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Plasma Cell Formation, Secretion, and Persistence: The Short and the Long of It

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Abstract

B cells can be activated by cognate antigen, anti-B-cell receptor antibody, complement receptors, or polyclonal stimulators like lipopolysaccharide; the overall result is a large shift in RNA processing to the secretory-specific form of immunoglobulin (Ig) heavy chain mRNA and an upregulation of Igh mRNA amounts. Associated with this shift is the large-scale induction of Ig protein synthesis and the unfolded protein response to accommodate the massive quantity of secretory Ig that results. Stimulation to secretion also produces major structural accommodations and stress, with extensive generation of endoplasmic reticulum and Golgi as part of the cellular architecture. Reactive oxygen species can lead to either activation or apoptosis based on context and the high or low oxygen tension surrounding the cells. Transcription elongation factor ELL2 plays an important role in the induction of Ig secretory mRNA production, the unfolded protein response, and gene expression during hypoxia. After antigen stimulation, activated B cells from either the marginal zones or follicles can produce short-lived antibody secreting cells; it is not clear whether cells from both locations can become long-lived plasma cells. Autophagy is necessary for plasma cell long-term survival through the elimination of some of the accumulated damage to the ER from producing so much protein. Survival signals from the bone marrow stromal cells also contribute to plasma cell longevity, with BCMA serving a potentially unique survival role. Integrating the various information pathways converging on the plasma cell is crucial to the development of their long-lived, productive immune response.

Keywords

immunoglobulin; antibody secreting cells; plasma cells; B-cell differentiation

I. INTRODUCTION

The clonal nature of antibody formation was clearly demonstrated in the early 1970s.[1,2] Mature B lymphocytes develop in the bone marrow and migrate to lymph nodes or the white pulp of the spleen. These resting B cells express specific immunoglobulin receptors for antigen on their surfaces, i.e., the B-cell antigen receptor or BCR. It has been estimated that each B cell carries a low number, about 10, identical receptors (IgM and IgD) in association with the co-receptors CD79a and CD79b. Following stimulation with a number of different

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agents, B cells are activated to proliferate, differentiate into plasma cells, and secrete copious amounts of antibody molecules. These antibody secreting cells make immunoglobulins (Ig) that carry the specificity of the original responding B cell. A large amount of Ig in the gamma globulin fraction of serum is made by B and plasma cells, so much so that the amount approaches ~25% of the serum level of albumin, made by the ~three pound liver. There are both short-lived antibody secreting cells that only live for days and long-lived plasma cells, thought to be important contributors to the large amount of antibody that is made, reviewed in Ref. 3. Plasma cells are arrested in G1 of the cell cycle, held there by the cyclin dependent kinase inhibitor p18INK4c that acts on cdk4 and 6.[4] Tumors of plasma cells like myeloma are able to bypass growth arrest and continue to secrete Ig while proliferating. The time course of mouse B-cell stimulation with naïve splenic B cells plus 10–50 ug/ml of LPS *in vitro* are summarized in the literature[5–8] and here in Table 1. Approximately 25–50% of the naïve B cells are converted to B220loCD138+ cells in 72–86 h after LPS treatment.

Addition of other exogenous factors besides LPS can induce Ig secretion *in vitro*. These include CD40:CD40L engagement;[9] CD40:CD40L+ IL10, which also induces isotype switching (10); CD27:CD70 interaction, which acts later than CD40 engagement, after proliferation, to promote IgE secretion;[11,12] OX40 ligand cross-linking;[13] addition of IL-5 (Igh mu production only) versus IL-5 plus IL-2, where Igh mu and J chain are both induced;[14] addition of TNF-alpha to tonsular B cells within the first 24 h of culture;[15] and IL-6 (BCDF) addition to human CESS or SKW B cells.[16] IL-21 and Stat3 have emerged as potent inducers of terminal differentiation to antibody secretion in human cells.[17] Exogenous inhibitors of Ig secretion include: LPS plus anti-Ig,[18,19] LPS plus anti-Ig mu,[20,21] and Interferon gamma.[20]

II. FACTORS LEADING TO ANTIBODY SECRETION

A. Early Drivers of Differentiation

Early on, opposing suites of transcription factors were shown to either maintain the B-cell program (e.g., Pax5, Bach2, Bcl6) or promote and facilitate differentiation to antibody secreting cells (e.g., IRF4, Blimp-1, XBP1).[22] A number of excellent reviews on the action of IRF4, Blimp-1, and XBP1 have been published.[17,22,23] Recently, several newly described pathways of inhibitors[24,25] and activators[26–28] of plasma cell differentiation have emerged. In Table 2, we list the genes described in the text that are involved in production of the various classes of B cells and long-lived plasma cells and briefly summarize their mode of action.

Based on work with conditional knockout mice by a number of investigators, activation of IRF4 and Blimp appear very early in the differentiation of B to plasma cells. IRF4 is necessary for lymphocyte activation and immune responses in B and T cells;[29] mice with a deletion in IRF4 in germinal center B cells are unable to differentiate into memory B cells and plasma cells.[30] IRF4 is one of a few specific transcription factors necessary for myeloma cell survival, along with Blimp-1, ELL2, sub1, and CCNC/ cyclin C.[31]

High levels of IRF4 are required for the development of antibody secreting cells in a B-cell intrinsic fashion[32] and to control the transcription of a number of plasma cell specific genes with an “interferon-like” signature.[33] Low levels of IRF4 promote germinal center formation and class switch recombination.[34] IRF4 can bind with high avidity to the 3′ enhancers of both kappa and lambda Ig light chains in conjunction with the ETS-family transcription factor PU.1 or the closely related factor Spi-B.[35,36] IRF8 and IRF4 recognize the same DNA sequence and can both interact with PU.1. However, the outcome of these interactions is different; the complex of IRF8 and PU.1 (high in B cells) promotes Bcl6 and Pax5 gene expression while repressing Aicda and Blimp-1.[24] Thus, the competition between IRF4 and IRF8 for PU.1 binding is in part responsible for controlling terminal B-cell differentiation.

