop-dependent, RPA-independent manner as well as via an RGYW and RPA dependent mechanism[28]. We note, however, that RPA recruited to S regions may have additional roles during CSR, downstream of DNA deamination. Indeed, RPA interacts with UNG and is involved in MMR[29], proteins and pathways that play critical roles in processing deaminated DNA during CSR and SHM[3,16,17]. However, while the proposed role of RPA during SHM and/or CSR *in vivo* remains to be formally proven, studies of AID phosphorylation outlined below have provided further support for this model and provided an approach that might be used to distinguish the relative importance of AID phosphorylation dependent and independent mechanisms for CSR.

11.4. Post-Translational Modifications of AID

We have argued that phosphorylation may augment AID activity *in* vivo[7], a proposal confirmed by recent studies from our lab and those of others. A portion of AID purified from B cells is phosphorylated at two sites, Ser-38

and Tyr-184[30–32]. Notably, Ser-38 in AID exists in the context of a cAMP-dependent protein kinase A (PKA) consensus motif. AID can be phosphorylated *in vitro* by PKA at Ser-38 and co-expression of PKA with AID in fibroblast cells leads to phosphorylation of AID at Ser-38[30,31]. In this context, pharmacological inhibition of PKA in B cells decreased CSR; whereas conditional deletion of the regulatory subunit of PKA led to increased in CSR[31], strongly suggestive that such phosphorylation is relevant *in vivo*. Furthermore, mutation of the AID S38 to alanine (S38a mutation), greatly reduced (to 10−20% of wild type levels) the ability of AID expressed from a retroviral vector to restore CSR activity to AID-deficient B cells stimulated to undergo CSR in culture[30]. Other groups found similar effects of the S38a mutation[31,32]. Yet, we note that the S38a mutant form of AID had no effect on overall AID enzymatic activity on ssDNA *in vitro*, suggesting it did not adversely affect the catalytic site[30]. Together, this work strongly argues that AID phosphorylation is important for augmenting ability of AID to function in CSR, likely through an RPA-mediated mechanism for accessing transcribed S regions. The fact that S38a mutant AID retained full catalytic activity on ssDNA may further indicate that the residual CSR activity observed in these studies reflects that occurring via an R-Loop mechanism[27].

Recently, Honjo and colleagues reported, based on the same type of retroviral rescue assay, that an AID S38A mutation generated CSR levels that approached those of wildtype AID[33]. How can we rationalize these findings with those from our laboratory and others that show a dramatic reduction in CSR of AID S38a mutants compared to those of wild-type AID[30,32]? To resolve this issue, we compared CSR activity and expression of wildtype and the corresponding S38a mutant AID constructs that were generated either in our laboratory[30] or generously provided to us by Honjo and colleagues. The vector of Honjo et al was expressed from a different viral backbone than ours[33] (Figure 11.1A,B). We found that the Honjo et al. constructs generated slightly higher (perhaps 2-fold) expression of the AID protein than ours (Figure 11.1B and data not shown), but that both wildtype constructs, despite the difference in AID protein expression, supported the same level of CSR (e.g. about 50%; Figure 11.1B). Thus, expression of wild-type AID by both of these vectors exceeds levels necessary to saturate CSR, consistent with findings that this retroviral approach generates a great excess of AID compared to endogenous levels.[34] However, in our hands, while the Honjo et al S38a mutant construct generated about 2 fold more CSR activity that ours (17% versus 8%; Figure 11.1), both S38a mutant constructs clearly had substantially reduced capacity to support CSR as compared to either wildtype construct. A plot of this approximate data reveals how it may be possible to over-estimate relative activity of hypomorphic mutant versus wildtype AID in such an assay–by assaying expression over a range of protein levels in which wildtype activity has reached plateau but mutant activity is still on a linear rise Figure 11.1C).

In the retroviral approach, AID levels are much higher than the normal physiological levels[32,34]. Thus, even our studies may overestimate the relative

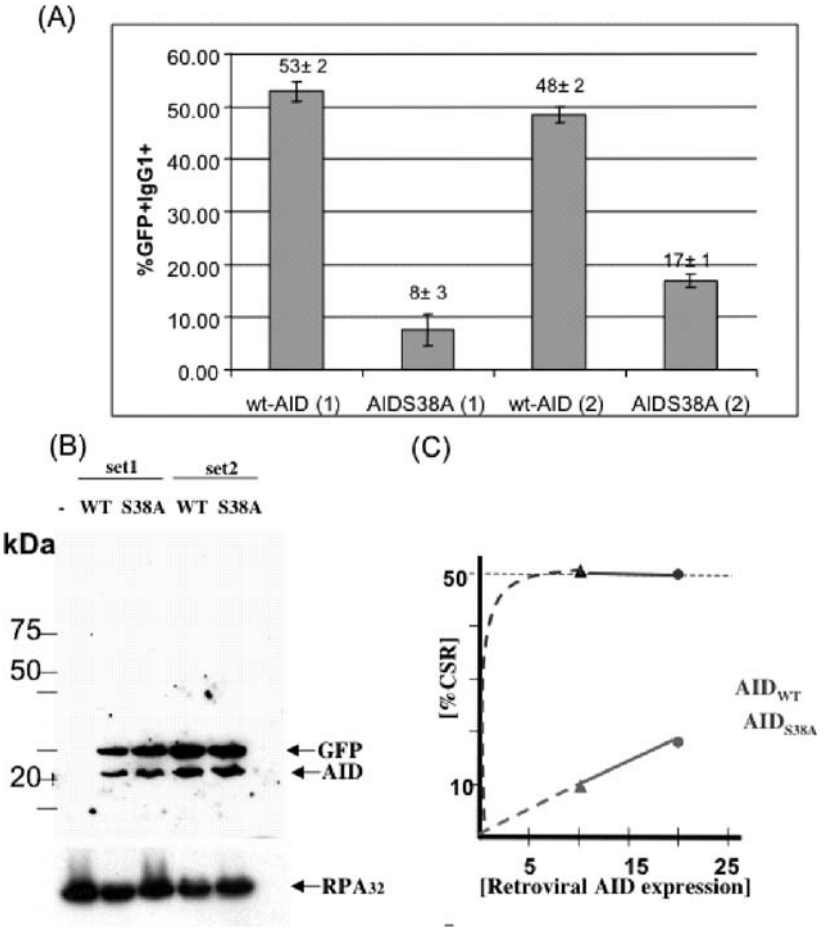

FIGURE 11.1. AID phosphorylation mutant (S38A) functions as a hypomorph when expressed in ex-vivo complementation assays. (A) AID-deficient B cells were stimulated with anti-CD40 and IL4 and infected with a retroviruses expressing wild-type AID (wt-AID) or an S38A phosphorylation site mutant (AIDS38a). The percentage of GFP -positive cells that had undergone CSR to IgG1 from several independent experiments is plotted as a bar graph. The retroviral vector pairs wt-AID (1) and AIDS38A(1) contain wild-type and S38A mutant AID ORFs expressed from matched vectors. Similarly, wt-AID(2)and AIDS38A (2) are also matched in their vector backbones and were kindly provided by T. Honjo.[33] (B) Equivalent amounts of each vector were transfected into 293T cells and 50μg of protein extract from these cells analyzed by western blotting using anti-AID, anti-GFP (an infection control) and anti-RPA (loading control) antibodies. (C) A graph representing the relationship between the percentage of CSR in WT and S38a mutant proteins when expressed at increasing levels from different vectors is diagrammed based the data in panels B and C. The Y-axis represents the percentage of GFP+ cells that express IgG1 in the assay represented in Panel A, and the X-axis represents the relative amount of AID protein expressed in arbitrary units, where the level of AIDS38a

activity of the S38a mutant AID in CSR as compared to that of wildtype (Figure 11.1B). In this regard, the relative contribution of phosphorylation-independent versus phosphorylation-dependent mechanisms to CSR might be most accurately evaluated via a knock-in approach that would allow mutant expression to occur at normal physiological levels. In any case, our findings with the retroviral approach suggest that the phosphorylation-dependent mechanism may be more dominant[30]. A knock-in approach should also allow a more accurate assessment of the contribution of AID phosphorylation to SHM. In that regard, a retroviral rescue approach recently was used to demonstrate that phosphorylation of AID also facilitates AID mediated mutations on artificial somatic hypermutation substrates expressed in non-lymphoid cells[32], as predicted from our studies that showed the dependence of RPA association on phosphorylation[7,30].

11.4.1. Other Implications of AID Phosphorylation

Multiple reports indicate that the majority of AID present in B cells is sequestered in the cytoplasm of B cells[27,30,35]. Considering that AID directly deaminates DNA at the Ig locus, the cytoplasmic sequestration of AID may be a mechanism to control its nuclear accumulation. In this regard, AID shuttling between the nucleus and the cytoplasm of B cells may be required for optimal regulation of CSR and SHM. AID possesses a defined nuclear localization signal (NLS) at its N–terminus, in addition to a nuclear export signal (NES) at its C-terminus[36]. Mutation of either of these motifs leads to the abrogation of nucleo-cytoplasmic shuttling of AID[36–38]. Recent work also indicated that increased cellular AID levels in mouse B cells led to decreased CSR[39]. How the cellular level of the AID protein would alter AID activity in CSR is unclear. Perhaps, higher cytoplasmic AID levels may inhibit AID nuclear entry. Notably, phosphorylated AID appears to be preferentially present in the nucleus of B cells as opposed to the cytoplasm[30]. Moreover, phosphorylated AID deaminates transcribed dsDNA in vitro[7,30] and binds chromatinized DNA[32], raising the possibility that phosphorylation of AID may directly influence its nuclear levels[30]. Recent studies with a phospho-AID specific antibody have shown that phosphorylated AID is enriched in the chromatin-bound AID fraction[32]. As discussed earlier, phosphorylated AID interacts with RPA and stimulates transcription dependent dsDNA deamination[7]. Thus phosphorylation dependent interaction of AID with RPA may promote chromatin association of AID in B cells.

FIGURE 11.1. *(Continued)* (1) is set at 10. The symbol (σ) approximates values obtained with wt-AID (1) and AIDS38A (1). The symbol (λ) represents the values from wt-AID (2) and AIDS38a (2), where AID expression was estimated to be 2-fold higher than that of our vectors (see Panel B). As illustrated, comparison of the relative activity of mutant and WT AID at the higher concentrations than physiological levels would give the misleading impression that their activities are more similar than they actually are.

11.5. Material and Methods

11.5.1. Antibodies.

The sources of the antibodies used are as follows: anti-RPA_{32} are from Santa Cruz Biotechnology; anti-GFP from Clontech and anti-AID is as described earlier[6].

11.5.2. Recombinant Retroviral Production and Infection of B cells.

The plasmids expressing wild-type and S38A mutant AID was transfected into virus packaging cell line (phoenix cells, Orbigen) using the Trans-It reagent (Mirus). The virus was mixed with pre-activated B cells (activated by anti-CD40 antibodies, IL4) for 3 days at a density of 10^6 cells per ml supplemented with 16μg per ml of polybrene. These cells were centrifuged at 2500 rpm for 30 min at 30° C and incubated for 48−72 hrs. These cells were assayed for surface IgG1 production using flow cytometry. B cells were isolated from wild-type or $AID^{-/-}$ mice spleens through negative purifications of B cells in MACS columns with antiCD43 antibodies coated magnetic beads (Miltenyi Biotec). The isolated B cells were incubated in RPMI medium containing 50 uM β Mercaptoethanol with 15% fetal calf serum (FCS) stimulated with anti-CD40 antibodies and IL4 for 24 hrs before infection.

11.5.3. Flow Cytometric Analysis

The antibodies PE-Cy5 conjugated anti-mouse CD45R (B220) (eBioscience) and PE anti-mouse IgG1 (A85-1) (BD Pharmingen) were used for staining of B cells used for flow cytometric analysis. The analysis was performed with BD FACScaibur.

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12
Modulation of MHC Class II Signal Transduction by CD19

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12.1. Introduction

Initial B cell encounter with cognate thymus-dependent (TD) antigen initiates a complex array of events facilitating collaboration with $CD4^+$ T cells. Signals transduced following B cell antigen receptor (BCR) ligation prepare B cells to induce T cell help by both increasing the expression of costimulatory molecules (including CD80 and CD86) and enabling the acquisition, processing, and presentation of antigenic peptides in the context of MHC class II molecules[1]. In addition, antigen encounter also induces B cells to migrate to the edge of B and T cell zones of secondary lymphoid compartments, where initial T-B collaboration is thought to occur[2,3]. Thus, antigen receptor signals prepare naïve B cells to induce and receive $CD4^+$ T cell help, and facilitate co-localization of rare antigen-specific lymphocytes.

