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Abstract: The ability of cells to sense and adapt to changes in oxygen is mediated by hypoxia-inducible factor (HIF). Immune cells function in physiologically complex and varying environments whereby oxygen, pH, nutrients, metabolites and cytokines are continuously fluctuating. HIF is well known to play an important role in coordinating the adaptation and function of both innate immune cells and T cells in these complex environments. This review summarises recent discoveries concerning how hypoxia and HIF control B cell behaviour, and regulate antibody quality and decisions concerning tolerance. Hypoxia and HIF activation may provide an important context; coordinating metabolism with variable demands for quiescence, rapid proliferation, and differentiation. Understanding when and how HIF is activated during B cell development and response is important as drugs targeting HIF could influence antibody responses, providing novel therapeutic opportunities for vaccine adjuvants and in treating autoimmunity.

UNIVERSITY OF CAMBRIDGE

Professor Urban Lendahl
Editor-in-Chief
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27 February 2017

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RE: Submission of review article 'Hypoxia and B cells' in memory of Professor Lorenz Poellinger.

Dear Professor Lendahl,

We thank you for the invitation to write a review for this special issue on cellular hypoxia in memory of Professor Poellinger. Our review discusses the recent discoveries regarding the role of hypoxia in B cell immune responses. This is a field that is both of great interest to the lab, and is in its infancy, and we believe that recent findings have paved the way for many significant discoveries to come. A better understanding of HIF in B cell physiology and disease may lead to novel immunological therapies since the HIF pathway is pharmacologically tractable.

This review was written by myself and Professor Patrick Maxwell, whom I am submitting for on his behalf.

With very best wishes,

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Hypoxia and B cells

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Abstract

The ability of cells to sense and adapt to changes in oxygen is mediated by hypoxia-inducible factor (HIF). Immune cells function in physiologically complex and varying environments whereby oxygen, pH, nutrients, metabolites and cytokines are continuously fluctuating. HIF is well known to play an important role in coordinating the adaptation and function of both innate immune cells and T cells in these complex environments. This review summarises recent discoveries concerning how hypoxia and HIF control B cell behaviour, and regulate antibody quality and decisions concerning tolerance. Hypoxia and HIF activation may provide an important context; coordinating metabolism with variable demands for quiescence, rapid proliferation, and differentiation. Understanding when and how HIF is activated during B cell development and response is important as drugs targeting HIF could influence antibody responses, providing novel therapeutic opportunities for vaccine adjuvants and in treating autoimmunity.

1. Introduction

The hypoxia-inducible factors (HIFs) are transcription factors stabilised under low oxygen (hypoxia), enabling cellular adaptation. Hypoxia and HIF play important roles in innate immunity and inflammation. HIF acts as a prosurvival signal in neutrophils and promotes migration, invasion and bactericidal activity of macrophages. In adaptive immunity, HIF has differing effects on T cell differentiation and function, depending on T cell subset and local stimuli (1). There is clear evidence that HIF is modulated in B cell malignancies, but the role for hypoxia and HIF in normal B cell physiology has received much less attention. B cells are exposed to a range of oxygen tensions as they develop, migrate and differentiate. Yet the hypoxic control of B cell fate is not well understood. B cell development and function depend on stage-specific proliferative bursts, rapid growth and differentiation, which requires metabolic reprogramming and cell-cycle regulation. HIF is emerging as an integral part of this. This review discusses how hypoxia and HIF shape B cell function, regulating antibody quality and tolerance. Hypoxia and HIF could provide an important context as to whether antigens that activate B cells are from pathogens or self, and in coordinating metabolism with variable demands for quiescence, proliferation, and variable efficiency of oxidative metabolism. Furthermore, hypoxia and HIF may modulate immunoglobulin (Ig) class preference and affinity by affecting DNA repair. Understanding when and how HIF is activated during B cell development and response is of interest as the HIF pathway is pharmacologically tractable; HIF activators or inhibitors may influence antibody responses, providing novel therapeutic implications for vaccine adjuvants and in treating autoimmunity.

2. Immunity

Host defences are grouped under two arms of immunity; innate and adaptive. Innate immunity provides the first line of defence against infection. It is rapid, non-specific and blocks pathogen entry and eliminates pathogens and toxins. Innate immune cells include natural killer cells, neutrophils, monocytes, macrophages and dendritic cells. Adaptive immunity develops more slowly and involves the expansion and differentiation of antigen-specific lymphocytes. It is characterised by the production of high affinity, class-switched antibodies and immunological memory. High affinity antibodies are produced by affinity maturation. This process involves somatic hypermutation (SHM) of variable regions of Ig genes altering affinity to antigen. Affinity increases in response to prolonged and repeated antigen exposure, with selective survival of high affinity B cells. B cells also change their Ig isotype by class-switch recombination (CSR), producing antibodies with different effector functions. Memory cells generated can undergo rapid expansion on subsequent pathogen encounters providing long-term protection. The random recombination and high mutation frequency that occurs during SHM, and in other gene editing events during B cell development, also yield self-reactive B cells. Under these circumstances, a sophisticated selection mechanism exists to eliminate or modify these B cells, reducing the risk of attacking self, whilst permitting effective defence. This is a delicate balance, which when tipped may lead to autoimmunity, a condition underlying many diseases.