Blimp-1 (aka PRDM1) is a negative transcription regulator that represses beta-interferon expression in other cells; in activated B cells, it is necessary to turn off Pax5, CIITA for MHC class II expression, c-myc,[37] and Ets-1.[38] Turning off c-myc stops cell proliferation; c-myc conditionally deficient B lymphocytes hypersecrete IgM and do not undergo Ig class switch recombination.[39] Blimp-1 is thought to work by downregulating Pax5 expression, which then turns on XBP1, a transcription factor important in the upregulation of unfolded protein response (UPR) components.[40] Ig secretion in a Blimp-1 knockout is low; this is thought to be indirectly through the downstream effects of Pax5,[41] but may also involve the persistence of myc allowing cell cycle progression.

The micro-RNA, miR-155, is required for the initiation of plasma cell development from B cells because it normally inhibits PU.1 expression through a sequence in the 3’UTR of PU.1 mRNA; this then leads to Pax5 downregulation with the subsequent downstream events as in Blimp-1 mediated suppression of Pax5.[28] Removing the target in PU.1 mRNA obviated the repression by miR-155. This study was important in that it also established that there is a network of cellular adhesion genes modulated by PU.1 controlling B- and T-cell interactions.

The AP-1 transcription factor Fra1 appears to inhibit Blimp-1 expression by binding to its promoter. When Fra1 is deleted in B cells, there are more plasma cells and an exacerbated antibody response; so Fra1 acts as a negative regulator of plasma cell differentiation.[25]

In mature plasma cells, the endoplasmic reticular (ER) response is unique from that seen in other cells.[42] The unfolded protein response (UPR) in many cells typically has three arms, i.e. the IRE-1/ XBP1 pathway, an ATF6 pathway, and the PERK pathway.[43] But PERK knockout mice secrete normal amounts of Ig, while PERK protein expression is not changed significantly between B and plasma cells.[44,45] In addition, ATF6 (activating transcription factor 6) is not necessary for the development of antibody secreting cells; thus, when B cells are stimulated to secrete antibody, the primary pathway for ER remodeling appears to reside in the IRE1 to XBP1 pathway.[46] Aggregation and then auto phosphorylation of IRE1 causes it to acquire the ability to specifically cleave and then splice XBP1 mRNA; the newly spliced XBP1 RNA species encodes a novel XBP1 protein with transcriptional activity on its own promoter and other UPR promoters containing the element UPRE.[44] In an XBP1 conditional deletion, the mice show defects in plasma cell development[47] and low levels of secretory Ig.[48] But it has been argued that the consequences of XBP1 deletion alone

are relatively mild.[49] Antibody secreting cells are present in normal frequencies in resting and immunized animals and Ig secretion is reduced but not eliminated in conditional XBP1 knockouts. Thus, the gene regulatory program controlling plasma cell differentiation may proceed relatively normally in the absence of XBP1.[49]

On further analysis, the low levels of Igh mRNA in XBP1$^{-/-}$ mice result from the eightfold increased levels of IRE1-P over control; the highly abundant IRE1-P cleaves the Igh mu secretory mRNA.[50] This is a process similar to the previously described pathway[51] in which IRE1-P can act to cleave its own mRNA, as well as other RNAs in a process called RIDD.[52] Only XBP1 mRNA is spliced, not cleaved, by IRE1-P to form a new functional RNA.[53] A double deletion of XBP1 and IRE1 restores IgM secretion by inhibiting Ig mRNA degradation.[50] Mutations in the IRE1 nuclease function cause only a twofold reduction in Ig secretion.[54] Taken together, this leads to a conclusion that some Ig secretion can occur without the unusual cleavage and splicing of XBP1 and there may be other proteins that allow for the upregulation of the UPR besides the spliced mRNA encoded XBP1. As we discuss below, ELL2, a transcription elongation factor, has a role in enhancing the transcription of other UPR proteins through the UPR element[26] thereby linking production of the Igh secretory mRNA and the buildup of the UPR. Activation of the mTOR pathway can also bypass XBP1 for Ig secretion.[27]

B. Production of Igh Secretory mRNA and Transcription Elongation

Antibody secreting cells differentiate from B cells, in which the Ig molecules are predominantly on the surface of the cell as antigen receptor, into antibody secreting factories. The shift in protein expression is reflected in the level of the production of the two Ig heavy chain mRNA isoforms, encoding either the secretory-specific or the membrane-specific forms; these are derived by alternative processing of the primary transcript of the immunoglobulin heavy chain (Igh) gene. The regulation of alternative Igh RNA processing has served as a model system for revealing the competition between *cis* and *trans*-acting RNA factors influencing splicing and polyadenylation, reviewed in Refs. 55 and 56.

ELL3, a shorter transcription elongation factor and homolog of ELL2, is expressed at a higher level in B cells and is reduced when B cells begin to secrete Ig. After IRF4 and Blimp-1 induction, there is a rise in the level of the transcription elongation factor ELL2 (eleven-nineteen lysine rich leukemia gene 2). ELL2 binds to the RNAP-II on the Igh gene, impels high levels of Igh mRNA production, and drives alternative processing at the promoter proximal, secretory-specific poly(A) site {sec poly (A)}.[57] It does this by enhancing modifications to RNA polymerase II, activating histone modifications, and subsequent downstream events.[58] The Igh sec poly(A) site, essentially hidden in B cells, is found by a complex of elongation factors including ELL2 and polyadenylation factors in plasma cells. Thus, elongation enhances the production of the shorter Igh secretory mRNA because the poly(A) site comes first in the transcript. This leads to a massive increase in efficient mRNA processing from only a twofold increase in the number of polymerase passes over the gene.[59] Both the 5’ splice site in the last sec exon and the sec poly(A) sites in Igh are weak and in competition;[60] but splicing is decreased in plasma cells.[61] A shift in the relative distribution of serine/arginine-rich proteins is seen from the activating type

(ASF/SF2) in B cells to the repression type (SRp20) in plasma cells.[61] The factors hnRNP F and snRNP U1A protein, both of which block the secretory-specific poly(A) site from functioning, are reduced in plasma cells,[62–65] thereby allowing the Igh secretory poly(A) site to function better. Numerous genes contain multiple poly(A) sites;[66] many are subject to regulation;[67,68] Blimp1 is one such gene[69] although the functional significance of the different isoforms is not clear. It has been estimated that up to 20% of the human genes may be arranged with a competition of splicing and polyadenylation sites as seen in the Igh genes.[70,71] The advancing developmental stage is generally correlated with use of promoter distal poly(A) sites in those multi-poly(A) site genes.[72,73] Yet, the opposite situation applies as the B cell terminally differentiates into plasma cells; thus, the changes seen in the expression of elongation factors may hold the key to understanding this poly(A) site choice paradox. The amounts of the polyadenylation factor CstF-64 also increase following B-cell stimulation[74] as cells go from Go to an actively growing state[75] that can stimulate overall polyadenylation.