The activation signals transduced following BCR ligation are primarily a consequence of protein tyrosine kinase recruitment/activation via Ig-α/β heterodimers. However, BCR-associated accessory molecules (such as CD19, CD21, CD22, and CD72) influence qualitative and quantitative aspects of Ig-α/β signals[4]. The BCR accessory molecule CD19 complements Ig-α/β signaling by enhancing the recruitment/activation of several key effectors, including the Src-family kinase Lyn, class I PI3K molecules, and the Rho-family GEF molecule Vav[5–7]. In *ex vivo* experiments, CD19-deficient B cells exhibit diminished BCR-mediated whole cell tyrosine phosphorylation and proliferation responses[7,11]. In addition, two reports indicate that CD19 transduces signals following LPS encounter and CD40 ligation, indicating that CD19 may regulate responses downstream of receptors other than the BCR[8,9]. However, in the

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absence of CD19, responses to thymus independent type 2 (TI-2) immunogens are unimpaired at moderate to high antigen doses, but TD responses are nearly ablated[10,11]. These findings are somewhat surprising since CD19 is primarily known to participate in BCR signaling, which is more important during TI-2 than TD responses.

Although lymphocyte conjugation involves interaction of multiple receptor-ligand pairs, the antigen-specificity and biologic outcome of T-B collaboration is determined by relatively low affinity/abundance interactions between TCR and MHC class II-peptide complexes. In addition to their role as antigen presentation platforms, it is clear that MHC class II molecules transduce signals leading to B cell activation. In naïve B cells aggregation of MHC class II mediates the production of cAMP, activation of PKC, and apoptosis[12]. In antigen or IL-4 primed B cells, however, MHC class II ligation induces activation of protein tyrosine kinases, mobilization of intracellular calcium, f-actin-dependent morphologic changes, and proliferation[13]. These responses in "primed" B cells occur because of the induced association of signal-transducing Ig-α/β heterodimers with cell-surface MHC class II complexes[14]. Because Ig-α/β heterodimers are also primary signal-transducers following BCR ligation, these findings suggested the possibility that, in primed B cells, MHC class II/Ig-α/β signals are also modulated by the BCR accessory molecule CD19. Although we and others have found that CD19 is phosphorylated following MHC class II aggregation[15,16], the importance of this accessory molecule for MHC class II/Ig-α/β signals in primary B cells has not been examined.

In this report we investigated whether the BCR accessory molecule CD19 participates in MHC class II signaling in primary B cells. We find that, unlike Ig-α/β, CD19 co-localizes with cell-surface MHC class II complexes in both naïve and IL-4-primed primary B cells. Following MHC class II ligation, however, CD19 molecules are only phosphorylated in primed B cells. Experiments using CD19-deficient B cells reveal that optimal MHC class II-mediated induction of whole cell protein tyrosine phosphorylation, sustained Akt activation and proliferation, all depend on CD19. The observation that CD19 is required for normal MHC class II-mediated responses, coupled with the TI-2 and TD immune response phenotypes of CD19-deficient mice, suggests that MHC class II-mediated signals may be critical for TD responses, and indicates that altered MHC class II/Ig-α/β signaling at least partially accounts for impaired TD responses in the absence of CD19.

12.2. Results

12.2.1. CD19 is Associated with MHC Class II Molecules in Resting and IL-4-Primed B Cells

As noted above, in IL-4 primed B cells MHC class II molecules transduce signals via Ig-α/β dimers. We explored the possibility that the BCR-associated

accessory molecule CD19 is also associated with MHC class II complexes in primed B cells by analyzing the localization of CD19 molecules in naïve and IL-4-primed primary B cells following aggregation of cell-surface MHC class II. Surprisingly, a portion of the cell-surface CD19 was enriched in MHC class II aggregates in both naïve and primed B cells following MHC class II ligation (Figure 12.1A). As expected from previously reported findings[14], Ig-α/β dimers were recruited to MHC class II aggregates in primed but not naïve B cells (Figure 12.1B), and MHC class II aggregation did not alter IgM localization (Figure 12.1A).

Because MHC class II molecules are ligated *in vivo* by TCR/CD4 molecules during cognate T-B collaboration, we investigated whether CD19 and MHC class II spatially co-localize in T-B synapses. To address this issue, we used

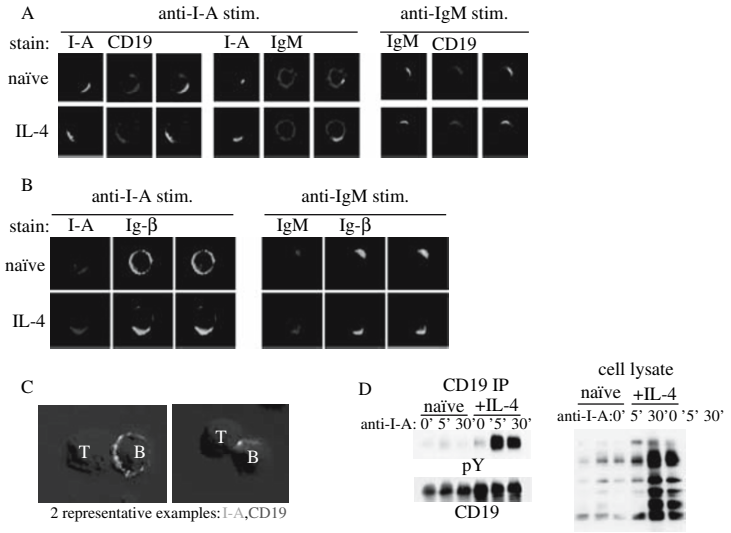

FIGURE 12.1. **CD19 associates with MHC class II in resting and primed B cells, but only becomes tyrosyl phosphorylated following MHC class II ligation in primed cells.** (A and B) Primary B cells were purified from WT mice and cultured for 18 hours in medium alone or IL-4. Samples were stimulated with the indicated ligands for 15 minutes at 37° C, fixed with paraformaldehyde, permeablized with Triton X-100, and stained with the indicated antibodies. Results are representative of three independent experiments. (C) K46 lymphoma B cells were pulsed for 18 hours with 50 □g/ml ovalbumin at 37° C. Cells were incubated with ova-restricted DO.11.10 T hybridoma cells (at a 1:1 ratio) for 15 minutes at 37° C and fixed/permeablized/stained as described above and in the Materials and Methods. Two example conjugates are shown representing two independent experiments. (D) Primary WT B cells were purified, primed, and stimulated for the indicated times as in A. CD19 molecules were immunoprecipitated from cell lysates, and samples were fractionated via SDS-PAGE and immunoblotted with the indicated primary antibodies (phosphotyrosine immunoblots of whole cell extracts are shown as a control for B cell priming).

immunofluorescence to analyze the location of CD19 and MHC class II following conjugation of ovalbumin-pulsed K46 B lymphoma cells (which constitutively exhibit a primed phenotype) and DO.11.10 T hybridoma cells. Consistent with data presented in Figure 12.1A, we found that CD19 and MHC class II were co-recruited to foci at the center of T-B contact regions (Figure 12.1C), which resembled supra-molecular activation clusters observed by others[17]. Thus, CD19 and MHC class II are spatially co-localized in resting and primed B cells, and are co-recruited to T-B synapses.

12.2.2. MHC Class II Aggregation Induces Tyrosyl Phosphorylation of CD19 Molecules in Primed, but not Naïve B Cells

Because CD19 is co-localizes with cell-surface MHC class II complexes, we wondered if ligation of MHC class II in naïve or IL-4-primed splenic B cells induces signal transduction via associated CD19 molecules. To explore this question, we analyzed tyrosyl phosphorylation of CD19 molecules in naïve and IL-4-primed B cells following MHC class II aggregation. Interestingly, although CD19 molecules co-localized with MHC class II in naïve B cells, they were not phosphorylated following aggregation of MHC class II (Figure 12.1D). In primed B cells, however, MHC class II aggregation induced robust and sustained CD19 phosphorylation. Taken together, these data demonstrate that CD19 molecules are associated with MHC class II aggregates in resting and in IL-4-primed B cells, but only transduce signals following MHC class II aggregation in primed cells.

12.2.3. CD19 is Required for Optimal Induction of Tyrosyl Following MHC Class II Aggregation

CD19-deficient B cells exhibit deficits in inducible tyrosyl phosphorylation and proliferative responses following IgM ligation[7,11]. To examine the importance of CD19 in MHC class II signaling we compared the pattern of whole cell protein tyrosyl phosphorylation following MHC class II aggregation in IL-4-primed wild type and CD19-deficient primary B cells. Interestingly, I-A-mediated induction of tyrosyl phosphorylation was globally reduced in the absence of CD19 (Figure 12.2A). As reported by Tedder and colleagues, CD19-deficient B cells also displayed impaired phosphorylation responses following aggregation of IgM. Thus, CD19 is the first molecule known to be required for normal MHC class II/Ig-α/β-mediated tyrosine kinase activation.

12.2.4. CD19 is Required for Sustained MHC Class II-Mediated Akt Activation, but not Calcium Mobilization

Current data indicate that a principle biochemical role of CD19 is recruitment of PI3K to signaling complexes[6]. The downstream products of PI3K, $PI^{3,4,5}P_3$ and $PI^{3,4}P_2$, are important for activation of plextrin homology domain-containing

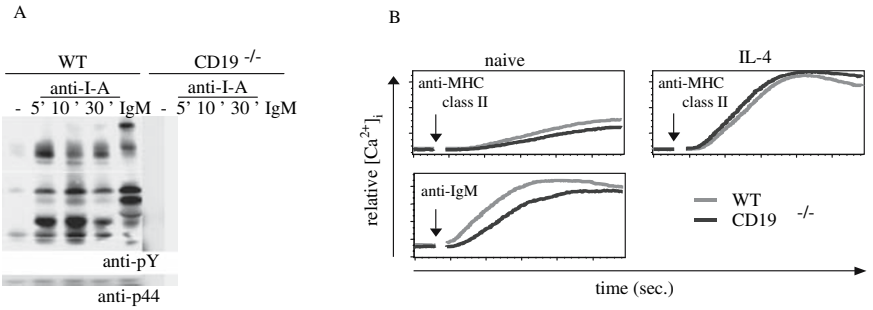

FIGURE 12.2. **CD19 is required for optimal MHC class II-mediated tyrosyl phosphorylation, but dispensible for calcium mobilization.** (A) Primary B cells were purified from WT and CD19$^{-/-}$ mice. Samples were stimulated for the indicated times with either anti-I-A or anti-IgM, lysed with 1% CHAPS buffer, fractionated via SDS-PAGE, and immunoblotted with the indicated antibodies (either anti-phosphotyrosine, anti-p44). (B) B cells were purified from WT and CD19$^{-/-}$ mice as described in the Materials and Methods. Samples were loaded with Indo 1-AM and calcium mobilization was assessed via flow cytometry following administration of the indicated antibodies. Results are representative of three independent experiments.

receptor-proximal signaling effectors such as Akt, Btk, and PLCγ2[4]. Accordingly, CD19 is critical for optimal activation of Akt and Btk following BCR aggregation[18,19]. Since Btk and PLCγ2 are critical mediators of calcium mobilization following IgM ligation, we and others hypothesized that CD19-deficient B cells would display impaired IgM-mediated calcium responses. However, for reasons that are unclear, calcium mobilization induce by aggregation of total surface Ig (but not IgM alone) is impaired in CD19-deficient B cells.

To address whether CD19 participation mobilization of calcium following MHC class II aggregation, we compared I-A-mediated calcium mobilization in wild type and CD19-deficient B cells. We found that, like anti-IgM-induced responses, MHC class II-mediated calcium mobilization was normal in IL-4-primed CD19-deficient B cells (Figure 12.2B). Thus, although CD19 is robustly phosphorylated following MHC class II aggregation, receptor-proximal signals leading to calcium mobilization occur normally in the absence of CD19. This indicates that IL-4 primes WT and CD19-deficient B cells equivalently for some responses (calcium mobilization), but not others (Akt activation and proliferation).

12.2.5. Global MHC Class II-Mediated Changes in Gene Expression do not Depend on CD19

Given the aforementioned requirements for CD19 in MHC class II-mediated Akt activation and proliferation, we predicted that global changes in gene expression following MHC class II aggregation might also be regulated by CD19. Thus,

we used Affymetrix microarrays to analyze global changes in gene expression profiles in primed WT and $CD19^{-/-}$ B cells cultured in medium alone or on anti-I-A-coated plates. We found no global differences in I-A-induced/reduced gene expression programs between WT and $CD19^{-/-}$ B cells (data not shown). Thus, the effects of CD19 on MHC class II-mediated proliferation (and possibly B cell response *in vivo*) are likely due to altered protein function and not globally altered gene expression profiles.