3. The life of a B cell

B cells consist of two lineages, B1 and B2. B1 cells (located in the peritoneum, spleen and at mucosal sites) are principally generated in the foetal liver and are sustained by self-renewal. B2 cells are generated in the bone marrow (BM) throughout life, forming the majority of the B cell pool. Haematopoietic progenitors proliferate, generating large numbers of proB cells that develop into preB cells by undergoing VDJ recombination at the Ig heavy-chain locus. The resulting Ig μ heavy-chain protein binds to surrogate light-chains and, along with Igα/Igβ signalling proteins, forms the membrane-associated pre-B cell receptor (preBCR). The preBCR triggers rearrangement at the light-chain locus and the resulting protein associates with the μ heavy-chain yielding the complete BCR on the surface of an immature B cell. Immature B cells egress from the BM as transitional B cells that migrate via the circulation to the spleen to complete maturation. There are two mature B2 subsets; follicular (FO) and marginal zone (MZ). FO B cells are recirculating and found in the spleen and lymph node (LN) follicles, whilst MZ B cells reside at the margins of splenic follicles. Maintenance of the naïve mature B cell pool depends on survival cytokines such as B-cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL), recognition of self-antigen and BCR signalling strength (2, 3).

Checkpoints occur throughout B cell development to eliminate self-reactive B cells. In the BM, B cells binding self-antigen change their receptor specificity (receptor editing), become anergic or are deleted by apoptosis, in a process called central tolerance. Similarly, autoreactive B cells in the periphery may apoptose or inhibitory receptors may engage, preventing activation. B cells recognising antigen in the absence of T cell help become anergic. Peripheral tolerance is more flexible as it also depends on interclonal competition of BCR signalling strength and on cytokine signals (2, 3).

B1 and MZ B cells respond to T cell-independent (Ti) antigens, whilst FO B cells mainly respond to T cell-dependent (Td) antigens. Td responses occur in two steps, providing immediate and persistent protection. First, B cells undergo class-switch recombination (CSR) and differentiate into short-lived plasmablasts that secrete low-affinity antibodies (extrafollicular response). Second, activated B cells proliferate extensively under the influence of T follicular helper (Tfh) cells forming a germinal centre (GC). GCs are dynamic anatomic sites where B cells undergo diversification of their antigen-receptors by somatic hypermutation (SHM) and selection, producing long-lived plasma cells (PCs) that secrete high-affinity antibodies, and memory B cells (4). Tolerance mechanisms similarly exist in GCs to eliminate self-reactive B cells produced following SHM (2).

4. Hypoxia-inducible factor (HIF)

There is a continuously operating oxygen-sensing system present in all metazoan cells that enables adaptation to changes in oxygenation. The system centres around Hypoxia-inducible factor (HIF), of which there are two isoforms; HIF-1 and HIF-2. HIFs are heterodimeric transcription factors consisting of a constitutively expressed HIF-1β subunit and an oxygen-dependent subunit (HIF-1α or HIF-2α). Under normoxia, HIF-α is hydroxylated on two proline residues by one of three prolyl hydroxylase domain enzymes (PHD1-3) (5). The PHDs use oxygen and 2-oxoglutarate as co-substrates, acting as the oxygen

sensors to HIF. Upon hydroxylation, the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex binds HIF-α leading to ubiquitination and proteosomal degradation (6). HIF-α is also hydroxylated on an asparagine residue in the C-terminal transactivation domain by Factor inhibiting HIF-1 (FIH-1), another oxygen-dependent dioxygenase. This blocks interactions with the transcriptional co-activators CREB-binding protein (CBP)-p300 (7). Hypoxia reduces hydroxylation leading to stabilisation and activation of HIF, which modulates the expression of hundreds of genes (8). The hydroxylation rates of PHDs and FIH are sensitive to oxygen concentrations throughout the physiological range (9). As a result HIF contributes to many physiologic (development, cell metabolism, apoptosis, inflammation and erythropoiesis) and pathologic conditions (ischemia, anaemia, atherosclerosis and cancer).

HIF can also be stabilised by O_2-independent mechanisms as a result of genetic mutations in *VHL* (10), *PHDs* (11) and *HIF2A* (12). A complex bidirectional relationship exists between HIF and other signalling pathways. Of particular relevance to the immune system are the Phosphoinositide-3-kinase (PI3K), Mitogen-activated protein kinase (MAPK) and Nuclear factor-κB (NF-κB) pathways. The PI3K and MAPK pathways increase translation of HIF-α, and HIF transactivation through phosphorylation of HIF-α (13). In turn HIF inhibits PI3K signalling by upregulating REDD1, which inhibits mechanistic target of rapamycin complex 1 (mTORC1) via activation of the tuberous sclerosis 1(TSC1)-TSC2 complex (14). During inflammation, Tumour necrosis factor-α (TNF-α) activates NF-κB that increases transcription of HIF-1α, leading to stabilisation under normoxia (15). HIF has similarly been reported to increase NF-κB activation in anoxic neutrophils (16).