Transfection of B cells with either Blimp-1 or IRF4 cDNA caused elevated expression of the mRNA for transcription elongation factor ELL2.[31,34,41] But in luciferase constructs driven by the ELL2 promoter, IRF4 was most efficient at driving its transcription, along with NF-kB p65.[26] Studies of the ELL2 promoter (–1142 to +154) show the presence of several putative NF-kB binding sites and cyclic AMP response elements; the promoter responds to these and to an HTLV-1 viral oncoprotein, Tax, in T cells.[76] The effect of Blimp-1 on ELL2 transcription may be more indirect.

The addition to B cells of exogenous cDNA for ELL2 stimulates alternative RNA processing resulting in the use of the Igh secretory-specific poly(A) site and the skipping of weak alternative exons in Igh and test substrates.[57] ELL2 drives the association of positive transcription elongation factor b (pTEFb) to RNAP-II, thereby increasing phosphorylation of ser-2 on the carboxyl-termini of the elongating polymerase near the start of the Igh locus.[57–59] The binding of ELL2 with the highly phosphorylated carboxyl-terminal domain of RNA polymerase II, polyadenylation factors, Dot1L (the histone H3K79 methylase), and the histone H3 K79me3 modifications are seminal to the changes in RNA processing seen at the Igh locus.

Using deep mRNA sequencing, the knockdown of ELL2 by siRNA in a plasma cell line was shown to influence several other genes besides Igh secretory specific mRNA processing, namely, several splicing factors, cyclin B2 (Ccnb2), and the B-cell maturation antigen (Tnfrsf17) aka BCMA.[77] Long-term survival of plasma cells is impaired by the lack of BCMA in a knockout mouse.[78] But loss of BCMA alone in $^{-/-}$ mice does not alter humoral responses (T-independent or T-dependent) nor the formation of short-lived plasma cells,[79] yet loss of ELL2 in mice does.[26]

The B-cell-specific ELL2 conditional knockout mice (ell2$^{loxp/loxp}$ CD19cre/+) exhibit curtailed humoral responses both in NP-ficol and NP-KLH immunized animals; recall responses were also diminished.[26] The number of immature and recirculating B cells in the bone marrow was increased in the conditional knockouts while plasma cells in spleen and bone marrow are reduced relative to control animals. Production of LPS *ex vivo* stimulated

B220loCD138+ cells from ELL2 deficient mouse spleens is fourfold less than from control splenic B cells. The resulting cells have a paucity of secreted Igh, and distended, abnormal appearing ER. IRE1-alpha is efficiently phosphorylated but the amounts of Ig kappa, ATF6, BiP, Cyclin B2, OcaB (BOB1, Pou2af1), and XBP1 mRNAs, unspliced and spliced, are severely reduced in ELL2 deficient cells. Transcription from the cyclin B2 and the canonical UPR promoter elements in luciferase reporters are upregulated by ELL2 cDNA. Thus, ELL2 has direct effects on those genes. BCMA expression is curtailed in the knockouts by as yet undefined mechanisms.

C. Other Genetic Factors for Secretion

Bright, for B-cell regulator of Ig heavy chain transcription, binds to the A:T rich sequences in the Igh enhancer region and the 5' flanks of some Vh promoters; Bright is induced after B-cell stimulation[80] to enhance Igh transcription. Constitutive Bright expression skews B cells toward marginal zone cells at the expense of follicular cells[81] while deletion of Bright causes embryonic lethality and loss of B lineage development.[82]

Recently ABF-1, aka activated B-cell factor or musculin, was found to suppress plasma cell differentiation.[83] ABF-1 was previously shown to bind to the E-box element in DNA, form a homo- or heterodimer with E12 or E47, and repress E47 transcriptional activity.[84] While plasma cell differentiation was suppressed by overexpression of ABF-1, memory and germinal center cell formation was enhanced; Blimp-1 downregulates ABF-1 expression.[83] Thus, the E-box in the Igh enhancer and modulation of Igh transcription may play a role in the germinal center, memory versus plasma cell fate decision. Interestingly, Myc has also been hypothesized to act through binding to E-box elements,[85] which represent a crucial point of regulation.

In contrast to ABF-1, ectopic Zbtb20 expression in primary B cells facilitates terminal B-cell differentiation to antibody secreting cells. In plasma cell lines, Zbtb20 induces cell survival but blocks cell cycle progression. Zbtb20 induction depends directly on IRF4 binding to the Zbtb20 promoter; but not on Blimp-1 expression.[86] Depending on the adjuvant used for immunization with an antigen, Zbtb20 deficient cells responded differently. Adjuvants that activate TLR2 and TRL4 restored antibody production through induction of other survival programs,[87] leading to the conclusion that plasma cell lifetimes are driven by the primary activation conditions. Zbtb20 represses IkB alpha after TLR activation, suppressing a suppressor to promote NF-kB activation.[88]

In chicken DT40 B cells, a knockout of histone deacetylase 2 (HDAC2), but not histone deactylase −1, caused the B cells to begin to secrete Ig mu, changing transcription and mRNA levels.[89] Histone deacetylase-2 does this by controlling the expression of the Igh and light chain genes through EBF1, Pax5, and Aiolos downregulation and by E2A upregulation of their expression.[90] In chicken DT40 B cells, deficiency of GCN5, a ubiquitous histone acetyl transferase that normally promotes transcriptional activation, caused a decrease in IgM surface and secreted protein expression; light chain was unaffected. By chromatin immuno-precipitation, GCN5 was bound to the Igh mu gene but not the light chain gene.[91] Thus, the acetylation state of the histones also regulates Igh expression.