12.3. Discussion

These studies were undertaken to determine whether CD19 associates with MHC class II signaling complexes in naïve and/or primed primary B cells, and to explore whether optimal MHC class II-mediated signals depend on CD19. We find that CD19 is recruited to MHC class II aggregates in both primed and naïve B cells, but only becomes phosphorylated upon MHC class II ligation in primed cells. This is consistent with the current paradigm that CD19 does not associate with Src family kinases in the resting state, and becomes tyrosyl phosphorylated only upon colocalization with kinase-associated signaling constituents such as Ig-α/β.

Our findings that CD19 is required for optimal MHC class II-mediated tyrosyl phosphorylation, Akt activation and proliferative responses are entirely symmetrical with the known requirements for CD19 during BCR-induced B cell activation. In addition, optimal LPS and anti-CD40-mediated proliferation of primary B cell populations depends on CD19. However, these studies did not eliminate the possibility that enhanced proliferation of WT cells may reflect selective responses of marginal zone B cells, which display heightened proliferation upon LPS/anti-CD40 treatment and are absent in $CD19^{-/-}$ mice. We find that marginal zone and follicular B cells proliferate comparably following MHC class II ligation, and that depletion of marginal zone cells from WT samples does not affect proliferation deficits observed in the absence of CD19 (data not shown). Thus, CD19 appears to play similar roles during MHC class II, BCR, and possibly RP105 and CD40 signaling. Future studies will explore whether CD19 plays any unique roles in MHC class II signaling.

The vast majority of literature describing the role of CD19 in B cell responses focuses on modulation of BCR signal transduction. The fact that CD19 controls signaling downstream of MHC class II, however, raises the possibility that immune response deficits observed in $CD19^{-/-}$ mice reflect combined diminished responses to multiple ligands *in vivo*, including antigen and TCR. Additionally, the importance of CD19 is highlighted by the observation that CD19 overexpression induces autoimmunity in mice[20]. Recent studies demonstrate that autoimmunity in CD19 transgenic mice depends on MHC class II expression, supporting the hypothesis that augmentation of MHC class II signals by CD19 is critical *in vivo*[21]. However, unequivocal evaluation of the importance of CD19-MHC class II association *in vivo* will require mutational analyses to define molecular determinants critical for coupling MHC class II and CD19.

12.4. Materials and Methods

12.4.1. Cell Preparation and Culture

B cells were isolated using magnetic bead-based negative selection. Single cell suspensions were prepared from excised spleens and depleted of erythrocytes using Gey's Solution. Leukocytes were suspended at 10^8cells/ml in PBS/2% fetal bovine serum (FBS), and incubated for 15 minutes at 4° C with 60□l/10^8cells anti-CD43 microbeads (Miltenyi Biotech). Cells were washed once, resuspended in 15ml PBS/2% FBS, and sorted using MACS LS columns (Miltenyi Biotech) according to manufacturer protocol. Cells were cultured in IMDM with 10% FBS containing 100 U/ml penicillin, 100 □g/ml streptomycin, 50 □g/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 □M 2-ME, 10% FCS (HyClone Laboratories, Logan, UT). Priming of B cells for MHC class II signal transduction was accomplished by culturing for 12–18 hours in 50 U/ml recombinant murine IL-4. All samples were incubated for 15 minutes with anti-Fc□ antibodies prior to stimulation to block IgG binding to these receptors. For plate-bound stimulations, tissue culture plates were coated with anti-MHC class II, antibodies (diluted in PBS) for 18 hours at 37° C. Wells were washed 3 times with PBS to remove non-bound antibody molecules.

12.4.2. Antibodies and Reagents

The following monoclonal antibodies were purified from hybridoma supernatant and used for stimulation, immunoprecipitation, and staining experiments: anti-I-$A^{d/b}$ (D3.137), low-affinity anti-IgM (Bet-2), high affinity anti-IgM (b-7-6), anti-FcγRII/III (2.4G2), and anti-H-$2K^{d/b/k}$ (M1.24.3.9.8). Purified polyclonal rabbit anti-SHP-1 used for immunoprecipitation and immunoblotting was from Upstate Biotechnology, Lake Placid, NY. Anti-CD19 cytoplasmic tail antisera used for immunoblotting were raised in Australian White rabbits. Anti-MHC class II (I-Aα) for immunoblotting was kindly provided by Dr. Ira Mellman (Yale University). Additional reagents used for immunoblotting and staining were: donkey anti-mouse IgG1-HRP (Zymed, San Francisco, CA), anti-mouse IgM-HRP (Zymed), protein A-HRP (Zymed), Cy3-streptavidin (Caltag, Burlingame, CA), Cy3-goat anti-mouse IgG1 (Caltag), Cy5-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and Cy5-donkey anti-rat IgG (Jackson). Recombinant IL-4 was purified from J558L-IL-4 plasmacytoma supernatant. Protein A-Sepharose used for immunoprecipitation was from Amersham Pharmacia Biotech (Upsala, Sweden).

12.4.3. Analysis of Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$) Mobilization

Cells were resuspended at 5×10^6/ml in IMDM (2.5% FCS) and incubated for 45 minutes at 37° C in 5 □M Indo 1-AM (Molecular Probes, Eugene, Oregon). For

aggregation of MHC class II, cells were incubated with biotinylated anti-MHC class II (1□g/ml) for 15 minutes at room temperature. Cells were washed with IMDM and resuspended at 1.5×10^6/ml for analysis on an LSR flow cytometer (Beckton Dickinson, Mountain View, CA). Signaling was triggered by addition of avidin and anti-IgM as indicated in figure legends. Data analysis was performed using FlowJo software (Tree Star, Inc., San Carlos, CA).

12.4.4. Immunoprecipitation and Immunoblotting

Naïve or IL-4 primed splenic B cells were resuspended at 20×10^6/ml in IMDM (10% FCS). Cells were incubated with biotinylated anti $-$ I $-$ $A^{b/d}$ at 20 □g/ml for 20 minutes at room temperature. Samples were washed once and resuspended at 20×10^6 cells/ml in IMDM. Stimulations were performed for 5 minutes at 37° C using 5 □g/ml anti-IgM or 20 □g/ml avidin. Samples were lysed for 10 min on ice in 1 ml 0.5% CHAPS lysis buffer (10 mM NaF, 0.4 mM EDTA, 2 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate,1 mM PMSF, 1 mM aprotinin, 1 mM □-1-antitrypsin, and 1 mM leupeptin) followed by centrifugation at 14,000 rpm for 10 minutes. CD19 was immunoprecipitated using 10 □l of anti-CD19 rabbit serum prebound to 20 □l protein A-Sepharose beads. Immunoprecipitations were performed for 1 hour at 4° C with constant mixing. Beads were pelleted by brief centrifugation and washed 3 times with 1 ml lysis buffer. Samples were boiled in reducing sample buffer and fractionated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes and visualized using specific antibodies followed by enhanced chemiluminescence (NEN, Boston, MA).

12.4.5. Fluorescence Microscopy

Primed B cells were incubated with biotinylated anti-MHC class II for 20 minutes at room temperature and washed once with IMDM. Samples were stimulated with either polyclonal rabbit CY5-anti-IgM, FITC-streptavidin, or Cy3-streptividin 30 minutes at 37° C. Cells were placed on poly-L-lysine coated cover slips for 2 minutes at 37° C and fixed with 4% paraformaldehyde, followed by permeabilization for 5 minutes with 0.2% Triton X-100. Cover slips were blocked with PBS (2.5% fetal bovine serum) for 5 minutes and probed with Cy5-anti-IgM, biotinylated anti-MHC class II, anti-CD19 rabbit serum, or anti-Ig-β (HM79) for 1 hour at room temperature. Samples were washed several times in PBS and probed with appropriate secondary reagents for 1 hour at room temperature. After washing several times, cover slips were mounted on microscope slides with mounting solution (2mg/ml OPDA in 90% glycerol). Images were captured and analyzed using a Leica DMRXA microscope, a Sensicam^{QE} camera (CooKe, Auburn Hills, MI), and Slidebook imaging software (Intelligent Imaging Innovations, Denver, CO)

12.4.6. Proliferation

Splenic B cells from wild type and CD19$^{-/-}$ mice were incubated at 5×10^6/ml in HBSS with 2□M CFDA-SE (Molecular Probes, Eugene, OR) for 5 minutes at 22° C. Cells were then washed with HBSS and cultured 18 hours at 2×10^6/ml with low affinity anti-IgM (bet-2, 2.5 □g/ml) and IL-4 (50 U/ml) in IMDM (10% fetal calf serum, 2% non-essential amino acids, 50□m □-ME). Samples were then transferred to non-coated or anti-I-A-coated 24-well plates and cultured for 72 hours. Proliferation was analyzed using a FACScan cytometer (Beckton Dickinson, Mountain View, CA) and FlowJo analysis software (Tree Star, Inc., San Carlos, CA). Data are presented as % of maximum population

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13
Role of NFκB Signaling in Normal and Malignant B Cell Development

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13.1. Introduction

Early B cell development is governed by a program of gene rearrangements in the immunoglobulin (Ig) loci, through which the cells ultimately acquire the ability to express an antibody as part of the B cell antigen receptor (BCR) complex. Gene rearrangements are also critical in the counter-selection of autoreactive specificities in the antibody repertoire expressed by the newly generated cells, through a process called receptor editing. Expression of a BCR is required for the generation of B cells and remains a survival determinant for mature, peripheral B cells, monitoring their functional integrity as antigen-responsive cells. When the cells enter the pool of long-lived peripheral B cells, they come under the control of a second survival factor, namely BAFF, a cytokine of the tumor necrosis factor (TNF) family. BAFF is produced by non-B cells in the environments where peripheral B cells accumulate, delivers a survival signal to the cells through interaction with BAFF-receptor (BAFF-R), and is considered to be critical for the control of B cell numbers in the peripheral immune system, with the exception of the compartment of B-1 cells[5].

A main signaling cascade activated by both BCR and BAFF-R engagement is the NFκB signaling pathway. While the BCR appears to activate mainly so-called canonical NFκB signaling through activation of a complex of two kinases, IKK1 and IKK2, and the scaffold protein NEMO. BAFF-R appears to signal mainly, although perhaps not exclusively, through the so-called alternative pathway, whose activation depends on IKK1 and a second kinase, NIK. The two pathways have very different kinetics of activation, use distinct members of the NFκB transcription factor family, and control overlapping,

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but partially distinct sets of target genes. NFκB signaling has been shown to be involved in the control of a wide variety of cellular functions, the most prominent of which are inflammatory reactions, cell survival and cellular proliferation[2].

In the present study we have addressed to which extent canonical NFκB signaling is required for the generation of mature B cells and whether its enforced activation liberates B cells from BAFF control. We have also analyzed in this context whether constitutive canonical NFκB signaling promotes B cell proliferation. The latter two questions relate to the role of NFκB signaling in B cell lymphomagenesis, given that several classes of B cell lymphomas display constitutive NFκB signaling and depend on it for survival[3]. A full account of these data, which are summarized below, can be found in[10].

13.2. Canonical NFκB Signaling is Dispensable for Early B Cell Development

Using B cell-specific gene targeting, we had previously shown that canonical NFκB signaling is essential for the survival of mature B cells,[8] quite like BCR expression and BAFF:BAFF-R interaction. We have now extended this analysis by ablating the canonical NFκB pathway in the B cell lineage from the pro-B cell stage onward. This was done by combining a conditional NEMO allele, which we had generated earlier,[11] with a cre transgene controlled by the mb-1 promoter, generated in the laboratory of M. Reth (Hobeika et al., unpublished). We found that in this situation the NEMO gene was completely and selectively deleted in the B cell lineage as analyzed by Southern blotting, and neither NEMO message nor protein was detectable from the pro-B cell stage on. Not withstanding, B cell development was undisturbed in the mutant mice in quantitative terms up to the transitional 1 (T1) stage in the spleen. However, there was a strong developmental block at the T1 to T2 transition, and mature follicular B cell numbers were about twenty-fold reduced. In addition, marginal zone (MZ) and B-1 cells in spleen and peritoneal cavity, respectively, were undetectable. The development of mature peripheral follicular, but neither MZ B nor B1 cells could be rescued by a bcl2 transgene, indicating that NFκB signaling keeps mature B cells alive through up-regulation of pro-survival genes of the bcl2 family.

Given that NEMO deficiency is known to completely abolish canonical NFκB signaling, we conclude from these results that in terms of cell numbers, the development of follicular B cells is independent of this signaling pathway up to the T1 stage, but relies on it subsequently, in accord with our previous work. It remains a possibility that other modes of NFκB transcription factor activation operate in early B cell development. Experiments are underway to address this possibility, as well as the question whether receptor editing is normal in the mutant animals.

13.3. Constitutive Canonical NFκB Signaling Rescues BAFF-R Deficiency and Promotes B Lymphomagenesis

The striking co-incidence of BAFF dependency and dependence on canonical NFκB signaling in B cells beyond the T1 developmental stage raises the question whether BAFF or BAFF-R deficiency can be rescued by NFκB activation. This is of particular interest in connection with the problem of lymphomagenesis in mature B cells, because several classes of mature B cell lymphomas including Hodgkin's disease exhibit constitutive NFκB activity, and there is evidence that at least in some cases the survival of the tumor cells in vitro depends on this feature[1,4]. Thus, constitutive NFκB signaling might make B lymphoma cells independent of BAFF control for their survival, and perhaps also contribute to their proliferative expansion.

