THE ROLE OF HYPOXIA AND COMPLEMENT RECEPTOR 2 OR TOLL-LIKE RECEPTOR 2 ON B1 B CELL EFFECTOR FUNCTION

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Abstract

Professional phagocytes play a critical role in maintaining homeostasis within a host through phagocytic, microbicidal, and inflammatory activity. Complement receptors (CR) and toll-like receptors (TLRs) aid in phagocytosis and stimulate these cells to enhance the immune response. Environmental factors such as hypoxia, prevalent at sites of tissue damage or infection, induce a similar effect. Systemic components such as opsonins may further enhance phagocyte activity. Similar to professional phagocytes, B1 B cells exhibit a broad range of immunological activity as well as expression of CRs and TLRs. Despite extensive studies with other phagocytes, the effects of CRs and TLRs expression, hypoxic stimulation, or opsonization on B1 B cell function remain unclear. We tested the hypothesis that TLR2 stimulation, hypoxia, CR2 expression, or opsonins would enhance B1 B cell phagocytic and inflammatory activity. Negatively selected peritoneal cavity B1 B cells from the (PerC) of wild type, *Tlr2*$^{-/-}$, and *Cr2*$^{-/-}$ mice, or a B1 B-like cell line, Wehi 231, were subjected to normoxia or hypoxia with or without particles for phagocytosis, TLR2 agonists, or CR2 ligands. The PerC of *Tlr2*$^{-/-}$ mice contained an altered B1 B cell subset distribution while *Cr2*$^{-/-}$ mice exhibited a normal repertoire. We demonstrated that hypoxia significantly downregulated inflammatory cytokine production by B1 B cells, while upregulating phagocytic activity in a TLR2 or CR2 dependent manner. TLR2 or CR2 deficiency altered constitutive production of B1 B cell associated cytokines. The CR2 ligand C3d, an opsonin, significantly enhanced the phagocytic activity of B1 B cells but failed to stimulate cytokine production. However, *Cr2*$^{-/-}$ B1 B cells phagocytosed C3d-coated particles suggesting multiple CR may play a role in B1 B cell phagocytosis. Overall, the data suggest TLRs, CRs, hypoxia, and opsonization all contribute to B1 B cell effector function similar to professional phagocytes.

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-k

Chapter 1 - Introduction

Immunity may be divided into two distinct but overlapping responses: the innate and adaptive immune response. The innate response consists of an early, non-specific line of defense against immune challenges. In contrast, adaptive immunity occurs in a delayed manner, requiring time to mount an immune response. However, adaptive immunity confers an antigen-specific response. Additionally, the antigen-specific response may result in immunological memory, a form of long lasting immunity [1]. Historically, many components of the immune response fell into either the innate or adaptive response, which together interact to mount a complete response within a host. However, with the discovery of new immune cell subsets and effector functions, immunologists must constantly reevaluate where the components of the immune system play a role in this schematic. Within the past 20 years a specialized subset of B cells, known as B1 B cells, was discovered and partially characterized. B cells generally participate in the adaptive response but characterization of B1 B cells indicated they may act as innate cells. This specialized subset shares cell surface markers and effector functions associated with other lineages of immune cells. Several recent studies reveal that B1 B cells participate in immune responses previously attributed to other cells such as macrophages. Recent advances in the field, including technological advances such as with flow cytometry, allow for precise study of this subset. However, the full scope of the role of B1 B cells in the immune response remains incomplete.

B cells

Multiple immune cells participate in the adaptive response. B cells that develop in the bone marrow, termed B2 B cells, originate from a lymphoid progenitor common to both T cells and natural killer (NK) cells (reviewed in [2]). Historically, B cells have been associated with the adaptive immune response due to recognition of antigens via membrane bound immunoglobulin (Ig) or the secreted form known as antibody (Ab) [1, 3]. Membrane Ig and Ab exhibit a high level of specificity, a critical component of adaptive immunity.

B cell Ig consists of two light chains and two heavy chains, each containing a variable and constant region. Membrane Ig associates with a signaling heterodimer composed of the Ig-α and Ig-β

subunits to form a functional B cell receptor (BCR). Through recombination events, a vast array of *V-D-J* immunoglobulin genes will be spliced to encode a single Ig. B2 B cells undergo an extensive selection process as they develop membrane bound Ig and mature. The B cell selection process occurs in two stages, positive and negative selection, to ensure B cells with functional Ig capable of recognizing pathogenic, but not host, antigens develop. The first selection step, positive selection, ensures that gene rearrangement produces functional BCR capable of recognizing a specific antigen. Negative selection follows to eliminate B cells which express BCRs capable of recognizing host antigens. Defects in negative selection allow B cells expressing autoreactive Ig to escape into the periphery, potentially leading to autoimmune disease.

Immature B cells with productive BCRs migrate from the bone marrow into circulation or peripheral lymphoid organs where they may mature [4]. Peripheral naïve B2 B cells undergo apoptosis if they fail to encounter an antigen. The naïve B2 B cells also require co-stimulatory signals through T cell interactions [5, 6]. However, recognition of a BCR-specific antigen in the blood or germinal centers of the spleen and lymph nodes, plus T cell co-stimulatory signals, initiates B2 B cell proliferation and activation [7, 8]. If an antigen crosslinks BCRs on a B2 B cell, a co-receptor complex consisting of CD19, CR2, and CD81 associates with the BCR in a lipid raft [9]. While crosslinking the BCR induces signaling through Ig-α/Ig-β, the BCR-co-receptor complex significantly enhances signaling and reduces the threshold for activation [10, 11]. Signal transduction occurs through phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) on the cytoplasmic tails of Ig-α/Ig-β and CD19 by multiple tyrosine kinases (Reviewed in [12]). B cell activation results in Ca^{2+} flux, generation of messenger molecules such as diacylglycerol, and induction of transcription factors [13, 14].

Once activated, a major effector function of B cells becomes antibody secretion. Approximately four to seven days after antigen induced activation, the primary adaptive immune response increases [15]. During this period, B cells undergo class switching, affinity maturation, and clonal proliferation [16-18]. These processes drive the antibody response to neutralize a particular antigen with greater efficiency. IgM acts as the first form of B cell derived Ab detected in circulation following antigen challenge. Generally, B2 B cells class switch to IgG secreting cells which peak approximately 14 days after the initial challenge [16]. However, different types of antigens may elicit other Ig isotypes, such as

IgA and IgE, instead of an IgG response [16]. Of the antigen activated B cells, some will develop into plasma cells. These cells may occur as short or long lived cells [19, 20]. Plasma cells generally lose membrane Ig expression and function to secrete large quantities of antibody [21]. In addition to differentiation into plasma cells, a number of activated B cells may develop into memory B cells [19, 20]. Immunological memory may be conferred in part through the generation of memory B cells. Memory B cells act as long lived cells with improved antigen affinity and recirculate to the bone marrow, spleen, and lymph nodes awaiting subsequent antigen challenge [22]. These cells drive the secondary adaptive response upon challenge with the same antigen. The secondary response, primarily IgG, exhibits enhanced kinetics, occurring much more quickly than 14 days, compared to the primary response [20]. The rapid production of IgG allows for switft clearance of an antigen [20]. In addition to antibody production, activated B2 B cells interact with CD4+ T cells to initiate and enhance the cell mediated adaptive immune response [5, 6].

Two distinct subsets of B2 B cells develop from immature B cells leaving the bone marrow, marginal zone B cells (MZB cells) and follicular B cells (FOB cells). Both subsets derive from a common B2 B cell progenitor but reside in alternate anatomical locations and exhibit different mechanisms of activation [23]. Marginal zone B cells reside in the marginal region of the spleen whereas follicular B cells migrate to germinal centers in the spleen and lymph nodes [24]. In addition to differences in anatomical location, MZB cells express the surface markers CD1, CD9, and high levels of CR2 [25-27]. In contrast, FOB cells may be identified by expression of CD23 and low CR2 levels [28]. The location of MZB cells at the marginal sinus of the spleen allows for quick migration and response to blood borne pathogens [29]. FOB cells recognize antigens passing through lymphoid follicles and require more time than MZB cells to activate and migrate into circulation [30]. While FOB cells require T cell co-stimulation for activation, MZB cells may be activated through innate immune receptors [28, 30]. Despite these differences, B2 B cells vastly contrast to another member of the B cell lineage, B1 B cells. Compared to B2 B cells, B1 B cells undergo BCR recombination with a restricted set of V_H gene segments, and exhibit unique surface marker expression, effector function, anatomical distribution, as well as activation mechanisms [31].