5. Oxygen gradients in lymphoid tissues

B cells circulate between high oxygen levels in the alveolar capillaries (~13.2%), to lower oxygen tensions (pO_2) in mixed venous blood (~5.3%) and the microvasculature in lymphoid tissues, where pO_2 depends on the amount of oxygen extracted (17). Tissue oxygenation is determined by vessel size and density, distance from the nearest vessel, and density and metabolic demand of resident cells. These structural features differ between lymphoid organs, setting a varied hypoxic landscape between lymphoid tissues. Elegant studies using non-invasive imaging techniques of the BM in live mice provide direct evidence for the existence of hypoxic niches and gradients (0.6-2.8% oxygen in extravascular regions). The lowest average pO2 (1.3%) was located deep within the peri-sinusoidal regions, >40μm from the well-vascularised endosteum (18). Oxygen gradients exist in murine spleens and may range from 0.5%-4.5% oxygen (mean 2.3%), with oxygenation being highest near the splenic artery and decreasing with distance (19, 20). The pO_2 of human neck LNs containing metastatic cells ranges from 0.1-3.6%. Although these LNs are pathological, it is possible that normal LNs may be similarly hypoxic (21). These studies support that local hypoxia and oxygen gradients occur in lymphoid tissues, and that the oxygenation differs between lymphoid tissues. This is likely to influence both the level and duration of HIF activation in B cells. The extent to which B cells respond and adapt to changes in oxygenation as they encounter different lymphoid compartments is not well understood.

GCs are hypoxic and enriched for HIF-1α in lymphoid organs of mice and humans (22-25). As cells become progressively further away from vessels, oxygenation falls and cells located >30μm away experience <2% pO_2 (18, 26). Anatomically, the majority of GCs are located ≥40μm away from blood vessels in murine spleens (24) and human GCs are also poorly vascularised (22). An intriguing possibility is that hypoxic gradients could set the location for GC formation and thereby modulate anatomical colocalisation of immune cells and antigens, which is critical for mounting an effective immune response.

Thus over their life cycle, B cells are exposed to a wide range of oxygen tensions across the physiological range as they egress from the hypoxic BM into a well oxygenated circulation, and then encounter hypoxic microenvironments within lymphoid tissues, including GCs (Figure 1). As a consequence, HIF will be activated at key points in the life of B cells, and this could shape and control B cell fate and function.

6. HIF and B cells

6.1. Immune response and metabolism

On antigen encounter, quiescent B cells are activated and undergo clonal expansion and differentiation, eliminating antigen, at which point the response declines restoring homeostasis. These distinct cell phases have differing metabolic needs and fates, influenced by local stimuli, immune cell interaction and antigen type. In other cell types, HIF activation is important in coordinating complex metabolic changes particularly in hypoxia through promoting glycolysis, whilst reducing oxidative metabolism, minimising damage to proteins and DNA. This, combined with the fact that hypoxia-induced HIF is common in lymphoid tissues sets the scene for exciting discoveries that HIF modulates B cell immune responses. This has significant clinical implications, as pharmacological targeting of HIF may be useful in refining vaccine responses or in treating autoimmunity.

Recent studies in mice reveal that the GC light zone is specifically hypoxic, and that this has major effects on T-cell dependent B cell responses. Hypoxia increases glycolytic rate in GC B cells, which reduces proliferation, increases apoptosis, and impairs class-switching. Constitutive HIF stabilisation via deletion of *Vhl* reduces GC B cells and impairs affinity maturation, class-switching to the pro-inflammatory IgG2c isotype, recall response and early memory B cell formation. HIF activation inhibits activation-induced deaminase (AID) expression and MTORC1, a metabolic checkpoint kinase activated by receptors involved in B cell activation, CSR and differentiation. Interestingly, hypoxia has little effect on IgA switching (27). These studies suggest that HIF plays a major role in the quality of antibody response, with hypoxia having substantial influence on class preference and antigen affinity. In this model system, over activation of HIF is detrimental to B cell function. But the existence of hypoxia in normal GCs is likely to be time-limited and of functional benefit, with hypoxia slowing B cell proliferation, and modulating the threshold for T cell-dependent survival signals, clonal competition and affinity maturation.

In contrasting studies, B cells cultured in hypoxia and exposed to different IgG switching-stimuli showed *accelerated* CSR, leading to an early increase in IgG+ B cells (24). Hypoxia may therefore differentially modulate switching kinetics depending on local stimuli. In both studies, switched IgG1+ B cells failed to thrive under hypoxia (24, 27) with hypoxia markedly increasing IgG1+ PC differentiation (24). Possibly the negative effect on switched B cells is secondary to increased PC formation, which prevents over expansion of the PC pool. Collectively these studies argue that precise and appropriate HIF responses are required to maintain B cell homeostasis and optimise the immune response so that it does not become detrimental to the host.