To assess this problem, we generated a conditional gain-of-function system, allowing the activation of a given signaling pathway in the cells of interest in vivo. For this purpose, a cDNA encoding the constitutively active signaling molecule of interest (IKK2 in the present case), preceded by a loxP-flanked transcriptional STOP cassette and followed by an internal ribosomal entry site (IRES)-equipped indicator gene encoding green fluorescent protein (GFP), is knocked into an ubiquitously expressed genomic locus (ROSA26 in the present case) in mouse embryonic stem cells (Figure 13.1). Using cell type-specific or inducible transgenic Cre expression, the signaling molecule in question can then be conditionally turned on, together with the GFP indicator.

Using this approach, we conditionally induced constitutively active IKK2 (IKK2ca;[7]) in mature B cells with the help of the CD19-cre allele, which we had generated earlier[9]. In the compound mutant animals, essentially all mature B cells were GFP positive, as tested by flow-cytometric analysis. A detailed biochemical analysis demonstrated that IKK2ca was selectively expressed in these cells and indeed activated the canonical, but not the alternative NFκB signaling pathway. At the cellular level, this led to a mild B cell hyperplasia, due to prolonged survival, but not proliferation, of the mutant cells. When this genetic system was crossed onto BAFF-R deficiency,[10,12] a complete rescue of follicular and MZ B cells was observed, as shown at the level of flow cytometric analysis

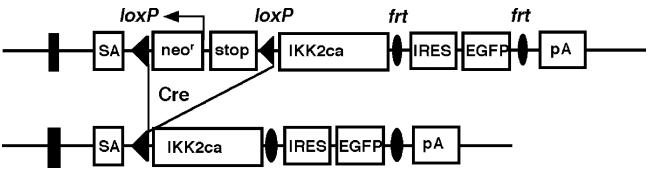

FIGURE 13.1. Schematic representation of a conditional allele encoding constitutively active IKK2 (IKK2ca) knocked into the ROSA26 locus. The ROSA26 promoter drives expression of IKK2ca and eGFP upon Cre-mediated deletion of a loxP-flanked STOP cassette (neo^r-stop). Vertical filled bar represents exon 1 of ROSA26, loxP and frt sites are indicated. IRES: internal ribosomal entry site; SA: splice acceptor; pA: polyA sequence.

in Figure 13.2. A complete rescue was also observed in terms of histological organization of B cell follicles and MZ in the spleen[10]. Overall, it became clear that canonical NFκB signaling can substitute for all known functions of BAFF:BAFF-R interaction.

As the survival function of BAFF-R had been linked to inhibition of pro-apoptotic nuclear translocation of Protein Kinase Cδ (PKCδ;[6]), we assessed the latter in the compound mutant B cells and found that IKK2ca expression, like BAFF-R signaling, kept PKCδ in the cytoplasm. This suggests that BAFF-R controls the cytoplasmic retention of PKCδ through NFκB.

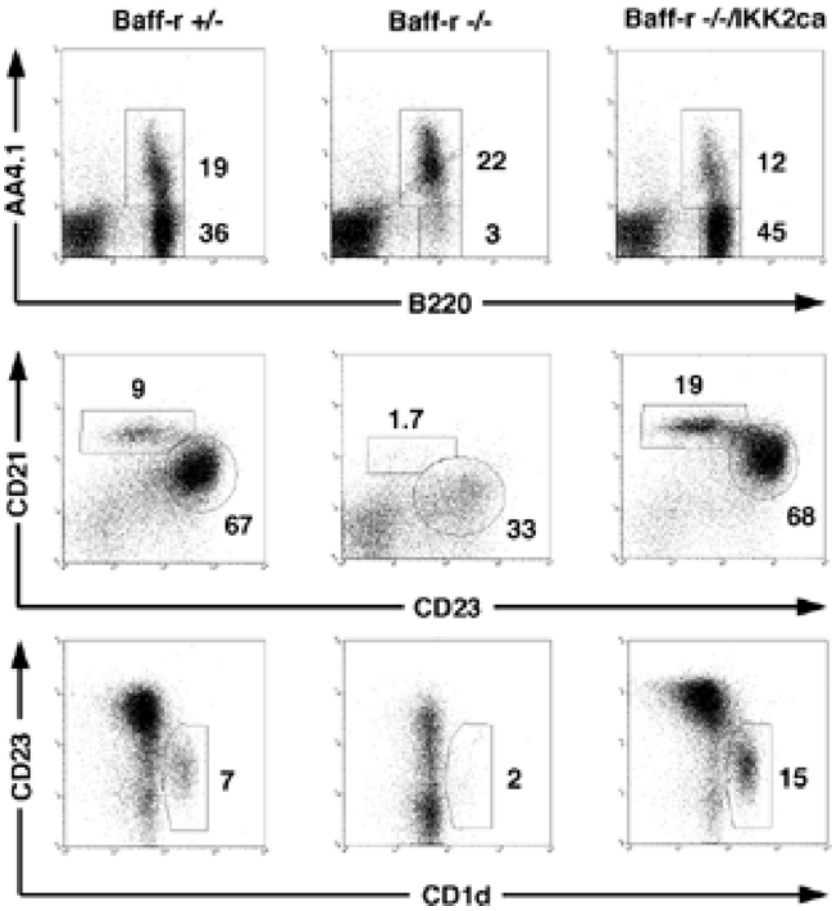

FIGURE 13.2. Rescue of follicular and MZ B cells in BAFF-R knockout mice by constitutively active IKK2 (IKKca). This is shown for mature follicular B cells ($B220^{+}AA4.1^{-}$ or $CD23^{high}CD21^{intermediate}$) in the upper and middle panel, and for MZ B cells ($CD23^{intermediate}CD21^{high}$ or $CD23^{intermediate}CD1d^{+}$) in the middle and lower panel. IKK2ca expression was induced by the CD19-cre transgene, which was carried by all three sets of mice.

As mentioned above, IKK2ca expression in B cells enhanced the persistence of the cells in vivo, but did not by itself induce cell proliferation. This was also true when the cells carried two copies of the IKK2ca transgene, which at the biochemical level led to enhanced canonical NFκB signaling with concomitant strong up-regulation of the ERK pathway. Prolonged cellular survival, but absence of proliferation was also observed when the cells were kept in culture. Strikingly, however, a dramatic enhancement of cell proliferation was seen when heterozygous and, in particular, homozygous IKK2ca mutant cells were stimulated by mitogenic stimuli through the BCR or TLRs like TLR4 or 9.

The latter results suggested that canonical NFκB signaling not only makes B cells independent of BAFF control, but might also promote B cell lymphomagenesis by enhancing proliferation in combination with some additional transforming event. In support of this notion, we have shown in unpublished experiments that transfer of homozygous IKK2ca transgenic B cells into syngeneic wild-type recipients regularly results in the outgrowth of oligo- or monoclonal B cell lymphomas from the cell inoculum. We have thus generated a mouse model of mature, NFκB-dependent B cell lymphomagenesis and are in the process of analyzing the non-NFκB-related transforming events involved in the generation of these tumors.

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14
Fc Receptor–like Proteins (FCRL): Immunomodulators of B Cell Function

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14.1. Introduction

The Fc receptor-like (FCRL) proteins belong to a recently identified group of type 1 immunoglobulin (Ig) superfamily orphan receptors. Five of the six FCRL family members are preferentially expressed by B cells. A variety of approaches led to the identification of these FCRL family members. Hatzivasilliou and co-workers identified FCRL4 and FCRL5 by sequencing a translocation breakpoint in multiple myeloma cell lines. They named these genes the immunoglobulin superfamily receptor translocation associated genes (IRTA)1 and 2[14]. Davis and co-workers, who identified six members of the FCRL receptor family on the basis of their sequence similarity to known Fc receptors named them Fc receptor homologs (FcRH)1-6[5]; D. Schreeder, unpublished observation. Members of the FCRL family have also been termed Ig-Fc-gp42 related genes (IFGP)[12], SH2 domain-containing phosphatase anchor proteins (SPAP)[40] and B cell cross-linked by anti-IgM activating sequences (BXMAS)[32]. Because of this confusing array of names, a new unified nomenclature has been proposed[24], which we will use in this review (Table 14.1).

14.2. Structure and Function of FCRL Family Members

The FCRLs share several properties with the well characterized Fc receptors (FcR) for IgG and IgE isotypes. Both *FcR* and *FCRL* family members are located

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Table 14.1. New nomenclature of FCRL-family proteins.

New nomenclature	Old nomenclature	Species
FCRL1	IRTA5, FcRH1, IFGP1, BXMAS1	Human
FCRL2	IRTA4, FcRH2, IFGP4, BXMAS2, SPAP1	Human
FCRL3	IRTA3, FcRH3, IFGP3, BXMAS3, SPAP2	Human
FCRL4	IRTA1, FcRH4, IFGP2	Human
FCRL5	IRTA2, FcRH5, IFGP5, BXMAS	Human
FCRL6	FcRH6, IFGP6	Human
FCRLA	FcRL, FREB, FcRX	Human
FCRLB	FcRL2, FREB2, FcRY	Human
Fcrl1	FcRH1, IFGP1, BXMAS1	Mouse
Fcrl5	FcRH3, mBXMH2, IFGP3	Mouse
Fcrl6	FcRH6, mIFGP6	Mouse
Fcrls	FcRH2, IFGP2	Mouse
Fcrla	FcRL, FREB, FcRX	Mouse
Fcrlb	FcRL2, FREB2, FcRY	Mouse

on chromosome 1q21-23. All of these genes have a characteristic split signal peptide coding region. The extracellular regions of FCRL family members are composed of 3-9 Ig domains with amino acid sequence similarity to the well characterized FcRs (15-31% identity). Despite having Ig domain subtypes that resemble the Fc-binding domains of classical FcR, the FCRL proteins have not been shown to have immunoglobulin binding capacity. The ligands for the FCRLs are presently unknown. In addition to the FCRL1-6 type 1 transmembrane receptors, two intracellular FcR-like proteins have been identified; these are named FCRLA and B.

The intracellular domains of the transmembrane FCRL proteins contain immunoreceptor tyrosine-based activation motifs (ITAM) and/or immunoreceptor tyrosine-based inhibition motifs (ITIM), thereby suggesting they have immunomodulatory function. The ITAM consensus motif, initially identified in the BCR signaling chains Igα and Igβ, has two conserved tyrosine residues within the consensus sequence E/D-x-x-Y-x-x-L/I-x_{6-8}-Y-x-x-L/I (with x indicating any amino acid). Upon phosphorylation of both tyrosine residues, kinases like the Syk tyrosine kinase and adaptor proteins like BLNK have been shown to associate with the ITAM motifs. ITIMs on the other hand have a single conserved tyrosine residue in the consensus motif I/V/L/S-x-Y-x-x-L/V. The ITIMs typically recruit lipid or protein phosphatases after tyrosine phosphorylation[34,36,37]. For example, FcγRIIb, the low affinity receptor for IgG immunoglobulins, mediates its inhibitory activity by recruiting the inositol phosphatase SHIP to attenuate BCR signaling[33]. Functional analyses of the immunoregulatory potential of FCRL family members have been conducted only for FCRL1 and FCRL4 thus far.

The intracellular domain of FCRL1 contains two ITAM-like sequences, thereby suggesting it has an activating role in B cell signaling. In keeping with this prediction, ligation of FCRL1 on naïve tonsillar B cells can induce a proliferative response, and co-ligation of FCRL1 with the B cell receptor enhances

the calcium mobilization and cellular proliferation initiated by BCR ligation[22]. Conversely, biomolecular analysis of the FCRL4 intracellular domain, which contains three consensus ITIMs, indicates a potent inhibitory potential for B cell receptor signaling[6]. Co-ligation of a fusion protein consisting of the extracellular and transmembrane domains of FcγRIIb and the intracellular domain of FCRL4 completely blocks BCR induced calcium mobilization and Erk activation. The two membrane distal ITIM sequences mediate this inhibitory activity via recruitment of the tyrosine phosphatases SHP-1 and/or SHP-2[6]. Memory B cells in the tonsils that express FCRL4 undergo proliferation in response to stimulation by T cell derived cytokines, but do not respond to BCR ligation in the same manner. On the other hand, tonsillar memory B cells that do not express FCRL4 undergo proliferation in response to BCR ligation[7]. It is tempting therefore to speculate that FCRL4 engagement may inhibit the proliferative response *in vivo*.