B1 B cells

Derived primarily from the fetal liver, B1 B cells represent a unique, self-renewing population within the B cell lineage accounting for approximately 5% of the total B cell pool [32]. B1 B cells reside predominantly within the peritoneal and pleural cavities with a small population found in the spleen [33, 34]. In addition to the pan-B cell marker CD19 and surface bound Ig, the presence of the marker CD11b, with or without CD5, identify B1 B cells. The expression of sialic acid-binding immunoglobulin superfamily lectin G (Siglec-G) and CD43 act as anatomical markers for the peritoneal cavity and spleen respectively [35-38]. B1 B cells also undergo a unique selection process when compared to conventional B2 B cells. B1 B cells often express an autoreactive B cell receptor (BCR) which promotes cell selection and development instead of inducing negative selection or anergy typically observed in B2 B cells. B1 B cell development depends on a high affinity interaction with an antigen, inducing stimulation signals through the BCR, resulting in positive selection of auto-reactive cells [39].

The repertoire of B1 B cells breaks into two subpopulations, B1a and B1b, based on the expression pattern of CD11b and CD5, with each subset exhibiting unique functional characteristics [32, 40, 41]. B1a B cells express the CD11b+ and CD5+ phenotype and produce the majority of naturally occuring IgM antibody (NAb). The NAbs exhibit limited diversity due to restricted V_H usage and a lack of terminal deoxynucleotidyl transferase (TdT) expression during fetal development when the majority of the B1 B cell pool arises [42-44]. As the major constituent of circulating antibody, B1a B cell derived IgM exhibits polyspecificity, allowing for recognition of multiple antigens but with low affinity [42]. The polyspecificity of this pool allows for an early, innate antibody response to a wide range of pathogens such as *Streptococcus pneumonia* until an adaptive response occurs [41]. The presence of NAb acts to reduce the bacterial load [41]. However, the polyspecificity of NAb may result in circulation of self-reactive autoantibodies [45].

In contrast to B1a B cells, the CD11b+ CD5- B1b B cell subset confers immunological memory and long lasting immunity [41, 46]. The B1b B cell memory response generally recognizes the same pathogens initially controlled by B1a B cells [41, 46]. Two mechanisms may account for the functional difference between the two subsets. The expression of CD5 may regulate B1a B cell Ab responses [47-

49]. Additionally, B1b B cells exhibit increased N region additions between VH-D and D-JH gene segments [47-49].

The mechanism of B1 B cell activation differs from that of conventional B2 B cells. Crosslinking a BCR with cognate antigen activates B2 B cells, inducing intracellular signaling and endocytosis of the BCR for antigen processing [50]. Unlike B2 B cells, stimulation via the BCR induces little to no level of activation in the B1 B cell population and potentially induces apoptosis [51]. Several factors contribute to the reduced BCR signaling observed in B1 B cells. B1 B cells exhibit a decreased ability to mobilize Ca+, possess defective CD19 signaling, and strong negative regulation occurs through the expression of CD5 and Siglec-G [49, 52-54]. In contrast to BCR engagement, antibody production by B1 B cells may occur after activation with mitogens or via TLR stimulation [55, 56]. TLRs not only activate B1 B cells, TLR2 and TLR4 expression play a role in shaping the Ab response, inducing IgG or IgM production respectively [57]. TLR2 or TLR4 deficiency leads to a reduced natural IgG and IgM titer to bacterial pathogens [57].

In addition to antibody production, activation of B1 B cells results in unique effector functions including microbicidal and phagocytic activity, potent antigen presentation capability, and secretion of high levels of IL-10 [58-63]. In response to TLR agonists, B1 B cells produce and release nitric oxide, a mediator of inflammation with potent antimicrobial activity [60, 64]. A small percent (<30%) of phagocytic B1 B cells reside in the peritoneal cavity (PerC B1 B cell) [63] [65]. These cells may phagocytose and present antigens loaded in MHC II molecules to T cells with higher stimulatory capacity that professional phagocytes such as macrophages [63, 66].

Phagocytosis

In the 1880s, Elie Metchnikoff first described the uptake of foreign particles by starfish larvae and subsequently by mammalian white blood cells [67]. Much later this phenomenon would be identified as the biologically important process of phagocytosis. Phagocytosis, the cellular uptake of particulates at least 0.5 micron in size, is an evolutionarily conserved process observed in a vast array of eukaryotes from unicellular organisms to higher vertebrates (Reviewed in [68]) [69]. Mammalian phagocytes play a pivotal role in homeostasis as phagocytosis influences embryonic tissue remodeling, wound healing and clearance of cellular debris and aged cells [70, 71]. Additionally, phagocytosis of pathogens allows for antigen presentation to lymphocytes and initiates adaptive immunity [72].

Phagocytes include a large array of cells. Derived from the bone marrow, several types of leukocytes comprise the pool of professional phagocytes including myeloid granulocytes, mononuclear phagocytes such as monocytes and macrophages, and dendritic cells [73]. Historically, B cells were excluded from the group of professional phagocytes but observations in fish and mice indicate a population of B1 B cell are phagocytic [63, 66, 74]. It has been proposed these phagocytic B1 B cells may provide an evolutionary connection between macrophages and conventional B2 B cells, linking innate and adaptive immunity [58].

The process by which phagocytosis occurs has been extensively studied and characterized into a series of sequential steps largely based on the "zipper hypothesis" proposed by Silverstein et al. in the 1970s [75]. The zipper hypothesis describes phagocytosis as the ligand-mediated spreading of a phagocyte's cell membrane around a particulate which leads to internalization of the ligand [75]. The initial step in phagocytosis depends on phagocyte recognition of the ligand, often mediated in part by phagocytic receptors on the plasma membrane. Contact between the ligand and phagocytic receptor induces the second step, a signal transduction cascade resulting in clustering of the phagocytic receptor(s) and localized F-actin polymerization [76-78]. Phagocytosis fails to proceed if inhibition of actin polymerization occurs as demonstrated with studies using cytochalasins, a class of compounds which inhibit actin rearrangement [79]. Internalization of the particle due to membrane reorganization follows ligand-receptor interactions and actin polymerization. Dependent on the signaling cascade initiated by particle recognition, the third step involves the plasma membrane engulfing the ligand which forms an endosome known as the phagosome [80]. Finally, the phagosome proceeds through a series of maturation steps and eventually fuses with a lysosome forming the phagolysosome [81, 82]. Within the phagolysosome various mechanisms allow for pathogen killing and antigen processing by the phagocyte (Reviewed in [83]).

Phagocyte function may be affected by various factors within the host such as the microenvironment. Phagocytes migrate to infected or damaged tissue, where a localized zone of hypoxia may occur due to reduced oxygen availability [84]. Hypoxia upregulates expression of the pro-inflammatory transcription factor Hypoxia Inducible Factor-1 (HIF1) and induces phosphorylation of p38-Mitogen-Activated Protein Kinase (MAPK) which enhances phagocytosis of macrophages and neutrophils

[85, 86]. Systemic factors also play a role in enhancing phagocytosis. In a process known as opsonization, soluble plasma proteins, opsonins, coat invading pathogens or particulates. The major plasma opsonins consist of immunoglobulin, complement, pentraxins, and collectins [87-90]. Opsonization enhances the ability of phagocytes to recognize and ingest the coated target through expression of opsonin receptors [91].

Different families of membrane receptors play a role in opsonin mediated phagocytosis. Two major pathways of opsonin-mediated phagocytosis, type I and II, exist and have been described extensively. When immunoglobulin G (IgG) binds and opsonizes an antigen, the Fc region (heavy chain constant domain 2 or 3) of IgG extends outward and may bind to Fc receptors present on a phagocyte's plasma membrane, enhancing phagocytosis [92, 93]. Type I phagocytosis occurs through Fc gamma (γ) receptor (FcγR), which interacts with the immunoglobulin coated particulates (Reviewed in [94]). IgG opsonized particulates recognized by FcγR on a phagocyte results in signal transduction through immune-tyrosine activation motifs (ITAMs) [95, 96]. ITAM activation initiates the critical step of actin polymerization in the process of phagocytosis (Reviewed in [97]). Complement receptor (CR) mediated phagocytosis, type II phagocytosis, recognizes particulates opsonized with complement component C3 degradation products (Reviewed in [88]). Complement activation, C3 opsonization, and complement receptors will be described in detail below.

In addition to receptors recognizing opsonized particles, nonopsonic receptors such as pattern recognition receptors (PRR) play a role in regulating phagocytosis [98]. TLRs fail to directly aid in internalization during phagocytosis. Instead, these receptors act as mediators of inflammation through recognition of pathogens prior to or following internalization in the phagosome to enhance phagocytosis [99, 100]. Another class of receptors, integrin receptors, may internalize particulates after direct binding to a pathogen associated molecular pattern without opsonization (Reviewed in [98]). In the clearance of host particulates such as apoptotic cells, scavenger receptors such as CD36 aid in phagocytosis through recognition of lipids such as phosphatidylserine, again without opsonization [101, 102]. However, internalization of non-opsonized particulates by intergrin and scavenger receptors requires multiple receptor engagement or an additional stimulus.