There are two cannabinoid receptors, i.e., CB1 and CB2; while CB1 is expressed in the nervous system, CB2 is expressed predominantly in B and T lymphocytes and in monocytes with B cells having the highest levels of the receptor. Cannabinoids directly induce B-cell class switching from IgM to IgE through a mechanism involving CB2 receptors, thereby biasing toward Th2-type immunity.[92] Initially, both delta9-tetrahydrocannabinol (THC) and anandamide (an endogenous cannabinoid) were shown to be immunosuppressive in primary and secondary antibody formation *in vitro* via their dose-dependent action on the CB2 receptor.[93] But the opposite conclusion was reached using a human B-cell line that required IL-6 to induce Ig secretion; antagonism of the CB2 pathway suppressed IgM secretion implying that activation of CB2 stimulates secretion.[94] With the recent interest in the medical uses of marijuana in myeloma therapy, this topic should be explored further.

D. Expansion of the Endoplasmic Reticulum and Chaperones

It has been assumed that expansion of the endoplasmic reticulum and chaperones in the unfolded protein response are independent of the shift to Igh sec mRNA production but recent data indicate there is a tighter linkage in their control than was previously thought. When a B-cell line (L29mu+) was stimulated to convert to the plasma cell phenotype, it did so in a multistep process; proteomic analyses showed that the metabolic capacity and secretory machinery were put into place prior to the mass production of Ig that normally follows (Table 1 and Ref. 8). When B cells deficient in secretory-specific mu protein ($AID^{-/-}$ $mus^{-/-}$ or $mus^{-/-}$) were stimulated with mitogens, they showed reduced ability to differentiate into B220loCD138+ antibody secreting cells and reduced survival.[95] But the absence of secretory Ig in the knockouts did not prevent XBP1 accumulation, or XBP1 splicing, which was then assumed to precede upregulation of secreted Ig.[44,95] However, in the ELL2 conditional knockouts, overall XBP1 production and its cytoplasmic splicing by IRE1-P, as well as ATF6, BiP, and cyclin B2, are reduced along with Igh. ELL2 may influence a potential coordination of regulation in Ig secretory protein production and the secretory apparatus itself.

The mechanistic protein target of rapamycin (aka mTOR) acts as a serine-threonine kinase on several proteins including: ribosomal protein S6, Akt, and 4EBP1, a binding protein for eukaryotic translation initiation factor eIF4E; dissociating eIF4E from the binding protein on phosphorylation activates mRNA translation. When mTOR was either knocked out in B cells or expressed at reduced levels by a hypomorphic knockin mutation, the mice showed decreased germinal center formation, lack of both somatic hypermutation and heavy chain class switching, and reduced ability to develop high-affinity antibodies for specific antigens.[96] Germinal center functions are therefore subject to regulation via mTOR.

The kinase mTOR is negatively regulated by the tuberous sclerosis complex (TSC) and deletion of it makes mTOR hyperactive. When mice were conditionally deleted for XBP1 and the tuberous sclerosis complex there was enhanced Ig synthesis and differentiation into plasma cells by the double knockouts.[27]

The typical abnormalities in the ER seen in XBP1 and ELL2 knockout CD138+ plasma cells were gone in the XBP1 and tuberous sclerosis complex double knockouts. Thus, hyperactive mTOR may compensate for loss of XBP1.[27]

The balance between Igh and light chain protein synthesis must be maintained as well. The treatment of plasma cells with an siRNA to lambda light chain caused an imbalance of H and L amounts, reduction in Ig secretion, and induction of effector caspases leading to cell death.[97] Thus, if the unfolded protein response is not fully balanced, or perhaps too vigorously induced, antibody secreting cells may die.

III. FACTORS IN LONGEVITY OF ANTIBODY SECRETING CELLS

The intrinsic survival capacity of short-lived antibody secreting cells from the spleen versus those found in the bone marrow was found to be similar *in vitro* in a supportive stromal environment; this suggests that extrinsic factors in the environment of the spleen versus bone marrow may have the greatest contribution to their differential survival.[98] But long-lived plasma cells have intrinsic CXCR4, which allows them to interact with CXCL12 in bone marrow stroma, retaining them there. However, manipulating CXCL12 by addition or neutralization had minimal effects on the survival of the antibody secreting cells *in vitro*;[98] thus, other factors also must play a role. Kruppel-like factor 2 controls homeostasis of B cells and homing of plasma cells to the bone marrow presumably through its action on beta-integrin expression.[99] Soluble survival factors like BAFF and APRIL (tumor-necrosis factor ligands) in the bone marrow help antibody secreting cells so they do not undergo apoptosis and seem most effective in the bone marrow.[100] Most striking is that most of the long-lived plasma cells appear to come from the follicular B cells and undergo a series of steps to arrive in the bone marrow where the environment is hypoxic. This leads to the question—are Ig secretion, location, and hypoxia linked in long-term plasma cell survival?

A. The B-Cell Location and History Influence Longevity

1. Marginal Zone Cell Responses—B cells mature in the bone marrow and travel to specific regions of the lymph node or white pulp of the spleen. Specific surface markers are associated with the B cells located in different regions of the spleen or lymph node, which may alter their functioning,[23] for example, the marginal zone (MZ), B cells are CDd1+, CD9+, and CD21+, versus those in the lymph node or splenic follicles FO. CD1 is involved in presentation of lipids to T cells. CD9 can modulate cell adhesion, while CD21, in association with CD19, is the C3d complement receptor, which can co-activate B cells when engaged along with antigen: Ig complexes. In the marginal zone, there are also dendritic cells and macrophages to produce cytokines and cell surface ligands. B cells entering the MZ tend to react to T-independent, bacterial cell wall antigens, produce primarily IgM, and display a lower activation threshold than their follicular B-cell counterparts, most likely through the co-receptor engagements. This leads to a heightened propensity for cell differentiation that may contribute to an accelerated primary antibody response.[101,102] If activated plasmablasts arise and remain in the marginal zone of the lymph node, there is a high probability that they will produce short-lived plasma cells, which will undergo apoptosis in a brief period of time (days) following stimulation. This may be because they either self-destruct through failure to remove an overload of internally derived reactive oxygen species (ROS) or because they lack receptors for external survival signals, or both. The apoptosis of most short-lived plasma cells is effector caspase-3 and −9 independent.