14.3. Expression Profiles of the FCRL Family Members

All of the FCRL family members, except for FCRL6, are preferentially expressed by B cells at different stages in their development. Unlike the CD19 pan B cell marker, the FCRL family members mark different stages in the B cell differentiation pathway. Immunofluorescence analysis of FCRL1 expression on bone marrow and tonsillar cells indicates that FCRL1 expression appears on preB cells, is upregulated on naïve B cells, downregulated on germinal center B cells and re-appears on memory and plasma B cells, albeit at levels below those seen on naïve B cells[22]. The heightened expression of FCRL1 on naïve B cells accords with *in situ* hybridization analysis of tonsillar sections, which detects FCRL1 mRNA primarily in cells within the mantle zone surrounding germinal centers[28]. FCRL2 expression appears to be restricted to a more discrete subset of B lineage cells[29,5,30]; Polson, A., personal communications; our unpublished observations. *In situ* hybridization experiments indicate FCRL3 transcription primarily by cells in the light zone of tonsillar germinal centers, an area occupied by centrocytes[29]. Analysis of FCRL4 mRNA identifies cells both in the tonsillar perifollicular zone and in the tonsillar epithelium[14,29]. This localization pattern is consistent with quantitative RT-PCR analysis, which detects FCRL4 mRNA predominantly in the $IgD^-/CD38^-$ memory compartment of tonsillar B cells[6]. The analysis of FCRL4 protein expression by immunohistochemistry and flow cytometry confirms this expression pattern[7]. It is noteworthy that the FCRL4-positive B cells are easily detected in tonsillar tissues, intestinal Peyer's patches and reactive lymph nodes from patients with infections such as toxoplasmosis, but are rarely seen in the bone marrow, peripheral blood or spleen[7,10,19]. Interestingly, a careful analysis of tonsillar B cell subsets indicates that FCRL4 expression characterizes a subpopulation of memory B cells with distinct morphological and functional characteristics. In contrast to the memory B cells that are FCRL4-negative, the FCRL4-positive B cells are larger, express relatively high levels of the chemokine receptors CCR1 and CCR5, and usually do not express the

CD27 memory B cell marker. FCRL4 expression thus identifies a tissue-based B cell population in mucosal or inflammatory lymphoid tissues that have been characterized by immunopathologists as monocytoid B cells[21].

FCRL5 expressing cells are detected by *in situ* hybridization in the light zone of germinal centers as well as in the interfollicular and intraepithelial regions [14,29]. FCRL5 is also expressed by tonsillar plasma cells[5]. These findings suggest that FCRL5 expression is upregulated in germinal center B cells and maintained as they undergo plasma cell differentiation. This interpretation is supported by flow immunocytometric analysis using FCRL5-specific monoclonal antibodies, in which FCRL5 expression is detected on post-germinal center cell lines, B lymphocytes from patients with hairy cell leukemia and plasma cells[18]. FCRL5 expression has recently been shown to be enhanced by expression of the EBNA2 protein[30].

In contrast to FCRL1-5, FCRL6 is not a B lineage marker. Instead, FCRL6 is expressed on discrete subpopulations of $CD8^+$ T cells, NK cells, $CD56^+$-T cells and γδT cells.[8]; D. Schreeder, unpublished observation

The intracellular FCRLA and FCRLB proteins are more closely related to the FcR family members then they are to the FCRL[4]. They have two (FCRLA) or three (FCRLB) Ig domains and carboxy terminal mucin-like domains[9,39]. In addition to *FCRLA* expression by B lineage cells in hematopoietic tissues[4,28], *FCRLA* mRNA is also expressed by normal and neoplastic melanocytes[17]. Immunohistochemical studies indicate that FCRLA is expressed in germinal centers in the tonsils[26,27]. and in the germinal centers and marginal zone of the spleen. The FCRLA protein is also found in certain B cell neoplasms, but not in T and NK cell malignancies[9]. FCRLB is likewise expressed in germinal center B cells and melanoma cell lines[2,27,39]. Interestingly, *FCRLB* mRNA is upregulated in response to LPS or BAFF stimulation of splenic B cells[27].

14.4. The Role of FCRLS in Disease States

The immunoregulatory potential of FCRL family members suggests that they may be involved in B cell mediated disorders. Other immunoregulatory genes for which polymorphisms have been associated with autoimmune disorders include FcγRIIb[11,38], PD-1[31], SHIP[23], CD22[3] and CD72[13]. The chromosome 1q21-25 region, where the *FCRL* family genes are located, has been recognized as a "hot-spot" for abnormalities observed for various autoimmune disorders, including systemic lupus erythematosus (SLE) and insulin-dependent diabetes mellitus (IDDM)[11,31,38]. Recently, an extensive analysis of single nucleotide polymorphisms (SNP) revealed an association between rheumatoid arthritis (RA) and a promoter region SNP for the *FCRL3* gene in a Japanese population[16,20]. This SNP is located within an NFκB-binding site and results in increased transcription of the *FCRL3* gene. A follow-up study of North American Caucasians revealed no association of *FCRL3* polymorphisms with RA,[15] while a third study on a Spanish cohort detected an association of *FCRL3* polymorphisms with RA only

after the samples were grouped by NFκB phenotypes[25]. Another study of an English population indicated a weak association of three SNPs in the *FCRL3* gene with Graves Disease (GD)[35]. It seems likely that polymorphisms in the other FCRL family members will be found to correlate with immunopathological states. The identification of highly expressed sequence tags (EST) for FCRL1-4 in certain B cell malignancies[1] also suggests involvement of FCRL family members in human disease and calls for further investigation into the function of these receptors. Support for this possibility was provided by Ise and co-workers who determined that FCRL5-positive cells could be detected in peripheral blood from hairy cell leukemia patients but not in healthy control donors[18].

14.5. Conclusions

The FCRL family includes an intriguing group of cell surface receptors with preferential B cell expression. Their selective expression during distinct phases of B cell differentiation makes them attractive B cell differentiation markers. The presence of ITIM and/or ITAM sequences in their intracellular domains of FCRL proteins implies immunomodulatory capability, a prediction that has been substantiated experimentally for FCRL1 and FCRL4. The discrete expression patterns of the FCRLs also make them attractive therapeutic targets for intervention in B cell disorders.

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15
Tolerance Mechanisms in the Late Phase of the Antibody Response

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15.1. Introduction

Immunological tolerance has historically been considered as arising primarily by elimination or inactivation of immature lymphocytes with self-reactive antigen receptors, during the early phase of repertoire formation in the thymus or bone marrow. Indeed, the clinical importance of thymic repertoire formation has now been established by two devastating genetic disorders - autoimmune polyendocrine syndrome 1 (APS1 or APECED) and immunodeficiency polyendocrinopathy enteropathy X-linked syndrome (IPEX) - and their mouse counterparts. In APS1 there is a failure to delete organ-specific T cells in the thymus caused by mutation of the *AIRE* gene[1,2], while defects in the *FOXP*3 gene in IPEX render the thymus unable to form a specific class of suppressor CD4 T cells[3,4], in both cases resulting in inexorable and severe autoimmunity.

Tolerance mechanisms that act later, on mature lymphocytes in the peripheral lymphoid organs and circulation, have garnered less attention – not because they are less important but because they have proved much less experimentally accessible. Two mechanisms that stop B and T cells from proliferating and clonally expanding - anergy and activation-induced cell death - have been studied in some detail[5,6]. Defects in one form of activation-induced cell death cause the severe autoimmune lymphoproliferative syndrome (ALPS) when the *FAS* gene is defective in man or mouse[7,8].

Arguably the two most critical steps for preventing autoantibody secretion, however, are the penultimate processes of affinity maturation in germinal centres and terminal differentiation into plasma cells. Tolerance mechanisms acting at these steps are nevertheless poorly characterized. Here we summarize recent work from our group providing insights into these late-stage tolerance mechanisms.

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15.2. Tolerance Mechanisms in Germinal Centres

Germinal centres play two pivotal roles in antibody responses.[9] They are the sites where high affinity antibodies are produced and selected through the linked processes of somatic hypermutation and positive selection for high affinity variants. They are also responsible for producing long-lived memory B and T cells and plasma cells. In concert, these actions transform antibody responses from transient, low titre affairs into high-titre, sustained antibody secretion. This transformation is critical for immunity to infectious agents, but it is equally crucial that any transient, weak autoantibody response does not evolve into sustained, high-titre production.

Most of the autoantibodies that are diagnostic for specific autoimmune diseases have nevertheless made this transformation. They typically have high affinity and exquisite specificity for particular autoantigens, making them much sought after as cell biological research tools. Where they have been analysed, these autoantibodies show the patterns of somatic hypermutation and selection that normally characterize post-germinal centre antibodies to foreign agents[10]. Thus, in clinical autoimmunity there has been a breakdown in the tolerance mechanisms that normally prevent selection of autoantibodies in germinal centres. In some cases where histopathology is available, such as autoimmune thyroiditis, germinal centres are often found within the target organ.

Failure to control the progress of self-reactive B cells into and through the germinal centre reaction appears to be a fundamental aspect of human systemic lupus erythematosus. This finding emerges from studies that take advantage of a particularly frequent Vh clonotype, recognized with a monoclonal antibody, which displays self-reactivity with Ii-type carbohydrate epitopes on ubiquitous proteins such as CD45[11,12]. In normal individuals, B cells with this clonotype are frequent in the immature and recirculating follicular mantle (IgD+ naïve) repertoire, but very few are found in the germinal centre centroblast or centrocyte subsets or in secreted antibodies and plasma cells. By contrast, in people with lupus, these self-reactive BCRs become equally frequent in centrocyte and long-lived plasma cell compartments and in secreted antibody, indicating a complete failure of control mechanisms that normally stop progression through these late phases of the antibody response[13].

Three basic mechanisms can be envisaged for tolerance in the germinal centre phase of the antibody response. First, binding of self antigens may transmit a negative signal into germinal centre B cells (centrocytes and centroblasts), triggering apoptosis or diminishing their proliferation. Second, self-reactive T cells may be eliminated or inactivated from the crucial subset of CD4 helper cells – T follicular helper or T_{FH} cells - that deliver growth and survival signals to centrocytes, so that self-reactive cells fail to receive these positive selection signals. Third, self-specific suppressor T cells may preferentially interact with self-reactive centrocytes and deliver an inhibitory signal.

Our group and others have shown that one or both of the first two mechanisms exist, at least under somewhat contrived experimental circumstances[14,15]. To

trace selection of antigen specific B cells through the germinal centre reaction, we immunized mice with a foreign protein, duck egg lysozyme (DEL), and at the same time introduced a defined population of traceable B cells bearing BCRs with a moderate affinity for duck lysozyme[14]. These marked B cells efficiently participated in the ensuing germinal centre response against the foreign protein DEL, where they could be readily enumerated by flow cytometry and by immunohistochemistry. To recapitulate a somatic mutation that changed the BCR so that it also had high affinity for a self antigen, we infused hen egg lysozyme (HEL) at the height of the germinal centre response. All of the marked B cells were now confronted with two different antigenic stimuli within the germinal centre: a moderate avidity foreign antigen (DEL) mostly immobilized on follicular dendritic cells, which could be processed by the centrocytes and presented to an existing population of DEL-specific T_{FH} cells; and a higher avidity circulating antigen (HEL) which was not recognizable by the T_{FH} cells. Within several hours, the crossreaction with HEL triggered a massive wave of apoptosis that selectively eliminated the HEL-reactive centrocytes[14].

In unpublished work, we have found that the same negative selection in germinal centres also occurs when germinal centres are induced by DEL immunization in mice that express soluble HEL as a neo-self antigen (JA Campbell and C Goodnow, unpublished data). Thus, there exists a very efficient and powerful mechanism to selectively eliminate from germinal centres any B cells whose BCR binds too avidly to a circulating self antigen.

Does HEL induce negative selection by delivering a death signal via the BCR, or by outcompeting with DEL for the antigen receptors and thus depriving the centrocytes of survival signals from T_{FH} cells? The short answer is that we don't yet know. The rapid induction of death, within 4 hours, implies a direct death signal, but remains compatible with deprivation from help given the very rapid cycling between centroblasts and centrocytes that may exist in the germinal centre[9].

Evidence for the existence of a new and critical tolerance mechanism acting at the level of T_{FH} cells has emerged from a genetic screen for inherited variants in mice that cause susceptibility to systemic lupus[16]. To discover new mechanisms, we developed a strategy of inducing genomic sequence variability at a rate of 1 basepair per million for a single generation in an otherwise homogeneous, known genome sequence, namely the C57BL/6 mouse. We bred libraries of individual pedigrees, each segregating a unique set of ~ 3000 single nucleotide variants and ~ 30 loss-of-function gene variants, and bringing 12.5% of these variants to homozygosity in any one of the third generation offspring[17]. The clinical immunology test for antinuclear autoantibodies (ANAs) on multiwell slides of permeabilized HEPG2 cells, was used to test sera from each mouse in hundreds of pedigrees, yielding a large panel of true-breeding ANA-susceptible variant strains[18].