Reinforcing this, studies in hyperoxic mice revealed that the GC reaction was suppressed, and CSR, switched GC B cells, PCs and antibody titres were reduced (24). That hyperoxic mice, where HIF is presumably suppressed, show similar effects to mice with enhanced HIF activation highlights the necessity of getting the hypoxic response "just right" in order to mount an optimal immune response. When oxygen gradients, or the normal response to them, are altered then B cell function is dramatically distorted.

Whilst resting B cells have low metabolic requirements, activated B cells undergo marked changes in their metabolic phenotypes to support rapid proliferation, growth and differentiation. As mentioned, HIF increases glycolytic rate in GC B cells by inhibiting the metabolic checkpoint kinase MTORC1, which reduces proliferation, increases apoptosis and modulates antibody production (27). Supporting studies reveal that rapid proliferation in GCs occurs under both a hypoxic and nutrient limited environment. Collectively these two environmental stressors have major consequences on B cell metabolism and behaviour. GC B cells undergo metabolic adaptation via induction of HIF-dependent glycolysis, increased glucose uptake and mitochondrial biogenesis. This is via Gsk3 which prevents metabolic collapse by restraining c-Myc-dependent glycolysis, and ROS-induced apoptosis in the glucose limited, hypoxic microenvironment (25). Further support for the role of HIF in regulating B cell metabolism comes from studies in mice where the HIF downstream target *Glut-1* was deleted in the B cell lineage. Mice had fewer splenic B cells and reduced antibody titres (28). These data indicate that HIF modulates metabolism where high metabolic demand meets low nutrient availability. Limited oxygen and nutrients may impose a metabolic checkpoint on GC B cells to prevent excessive proliferation, whilst supporting antigen-driven clonal selection and differentiation (25).

Extracellular ATP accumulates in GCs due to the high frequency of proliferation and apoptosis. CD39 converts ATP to AMP, which is converted to adenosine by CD73. Adenosine activates A_2 adenosine receptors on immune cells, leading to immunosuppression. CD73 expression increases on GC B cells and Tfh cells, which is required for the generation of long-lived PCs (29) and in modulating T-B cell interactions (30). Under hypoxia, extracellular adenosine increases owing to altered cell metabolism and upregulation of CD73. These data point to a potential role for hypoxia in ATP metabolism in the GC, which may contribute to shaping B cell responses to antigen.

HIF activation likely has other consequences beyond the immediate GC centre microenvironment. For example, the HIF-induced target Vascular endothelial growth factor (VEGF) is produced in inflamed tissues or draining LNs, leading to expansion of the vascular network, enhancing the transit and

distribution of dendritic cells (DCs) and antigen into draining LNs. Mice with B cells overexpressing human VEGF have enlarged LNs, increased lymphatic and endothelial vessels and accumulation of CD11b+ cells, splenomegaly and disorganisation of splenic structure. These mice have reduced antigen-specific antibody titres when immunised with ovalbumin and reduced pro-inflammatory cytokines in response to LPS (31). Evidence for HIF-induced VEGF in B cells comes from studies where *VEGF* expression was increased in Burkitt's lymphoma cells cultured under hypoxia (32) and in chronic lymphocytic leukaemia B cells that have low VHL levels (33). Further we observe a substantial increase in *VEGF* mRNA in human peripheral blood B cells cultured under hypoxia, and in all B2 cell subsets examined in multiple mouse models of B cell-specific HIF activation (unpublished). Thus HIF activation in B cells may regulate immune responses by modulating angiogenesis and lymphangiogenesis through induction of VEGF.

6.2. Tolerance

HIF is likely to influence tolerance in B cells through dynamic effects on metabolism, cell cycle and differentiation. Early studies in RAG-2-deficient chimeric mice with *Hif1α*-deficient T and B cells showed evidence of autoimmunity, attributed to increased B1-like cells. B cell development in the BM was abnormal, possibly through an inability to maintain sufficient glycolysis (34). Using chimeric mice did not allow distinction between the direct effects of *Hif1α*-deletion in B or T cells but point to the possibility that HIF may negatively regulate B cells preventing autoimmunity. Hypoxia-induced cell quiescence is necessary for maintaining viability in many cell types. In murine splenic B cells, hypoxia induced cell cycle arrest HIF-1 dependently (35). Since B cell development and function depend on stage-specific proliferative bursts, rapid growth and differentiation, these studies highlight the potential role for HIF-1α in regulating B cell quiescence and homeostasis. The hypoxia-induced MicroRNA-210 (MiR-210) that down-regulates proliferation promoting genes, is upregulated in activated B cells by HIF-1α and Oct-2. Mice with B cells overexpressing MiR-210 have impaired proliferation, cell cycle entry and CSR, whilst mice with MiR-210 deficient B cells develop autoimmunity (36). These data further support a role for HIF in controlling B cell activation through the cell cycle, enabling a balance between pathogen clearance and autoimmunity, indicating a role for HIF in maintaining tolerance.