Cell death occurs in a poorly understood mechanism that is triggered by excessive protein production; meanwhile, caspase-12 links ER stress and apoptosis of plasma cells.[103]

2. Follicular B-Cell Responses—Follicular B cells (FOs) express lower levels of CD21 (complement receptor 2) than do MZ B cells and can be divided into two types, i.e., type I and type II.[104] Follicular type I B cells express high IgD and low IgM. The type II follicular B cells express high levels of surface IgM, IgD, and CD23a; their unique responses to calcium require the Kruppel-like factor 2 gene to distinguish them from marginal zone B cells.[99] CD23a is the low-affinity receptor for the Fc portion of IgE; it is not clear how this plays into the reactivity of these cells but it may facilitate antigen presentation to B cells by CD11c+ cells. Follicular B cells require CD40-CD40L dependent T-cell help to promote effective primary immune responses, to undergo antibody isotype switching, and to establish high-affinity B-cell memory[105] as they pass through the germinal center. Once activated, FOs undergo class switching and acquire homing signals like CXCR4; they may then travel to specific niches in the bone marrow where CXCL12 expressing stromal cells and soluble factors provide for their survival (months to years) as long-lived plasma cells or memory cells.[106]

The relationship of B-cell memory and long-lived plasma cells (LLPC in Table 2) is still a matter of active investigation. It is clear the initial interactions at the cell surface and in the germinal center are key.[107] Expression of CD73, which catalyzes the conversion of extracellular nucleosides to adenosine, is high in mature germinal center cells and influences the establishment of the long-lived plasma cell compartment.[108] Memory B cells are long lived and retain the ability to self-renew and can be divided into at least two varieties. CD80+ PD-L2+ memory cells differentiate into antibody secreting cells rapidly without new germinal center formation while CD80-PD-L2 memory cells robustly seed the germinal centers and produce antibody secreting cells more slowly.[109] Both CD80 and PD-L2 are members of the B7 family of surface molecules and can be co-inhibitory or co-stimulatory. Viral particles can also drive memory B cells to differentiate into secondary plasma cells,[110] presumably through interactions with surface receptors.

T:B interactions are important not only for activating plasma cells, but also for modulating their activities. CD28, B7.1, and B7.2 deficient mice each produce higher levels of antibody than do wild-type mice. They do this by increased production of both long- and short-lived plasma cells, more antibodies per cell, higher levels of survival factors, and lower levels of the apoptosis inducer caspase-12.[111]

The widely studied lipopolysaccharide (LPS) activation of splenic B cells *ex vivo* through TLR4 may most closely resemble the MZ plasma-blast reactions while B-cell receptor engagement by anti-IgM may mimic follicular cell responses, based on surface markers engaged. It is interesting that in murine lupus models, the anti-DNA antibodies arise from short-lived plasma blasts (TLR activation) while the anti-RNA and anti-cardiolipin antibodies arise from long-lived plasma cells,[112,113] so in this case the triggering antigens and the longevity of the antibody secreting cells differ based on their anatomical location and the nature of receptors engaged.

3. Deciding Where to Go and What to Do—A few transcription factors have recently been implicated in directing the MZ versus FO fate. Overexpression of the Bright transcription factor for Igh drives cells to the marginal zones versus the follicles,[81] so the level of surface Igh may play a role in where to go. Bcl-3 functions as a nuclear transcription cofactor and may promote or repress expression of some genes. Loss of Bcl-3 in B cells increased the marginal zone pool while decreasing follicular cells; both types were more responsive to LPS but more prone to apoptosis following B-cell receptor stimulation.[114] Early B-cell factor 1 (EBF1) binds DNA and functions as a tissue-specific regulator of chromatin structure at B-cell-specific genes.[115] While EBF1 is important in early B-cell development, it is also responsible for enhancing the production and maintenance of marginal zone, B-1 B, follicular, and germinal center B cells.[116] EBF1 deficiency blocks signaling via the BAFF receptor and B-cell receptor dependent Akt pathways; the deficiency can be rescued by c-myb or Bcl-xL overexpression.[117] It is also a previously unrecognized target of somatic hyper-mutation in lymphomas.[118]

B. The Role of Reactive Oxygen Species (ROS)

As mature B cells transition to activated antibody secreting cells, they are signaled through a variety of ligands and corresponding receptors that may differ based on their expression in various locations, MZ versus FO, for example. But all antibody secreting cells undergo rapid and extensive enlargement of their endoplasmic reticulum in order to accommodate increased immunoglobulin production and secretion, the hallmarks of the antibody secreting cell. The increased production of immunoglobulin results in the accumulation of reactive oxygen species such as H_2O_2. Several redundant antioxidant pathways are activated in response to the H_2O_2; these pathways may be necessary to the process of plasma cell differentiation, immunoglobulin secretion, and long-term survival.119 But the cellular sources of the ROS and the functional role of antioxidants (internal and external) in the response are not clear. It is also not well understood how ROS-mediated induction of endoplasmic reticular stress contributes to longevity since there are both positive and negative effects of reactive oxygen species on NF-kB expression.[120]

Mitochondrial metabolism and misfolded protein formation in cells secreting large amounts of protein can induce stress and block cell survival. Mitochondria make superoxide, aka hyperoxide or O_2^-, as a result of the activation of the electron transport chain making ATP; superoxide is converted to H_2O_2 and further converted to H_2O and O by superoxide dismutases, catalases, or peroxidases. Small molecules such as vitamin C, uric acid, glutathione, and polyphenol antioxidants scavenge free radicals to prevent damage from these reactive oxygen species. While their levels are key to survival, they have not been well studied in plasma cell differentiation. Damage to mitochondria by H_2O_2 can cause apoptosis. Bcl-2 proteins are layered on the surface of mitochondria and activate Bax, which punches holes in mitochondrial membranes causing cytochrome C to leak out; Bcl2 binds to Apaf-1 to form apoptosomes. Meanwhile, Bcl6 suppresses Bcl2 via Miz1 and this can lead to apopotosis in lymphoma cells.[121]

In cell membranes, NADPH conversion to NADP+ also generates H_2O_2; NADPH is also found in mitochondria and the endoplasmic reticulum. Lipoxygenase action also stimulates