The first strain identified by this screen was named *san roque* after the patron saint of the buboes, because the ANA+ mice also developed greatly enlarged lymph nodes[16]. These mice develop a range of cardinal signs of lupus, and

when crossed to a TCR transgenic strain also show failure of tolerance to organ-specific antigens. The *san roque* mutation does not disrupt known tolerance mechanisms, but instead acts within mature T cells to allow them to form an excessive number of T_{FH} cells, leading to massive germinal centres that form spontaneously and appear to be directed selectively against self antigens. Consequently, extraordinarily high titre autoantibodies are made in the *san roque* mice, whereas antibody responses against foreign immunogens appear normal in titre and quality.

What does the *san roque* mutation do to dysregulate T_{FH} cells and apparently break down tolerance in the germinal centre? T cells with the gene defect express higher then normal levels of ICOS mRNA and protein both as naïve cells and as activated and memory cells[16]. ICOS is highly expressed on T_{FH} cells[19] and ICOS deficiency causes a loss of T_{FH} cells and a failure of germinal centre responses in man and mouse[20–24], while transgenic expression of soluble ICOS ligand causes a plasma cell dyscrasia resembling the *san roque* mice. It is striking that ICOS, which appears to take the place of CD28 in T_{FH} cells, is tightly controlled in its expression normally, whereas its ligand is expressed more widely. The defective gene, *Roquin* (*Rc3h1*) encodes an E3 ubiquitin ligase family member that contains a putative RNA-binding CCCH zinc finger and localizes to cytoplasmic RNA stress granules, where mRNAs are held in translationally silent form or degraded[16]. This pathway may selectively repress ICOS expression in self-reactive T cells in order to bar them from becoming T_{FH} cells. When this control is defective, self-reactive T_{FH} cells may now accumulate and select high titre autoantibodies.

15.3. Tolerance Mechanisms Regulating Plasma Cell Differentiation

The final step in the antibody response involves a dramatic reprogramming of extrafollicular B lymphoblasts or intrafollicular centrocytes to differentiate them into plasma cell factories for antibody secretion. In the last several years, the transcriptional reprogramming that brings about plasma cell differentiation has been elucidated[25]. At its core is the switching on of the *prdm1* gene encoding a transcriptional repressor, Blimp-1, and the switching off by Blimp of two key transcription factors for B lymphocytes and centrocytes, *Pax5* and *Bcl6*. Relatively little is known about how Blimp is induced, although TLR signalling via NFκB and cytokine signalling via Stat3 may play direct roles.

Recently, we have discovered that the ERK serine threonine kinase signalling pathway plays a central role in integrating opposing signals to regulate the induction of Blimp and plasma cell differentiation[26] (Rui L and CCG, manuscript submitted). When the BCR is constantly engaged by antigen, as occurs in self reactive B cells, it displays the intriguing property of not desensitising with respect to signalling the activation of ERK[27]. This constant activation of ERK actively blocks the induction of Blimp and all of the subsequent events of plasma

cell differentiation, providing a final control mechanism to block secretion of autoantibodies. In B cells that are not self-reactive, two differences intervene to allow Blimp expression and antibody secretion. First, antigen binding is typically transient so that when it ceases so does ERK activity, and Blimp expression follows within a day or two. Second, if helper T cells deliver the maturation promoting B cell factors, IL-5 and IL-2, these induce a dual specificity phosphatase that inactivates ERK even in the presence of continued BCR engagement. This control mechanism is likely to be critical for preventing autoantibody secretion induced by bacterial antigens that bypass tolerance in the T cell repertoire, such as lipopolysaccharides, DNA and other bacterial components that activate B cell proliferation and differentiation via Toll like receptors.

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16
Role of RS/κDE in B Cell Receptor Editing

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16.1. Introduction

B lymphocytes that are autoreactive can undergo central tolerance by deletion or receptor editing. Editing allows the cells to alter receptor specificity and remain viable-and useful. It is unknown if a defect in receptor editing leads to problems with self tolerance, but it is assumed to play some role. Receptor editing stimulated by immune tolerance takes place in the bone marrow and perhaps in other tissues[1,2]. A major pathway of editing involves secondary rearrangements in the Ig kappa locus, which can displace primary VJ rearrangements by recombining upstream Vs with downstream Js[3]. In mouse there are only 4 functional Jk elements, Jk1, 2, 4, and 5 (Jk3 is a pseudogene), but even VJk5 rearrangements can be silenced by editing through rearrangements to the recombining sequence (RS). RS and its human homologue, the kappa deleting element (kde), are found ~25kb downstream of the Ck exon[4,5]. RS can recombine to Vk elements or to a site in the Jk-Ck intron, leading to the functional inactivation of the k-locus [4–6]. RS and kde deletions occur in the vast majority of B cells that go on to express lambda L-chain[7–9] and also on the non-functional allele in a significant subset of kappa expressing cells[10]. We found that in 40–50% of normal mouse lambda+ B cells RS rearrangements had silenced previously functional VJk joins, suggesting that much RS recombination was induced by self tolerance[8]. Similar findings in human B cells[11] suggest that RS/kde mediated editing is common.

The RS element has been proposed to have one of three, non-mutually exclusive functions. First, as originally suggested by Selsing and colleagues, RS could play a role in promoting activation of lambda rearrangement, explaining the correlation between RS and lambda rearrangements[4,5]. Alternatively, RS rearrangements could play a role in mediating allelic exclusion through a stochastic process of kappa gene inactivation[7–9]. Finally, as we have proposed, RS rearrangements may mediate receptor editing, eliminating kappa genes that

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promote autoreactivity.[8] To see if inactivation of RS recombination has phenotypic consequences, we generated mice with defective RS recombination.

16.2. Generation and Analysis of RS Mutant Mice

We knocked out the recombination heptamer/nonamer signal sequences adjacent to RS in ES cells by replacement with a neomycin resistance gene, and the mutation was introduced to the germline by blastocyst injection and selection of chimeric mice. The neo gene was flanked by loxP sites, allowing its subsequent removal by breeding with mice expressing cre in the germline. PCR and southern blotting experiments confirmed that the targeting and neo deletion was successful.

The knockout mice were then analyzed for phenotypic differences from littermates lacking the mutation. The results are preliminary, but several conclusions are already possible. First, we find a clear, statistically significant, reduction in the frequency of lambda+ B cells. On average, 40–50% fewer lambda cells were found in all tissues. In addition, in spleen there was a slight reduction in overall B cell numbers.

As the loss of a proportion of the lambda+ cells was compatible with all of the models of possible RS function described above, we next explored the kappa allele usage adjacent to the defective RS locus itself by breeding mice with mice carrying a human Ck exon in place of the mouse version. These mice were then RS+/−, with the human Ck allele (hCk) carrying an intact RS recombination signal and the mouse allele was RS deficient (mCk.RS-)[12]. As controls, we compared human Ck mice carrying a wildtype mouse RS allele. If RS rearrangement affected the proportion of wt vs knockout kappa usage, then we could conclude that RS rearrangement has consequences independent of lambda activation. The result of the analysis suggested that this was the case: in mCk.RS-/hCk mice, the frequency of human Ck+ B cells was significantly lower compared to mCk/hCk controls. This skewing was apparent already among newly formed B cells in bone marrow. We believe that this reduction is the result of counterselection against mouse Ck cells that would normally inactivate Ck by RS recombination and continue to rearrange and express hCk. We estimate a reduction of hCk+ cells of ca. 14%. Furthermore, in all of the RS−/− and RS+/− mice examined we failed to detect an increase in the proportion of cells carrying two distinguishable L-chains, either on the cell surface or in the cytoplasm. We conclude that in cells that fail to edit owing to autoreactivity, B cells preferentially die in the bone marrow.

16.3. Estimate of the Normal Extent of Receptor Editing

Because the reduction in B cell output in RS deficient mice is likely the result of deletion because of tolerance, it allows an estimate of the overall likelihood that a B cell undergoes editing as a result of immune tolerance. We estimate that

the proportion of B cells lost in RS−/− is ca. 16% above normal. This value is quite close to the theoretical maximum since ∼15% of k+ cells normally carry an RS rearrangement on the silent allele and ∼75% of lambda+ cells have one or two RS rearrangements. As the normal proportions of kappa and lambda are 94:6, this indicates that 16% ÷ [(15% X 94%) + (75% X 6%)]=∼86% of cells that normally undergo an RS rearrangement are counterselected. A problem with this conclusion is that measurements of the likelihood that RS rearrangement to the J-Ck intronic site deletes an in-frame rearrangement is only 30–50%[8,11]. However, the likelihood that the more common RS-to-Vκ editing eliminates in frame rearrangements is unknown, and difficult to measure. Perhaps a much higher percentage of Vk-to-RS rearrangements are induced in autoreactive cells and eliminate in-frame kappa VJ rearrangments. An alternative possibility is that RS-deficient alleles are better able to generate non-autoreactive B cells carrying kappa chains, however, that would predict that positive selection is inefficient relative to rearrangement, and would in turn predict that there should be an increase in the proportion of cells carrying two L-chains, a prediction that we have so far not been able to confirm. Therefore, we tentatively conclude that in cells with defective RS rearrangement, cells that would otherwise edit are usually eliminated.

Assuming that the forgoing conclusions are correct, from these data one can also attempt an estimate of the likelihood that a random B cell is autoreactive and regulated by editing. Since RS-to-JCk-intron rearrangements occur 80% of the time in alleles that have used up Jks and carry a VJk5 rearrangement[8], and assuming that only 25% of rearrangements involve Jk5[13], then an estimate of the likelihood that a cells is autoreactive and undergoes editing is as follows:

16% (reduction in B cell output in RS−/−) X 4 (correction for Jk5 usage rate, since RS inactivates mainly this subset of rearrangements) / 0.8 (correction for RS rearrangements eliminating non-VJk5 joins)=∼80%

In other words, our data suggest that in the normal immune system, roughly 80%–90% of B cells are autoreactive and must be tolerized by editing. This value is higher than our own and others' previous estimates[12,14−16]. Our finding underscores the purpose and importance of receptor editing, which is to eliminate the bottleneck of development that is caused by the high probability of BCR autoreactivity, and to minimize cell loss.

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17
The Regulation of Receptor Editing

Mark S. Schlissel*

17.1. Introduction

Antigen receptor genes are assembled during lymphocyte development by the site-specific V(D)J recombination machinery.[8] The virtue of this manner of encoding diversity in the genome is one of economy—a relatively small investment of genetic material can encode a near-limitless repertoire of immunoglobulin (Ig) or T cell receptor (TCR) genes. There are two consequences of this combinatorial joining process which are potentially deleterious, however. First, the generation of dsDNA breaks during V(D)J recombination is a source of genomic instability.[14] Inappropriate joining of broken-ended reaction intermediates can result in the translocations, deletions and transpositions associated with lymphoid malignancy. Second, the random nature of V(D)J recombination frequently generates self-reactive B or T cells.[12] These cells must either be altered or destroyed in order to prevent autoimmunity. This brief review will consider recent approaches to understanding a major mechanism of self-tolerance in the B cell compartment, receptor editing.

The earliest genetic models of the B cell tolerance problem were established independently by Goodnow and Nemazee and their colleagues.[6,15] These consisted of Ig heavy + light chain transgenic mice which expressed a potential auto-antibody. In the Goodnow system, it was an anti-HEL antibody (hen egg lysozyme, the well-studied model antigen) and in the Nemazee system it was an anti-H-2K^b MHC I antibody. Goodnow could then introduce various forms of HEL by transgenesis and Nemazee could introduce the target MHC antigen by breeding to mice of various MHC haplotypes. These "first-generation" models led to the conclusion that B cell tolerance occurred either by apoptosis of the offending cells or by the induction of anergy depending upon the affinity, location, or effective valence of the auto-antigen. More subtle follow-up investigations revealed that anergic B cells competed poorly for survival niches and were often lost, presumably due to apoptosis.[4]

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Nemazee and Weigert working independently first reported that auto-immune B cell receptors could be edited through the expression of newly rearranged Ig light-chain genes which could replace the existing light-chain and avoid self-reactivity.[5,22] This mechanism has been termed receptor editing. Subsequent work by a number of labs has shown that receptor editing occurs very frequently and is arguably the most important mechanism of B cell tolerance.[1,7]

A significant amount of attention is now being focused on the mechanisms which regulate receptor editing and its potential consequences. Important questions include a) how the RAG genes are regulated in immature self-reactive and self-tolerant B cells; b) whether Ig HC genes undergo receptor editing; and c) how allelic exclusion can be enforced in the setting of receptor editing.