6.3. B-T cell interactions

How GC hypoxia modulates the behaviour of Tfh cells and T-B cell interactions is largely unknown. Hypoxia and HIF have opposing effects on CD4+ T cell proliferation, differentiation and function: HIF promotes differentiation of Th17 cells, whilst Treg differentiation is suppressed under certain environments. The differential effects of HIF on CD4+ T cells appear to be dependent on the local microenvironment (1). A recent study reported that hypoxia increased the frequency and activity of Tfh cells, with the opposite observed in hyperoxic mice (24). The authors proposed this effect to be secondary to that of GC B cells, which may support maintenance of Tfh cells (24, 37). These preliminary findings are first to highlight a potential role of hypoxia on adaptive immune cell interactions. In the GC,

Tfh cells secrete cytokines that support B cell survival (38). Hypoxia differentially modulates many of these cytokines, depending on T cell subset. Hypoxia increased IL-2, IL-4 and IFN-γ in stimulated CD4+ T cells (39), whilst IFN-γ was reduced and IL-10 increased in Th1 cells cultured under similar conditions (40). GC-Tfh cells produce high levels of IL-4 and IL-21 (41), yet how the hypoxic microenvironment of the GC influences cytokine production by Tfh cells remains to be determined. Similarly, the effects of hypoxia on other GC-associated immune cells such as follicular dendritic cells (FDCs) and plasmablasts, is unknown. GC B cells are rapidly cycling and apoptotic, with Tfh cells acting as the principle source of survival cytokines. One possibility is that GC hypoxia may be an environmental requirement that enables different immune cells to perform their distinct but complimentary functions (24).

6.4. Oxygen-independent activation of HIF in B cells

In B cells, HIF-α is likely to be stabilised via signalling cascades activated downstream of the BCR, Toll-like receptors (TLRs) and cytokine receptors. Precedents for this come from studies in T cells and other immune cells: T-cell receptor (TCR) ligation in Th17 cells increases *Hif1α* mRNA via a STAT3-dependent mechanism and HIF-1α protein via activation of PI3K/MTORC1. In CD8+ T cells, TCR ligation stabilises both HIF-1α and HIF-2α protein and cytokine receptor signalling differentially modulates the HIF isoforms: IL-4 induces HIF-2α stabilisation, whilst IL-2 favours HIF-1α. In myeloid cells, TLRs signal through NF-κB to increase *Hif1α* transcription (17). BCR ligation triggers activation of several downstream pathways that are likely to promote HIF stabilisation. These include MAPK, PI3K/MTORC1 and NF-κB (42). NF-κB is similarly activated by TLRs on B cells. Preliminary support for receptor-mediated HIF stabilisation comes from studies where HIF-1α protein was observed in normoxic LPS-treated B cells (28) and in purified GC B cells removed from the hypoxic microenvironment (25). How receptor signalling controls HIF expression is a timely topic in light of the recent publications highlighting the intricate relationship between hypoxia and metabolic reprogramming in controlling B cell fate in the GC. That B cell signalling may stabilise HIF oxygen-independently, indicates a potential role in fine-tuning the level and duration of HIF activation, which may have significant consequences for the nature and quality of the immune response.

6.5. B cell migration

An important requirement for B cell function is the ability to home to and migrate within lymphoid tissues. Hypoxia has opposing effects on B cell migration depending on their subset. Hypoxia increases expression of the chemokine receptor CXCR4 that promotes migration of peripheral blood B cells, whilst migration of GC B cells is inhibited. This is due to HIF-dependent CXCR4 receptor uncoupling that occurs specifically in GC B cells. Importantly, increased CXCR4 is normalised on reoxygenation (22) indicating a rapid and reversible response to changes in oxygenation. Subset-specific, differential regulation of CXCR4 function by hypoxia may contribute to B cell egress from lymphoid tissues. For example CXCR4 promotes mature B cell egress from Peyer's patches (43), but is required for the retention of immature B cells in the BM (44). Whilst B cell migration and egress depend largely on the activity of distinct

chemoattractants, oxygenation differs substantially between and within lymphoid organs, which may represent a mechanism for guiding B cell migration in microenvironments of conflicting chemo-attractants.

6.6. SHM and CSR

During SHM, Ig heavy and light-chain genes undergo numerous mutations at high frequency in their variable regions. The Ig heavy-chain can also be targeted for CSR, where exons of the IgM constant region are exchanged with those of downstream Igs. This deletion-recombination reaction involves the generation of DNA double-strand breaks (DSBs) in switch (S) regions, followed by DNA repair. This enables IgM antibodies to switch to IgG, IgA or IgE isotypes.

SHM consist of two steps, generation of DNA lesions and subsequent repair. AID catalyses deoxycytidine deamination resulting in a deoxyuridine lesion. The lesion is repaired by a number of error-prone DNA repair pathways, which are not fully understood. Described are four of the most well defined: First an error-free DNA polymerase may copy the Uracil (U; read as T) leading to a C:G to A:T transition. Second, the base-excision repair (BER) enzyme uracil-DNA glycosylase (UNG) may remove the U, allowing apurinic–apyrimidinic endonuclease 1 (APE1) to nick 5′ to the abasic site, enabling RNA-directed polymerase (REV1)-dependent transversion. Third, following UNG and APE1 activity, exonuclease 1 (EXO1) may resect downstream of the nick site leading to gaps that are filled by error-prone translesion DNA synthesis (TLS) polymerases. Fourth, the U:G mispair created by AID, is recognised by the mismatch repair (MMR) MSH2/MSH6 heterodimer, triggering mutagenic patch repair by error-prone TLS polymerases, leading to mutations at A:T. The substitution of error-free DNA polymerases with error-prone TLS polymerases plays a significant role in the high mutation frequency and in antibody diversification (45, 46).