Toll-like receptors

Pattern recognition receptors play a critical role in innate immunity. Major classes of PRR include TLRs but also C-type lectin receptors (CLR) and NOD-like receptors (NLR). PRR bind conserved sequences known as Pathogen Associated Molecular Patterns (PAMPs). The molecular sequences recognized by PRR include protein, lipid, carbohydrate, or nucleic acid-motifs common to a wide array of pathogens. However, damaged cells and tissue may express normally sequestered ligands, known as Damage Associated Molecular Patterns (DAMPs), which also activate PRR. With all PRRs, the ligand binds to the PRR, inducing a signaling cascade and subsequent inflammatory response. The broad specificity of PRR coupled with the widespread expression on both immune and non-immune cells allow for a rapid inflammatory response to occur following exposure to PAMPs or DAMPs.

Toll-like receptors, a family of plasma membrane and endosome bound PRR, play an important role in the innate immune response and may contribute to phagocytosis through the Nuclear Factor κ B (NFκB) pathway of inflammation. The cytoplasmic tails of TLRs contain two Toll/IL-1 receptor (TIR) domains [103]. TLR ligation induces recruitment of the intracellular adaptor molecule myeloid differentiation factor 88 (MyD88) to the TIR domains (Reviewed in [104]). Recruitment of interleukin-1 receptor-associated kinases (IRAKs) to the TIR/MyD88 complex results in the activation and translocation of NFκB to the cell nucleus [105]. Once in the nucleus, NFκB induces the transcription of numerous genes associated with the immune response including inflammatory enzymes, such as iNOS, and pro-inflammatory cytokines, TNFα and IL-6 (Reviewed in [106]).

Professional phagocytes include dendritic cells, monocytes, macrophages, and neutrophils (Reviewed in [107]). TLR stimulation may enhance phagocytosis by these cells (Reviewed in [107]). TLR-induced signal transduction augments phagosome maturation, enhances antigen processing, ligand presentation to T cells, and generates antigen specific lymphocytes [108-111]. In addition to MyD88 recruitment and NFkB activation, TLR signals may induce other pathways that enhance phagocytosis. TLR stimulation induces the phosphatidylinositol 3-kinase (PI3K) pathway [112]. Phosphorylation of PI3K activates another kinase, Akt, [113]. The pathway enhances phagocytosis, migration, and induces nitric oxide synthase in phagocytes [114, 115]. Activation through TLRs also induces opsonin-mediated

phagocytosis through CRs, leading to particulate internalization and processing during phagocytosis [116, 117].

As a member of the TLR family, TLR2 recognizes lipoteichoic acid and zymosan by forming a heterodimer with TLR1 or TLR6, respectively. Granulocytes, macrophages, dendritic cells, and lymphocytes all express varying levels of TLR2 which modulates phagocytosis. TLR2 expression enhances phagosome maturation in macrophages and increases neutrophil oxidative killing of bacteria [118, 119]. Normally residing in the plasma membrane, extracellular stimulation or particulate internalization may induce TLR2 migration to the phagosome [118]. TLR2 may recognize ligands on processed particulates to enhance inflammation [118]. Phagocytosis of several fungal pathogens depends on TLR2/TLR6 heterodimer expression [120]. Similarly, stimulation with lipopeptide agonists of the TLR2/1 heterodimer selectively enhances the phagocytosis of both Gram positive and negative bacteria [121]. Additionally, TLR2 heterodimers may recognize DAMPs, generally intracellular or sequestered cellular components such as heat-shock proteins and oxidized lipids (Reviewed in [122]).

B1 B cells express high levels of TLR2, known to play a prominent role in professional phagocyte-mediated phagocytosis [123]. TLR2 engagement activates B1 B cells to augment antibody secretion and in some cases TLR2 expression drives autoreactive antibody production [124]. The TLR2/1 agonist peptidoglycan or Pam_3CSK_4 strongly induce IL-6 and IL-10 secretion by B1 B cells [123]. Additionally, stimulation of B1 B cells via TLR2/1, with Pam_3CSK_4, or TLR2/6, with zymosan, augment nitric oxide production and induces migration, both of which are important processes in the phagocytic response [125]. While the effect of TLR stimulation, specifically TLR2, on professional phagocytes has been studied extensively, the effect on B1 B cell phagocytosis remains poorly characterized. [60].

Hypoxia

Hypoxia, a reduction in available oxygen, occurs not only as a normal physiological condition but also as a pathological condition. Generally, cells under normal physiological conditions are exposed to an adequate supply of O_2 to maintain homeostasis. However, multiple immune cells may develop under hypoxic conditions due to low oxygen levels in the bone marrow [126-128]. Interestingly, many of these cells require the expression of hypoxia induced factors for normal development [128]. Pathologically, an ischemic insult such as trauma or hemorrhage may induce systemic hypoxia due to massive blood loss,

thus a reduction in oxygen delivery [129]. A localized area of hypoxia, tissue hypoxia, may develop in healing wounds, poorly vascularized tumors, or at sites of infection. Cells exposed to hypoxia induce multiple stress response mechanisms such as autophagy and heat shock proteins (HSP) to alter metabolism, conserve oxygen, and inhibit apoptosis [130-132].

Hypoxia may induce the activation of the NFκB pathway, common to TLR activation, suggesting an overlap exists between the two stimuli. Additional transcription factors such as the Hypoxia Inducible Factor 1 (HIF1) pathway activate in order to maintain homeostasis and restore physiological oxygen levels [133, 134] (Reviewed in [135]). HIF1 exists as a heterodimer of HIF1α and HIF1β which exhibits a basic helix-loop-helix structure [136]. Under normal oxygen levels, cells constitutively express HIF1β. However, the HIF1α subunit contains three proline residues which act as targets for prolyl hydroxylases (PHD) [137]. In the presence of oxygen and iron, PHD continuously catalyze proteasome-mediated degradation of HIF1α, preventing formation of the functional HIF1 heterodimer [137]. Chelating agents or hypoxic conditions inhibit PHD activity allowing for an accumulation of HIF1α in the cytoplasm. Free HIF1α combines with HIF1β, forming HIF1, and translocates to the nucleus. Once in the nucleus, HIF1 exhibits activity by binding to Hypoxia Response Elements (HRE) to upregulate or inhibit gene expression, (Reviewed in [138]), recognizing a 5'-CGTG-3' sequence [136].

Despite mechanisms to avoid the damaging effects of hypoxia, cell death and tissue damage often occur. Hypoxia disrupts the normal lipid bilayer of cell membranes, flipping inner leaflet lipids such as phosphatidylserine and phosphatidylethanolamine, to the outer leaflet [139, 140]. Additionally, multiple intracellular proteins flip to the outer leaflet or diffuse out of apoptotic cells following the response to hypoxia. A few potential intracellular proteins exposed may include non-muscle myosin heavy chain II, annexin IV, and high-mobility group box-1 protein (HMGB1) [141-143]. Aside from lipids and proteins, cytoplasmic and nuclear RNA and DNA may escape through damaged cell membranes (Reviewed in [144]). These normally sequestered components act as neoantigens and DAMPs. The neoantigens and DAMPS possess immunogenic properties but due to their intracellular location, fail to elicit an immune response under normal physiological conditions. Immune cells already present at these sites of hypoxia respond to the DAMPs. When blood flow and reoxygenation occur, known as reperfusion, an augmented immune response against the newly accessible neoantigens and DAMPs may occur. Following

reperfusion, subsequent periods of intermittent hypoxia may reoccur at the site. Immune cells recruited to reperfused tissue may therefore experience hypoxia.

Reperfusion of ischemic tissue allows circulating natural antibody to bind newly exposed neoantigens [145, 146]. Deposition of IgM on the damaged tissue strongly induces C activation and high levels of C3 deposit on the membrane. The inappropriate activation of Ab and C on self cells results in an immune response, exacerbating the tissue injury endured from hypoxia alone (Reviewed in [147], [148]). Studies in murine models of ischemia/reperfusion (IR) injury demonstrate that antibody or C deficiency protect against IR-induced tissue damage [149-151]. The reconstitution of antibody deficient *Rag 1*$^{-/-}$ mice, which are resistant to IR-induced tissue damage, with monoclonal antibody against phospholipids or intracellular proteins restores injury [142, 152, 153]. These data suggest the antibody repertoire plays a critical role in facilitating IR injury. As natural antibody comprises the majority of the pathogenic antibody pool, mainly produced by B1 and MZ B cells, it suggests these cell types may play an integral part in shaping the response.