17.2. The Regulation of RAG Gene Expression in Immature B Cells

V(D)J recombination depends upon regulated expression of the genes encoding the lymphocyte-specific component of the recombinase, *RAG1* and *RAG2*. Various labs have contributed to the identification of the *RAG* promoters and a number of enhancers which are important for this regulation.[19] Initially it was thought that surface Ig expression invariably results in *RAG* gene inactivation. This turned out not to be the case in auto-antibody transgenic mice, however, leading to the idea that BCR signaling in immature B cells causes either reactivation or maintenance of *RAG* transcription.[18,24] The current controversy in this area is how the BCR on an immature B cell can signal either continued *RAG* expression or inactivation of *RAG* expression.

Melamed and Nemazee developed a widely used culture system to study *RAG* regulation in immature B cells.[13] This system consists of Ig transgenic mouse bone marrow cultured *ex vivo* in the presence of pharmacologic doses of rIL-7. After five days, the vast majority of surviving cells are sIg^+ immature B cell cells. IL-7 is withdrawn and the cells are returned to culture in the presence or absence of anti-BCR antibody (to mimic a self-antigen signal). Verkoczy et al showed that anti-BCR treatment resulted in the transcriptional activation of *RAG* expression which was dependent upon NF-κB.[24] Subsequent work discussed by Nemazee at this meeting is focused on understanding the signaling pathways involved in this phenomenon. A problem with this system, however, is the developmental history of the cultured, transgenic immature B cells. B cell development is disrupted in the presence of Ig transgenes, speeding passage through various regulated stages and resulting in cells which are mature with respect to surface phenotype but may nonetheless differ from "normal" immature B cells in terms of signaling pathways and gene expression. In addition, driving cell division with rIL-7, followed by its acute withdrawal may have other unknown effects on cultured cell metabolism.

Recent work from Behrens and colleagues has led to an alternative view. They argue that tonic (antigen independent) BCR signaling results in the inactivation of *RAG* expression and that antigen engagement causes the removal of self-specific BCR from the cell surface and loss of this tonic signal. Their experimental approach was to use the cre-recombinase to delete the floxed V(D)J exon of a targeted IgHC locus, thereby removing Ig from the surface of an immature B cells.[23] DNA microarray analyses revealed that immature B cells after losing surface BCR expression reverted to a gene activity pattern almost identical to that found in normal bone marrow pre-B cells. This included activation of *RAG* transcription and IgLC gene rearrangement. The same result was obtained when immature anti-HEL Ig transgenic B cells were treated with soluble HEL. In each case, these workers showed that the activation of *RAG* expression and receptor editing correlated with diminished surface Ig expression.

In a complementary report, Pelanda and co-workers showed that self-reactive Ig gets reversibly down-modulated from the cell surface (but continues to be expressed) in mature B cells which have successfully generated a self-tolerant BCR.[11] Culture of such cells in vitro in the absence of self-antigen results in the surface expression of two different Ig's on a single B lymphocyte, a violation of allelic exclusion (see below). This data is consistent with that of Behrens' group since loss of surface expression of the auto-antibody presumably activated receptor editing and the generation of a self-tolerant BCR.

17.3. Does Receptor Editing Act on the IgHC Locus?

Receptor editing in the Igκ locus involves either replacement rearrangement on the previously rearranged κ allele or de novo rearrangement of the second κ allele or of an Igλ allele. Since Igκ genes contain only Vκ and Jκ gene segments, upstream Vκ's can rearrange with downstream Jκ's replacing the previous recombinant exon. This is not the case in the IgHC locus where V(D)J-rearranged alleles lack D_H gene segments and thus can no longer undergo V_H-to-DJ_H rearrangement. Interestingly, in both the human and mouse genomes, a so-called "cryptic heptamer" exists in the 3' region of many V_H gene segments.[16] Using knock-in mice, various workers have shown that an upstream V_H gene segment can rearrange with a previously assembled VDJ exon resulting in editing of the heavy-chain knock-in.[2] These knock-ins, however, leave the entire V_H to D_H to J_H interval intact, resulting in quite a different genomic structure than what is found on an endogenously rearranged IgHC allele. Similar rearrangements have also been observed in leukemic cell lines.[9,17,25] While the existence of such V-gene replacement events is generally accepted, its significance is controversial. It is likely that these events are normally restricted to pro-B cells and serve to rescue cells with out-of-frame V(D)J rearrangements. This is because previous studies have shown that V-to-DJ rearrangement does not occur in cells at the

pre-B cell stage or beyond[3,21], presumably due to changes in V_H region recombinase accessibility. The definitive experiment in this area has yet to be done, however.

17.4. Is There a Bias in Receptor Editing to the Previously Rearranged Allele?

A reasonable definition of receptor editing is the replacement of a self-specific BCR with a self-tolerant one through further V(D)J recombination. Since the vast majority of peripheral B cell express only one Ig on their cell surface, the simplest way for the recombinase to accomplish this task would be to perform replacement rearrangement with an upstream Vκ rearranging to a downstream Jκ, deleting the offending IgLC variable exon (Figure 17.1A). This would avoid the situation where a single cell could express two different BCRs.

Recent data from our own lab has shown that at any given time, one of the two κ alleles in a pre-B cell is the preferred substrate for recombination and can undergo multiple rounds of rearrangement while the allelic locus remains in its germline state.[10] This observation suggests that there may be an inherent bias in Igκ rearrangement in favor of rearrangement on the previously rearranged allele (replacement rearrangement).

In an attempt to distinguish de novo (1°) from replacement (2°) Igκ locus rearrangement, we modified a previously described LM-PCR assay which detects dsDNA break intermediates in V(D)J recombination.[20] We compared the frequency of *de novo* and replacement RSS breaks involving Jκ2 in DNA purified from bone marrow pre-B or immature B cells and from an AMuLV-transformed cell line inducible for light-chain rearrangement (Figure 17.1B). We found that while the cell line had more *de novo* as compared with replacement RSS breaks and primary pre-B cells had similar amounts of each, immature bone marrow B cells had far more replacement breaks. We conclude from this preliminary experiment that there may be a bias in favor of rearranging the previously rearranged allele during Igκ locus receptor editing. This may be due to the remaining Vκ gene segments being far closer to Jκ gene segments on the previously rearranged allele as compared with the germline allele. Alternatively, the nearby Vκ promoter may enhance the accessibility of the Jκ gene segments on the previously rearranged allele, biasing recombination in its favor. Further work will be required to confirm this result and distinguish the various mechanistic possibilities.

Surprisingly, it may not be necessary to delete the genes encoding a self-reactive BCR in order to replace it functionally. As noted above, Pelanda and colleagues showed that autoreactive Ig continues to be expressed within mature B cells which express a second, self-tolerant BCR on the cell surface.[11] If generally true, this result has important implications for possible mechanisms of B cell autoimmunity.

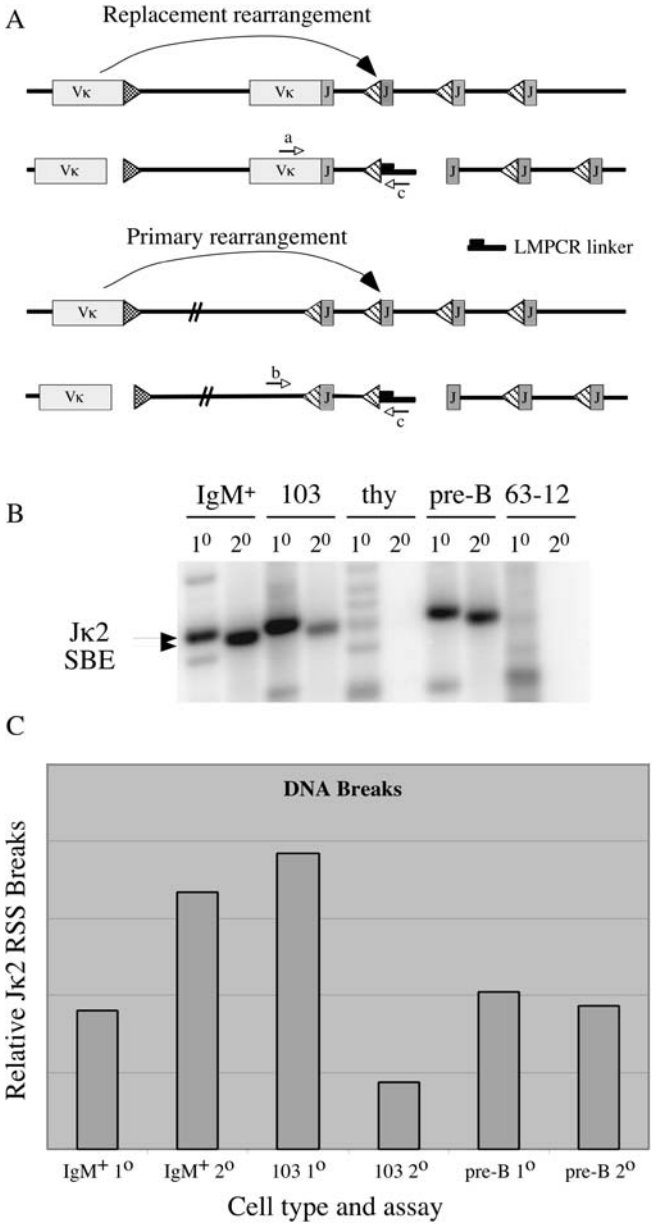

FIGURE 17.1. The regulation of two types of recombination in the Igκ locus. A. Diagram of a κ allele undergoing replacement (upper; 2°) as compared with primary (lower; 1°) Vκ-to-Jκ rearrangement. The lower of each pair of diagrams depicts the dsDNA RSS breaks which accompany each type of rearrangement and the PCR primers used to amplify linker-ligated DNA of each type. B. Phosphorimage of a Southern blot analysis of LMPCR products from the indicated DNA samples. 63-12 is a RAG null pro-B cell line and thy is thymus DNA. The reactions labeled 1° used primers b and c while the reactions labeled 2° used primers a and c. C. Quantitation of blot shown in part B.

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18
B Cell Hyporesponsiveness and Autoimmunity: A New Paradigm

Christine Grimaldi[1], Emil Nashi[1], Jeganathan Venkatesh[1] and Betty Diamond[1,2]

18.1. Introduction

Systemic lupus erythematosus (SLE) is a disease caused by autoantibody-mediated damage in multiple target organs. The hallmark autospecificity in SLE is nuclear antigen with double stranded (ds) DNA being most critical as antibodies to dsDNA are the most common anti-nuclear autoantibody and also contribute to pathogenicity. Thus, anti-DNA antibodies have been extensively studied and multiple murine strains have been genetically engineered which spontaneously develop anti-DNA antibodies.

The prevailing understanding of autoantibody production focuses on B cell hyper-reactivity with the ensuing production of immune complexes containing DNA and RNA. These, in turn, activate dendritic cells through interaction with toll-like receptors generating a proinflammatory cycle. We have been studying two models of SLE in which autoantibody production is not spontaneous, but rather is induced. These models lead us to an alternative model of autoantibody production, one in which autoreactivity derives from B cell hyporesponsiveness[2].

18.2. B Cell Repertoire Selection

Much has now been learned about the formation of the B cell repertoire. While a limited number of antibody specificities are part of the B1 cell repertoire generated in the fetal liver, the vast majority of B cells are continually generated within the bone marrow. These B cells undergo random rearrangement of both

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heavy chain variable region gene segments, V, D and J, and light chain variable region gene segments, V and J. It has been estimated that as many as 70% of the newly arising B cells generated produce autoantibodies[1]. Since a significantly smaller percent of mature B cells are autoreactive, the process of B cell maturation is linked to a process of selection with the elimination of autoreactive cells.

Three mechanisms have been identified that mediate negative selection: receptor editing, anergy and deletion[2–8]. Each of these mechanisms is initiated by engagement of the B cell receptor (BCR). Since self molecules are, in general, the antigens present in the microenvironment of the developing B cell that can engage the BCR, these processes lead to the elimination of autoreactive B cells. Receptor editing involves the generation of a new light chain which may alter antigenic specificity and render the BCR no longer autoreactive[2,3]. It is generally believed that when receptor editing fails, anergy and deletion become operative with anergy ensuing when there is less BCR cross-liking and deletion ensuing when more BCR cross-linking occurs[7,9]. Anergic B cells die within days unless B cell lymphopenia decreases the competition for entry into follicles[10,11]. This model that has been proposed predicts that the higher the affinity of the BCR for self antigen, the more likely that the B cell will be deleted.

Tolerance mechanisms are operative within the bone marrow, and subsequently as B cell maturation continues, in the spleen[12–14]. The transitional stage of development which occurs in the spleen has been subdivided into the T1 and T2 stage, based on the expression of cell surface markers[15,16]. There is general agreement that negative selection occurs in the T1 stage[17–19], but there is some disagreement regarding the impact of negative selection on cells within the T2 compartment[20]. A T3 compartment has also been proposed but it is not clear if cells that have a T3 phenotype represent cells in a stage of maturation to immunocompetence or cells that may be marked for deletion.