H_2O_2 production.[122] In large quantities, reactive oxygen species like H_2O_2, superoxide, NADPH oxidase, and disulfide related oxidative species (collectively ROS) can cause organelle and DNA damage leading to apoptosis. In smaller amounts. they are utilized by the cell for signaling and survival.[123] It is interesting to note that autophagy, most likely in response to the need to remove cells damaged by ROS, is necessary for sustainable immunoglobulin production in plasma cells.[124]

Many disulfide bonds in Igs are formed when massive amounts of protein are made. But B-cell differentiation per se does not cause massive thioldisulfide stress to the cells. The steady state levels of protein-glutathione mixed substrates are maintained at very low levels, even in fully differentiated cells, and the overall protein redox state is not affected until late in differentiation, when large-scale IgM production has started and the ER stress response has been activated.[125]

The B-cell receptor (BCR), CD40 and CXCR4 signaling pathways in B cells require reactive oxygen species to activate their downstream targets like JNK, p38, and Akt. High levels of ROS might contribute to excessive B-cell activation or to malignancy[123] versus cell death, which one might have expected. Thus, the role of ROS in differentiating short-versus long-term antibody secreting cells is still enigmatic.

C. Signaling through the BCR

At first glance, signal transduction through the surface Ig serving as antigen receptor should be different for MZ IgM+ plasmablasts and long-lived plasma cells since the bone marrow antibody secreting cells have typically undergone isotype switching and have down-modulated the expression of the alpha (CD79a) co-receptor.[126] The cytoplasmic tail of IgM is much shorter than that of IgG and is less able to interact with signaling molecules without the benefit of the CD79a and CD79b co-receptors. Restoring CD79a and b expression to plasma cells increases surface expression of the BCR by an order of magnitude while still allowing them to secrete copious quantities of Ig.[127] In addition, FO derived bone marrow plasma cells have higher affinity BCRs than MZ cells, having passed through the germinal centers and experienced affinity maturation, so the balance between affinity of the surface receptors versus amounts is different. Thus, long-lived plasma cells may not be stimulated until recall antigen rises to a high level or the epitopes are very specific, leading to the cell's inherent quiescence and thus survival.

D. Survival Factors

B-cell activating factor (BAFF, TNFRSF13B) is produced by monocytes, dendritic cells, and bone marrow stromal cells. BAFF is first expressed as a membrane-bound protein, which is cleaved to generate a soluble factor. It is the natural ligand of three tumor necrosis factor receptors named BAFF-R (BR3), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), and BCMA (B-cell maturation antigen, TNFRSF17), all of which have differing binding affinities for BAFF.[78] These receptors are expressed mainly on mature B lymphocytes, increase as cells mature, with BCMA expression on plasma cells most important for long-term survival.[78] TACI has higher affinity for a proliferation-inducing ligand (APRIL) than it does for BAFF.[100] BCMA displays an intermediate binding

phenotype and will bind either BAFF or APRI, but signaling through BAFF-R and BCMA stimulates B lymphocytes to undergo proliferation and to counter apoptosis.[128]

Mice with deficiencies in BAFF, APRIL, BAFFR, and TACI all have multiple deficiencies in B-cell maturation and development while the BCMA$^{-/-}$ mice show impaired survival only of long-lived bone marrow plasma cells,[128] indicating its unique function for that compartment. BCMA induces high levels of Mcl-1 in bone marrow but not spleen. Mcl-1 is an anti-apoptotic protein; in mice where it is deficient, the plasma cell compartment is depleted.[129] ELL2 expression links secretory Igh mRNA production and BCMA expression in CD138+B220 low cells.[26,77]

E. Autophagy in Maintaining Survival

The B-cell conditional knockout mouse of Atg5, autophagy related gene 5, had plasma cells with more endoplasmic reticulum, higher expression of Blimp-1, and more Ig secretion; this was associated with less intracellular ATP and fewer antigen specific long-lived bone marrow plasma cells.[124] The defects occur post germinal center; therefore, autophagy must play a role in maintaining plasma cell homeostasis. There is a balance between Ig synthesis with secretion and long-lived plasma cell viability. Secrete a lot and die, or control the secretion and reduce stress and live. The ER is the specific target of auto-phagosomal degradation (reticulophagy) by autophagy related gene 5 while mitochondria and ribosomes are spared. Autophagy related gene 5 transgenic mice are more tolerant to oxidative damage and cell death induced by oxidative stress, and this tolerance was reversible by treatment with an autophagy inhibitor.[130] In contrast to the experiments with culturing of different populations of antibody secreting cells with stromal cells, the autophagy results argue for cell intrinsic regulation of viability.

F. CHOP

CHOP (C/EBP homologous protein aka DNA damage inducible gene 3) is downstream of PERK in the ER stress pathway; as shown in Table 1, it is induced early after B-cell activation *in vitro* and then declines.[42] PERK$^{-/-}$ mice can secrete Ig. CHOP, BiP, and XBP1 share an ER stress response element in their promoters.[43] CHOP causes Bcl2 inhibition (thus inhibiting growth), activation of beclin1 (involved in autophagy), and upregulation of autophagy related gene 5.[131] It is not clear if all of these functions of CHOP are knocked out in the PERK$^{-/-}$ mice. CHOP deficient animals have plasma cells that secrete Ig at a lower rate than wild type, but antibody secreting cell differentiation and lifespan appear normal in the CHOP$^{-/-}$ knockout mice. This appears paradoxical since CHOP seems required for expression of genes like autophagy related gene 5 for plasma cell viability.[132] As a counter to CHOP, Bcl-x_L protects the plasma cells from CHOP-dependent apoptosis, connecting the UPR and cell death.[133] In summary, the interactions of the balance of the ER stress pathways and autophagy mediated by CHOP to control ROS damage to the ER are emerging as noteworthy but still unresolved pathways for understanding the survival of long-lived plasma cells.

G. Hypoxia

1. The Bone Marrow Environment

Following stimulation and germinal center passage, long-lived plasma cells reside in bone marrow niches, which are believed to be hypoxic.[134,135] Hypoxia may be a key contributor to long-lived plasma cell survival through degradation of c-myc, a key cell cycle regulator,[134] to balancing ROS production and autophagy,[135] and manipulating the bio-energetic profile of the cells. In many tissues HIF-1, hypoxia inducible factor, suppresses mitochondrial function in tumor cells, induces glucose transporters and hexokinase, and may modulate glycolytic flux.[136] The switch between glycolysis and oxidative phosphorylation is controlled by the relative activities of two enzymes, i.e., pyruvate dehydrogenase and lactate dehydrogenase. More ATP is produced through oxidative phosphorylation in mitochondria than through anaerobic glycolysis in the Krebs cycle, which dominates when oxygen is limited.