Toll-like receptors play a multifaceted role in the response to hypoxia. A deleterious role for TLRs occurs during oxygen deprivation as hypoxia leads to DAMP expression. DAMPs act as TLR ligands to induce inflammation (Reviewed in [154]). The major TLR signaling pathway, NFkB, overlaps with hypoxia-induced signaling via HIF1, inducing transcription of many of the same genes. Interestingly, TLR stimulation may stabilize HIF1 under normoxic conditions to upregulate pro-inflammatory cytokine production [155]. Deficiency in TLR2 or TLR4 confers protection from tissue injury in multiple models of IR demonstrating the important role for these receptors following hypoxia [156-158]. However, the full contribution of TLRs to hypoxia-related tissue injury remains unclear. A direct effect induced by hypoxia includes upregulation of inflammation through TLR signaling. An indirect contribution occurs as TLR expression alters the NAb repertoire which recognize neo-antigens (neoAg).

Despite the damaging effects, hypoxia proves to be beneficial in certain circumstances. Phagocytes often migrate to sites of transient or chronic hypoxia. Granulocytes and macrophages appear at sites of tissue trauma, dermal wounds, tumors, atherosclerotic plaques, bacterial infection, and arthritic joints (Reviewed in [159, 160]). All of these locations generally exhibit low oxygen levels (<1%, 10 mmHg) due to poor or irregular vascularization, far proximity to a blood supply, or depletion resulting from

increased O_2 demand [126, 127]. Hypoxia-induced upregulation of phagocytosis occurs due to activation of transcription factors and MAPKs. Hypoxia upregulates phosphorylation of p38 MAPK in phagocytes [161, 162]. Activation of p38 plays a critical role in the phagocytic process as inhibition prevents actin polymerization [163]. Additionally, p38 inhibition downregulates HIF1 expression, an important finding as knockdown of HIF1 in phagocytes reduces phagocytic activity [85, 163]. Together the data support hypoxia and downstream signaling pathways play an important role in enhancing phagocytosis.

Compared to other immune cells, the effect of hypoxia on B cells remains poorly characterized. However, the studies discussed below reveal a role for hypoxia and HIF1 expression in B cell development and immunity. Conditional knockout of HIF1α leads to a severe reduction in the number of B2 B cell progenitors in the bone marrow [128]. However, in the B1 B cell lineage, HIF1α deficiency significantly increases the percentage of CD5+ B1 B cells, while also increasing titers of anti-dsDNA autoantibodies [128, 164]. The subsequent result leads to autoimmune disease with a high level of renal immune complex deposition [128, 164]. The contrasting effect of HIF1 expression between B1 and B2 B cells may occur due to the different anatomical sites of development, with B2 B cells developing in the oxygen-poor bone marrow while B1 B cells originate in the fetal liver and subsequently, the PerC and pleural cavity. In addition to affecting B cell development, hypoxia alters the phenotype and function of B cells. Hypoxia induces CXCR4 expression, an important receptor in organ homing and tumor migration, on peripheral B cells and B cell lymphomas but not FOB B cells [165]. B1 B cells migrate to wounds where they act as phagocytes and enhance the wound healing process through production of IL-10 [58, 166]. Additionally, tumor infiltrating B1 B cells, likely exposed to hypoxia, significantly enhance the metastatic potential of melanoma cells [167]. While these studies on wound healing and tumor infiltration did not evaluate oxygen levels, transient hypoxia likely occurred at these sites and influenced the B1 B cell response. While hypoxia and HIF1 expression affect B cell development and immunity, the role of hypoxia in modulating TLR signals and phagocytosis in this lineage remains uninvestigated.

Complement, opsonization, and complement receptors

The complement (C) system plays an important role in innate and adaptive immunity [168]. C is a serine protease cascade that destroys pathogens through membrane lysis, enhances phagocytosis, augments antibody production, and aids in clearance of immune complexes [92, 169, 170](Reviewed in

[171]). More than 30 serum proteins comprise the complement system with the majority of proteins synthesized in the liver, although other cell types may produce C components (Reviewed in [172]). Four interacting C pathways exist: the classical, alternative, lectin, and lytic pathway, all of which converge on the C component C3. Activation of C3 occurs through spontaneous hydrolysis or via cleavage by the C3 convertase, C4b2a [173, 174]. Once activated, a positive amplification loop occurs as cleaved C3 combines with another serum protein, Factor B, to act as a C3 convertase, C3bBb.

Cleavage of C3 results in two fragments. The larger fragment, C3b, proceeds in the C cascade while the smaller fragment, C3a, diffuses away. C3a acts as an anaphylatoxin to recruit inflammatory cells to sites of C activation [175]. C3b functions as the major serum opsonin in addition to immunoglobulin and also allows the C cascade to proceed to induce cell lysis [176, 177]. Subsequent cleavage of C3b generates iC3b and C3d, additional opsonins [178]. Particulates coated with C3b and iC3b bind to complement receptor 1 (CR1, CD35) (Reviewed in [179]). Two integrin receptors recognize the same fragments as CR1, complement receptor 3 (CR3), a heterodimer of CD11b and CD18, and complement receptor 4 (CR4), composed of CD11c and CD18 (Reviewed in [179]). The fragment C3d acts as an opsonin which binds to CR2, a co-receptor which associates with the BCR [178]. C3d binding to CR2 plays an important role in antigen presentation by reducing the threshold of activation for B2 B cells, specifically MZ B cells, due to high-affinity receptor interactions [180-182]. While CR2 binds C3d opsonized particulates, no phagocytic activity has been associated with this receptor on B cells [180-182].

The distribution and function of complement receptors varies greatly. Complement receptor 1, also called CD35, belongs to the regulators of complement activity (RCA) family [183, 184]. The RCA family protects a host from inappropriate C activation through self/non-self discrimination [185]. Structurally, 30 short consensus repeats compose the extracellular domain of CR1 which bind C3 or C4 fragments and may also inactivate C3 and C5 convertase [185, 186]. C3b cleavage may occur through CR1 interactions with a co-factor, Factor I, to generate iC3b, with further cleavage generating C3d [187, 188]. Erythrocytes, professional phagocytes, and lymphocytes all express CR1. On B cells, CR1 functions as a regulatory receptor by inhibiting BCR-induced proliferation, preventing plasma cell differentiation, and reducing antibody secretion [189-191].

CR2 exhibits structural homology to CR1 and also belongs to the RCA family [184]. In mice, the *Cr2* gene encodes both CR1 and CR2 through alternative splicing [192]. The extracellular domain of murine CR2 consists of 15 to 17 short consensus repeats which bind the complement fragment C3d [193-195]. Aside from C3d, human CR2 exhibits additional binding activity, as interferon-α and an Epstein Barr virus coat proteins also act as ligands [193-195]. While predominantly expressed on MZ and B1 B cells, follicular dendritic cells may also exhibit surface expression of CR2 [196]. As part of the BCR-co-receptor complex described above, CR2 recognizes C3d-opsonized antigens bound by a cognate BCR to enhance the affinity of the interaction. CR2 expression also affects the IgM antibody repertoire derived from MZ and B1 B cells. When compared to wild type mice, *Cr2*$^{-/-}$ mice possess an altered natural antibody repertoire which confers protection from experimental models of ischemia/reperfusion injury, arthritis, and systemic lupus erythematosus [197-199]. *Cr2*$^{-/-}$ mice express a 30-40% decrease in the B1a B cell subset [198]. Previous studies demonstrate that B1 B cells phagocytose in a C dependent manner [63, 66]. However, it remains unclear if B1 B cells utilize CR2 as a phagocytic receptor for C3d-opsonized particulates or if an alternative phagocytic receptor such as CR3 performs this function.

CR3 occurs as an integrin receptor heterodimer, where CD18 acts as a β_2 integrin common to multiple receptors while CD11b (Mac-1, ITGAM, or α_M) functions specifically as part of CR3. Similar to CR1, CR3 binds C3b and iC3b but also exhibits a low affinity for the CR2 ligand, C3d. CR3 functions as a C mediated phagocytic receptor on professional phagocytes. CR3 may act as a non-opsonic PRR receptor, binding molecular patterns expressed on fungal pathogens and self-components such as myelin [200, 201]. CR3 ligand affinity may increase following TLR2 activation or co-receptor expression, such as CD14, to enhance phagocytic activity [202]. Additionally, the receptor plays a role in adhesion and migration of immune cells [203]. While ligand binding to CR3 induces conformational change in the receptor as well as upregulating phagocytic activity, no signaling capability has been described for CR3 in the upregulation of inflammatory mediators such as cytokines [200]. CR3 deficiency results in severe defects in neutrophil and NK cell immune responses and leads to leukocyte adhesion deficiency (LAD) [204-206]. Traditionally, CD11b acts as a marker for professional phagocytes such as macrophages but additional studies identified it as a surface marker, albeit with low expression, for B1 B cells [207]. However, studies have not confirmed if CR3 acts as a phagocytic receptor on B1 B cells.