Once the B cell matures through the transitional stage, it is an immunocompetent cell. At that time, engagement of the BCR sets in motion activation programs that lead to differentiation to short-lived plasma cells or to germinal center cells which undergo affinity maturation and class switch recombination and ultimately become either memory cells or long lived plasma cells which reside in the bone marrow (reviewed in[21,22]).

It is apparent that autoreactivity is generated within the germinal center response[23,24], but mechanisms governing the elimination of somatically mutated autoreactive B cells are poorly understood[25–28].

18.3. Lupus and Repertoire Selection

Analyses of anti-DNA antibodies in spontaneous models of lupus display extensive somatic mutation[23,29–31]. For this reason, most analyses of lupus B cells have focused on activation profiles and the function of B cells following antigen engagement. Little attention has been paid to the naïve B cell repertoire.

We have been exploring two non spontaneous models of lupus. Both occur in BALB/c mice; in one model the trigger for autoantibody production is estrogen, in the other, antigen. In each case, the autoimmune diathesis is established by a lack of stringency in B cell negative selection with enhanced autoreactivity in the naïve repertoire.

18.4. Estrogen-Induced Autoreactivity in R4A Transgenic Mice

R4A transgenic mice possess a γ2b heavy chain of an anti-DNA antibody. When BALB/c mice harbor the transgene, approximately 5% of the splenic B cells express the transgene; the rest express an endogenous heavy chain[32]. Those B cells expressing the transgenic heavy chain do so in conjunction with a spectrum of light chains. Thus, there are three populations of transgene-expressing B cells that arise in the bone marrows one with no detectable affinity for DNA, one with low affinity for DNA that matures to immunocompetence, and one with high affinity for DNA that is deleted[33,34]. Deletion of the high affinity autoreactive B cells enables the maintenance of self tolerance in the mice.

We elected to treat R4A transgenic mice with 17β estradiol as there is a large, albeit controversial, literature suggesting that estrogen exposure can trigger or exacerbate human lupus and clear data for an aggravating role of estrogen in the NZB/W lupus-prone mouse[35,36]. R4A transgenic mice implanted with estradiol pellets that deliver a sustained serum concentration of 75–100 pg/ml develop high titers of anti-DNA antibodies, glomerular immunoglobulin deposition and proteinuria[37]. Cell sorting followed by ELISpot analysis demonstrated that the DNA-reactive B cells reside within the marginal zone compartment and the analysis of hybridomas making anti-DNA antibodies showed that the high affinity DNA-reactive B cells were those that are normally deleted before maturing to immunocompetence[37,38].

Gene expression studies have demonstrated increased expression of the inhibitory BCR coreceptor CD22 in B cells of estradiol-treated mice and increased expression of its associated phosphatase, SHP1[39]. Transfection of a B cell line with a constitutively activated estrogen receptor (ER) α demonstrated that the increased CD22 and SHP1 expression was a direct consequence of estradiol acting on the ERs present within the B cell[39].

As expected from increased expression of an inhibitory coreceptor, engagement of the BCR by anti-IgM antibody, a surrogate for antigen, led to diminished calcium flux in transitional B cells of estradiol-treated mice and decreased phosphorylation of ERK[40]. As a result, a lower percentage of the transitional B cells are triggered for apoptosis. These studies demonstrated that the higher threshold for negative selection, i.e. the need for more antigen to trigger deletion that results from estradiol exposure, permits pathogenic DNA-reactive B cells to mature to immunocompetence and to become activated (Figure 18.1).

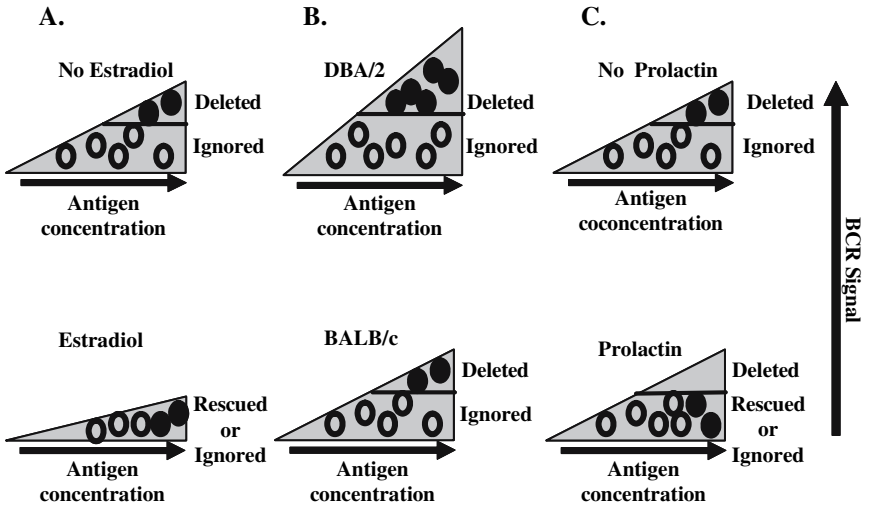

FIGURE 18.1. A reduced BCR signaling strength can permit the escape of autoreactive B cells. A. Treatment of R4A transgenic mice with estradiol lowers the strength of the BCR signal, which leads to the rescue of autoreactive B cells that would normally be deleted. B. The B cells of DBA/2 mice exhibit a stronger BCR signal than the B cells of BALB/c mice, which reduces the number of autoreactive B cells in the periphery of DBA/2 mice. The different BCR signaling strengths demonstrate that genetic determinants regulate the fate of autoreactive B cells. C. Unlike estradiol, treatment of R4A transgenic mice with prolactin results in the rescue of high affinity DNA-reactive B cells without changes in the signaling strength of the BCR.

To understand which check points for B cell selection are altered by estradiol, we examined by single cell PCR kappa light chain usage in transgene-expressing B cells in the transitional or mature splenic B cell compartment[41]. Our previous studies of hybridomas from these mice facilitated this analysis as we know which light chains associate with the R4A transgene to produce low or high affinity anti-DNA antibodies[34,37]. We found a higher percentage of low affinity DNA-reactive B cells in the transitional compartment of spleens of estradiol-treated mice, suggesting that these are normally subject to regulation in the bone marrow (Table 18.1).

We also identified a higher percentage of high affinity DNA-reactive B cells in the transitional compartment of estradiol-treated mice, suggesting that these are also normally regulated in the bone marrow. Placebo-treated mice showed a decrease in high affinity DNA-reactive B cells at the transitional mature interface, while showing no regulation of the low affinity population at this checkpoint. In contrast, there was no loss of high affinity DNA-reactive B cells in the estradiol-treated mice as they progressed from a transitional to a mature phenotype. Concomitant with the maintenance of the population of high affinity DNA-reactive B cells was an unexpected decrease in the percentage of low affinity DNA-reactive B cells that occurs between the transitional and the

TABLE 18.1. Frequencies of low and high affinity DNA-reactive B cells.

	Transitional[1]		Mature[1]	
	Low	High	Low	High
Estradiol	35%	40%	17%	38%
Placebo	23%	23%	22%	9%

[1]The percentage of transitional and mature B cells with known affinities for DNA was determined by single cell PCR as described by Grimaldi et al.,[41].

mature stage of development. This observation is strongly suggestive of positive selection in the transitional compartment. When high affinity autoreactive B cells escape deletion, they have an advantage in competition against low affinity B cells. Thus, there can be survival of high affinity autoreactive B cells without survival of low affinity B cells. This has, in fact, been in a murine model of lupus, C57BL/6 mice deficient in FcRγII, but is not predicted by models that do not allow for positive selection of transitional B cells[42].

Estradiol treatment, therefore, increases the autoreactivity in the naïve B cell repertoire by increasing the threshold for negative selection during B cell maturation. With increased activity of an inhibitory coreceptor, more BCR engagement is required for negative selection to occur. The autoreactive B cells that mature to immunocompetence can be activated to produce autoantibody, and apparently in the absence of low affinity autoreactive B cells that several studies have suggested contribute to the homeostasis of the immune system and to self tolerance[43,44]. Thus, progression to autoimmune disease begins.

18.5. Peptide-Induced Autoreactivity

In studies designed to identify fine specificity differences among anti-DNA antibodies, we identified a pentapeptide DWEYS which functions as a mimetope for DNA[45]. Approximately 60% of the DNA-reactivity in NZB/W serum is peptide inhibitable and soluble peptide can prevent glomerular deposition by anti-DNA antibodies. When BALB/c mice are immunized with the peptide in a multimeric configuration, they develop high titers of anti-peptide, anti-DNA cross-reactive antibodies, glomerular immunoglobulin deposition and proteinuria. The antibody response is T cell dependent and restricted by class II E^d [46]. When several other strains are immunized with multimeric peptide, they fail to develop either an anti-peptide or an anti-DNA response. Of particular interest, the DBA/2 mouse fails to develop an antibody response following immunization[47]. DBA/2 mice share MHC haplotype with BALB/c mice and generate a T cell response to multimeric peptide that, like the response in BALB/c mice, is E^d restricted. This model then permitted an exploration of non-MHC genetic loci that contribute to an autoimmune diathesis.

Adoptive transfer experiments demonstrated that B cells from BALB/c mice together with BALB/c x DBA/2 F1 T cells could be activated to mount an anti-peptide, anti-DNA response following immunization. B cells from DBA/2 mice together with F1 T cells did not mount an antibody response (Hicks ET AL., submitted[48]). This focused our attention on the differences in B cell function in the two strains. We were able to demonstrate that tolerance thresholds differed between the two strains. BALB/c mice had more DNA and peptide reactive B cells in the naïve B cell compartment, prior to immunization with multimeric peptide, than DBA/2 mice. Furthermore, DBA/2 mice exhibited a greater calcium flux in transitional B cells following incubation with anti-IgM antibody despite a similar surface expression of IgM. Consistent with this observation, more transitional cells from DBA/2 mice became apoptotic following incubation with a given concentration of anti-IgM. Thus, the data demonstrated more stringent tolerance in DBA/2 mice secondary to enhanced BCR signaling. The model, therefore, provided an opportunity to understand B cell intrinsic factors that govern selection of the naïve B cell repertoire.

A genetic backcross analysis revealed a dominant chromosomal locus in BALB/c mice involved in altering the BCR signal, and in simultaneously permitting an autoantibody response to immunization with multimeric peptide.

This model provides a second example of a lack of stringency in the naive B cell repertoire predisposing to autoimmunity. In each instance, the B cell function is not so dysregulated as to lead to spontaneous autoimmunity that ensues in the absence of any environmental trigger. Rather, the B cell function is capable of supporting protective antibody responses. Only when particular environmental triggers are present will autoimmunity ensue.

18.6. Prolactin-Induced Autoimmunity in R4A Transgenic Mice

Not all changes in the naïve B cell repertoire involve a B cell intrinsic factor or a change in strength of BCR signaling. When R4A transgenic mice are treated with prolactin, such that there is a doubling of the serum prolactin, they also display increased titers of anti-DNA antibodies, glomerular immunoglobulin deposition and proteinuria[40]. In this instance, it is the follicular B cell compartment that houses the DNA-reactive B cells. The antibody response depends on the presence of $CD4^+$ T cells. We have no evidence of a prolactin-mediated alteration in BCR signaling. Specifically, anti-IgM mediated calcium flux is unchanged. There is, however, an increase in CD40 expression on B cells and an increase in CD40 ligand expression on T cells. It has previously been shown that CD40 engagement can protect WEHI cells against anti-IgM mediated apoptosis[49,50]. It is not surprising that features of B cell function other than the strength of the BCR can alter thresholds for negative and/or positive selection.

18.7. Conclusions

These studies strongly suggest that B cell hyporeactivity constitutes a critical component of a genetic diathesis to autoimmunity and can represent an environmentally (hormone or antigen exposure) induced predisposition to autoimmunity. This represents a new paradigm for B cell function in SLE, where the B cell intrinsic abnormalities have previously focused on hyper-reactivity. This paradigm is, in fact, consistent with recent data from Nussenzweig and colleagues demonstrating increased autoreactivity in the transitional and naïve B cell repertoires of SLE patients[1,51]. Their studies do not demonstrate whether this is a B cell intrinsic property or whether the autoreactive repertoire reflects increased B cell rescue from apoptosis by cytokines or costimulation. The human studies also do not yet address the question of whether the increased autoreactivity in the naïve repertoire is genetically determined or represents the influence of an activated, pro-inflammatory immune system. They do, however, affirm the potential importance of B cell hyporesponsiveness as one contributor to an autoimmune diathesis.

Finally, it will be of fundamental importance to determine whether those genetic or environmental factors that alter the naïve B cell repertoire also affect negative selection in the germinal center response. Looking for genes that modulate the BCR signaling cascade or apoptotic pathways may reveal clues to the regulation of germinal center B cells and may suggest new therapeutic strategies in SLE.

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