In addition, CD73 has an important and previously unrecognized role in modulating the establishment of the long-lived plasma cell compartment through securing more adenosine for ATP.[108] Although multiple cell types express CD73 and adenosine receptors, CD73 is markedly upregulated on both germinal center B and T cells. Hypoxic environments and increased extracellular ATP define the germinal center compartment as well. HIF-1alpha expression is seen in germinal center B cells and promotes CD73 expression.

2. Lessons from Multiple Myeloma

Multiple myeloma is a tumor of plasma cells that is responsible for almost 10% of hematological malignancies and is characterized by bone lesions and excess Ig secretion. Concentrations of Ig in the serum in patients can approach that of serum albumin.[137] Most myelomas have a translocation of the Igh enhancer into genes like myc, ras, maf, cyclin D, etc., to activate expression of those as oncogenes[138] while still secreting Ig. Thus, a cessation of growth is not a requirement for Ig secretion. Extrinsic cytokines made by the bone marrow stroma that aid in myeloma survival are IL-6, IL-10, IL-11, ciliary neurotropic factor, and oncostatin M leukemia inhibitory factor.[139] IL-6 has been shown to be important for long-term survival of tonsular plasma cells expressing CD27 and CD38 in organ culture even from non-myeloma patients.[140] Too much IL-6 made by the myeloma cells can activate osteoclasts, erode the bone, and cause the characteristic lesions seen on X-ray; in a mouse model, this is driven by c-myc or Bcl-$_X$L.[141]

Intrinsic survival factors for myeloma cells include IRF4 and a limited set of transcription factors, namely, sub1, ELL2, CCNC, and PRDM1 (aka Blimp-1).[31] BCMA and caspase-3 expression in myeloma cells are also strong survival factors. Many genes active in multiple myeloma undergo alternative splicing or polyadenylation patterns.[31] As part of their survival strategy, these plasma cell tumors reside in hypoxic niches, which may present a unique target for anti-myeloma therapy.[142] CCNC or cyclin C, in both myeloma and long-lived plasma cells, interacts with CDK8 in the mediator complex to phosphorylate the carboxyl terminus of RNAP-II. CCNC is important not only for myeloma survival, but also for the expression of Hif1 alpha and beta regulated genes. In hypoxia, Hif1 and CCNC act, along with the super elongation complex, composed of pTEFb and ELL2, to accelerate

transcription of a subset of hypoxia inducible genes in many cell types.[143] This is illustrated in Fig. 1. Suppression of Hif1 blocks tumor growth and stops bone destruction by excess IL-6 production.[144]

IV. CONCLUSIONS

Ig secretion and longevity are influenced by a variety of parameters. Terminal Ig secreting cells are arrested in G1 in a normal immune response; tumor cells bypass this cell-cycle block and still secrete Ig. The evidence points to the fact that short-lived plasma cells "overcommit" to secretion and thereby cause damage to the ER with reactive oxygen species unless they receive signals from their environment to balance out secretion and stress. Use of antioxidants supplied exogenously may help to mitigate this damage. A balance between heavy and light chain protein synthesis is also crucial indicating tight coordinate control. Damage to the ER brought about by the stress imposed by secreting large amounts of Ig can be countered by autophagy, which helps to remove damaged organelles. The role of the cannabinoid receptor CB2 in influencing Ig secretion is also of interest based on the increasing legality of marijuana use.

Cells that have experienced T-cell help during their activation in the germinal centers are more likely to migrate to bone marrow niches by virtue of CXC4:CXCL12 interactions. Once in the bone marrow, expression of the surface receptor BCMA, in the Tnf family, stimulates survival from signals made by the stromal cells. Exogenous application of BAFF and APRIL survival signals in some critical circumstances may increase long-lived plasma cell longevity. Experiments point to the hypoxic environment of the bone as contributing to the long-term survival of plasma cells that reside there. The details of the relationship between hypoxia, autophagy, Ig secretion, and longevity will require further exploration. The transcription elongation factor ELL2 links secretion, the unfolded protein response, and hypoxia by virtue of its action on the RNA polymerase and interactions with mediator complex factors. ELL2 is important for normal plasma cell development and crucial for survival of myeloma cells. It may represent a promising target for therapy in myeloma, an as yet incurable disease.

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ABBREVIATIONS:

BCMA	B-cell maturation antigen, aka tnfrsf17, CD269
Blimp-1	B lymphocyte maturation protein 1, aka PRDM1
cKO	conditional knockout; for ELL2 this is CD19cre/+ ell2loxp/loxp in exon 1 or exon 3
c-myc	cellular myelocytomatosis proto-oncogene

ELL	eleven-nineteen lysine-rich leukemia gene in the ELL family of three genes (1, 2, 3), part of the biochemically defined super elongation complex
ER	endoplasmic reticulum
FO	follicular
Igh	immunoglobulin heavy chain
IRF4	interferon regulatory protein 4
LLPC	long-lived plasma cell
LPS	lipopolysaccharide, a pan B-cell activator
mTOR	mammalian target of rapamycin
MZ	marginal zone
sec (lowercase)	secretory-specific form of Igh mRNA or protein
SEC (uppercase)	super elongation complex
pTEFb	positive transcription factor b composed of cdk9 (a kinase) and cyclin T1 or 2, the enzymatic part of SEC
RNAP-II	eukaryotic RNA polymerase II
ROS	reactive oxygen species
UPR	unfolded protein response
XBP1	X-box protein 1