Cytokines and inflammatory mediators: TNFα, IL-6, IL-10 and nitric oxide

Cytokines act as pleiotropic molecules which influence the function of a cell by autocrine, paracrine, and endocrine mechanisms (Reviewed in [208]). Typically small proteins, 30 kilodaltons (kDa) or less, cytokines belong to one of four families: tumor necrosis factor (TNF), hematopoietin, interferon (IFN), or the chemokine (CC) family. Members within a family exhibit a high degree of structural homology. The biological activity of cytokines occurs through binding to a specific cytokine receptor. Regulation of cytokine biological activity occurs through the selective expression of cytokine receptors on a target cell and the amount of cytokine produced. Immune cells generally secrete cytokines and express cytokine receptors following stimulation. Signals induced through TLR ligation or hypoxic conditions may initiate the transcription, translation, and secretion of numerous cytokines and respective receptors. Several of the major cytokines and inflammatory mediators produced by activated phagocytes, especially B1 B cells, include TNFα, IL-6, IL-10 and nitric oxide. TNFα, IL-6, IL-10 and nitric oxide have all been previously associated with the B1 B cell inflammatory response as well as with hypoxic and TLR stimulation, suggesting they may act as factors in the B1 B cell immune resposne.

Tumor necrosis factor - α

Tumor necrosis factor α (TNFα) occurs as a homotrimer transmembrane protein. Cleavage of the membrane bound form by a metalloprotease, ADAM17, generates a biologically active soluble form [209, 210]. Originally identified and named for the ability to induce apoptosis of tumor cells, TNFα acts as an early, pro-inflammatory mediator [211-213]. TNFα stimulates the production of acute phase proteins, driving the early immune response in a host (Reviewed in [213]). Secretion and subsequent stimulation with TNFα rapidly induces the transcription and translation of another critical inflammatory cytokine, IL-6 (described below) [214]. Additionally, TNFα increases phagocytic activity [215]. Activated macrophages secrete the majority of TNFα, although B1 B cells, NK cells, and T cells may also produce the cytokine [216-218]. Production of TNFα occurs in response to numerous stimuli. Stimulation with the cytokine

interleukin 2 (IL-2) induces T cells to produce TNFα, while a combination of mitogens, such as LPS plus interferon γ (IFNγ), activate other cell types [219, 220].

TNFα secretion also occurs in response to TLR stimulation or during a stress response. TLR2 or 4 agonists induce TNFα production by macrophages [221]. TLR-induced TNFα secretion occurs through p38 MAPK activation [222]. Similarly, hypoxia enhances TNFα production through HIF1 and p38 MAPK activation. Exposure of macrophages to hypoxia upregulates secretion of TNFα, as well as expression of the TNFα receptor [223]. During an inflammatory response, a link exists between TNFα production and HIF1 expression under normoxic conditions. The hypoxia induced upregulation of TNFα occurs when HIF1 binds to a HRE in the promoter region of the *TNF-α* gene [222, 224]. Upregulation of TNFα occurs due to hypoxic activation of p38 [222, 224]. However, induction of TNFα due to other stimuli may activate the HIF1 pathway under normoxic conditions. Interestingly, stimulation with TNFα leads to the accumulation of stabilized, transcriptionally active HIF1 in the presence of oxygen [225].

TNFα activates cells through binding to one of two distinct TNF receptors (TNFR) [226]. As single pass transmembrane cell surface proteins, three TNFR monomers will localize to bind the TNFα homotrimer [227]. The TNFRs, designated TNFR-1 and TNFR-2, exhibit different molecular weights at 55 and 75 kDa respectively. Despite limited homology between TNFR-1 and TNFR-2, they both contain repeating cysteine clusters in the extracellular domain [228]. TNFR expression occurs on almost all cell types with a few exceptions such as erythrocytes (Reviewed in [229]). When TNFα binds to the TNFR, the complex internalizes in a clathrin coated pit. After internalization, TNFα induces activation of a wide range of proteins including transcription factors such as NFκB, caspases, MAPKs, and phospholipases (Reviewed in [230]). Downstream effects include gene transcription and production of oxygen and nitrogen radicals. Processing of TNFR1 reveals the presence of death domains which activate caspases to induce apoptosis [231]. TNFR2 lacks death domains and provides pro-survival signals instead [231]. An endogenous antagonist regulates the activity of TNFα. Soluble forms of TNFR-1 and TNFR-2 constantly circulate to limit the bioavailability and control inflammation [232].

Interleukin-6

Produced by a wide range of cell types, Interleukin 6 (IL-6) exhibits a vast number of biological functions. This became overly apparent as at one point prior to its definitive identification, the cytokine

accumulated 36 different descriptive names (Reviewed in [233]). In humans, IL-6 occurs as a 26 kDa secreted glycoprotein comprised of four alpha chains [234], and mice express a homologue approximately 22 kDa in size originally known as IL-HP1, [235]. The production of IL-6 may occur following transcriptional upregulation due to activation of the HIF1 or NFkB pathway and stimulation with IL-6 may induce multiple pathways, including the JAK/STAT, Ras and PI3K signaling pathways [236-238]. Major immunological functions of IL-6 include the induction of acute phase proteins by hepatocytes, B cell proliferation and differentiation, and cytotoxic T cell activation [236-238]. Other biological functions include regulation of serum lipid levels and modulation of bone growth via osteoclast-osteoblast stimulation [239-241].

In addition to secretion by a vast array of cells, IL-6 exhibits widespread biological activity due to the presence of two different signaling pathways. The receptor for IL-6 occurs as a heterodimer consisting of the high affinity IL-6Rα chain and ubiquitously expressed gp130 signaling subunit [242, 243]. Immune cells and hepatocytes express the high affinity IL-6Rα chain in conjunction with the gp130 unit to form a functional heterodimer [244, 245]. Non-immune cells, which do not express the IL-6Rα chain, may respond to IL-6 due to a process known as trans-signaling (Reviewed in [246, 247]). Immune cells and hepatocytes, which normally express the IL-6R, may release a soluble form of the IL-6Rα chain due to proteolysis of the membrane bound form or due to alternative splicing [246, 247]). The released soluble IL-6Ra chain may then associate with gp130 expressed on cells which normally do not produce IL-6Rα, allowing for a functional IL-6R to form [248, 249]. Circulating IL-6 then binds the soluble IL-6Rα complexed with gp130 on non-immune cells or hepatocytes, inducing signal transduction (Reviewed in [246, 247]). However, due to the widespread association of gp130 with multiple other receptor subunits, the availability to bind with the IL-6Rα subunit on non-native IL-6R expressing cells may be low.

Exposure to mitogens or the early pro-inflammatory cytokines TNFα and IL-1 induces transcription, translation, and secretion of IL-6 [250]. Much like TNFα, additional stimuli may induce the production of IL-6. PAMPs expressed on peptidoglycan significantly upregulate IL-6 secretion in a TLR2 dependent manner as studies with TLR2 siRNA abolished this activity [251]. TLR2 induced IL-6 production may occur through activation of the PI3K/Akt pathway which also plays a role in phagocytosis as described earlier [114, 115, 251]. TLR stimulation also proceeds through the NFκB pathway to

upregulate IL-6 secretion (Reviewed in [252]). IL-6 transcription increases following hypoxia in some cell types. However, the mechanism occurs through hypoxia induced NFkB activation and binding to the IL-6 gene promoter rather than via HIF1 and a HRE [253]. IL-6 secretion increases during phagocytosis but the upregulation may occur as an indirect effect of TNFα production following particulate internalization [214].

Interleukin-10

Discovered in the late 1980s and originally known as cytokine synthesis inhibitory factor (CSIF), Interleukin 10 (IL-10) plays a critical role in regulating the inflammatory response (Reviewed in [254]). The discovery of IL-10 occurred through research on CD4+ T cells to identify a T_H2 derived cytokine which crossregulated the T_H1 response [255]. The gene encoding human and mouse IL-10 share approximately 73% sequence homology and encodes an 18 kDa alpha helical protein [256, 257]. IL-10 monomers, each consisting of 178 amino acids, associate noncovalently to form a biologically active, secreted homodimer [258]. The major sources of IL-10 include T_H2 cells, B1a B cells, dendritic cells, NK cells, and cells of the myeloid lineage (Reviewed in [259]). Cells must express both chains of the IL-10 receptor (IL-10R), comprised of the high affinity IL-10R1 and low affinity IL-10R2 subunits, for signaling and stimulation [260]. Cells from the hematopoietic lineage constitutively express the IL-10R whereas induced expression occurs on epithelial cells, fibroblasts, and keratinocytes (Reviewed in [261]).