During CSR, AID and UNG activity in S regions produce abasic sites that are excised by APEs and/or MRE11–RAD50 lyase forming single-stranded breaks that either directly produce DSBs or are converted into DSBs by MSH2/MSH6 and EXO1. The MRE11–RAD50–NBS1 (MRN) complex senses DSBs and recruits ATM, which activates NBS1 and other downstream repair proteins. MRN processes break-ends for repair by either non-homologous end joining (NHEJ) that requires KU70, KU86, DNA-PKcs and other proteins including XRRC4-DNA ligase IV, or alternative end joining (A-EJ) that requires MRN, CTIP and DNA ligases. These two repair pathways create S-S junctions and class-switching. The choice of NHEJ over A-EJ depends on expression of 53BP1 that protects DSBs from resection for A-EJ, and KU70/KU86 that inhibits CTIP. NHEJ is essential for maintaining genomic stability over A-EJ, where chromosomal translocations are common (47).

Hypoxia and HIF have major effects on class-switching and affinity maturation (24, 27) yet the mechanism is unknown. In cancer cells, hypoxia markedly inhibits MMR proteins (MSH2/MSH6) and BER proteins (48), providing a potential mechanism for reduced affinity maturation reported under hypoxia (27). Conversely, HIF-1 upregulates TLS polymerases (48) that could lead to increased mutagenesis and B cell diversification under hypoxia. Which hypoxia-mediated effect predominates during SHM is yet to be

determined. It is likely that hypoxia influences the frequency and spectrum of mutations by exerting diverse effects on different DNA-repair proteins.

Acute hypoxia leads to replication stress, which activates ATM and subsequently 53BP1 and DNA-PKcs. This may function to slow cell cycle and favour DNA repair by NHEJ, increasing the efficiency of CSR. However, the effects of hypoxia on proteins involved in NHEJ (KU70, DNA-PKcs and DNA ligase IV) are conflicting (48) suggesting that hypoxic effects on CSR are likely to be influenced by B cell subset, level and duration of hypoxia and local stimuli. This may contribute to the opposing effects reported on CSR in hypoxic B cells cultured in different switching conditions (24, 27). Hypoxia has been reported to inhibit expression of AID (27) and NBS1 (48), which could be predicted to impair both SHM and CSR. Therefore it would be interesting to determine how hypoxia influences AID in response to different stimuli that modulate its expression (such as CD40L, TLR ligands and specific cytokines). These contrasting effects of hypoxia on DNA repair proteins, exemplify the potential for hypoxia to fine tune SHM and CSR, regulating switching kinetics and class choice in response to environmental cues.

In some reports GC hypoxia has been reported specifically in the light zone, whilst others report diffuse localisation. Since GCs are dynamic it is likely that the location and level of hypoxia may fluctuate leading to cyclic oxygenation and reoxygenation, which may accelerate DNA damage due to ROS production. This would activate ATM and CHK2 inducing a G2 cell cycle arrest. Thus changes in oxygenation in later stages of the GC response may serve to reduce excessive proliferation, maintaining the balance between effective pathogen clearance and autoimmunity.

6.7. Plasma cells (PCs)

Antibody generation is one of the most important parts of the immune response and forms the basis for the majority of successful vaccines. How hypoxia and HIF impact on the generation, function and maintenance of PCs is largely unknown. Further, the majority of long-lived PCs reside in the hypoxic BM compartment, yet the role of hypoxia and HIF in PC homing to and survival in the BM is unclear. A number of hypoxia-regulated genes are required for PC migration and survival but have yet to be linked with HIF: CXCR4 and CXCL12 are required for the recruitment and retention of PCs in the BM, and inducible nitric oxide synthase (iNOS) is required for the survival-promoting activities of APRIL and IL-6. PCs are highly susceptible to ER stress due to the high rate of Ig synthesis and secretion. Consequently, PCs have a constitutively active unfolded protein response (UPR) for detecting and handling large amounts of protein passing through the ER, which maintains ER homeostasis (4). Similarly, hypoxia activates the UPR, which functions to promote hypoxic tolerance. An interesting perspective would be that hypoxia promotes UPR activity in PCs supporting secretion of high antibody titres, whilst HIF activation enables adaptation, and survival in the hypoxic BM.