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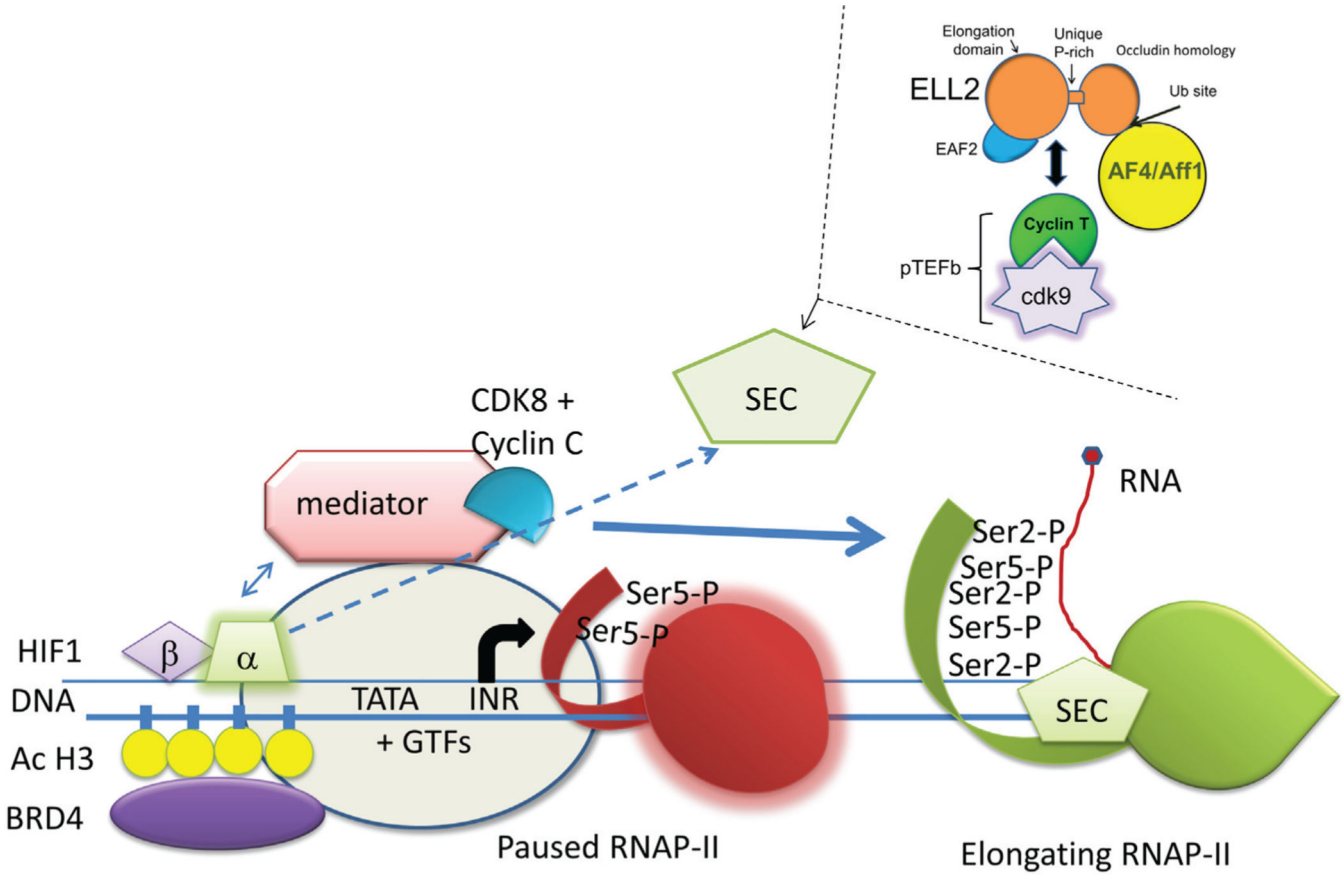

FIG. 1:
Hypoxia regulation with CCNC, cdk8, super elongation complex

TABLE 1:

Changes in naïve splenic B cells following LPS stimulation *in vitro*

			Igh mu									
Hours > LPS	**% S phase**	**^{3}H TdR incorp**	**J chain mRNA and protein**	**mRNA**	**% sec**	**Sec protein ng/ml***	**Redox balance**	**ER resident proteins**	**Metabolism**	**Cyto and mito chaperones**	**CHOP mRNA**	**ELL2 mRNA**
0	1.2	nd	1×	1	<10	–	1	1	1	1	1×	1×
24	8.5	1×	1×	1×	<10	0.9	4×	1.5×	1.9X	5×	7×	1×
48	28.4	18×	1×	5×	61	–	4×	2.7×	2.5×	2.5×	2×	6×
72	40	74×	4×	30×	>90	85	6×	4×	4×	2.5×	2×	10×
84–96	59	86×	118×	30×	>90	1100	11×	6×	3.7×	2×	nd	nd
120	nd	45×	400×			7300						

*Supernatant from 4 × 10^{6} cells/ml initial cell concentration. For references and further details see text.

TABLE 2:

Factors important for antibody secretion and/or survival

	Expressed in or important for				
Factor/gene	**B cells**	**MZ cells**	**FO cells**	**LLPC**	**Comments**
ABF1, GCN5	+				Blocks further development if not shut off
ELL3	+				On from stem cells stage, shut off after stimulation
EBF1	+	+	+		Mediates choice of MZ vs FO fate
TLRs, CD19/21		+			Engagement actives cells
Bcl3			+		Mediates choice of MZ or FO fate
CD40:CD40L			+		Interactions help activate cells
CHOP		+	+	+	Turns off Bcl2, turns on Atg5
Cytokines, BCR, NF-kB		+	+	+	Important for activation and survival
IRF4, Blimp-1		+	+	+	Help shut off Bcl6, pax5
ELL2		+	+	+	Drives Igh secretory mRNA, UPR, BCMA, involved in hypoxia
XBP1		+	+	+	mRNA spliced by IRE1P to make transcription factor for UPR genes
Zbtb20		+	+	+	Promotes NF-kappaB activation
CB2 stimulation		+	+	?	Necessary for secretion, effects on survival not measured
HDAC2 deletion		+	+	?	Done with chicken DT40 cells, effects on survival not measured
mTOR		+	+	?	Necessary for secretion, effects on survival not measured
Atg5				+	Important for autophagy
BCMA				+	Induces MCL-1, an anti-apoptotic protein
CXCR4:CXCL12				+	Important for retention in bone marrow
CD73			+	+	Converts extracellular nucleosides to adenosine
hypoxia			?	+	Promotes CD73 expression, modulates metabolism
Kruppel-like factor 2			+	+	Control beta integrin expression

MZ are marginal zone B cells, FO are follicular B cells, LLPC are long-lived plasma cells. See text for references and details.