IL-10 exerts strong anti-inflammatory activity on a number of immune cells through direct and indirect mechanisms. Stimulation of professional antigen presenting cells with IL-10 leads to reduced MHC II expression, decreased NO release, and downregulation of T cell co-stimulatory molecules such as B7.1 and B7.2 [262-264]. IL-10 directly inhibits the production of multiple cytokines such as IFNγ, IL-1, IL-6, IL-12, and TNFα [265, 266]. Simultaneously, IL-10 stimulation leads to production of endogenous antagonists to the IL-1 receptor and TNFR to enhance the inhibitory activity [267, 268]. The production of growth factors, G/M-CSF, and chemokines, IL-8 and MIP, also decrease following stimulation with IL-10 [265]. A negative feedback loop exists as stimulation with IL-10 may inhibit production of IL-10 itself. Multiple mechanisms facilitate the biological activity of IL-10 to inhibit these factors: mRNA destabilization, posttranscriptional alterations, and inhibition of upstream pro-inflammatory transcription factors [269-272]. Downregulation of all these factors indirectly inhibits the CD4+ T cell and NK cell response due to a lack

of both co-stimulatory signals and activating cytokines [262, 273]. Evolutionarily, pathogens developed mechanisms to exploit the immune inhibitory effects of IL-10. The Epstein-Barr virus genome encodes the protein BCRF1, a mimic of endogenous IL-10 in humans [274]. Similarly, the human cytomegalovirus genome contains an IL-10 open reading frame which encodes a biologically active viral IL-10 homologue [275].

Aside from inhibitory activity, IL-10 exhibits other immunological functions. Prolonged exposure of monocytes and macrophages to IL-10 resulted in increased surface expression of FcγR and scavenger receptors, enhancing their phagocytic ability [276]. IL-10 induces proliferation and differentiation of B2 B cells which supports a T_H2 driven humoral response [277]. B1a B cells require autocrine production and stimulation with IL-10 for self-renewal [278]. B1 B cell facilitated wound healing also depends on the production of IL-10 [166]. Similar to B cells, IL-10 acts as one of the few cytokines capable of enhancing mast cell proliferation and longevity [279]. Unlike CD4+ cells, CD8+ T cells benefit from IL-10 stimulation, increasing migratory, cytotoxic, and proliferative activity [280, 281]. IL-10 secretion plays a critical role in limiting inflammation and preventing autoimmunity as *IL-10*$^{-/-}$ mice develop inflammatory bowel disease and severe arthritis [282, 283]. In a model of multiple sclerosis, experimental autoimmune encephalomyelitis, the expansion of IL-10 producing cells, resulting in higher levels of systemic IL-10, correlated with increased remission of the disease [284].

IL-10 production occurs in response to multiple stimuli and depends on the specific cell type. C-type lectin and Dectin-1 stimulation initiate transcription and translation of IL-10 [285]. TLRs play a major role in induction of IL-10. PAMP recognition via TLR2 plays a critical role in the induction of IL-10 by APCs and acts as the major pathway for production of this cytokine (Reviewed in [286]). Stimulation of TLR3 in macrophages and TLR4 or TLR9 in APCs also induces IL-10 production [287]. Hypoxia influences secretion of IL-10 with up- or down-regulation depending on the cell type. Macrophages exposed to hypoxia increase angiogenesis through VEGF production in an IL-10 dependent manner [288]. However, dendritic cells downregulate the production of IL-10 and maintain secretion of pro-inflammatory cytokines following hypoxic treatment [289]. Despite these studies, interactions between hypoxia and IL-10 production remain poorly characterized in other cell types.

Nitric Oxide

While cytokines contribute heavily to modulating the inflammatory response, small molecules such as oxygen and nitrogen radicals also exhibit potent biological activity. Nitric oxide (NO) exists as a labile gas, composed of one nitrogen atom and one oxygen atom, stabilized by a double bond. The nitrogen radical contains a highly reactive free electron allowing the molecule to readily interact with several biological pathways. Production of NO occurs through an oxidation reaction regulated by expression of nitric oxide synthase (NOS) enzymes (Reviewed in [290]). NOSs, in the presence of the substrates NADPH and O_2, catalyze the oxidation of L-arginine [291]. The overall reaction yields NADP, L-citrulline, and NO [291].

Three isoforms of NOS exist which include neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) (Reviewed in [292]). nNOS and eNOS expression occurs in the respective cell types as the name implies [293, 294]. These cells constitutively express the NOS isoforms and cell stimulation may upregulate NOS expression [293-296]. In response to cell stress or inflammatory stimuli, immune cells, hepatocytes, and cardiac cells express the other isoform, iNOS, (Reviewed in [297]). The promoter region of the *iNOS* gene contains a HRE as well as a binding region for NFκB [298, 299]. Cytokine stimulation, TLR activation, or hypoxic stimulation induce the transcription of iNOS, resulting in increased NO production [60, 300].

Under physiological conditions, nitric oxide acts to maintain homeostatic conditions in the host. Constitutive, low level production of NO plays a role in vasorelaxation, neurotransmission, and regulates platelet adhesion and aggregation [301-303]. However, high levels of NO produced after iNOS expression play an inflammatory role in the immune response. NO exhibits cytotoxic activity against intracellular microbes and tumor cells [304, 305]. An increase in the level of NO within a cell induces an accumulation of cyclic GMP (cGMP) to inhibit apoptosis, extending the lifespan of numerous immune cells participating in the inflammatory response [306, 307]. High levels of cGMP also activate the metalloprotease ADAM17, resulting in membrane shedding of TNFα [308]. The presence of NO in the phagolysosome aids in degrading ingested particulates, and kills microbes [309]. This function also plays an important role in antigen processing for presentation on MHC II molecules, required to activate CD4+ T cells (Reviewed in [310]). Studies identified another critical role for NO in phagocytic activity. LPS stimulated macrophages

treated with iNOS inhibitors lost their phagocytic activity entirely [311]. Additionally, as a membrane diffusible gas, NO allows for rapid signaling between cells (Reviewed in [312]).

Summary

Almeida et al. first described murine B1 B cells as mononuclear phagocytes in 2001 [58]. Additional studies in the past decade indicate a microbicidal role for B1 B cells via nitric oxide production [60] [65]. Similarly, B1 B cells also produce cytokines historically associated with other immune cells such as TNFα, IL-6, and IL-10, which play both an inflammatory and autocrine role in B1 B cell regulation and activation [313]. In the past year, 2012, multiple studies evaluated the effector function of B1 B cells as phagocytes [63] [65] [66]. These studies revealed a number of novel results: 1) Phagocytic B1 B cells present processed antigen more efficiently than macrophages to T_H cells, 2) Size limits B1 B cell phagocytosis to particulates ≤3 microns, 3) B1 B cell phagocytosis of pathogens such as *Escherichia coli* require complement component C3. These findings enhance our understanding of B1 B cell effector function.

However, many factors known to affect the function of inflammatory cells and professional phagocytes remain uncharacterized. TLR activation significantly enhances the phagocytic and microbicidal activity of professional phagocytes [314]. TLR stimulation upregulates NO production by B1 B cells [60] but the effect on phagocytosis remains unclear. The effect of TLR expression on B1 B cell microbicidal and phagocytic function, specifically TLR2 which plays a role in B1 B cell activation and affects the specific antibody repertoire, have yet to be determined. While C plays a critical role in B1 B cell phagocytosis [63], which C3 opsonins and respective receptors required for this activity must be identified. Due to CD11b expression, a B1 B cell marker and part of the CR3 heterodimer, as well as expression of CR2, a single or multiple C3 degradation product(s) and CR may contribute to phagocytic activity. Much like TLR stimulation and opsonization alter and enhance professional phagocyte function, microenvironment conditions may also play a role in B1 B cell effector function. Specifically, the effect of hypoxia on the immune function of B cells in general, especially B1 B cells, remains unclear. Few studies elucidate the effect of the hypoxic microenvironment on B1 B cell inflammatory response and none evaluate phagocytosis under these conditions. Despite major advances in B1 B cell immunological function, multiple questions remain. We tested the hypotheses that both Toll-like receptors and

complement receptors play a role in B1 B cell inflammation and phagocytosis in response to hypoxia. The studies presented within evaluate the role of TLR2 expression on B1 B cell phagocytosis, subset distribution, and cytokine production under normoxic and hypoxic conditions. Similarly, the effect of opsonization and CR expression, specifically CR2, on phagocytic activity of B1 B cells was evaluated. Additionally, we characterized a B1 B-like cell line via phenotype and effector function, Wehi 231, for *in vitro* studies of this scarce cell population.