7. HIF and the innate-adaptive immune interface

How hypoxia and HIF impact on the innate-adaptive immune interface is an interesting and incompletely understood area. Studies into the role of hypoxia and HIF on dendritic cells (DCs) have yielded contradictory findings. Whilst there are reports of hypoxia/HIF inhibiting DC maturation, antigen presentation and stimulation, others report opposing findings (1). Of note, pharmacological stabilisation of HIF with a PHD inhibitor increased antigen presentation, and expression of major histocompatibility and costimulatory molecules on DCs. This enhanced T cell proliferation and increased antibody titres in Td immunised mice [10]. PHD inhibitors are currently in clinical trials for treating anaemia and show favourable safety profiles (49). Thus, it is feasible that PHD inhibitors could be used short-term as adjuvants to boost vaccination response, particularly in the elderly. Alternatively, genetic studies in mice imply that prolonged use of PHD inhibitors may be immunosuppressive, potentially representing a novel therapy for autoimmune disease, organ transplant rejection or cancer.

8. Concluding remarks

B cells are exposed to a range of oxygen tensions through their life cycle. The ability of B cells to sense and respond to hypoxia is emerging as an integral part of this (Figure 2). The importance of hypoxia in GC responses has recently been exposed; this has begun to scrape the surface and has raised many more intriguing questions. Interestingly it is not known if HIF-2α is expressed or functional in B cells. In macrophages, T cells and neutrophils, HIF-2α exerts differential and sometimes opposing roles to HIF-1α (1). HIF-2α is expressed in B cell lymphomas but to our knowledge has not been reported in normal B cells, and could be important in physiological and disease settings. Another major unexplored avenue is whether hypoxia influences regulatory B cells (Bregs). Bregs are immunosuppressive cells that support immunological tolerance. Bregs differentiate in response to environmental stimuli, namely inflammation and have been found in draining LNs at sites of inflammation in mice (50) suggesting that hypoxia could be important in the control of Breg differentiation and function. It will be interesting to determine when in the life of a B cell HIF is activated, and what the functional outcomes of this are. In this context hypoxia and HIF must not be approached as functional equivalents. First, because HIF can be activated by other stimuli than hypoxia, and in other immune cells hypoxic and cytokine mediated activation lead to different HIF-regulated transcriptional outcomes. Second, because hypoxia will have other effects besides HIF activation. A better understanding of this complexity could enable therapeutic manipulation of the HIF pathway to modulate immune responses in vaccination and disease.

9. Acknowledgements

This review was written in memory of Professor Lorenz Poellinger. This work was supported by the Wellcome Trust and the NIHR Cambridge Biomedical Research Centre Senior Investigator Awards.

Figure legends

Figure 1: Oxygen tensions and gradients encountered during the life of a B2 cell.

In the bone marrow (BM), haematopoietic stem cells commit to the B cell lineage producing large numbers of proB cells. ProB cells undergo VDJ recombination of the μ heavy-chain locus, and the resulting protein binds surrogate light-chains, which together with Igα/Igβ yield the preBCR at the membrane. Rearrangement of the light-chain locus yields a complete BCR and an immature B cell. The BM is a hypoxic organ (pO_2 1.3%), with extravascular hypoxic gradients of 0.6-2.8%. It is likely that HIF will be activated during these developmental stages and has been implicated in regulating glycolysis. Immature B cells egress from the BM where they enter the well oxygenated circulation as transitional cells (pO_2 13.2%) and enter the spleen to complete maturation. The spleen is less hypoxic than the BM (pO_2 2.3%), but oxygen gradients are thought to exist within the spleen (pO_2 0.5-4.5%), which may influence tolerance and migration. On Td antigen encounter, B cells undergo CSR and differentiate into short-lived plasmablasts that secrete low-affinity antibodies. Some of these activated B cells proliferate extensively under the influence of Tfh cells and follicular dendritic cells (FDCs), forming a GC. GCs are sites where B cells undergo diversification of their BCRs, and selection, producing long-lived plasma cells that secrete high affinity switched antibodies, and memory B cells. GCs are hypoxic and express HIF-1α that influences B cell metabolism, cell-cycle and antibody quality. Most GCs form >40μm from the splenic vessel (pO_2 1.3%), suggesting that hypoxic gradients set the location of GCs and thus control the anatomical colocalisation of immune cells and antigens, critical for mounting an effective immune response.

Figure 2: The role of hypoxia and HIF in B cell function

A: Immune response and metabolism. Hypoxia and HIF increase glycolysis in GC B cells, with constitutive HIF activation reducing SHM, CSR, high-affinity switched Ig titres, and memory recall. The effects of hypoxia and HIF are likely to be time-limited and of function benefit: Effects of hypoxia and HIF on the early immune response differ depending on local stimuli, and the level and duration of HIF activation, determined by tissue oxygenation and possibly through signalling cascades activated downstream of B cell-associated receptors. **B: Tolerance.** Hypoxia reduces splenic B cell proliferation HIF-1 dependently. HIF-1 may regulate B1 cells and prevent autoimmunity through abnormal B1-like cell activity. Upregulation of Mir-210 by HIF-1α in activated B cells reduces cell cycle entry, slowing proliferation and reducing CSR. Mice deficient in Mir-210 develop autoimmunity. **C: Migration.** HIF-1 upregulates CXCR4 which increases migration in peripheral blood B cell, whilst GC B cell migration is inhibited. HIF has functionally distinct roles in different B cell subsets that may help guide specific subsets in variable and conflicting chemoattractant gradients.