Chapter 2 - The role of TLR2 and hypoxia on B1 B cell effector function

Abstract

The two B1 B cell subsets, B1a and B1b, act as innate immune cells with effector functions typically associated with professional phagocytes. Phagocytes often migrate to sites of infection, trauma, or tumors where low oxygen levels, hypoxia, may occur. These same sites contain ligands for toll-like receptors which activate professional phagocytes as well as B1 B cells. Expressed on B1 B cells, Toll-like receptor 2 stimulation significantly upregulates phagocytic activity in other immune cells. While the effect of hypoxia and TLR stimulation has been extensively characterized for phagocytes, the effect on B1 B cell phagocytic activity and inflammatory response remains unclear. We tested the hypothesis that hypoxia, TLR2 stimulation, or a combination of both would enhance the B1 B cell pro-inflammatory response and upregulate phagocytic activity. Negatively selected B1 B cells from the peritoneal cavity of wild type or *Tlr2*$^{-/-}$ mice were subjected to 2 hours of normoxic or hypoxic treatment with or without TLR2 stimulation and polystyrene microspheres. We determined that hypoxia significantly upregulated the phagocytosis of microspheres by wild type B1 B cells although hypoxia decreased the production of pro-inflammatory cytokines, TNFα and IL-6, as well as anti-inflammatory IL-10. The hypoxia-induced upregulation of phagocytosis was dependent on TLR2 expression as *Tlr2*$^{-/-}$ mice exhibited no increase with treatment. Unexpectedly, *Tlr2*$^{-/-}$ mice contained a higher percentage of B1a B cells compared to wild type mice and constitutively produced significantly higher levels of IL-10. Despite the requirement for TLR2 expression, stimulation with the TLR2/1 agonist, Pam_3CSK_4, failed to enhance phagocytosis under normoxic or hypoxic conditions. The data suggest that similar to professional phagocytes, both TLRs and hypoxia play a role in B1 B cell effector function.

Introduction

Within the B cell lineage, two distinct populations exist termed B1 and B2 B cells. While B2 B cells function primarily in the adaptive immune response, B1 B cells exhibit unique effector functions which contribute to innate immunity. The pool of B1 B cells originates in the fetal liver and exhibits the capacity for self-renewal through autocrine stimulation with cytokines such as IL-10 [32]. B1 B cells express a unique repertoire of surface markers which identify distinct subsets and reveal the anatomical location of the cells. CD11b acts as a pan B1 B cell marker with expression of CD5 dividing the population into two subsets, the B1a subset expresses CD11b and CD5 while the B1b subset expresses only CD11b. The majority of B1 B cells reside in the peritoneal and pleural cavities, expressing Siglec-G as an anatomical marker whereas splenic B1 B cells possess CD43 [35-38]. The mechanism of B1 B cell activation differs from that of conventional B cells. Crosslinking the B cell receptor (BCR) with cognate antigen activates B2 B cells, whereas stimulation via the BCR of B1 B cells leads to a minimal level of activation and may even induce apoptosis [50, 51]. Instead, mitogen stimulation or pattern recognition receptor ligation induces activation of B1 B cells. Activation of B1 B cells augments nitric oxide release [60], the secretion of IL-6, IL-10, and TNFα [56], and antibody production [55]. Recently, multiple groups demonstrated murine B1 B cells may act as phagocytes in the peritoneal cavity and liver [63, 66].

Phagocytosis plays a critical role in maintaining homeostasis through clearance of apoptotic cells, immune complexes, pathogens, and other particulates. Professional phagocytes often function as antigen presenting cells (APC) to present peptide-loaded MHC II to lymphocytes and initiate adaptive immunity (Reviewed in [68])[72]. Historically, while B cells act as APC, they were excluded from the group of professional phagocytes. However, observations in fish and mice indicate a population of B cells display phagocytic activity [63, 66, 74]. The activity of professional phagocytes in particular niches has been characterized. However, the full extent of phagocytic activity by B1 B cells and the factors which influence effector function remain unclear.

Phagocyte function may be affected by various factors within the host such as the microenvironment. Phagocytes often migrate to sites of infection, trauma or tumors. Within these microenvironments, localized zones of hypoxia develop as a result of a reduction in available oxygen due

to increased O_2 requirements, decreased blood supply, or a decreasing O2 gradient [84]. Hypoxia upregulates expression of the pro-inflammatory transcription factor Hypoxia Inducible Factor-1 (HIF1) and induces phosphorylation of p38-MAPK, enhancing macrophage and neutrophil phagocytosis [85, 86]. In addition to the microenvironment, systemic factors such as opsonins play a role in modulating phagocyte function. Opsonization with immunoglobulin, complement, pentraxins, or collectins enhance phagocytosis through high affinity binding to opsonin receptors on the phagocyte's cell membrane [87-91].

Nonopsonic receptors such as pattern recognition receptors (PRR) also play a role in regulating phagocytosis [98]. Toll-like receptors (TLRs) contribute to phagocytosis through the NFκB pathway of inflammation (Reviewed in [107]). Toll-like receptor stimulation of professional phagocytes induces production of phagocytosis-enhancing cytokines, increases the average number of particulates ingested, and augments phagosome maturation (Reviewed in [107]). Additionally, TLR activation provides the necessary co-stimulatory signals to induce complement-mediated phagocytosis [116, 117]. Toll-like receptor-mediated signaling also enhances antigen processing and presentation to T cells to generate antigen specific lymphocytes [108-111]. Of the members of the TLR family, TLR2 has been identified as a critical PRR in modulating phagocytosis. TLR2 forms a heterodimer with TLR1 to recognize bacterial ligands while combining with TLR6 to recognize fungal pathogens (Reviewed in [315]). In addition to recognition of extracellular ligands, TLR2 may migrate to the phagosome and recognize internalized ligands, augmenting phagocytosis and induce inflammation [118]. TLR2 expression also enhances granulocyte oxidative killing of bacteria [118, 119].

While the effect of TLR stimulation on professional phagocytes has been studied extensively, the effect on B1 B cell phagocytosis remains poorly characterized. [60]. In professional phagocytes, phagocytosis of several bacterial and fungal pathogens depends on TLR2 expression in professional phagocytes [120]. Additionally, stimulation with lipopeptide agonists of TLR2 selectively enhances the phagocytosis of both Gram positive and negative bacteria [121]. B1 B cells express high levels of toll-like receptor 2 (TLR2) [123]. It is known that TLR2 stimulation of B1 B cells augments nitric oxide production and induces migration, both of which are important processes in the phagocytic response [125].

We hypothesized hypoxia and TLR2 stimulation would enhance the inflammatory and phagocytic activity of B1 B cells enriched from the peritoneal cavity (PerC). We evaluated the effect of short term

hypoxia, 2 hours of 1% O_2, on phagocytic activity and inflammatory response of PerC B1 B cells. Additionally, we evaluated the effect of TLR2/1 stimulation with the synthetic agonist Pam_3CSK_4 on B1 B cell phagocyte function. Finally, the requirement for TLR2 expression on B1 B cell inflammatory and phagocytic activity was determined by treating wild type and *Tlr2*$^{-/-}$ PerC B1 B cells with either normoxia or hypoxia. Here we demonstrate for the first time that short periods of hypoxia upregulate the percentage of phagocytic PerC B1 B cells and augment the amount of particulates ingested despite inhibiting inflammatory cytokines. Furthermore, we determined hypoxia induced phagocytic activity required TLR2 expression but was independent of stimulation with the TLR2/1 agonist Pam_3CSK_4. These data reveal a unique role for TLR2, independent of TLR2-1 stimulation via $Pam_3CSK_{4,}$ in the phagocytic response of PerC B1 B cells and indicates hypoxia increases B1 B cell phagocytosis.

Materials and Methods

Mice

Experiments were conducted with adult C57Bl/6 (B6)*,* and *Tlr2*$^{-/-}$ mice obtained from Jackson Labs (Bar Harbor, MA) and bred within the Division of Biology at Kansas State University. *Tlr2*$^{-/-}$ mice are on the C57Bl/6 background. Mice were maintained in a light to dark cycled, temperature controlled, specific pathogen free facility *(Helicobacter sp.*,mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus*, Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, and endo- and ecto- parasites). Food and water were provided *ad libitum.* All research was approved by Kansas State University's Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals.