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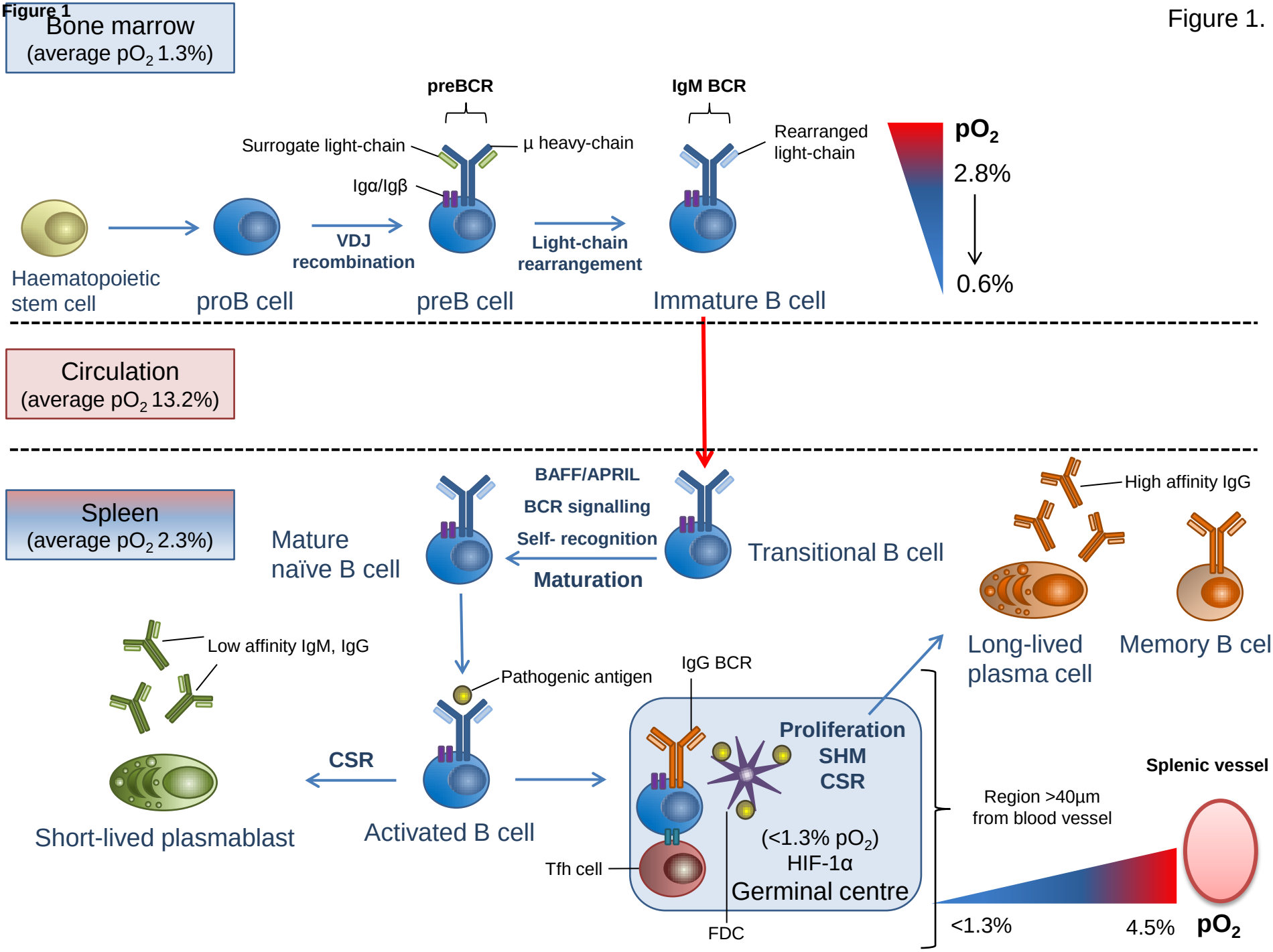
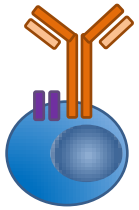
Figure 1

Figure 1.



A: Immune response and metabolism

Figure 2.



GC B cell

↑ Glycolysis

↓ SHM

↓ High-affinity Ig

↓ Memory recall

Constitutive HIF activation

↑↓ Survival

↑↓ Proliferation

↑↓ Plasmablasts & Ig

↑↓ CSR

Early immune response

HIF effect depends on;

- Local stimuli
- Local oxygenation
- Level & duration of HIF activation

B: Tolerance

HIF-1α;

Splenic B cells: ↓ Proliferation

B1 cells: - Regulates B1 phenotype

- Inhibitory role in autoimmunity

Activated B cells: ↑ Mir-210 → ↓ Proliferation

↓ Cell cycle

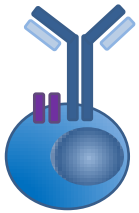
↓ CSR

C: Migration

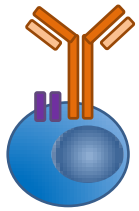
Hypoxia → HIF-1α → ↑ CXCR4

↑ **migration**

↓ **migration**



Peripheral blood B cell



GC B cell