Enrichment of peritoneal B cells and cell culture

Peritoneal cavity (PerC) B1 B cells were obtained from whole peritoneal exudate cells (PEC) and cultured in standard medium consisting of DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 10% Opti-MEM (Gibco), and 50 μM 2-Mercaptoethanol (Fisher Scientific, Waltham, MA) at 37°C in a humidified incubator with 5% CO_2. For phagocytosis experiments, cells were incubated in serum free standard medium. PECs were collected from 6 – 12 week old male and female C57Bl/6 or *Tlr2*$^{-/-}$ mice by peritoneal lavage with ice cold phosphate buffered saline containing KCl and KH_2PO_4 (PBS) and pooled by strain. PECs from 3-8 mice per strain were centrifuged and resuspended in 5 mL ice cold ammonium-chloride-potassium buffer and placed on ice for 5 minutes to lyse red blood cells. PECs were washed once in PBS and resuspended in standard medium without FBS. B1 B cells were negatively selected from total unelicited PECs. PECs were incubated with 0.5 μg Fc receptor blocking antibody (anti-CD16/32) per $1x10^6$ cells on ice for 15 minutes to prevent non-specific antibody binding. The following biotinylated antibody cocktail was added for another 15 minute incubation at 4° C: biotin anti-mouse CD3, biotin anti-mouse NK1.1, biotin anti-mouse CD115, and biotin anti-mouse Gr-1 (BioLegend, San Diego, CA). Each antibody was added at a final concentration of 0.25 μg per $1x10^6$ cells. Anti-biotin-anti-dextran tetrameric antibody complexes were

added and incubated for an additional 15 minutes at 4°C. Dextran coated magnetic beads (Stemcell Technologies, Vancouver, CA) were added and incubated for 5 minutes at room temperature. Cells were placed in an EasySep magnet (Stemcell Technologies) according to the manufacturer's protocol. The negatively selected cells were resuspended in standard medium without FBS and used as PerC B1 B cells for further experiments. Population purity, determined by flow cytometry analysis by gating on CD19, was >90% for all experiments.

Hypoxia

Cells were subjected to hypoxia as previously described [316]. Standard medium was deoxygenated for 15 minutes in a sealed vacuum flask at >15 inHg prior to hypoxia. For hypoxic treatment, cells were placed in deoxygenated standard medium and cultured in a hypoxia chamber (Billups-Rothenburg, Del Mar, CA), purged with 1% O_2 gas mixture for 5 minutes, and incubated at 37°C for 2 hours as an *in vitro* model of oxygen deprivation. Normal culture conditions with fresh standard media served as a normoxic control for all experiments.

Supernatant generation and secretion analysis

PerC B1 B cells from B6 or *Tlr2*$^{-/-}$ mice were seeded into 24-well tissue culture plates at $2x10^6$ cells/mL. Normoxic and hypoxic cells were unstimulated or stimulated with 1 µg/mL Pam_3CSK_4 (Invivogen, San Diego, CA) for 2 hours prior to supernatant collection and stored at -80°C until analysis for IL-6, IL-10, and TNFα. The concentration of IL-10 was evaluated with a multiplex bead assay on a Luminex 200 (Millipore, Billerica, MA) and analyzed with Xponent software (Millipore) while IL-6 and TNFα were determined using mouse ELISA MAX Standard kits (BioLegend) according to the manufacturer's protocol.

Preparation of FITC-labeled, uncoated or C57Bl/6sera-opsonized microspheres

Microspheres, 200 µL of 2.75% solids amino microspheres (0.97 micron) (PolySciences, Warrington, PA) were fluorescein isothiocyanate (FITC)-labeled. Under sterile conditions, microspheres were incubated in 0.1 M Carbonate-Bicarbonate buffer (pH 9.4) with 2 mg/mL FITC-Celite (Sigma-Aldrich) for 4 hours at room temperature with constant rotation. To sera-opsonize, FITC-labeled microspheres

were washed in PBS and incubated with previously frozen C57Bl/6 sera for 1 hour at 37°C to passively adsorb serum proteins. After labeling with or without sera-opsonization, microspheres were washed with 0.1 M Glycine in PBS to block non-specific binding and resuspended in 200 µL (final volume) of the same buffer to yield a suspension at 4.55×10^{10} particles/mL. The beads were stored at 4°C for use in phagocytosis assays.

Phagocytosis assay

A modified version of the phagocytosis assay described by Nakashima, et al. [63] was used to evaluate PerC B1 B cell phagocytic activity under normoxic or hypoxic conditions. PerC B1 B cells were pooled from three to eight B6 or *Tlr2*$^{-/-}$ mice and incubated on ice with 1 µg Fc receptor blocking antibody (BioLegend) per 10^6 cells for 15 minutes prior to use in the assay. Cells were resuspended in normal or deoxygenated standard medium at a concentration of 2.5×10^6 cells/mL, and 200 µL was aliquoted per well of a flat bottom 96-well plate containing 0.5 µg Fc receptor blocking antibody. Cells were incubated with or without 1.0×10^8 uncoated- or serum-opsonized microspheres. Where indicated, cells were incubated with microspheres in the presence or absence of 1 µg/mL Pam_3CSK_4 (Invivogen). Prior to 2 h normoxic or hypoxic incubation, the plates were centrifuged at 300 x g for 1 minute to settle microspheres onto the cells. After the 2 hour incubation, cells were centrifuged and washed with 5% FBS in PBS. The supernatants were discarded and cells were resuspended in warmed trypsin-ethylenediaminetetraacetic acid (Gibco) for a 5 minute incubation at 37°C to release non-specifically bound, external microspheres. An equal volume of 5% FBS in PBS was added to neutralize trypsin activity followed by three washes. Cells were resuspended in the same buffer at a concentration of 10^6 cells/mL for flow cytometry staining or microscopy slide preparation for immunocytochemistry.

Flow cytometry

After normoxia or hypoxia, 1×10^6 cells/mL, suspended in staining buffer (5% FBS in PBS) were aliquoted into round bottom 96-well plates and incubated with 10 µg/mL Fc receptor blocking antibody (Biolegend) for 15 minutes on ice. The plates were centrifuged at 300 x g for 3 minutes, supernatants discarded, and the cells were resuspended in staining buffer to contain a final volume of 100 µL per well after the addition of antibodies. Antibodies were used at the concentrations indicated in Table 1. Primary

or isotype antibodies were added to the appropriate wells and incubated with cells for 15 minutes on ice in the dark. Cells were washed in staining buffer three times before resuspending and transferring to 5 mL Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) in a final volume of 500 µL. Fluorescence was detected the same day on a BD FACScalibur (BD Biosciences, San Jose, CA) with CellQuest Pro software (BD Biosciences) without compensation applied. Analysis was performed with WinList 5.0 software (Verity Software, Topsham, ME). B cell populations were identified and analyzed by gating on $CD19^+$ cells displayed on a forward scatter and side scatter plot. For phagocytosis assays, untreated cells and unlabeled microspheres were used to determine background fluorescence of FITC-labeled microspheres. $CD19^+$ gated $CD11b^+$, $CD5^+$ and $FITC^+$ cells were considered to be phagocytic B1a B cells and $CD19^+$ gated $CD11b^+$, $CD5^-$, $FITC^+$ cells as phagocytic B1b B cells. Compensation was applied during data analysis in WinList 5.0 to account for fluorescent spillover between channels, adjusting for FITC with R-phycoerythrin (PE), PE with PE-Cyanine (Cy) 5.5, and PE-Cy5.5 with allophycocyanin (APC).

Immunocytochemistry

Slides were prepared by centrifuging $1.5x10^5$ cells onto double frosted glass slides (Fisher) using a Cytospin200. The slides were air dried, fixed in iced methanol, and washed with PBS. Non-specific binding was blocked by incubating the slides with 10% normal rat sera in PBS for 30 minutes at 37°C. Cells were stained overnight at 4°C with biotin anti-mouse IgM (BD Biosciences) and Brilliant Violet 421 anti-mouse/human CD11b (BioLegend), or with corresponding isotype antibodies, biotin rat IgG2a (BD Biosciences) and Brilliant Violet 421 rat IgG2b (Biolegend). The following day, slides were washed in PBS and incubated with strepavidin-Texas Red (Jackson ImmunoResearch) for 15 minutes in the dark. Slides were washed, dried, mounted with ProLong Gold anti-fade reagent (Life Technologies, Carlsbad, CA) and coverslipped. Fluorescent microscopy was performed with a Nikon 80i fluorescent microscope and images acquired using a CoolSnapCf camera (Photometrics, Tuscon, AZ) and MetaVue Imaging software (Molecular Devices, Sunnyvale, CA). Pictures were taken with FITC, Texas Red, and Brilliant Violet 421 filters of the same field of view and merged into a single image. The percent phagocytic B1 B cells (B cells ingesting at least one FITC-labeled particle/total B cells X 100) and Phagocytic Index (PI, Mean particles per phagocytic cells X percent phagocytic cells) were determined by counting 100 – 200 IgM^+, $CD11b^+$ total cells in multiple fields of view in merged microphotographs.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Data were analyzed with an unpaired t-test. Data are presented as the SEM and considered significant when $P<0.05$.

Table 1. Antibodies for Flow Cytometry

Antibody	μg/100 μL	Supplier
Purified anti-mouse Siglec-G	0.10 μg	Santa Cruz
FITC-anti-mouse IgG2	0.25 μg	BioLegend
FITC-anti-mouse IgM	0.25 μg	BioLegend
FITC-anti-mouse CD43	0.25 μg	BioLegend
PE-anti-mouse IgM	0.25 μg	BioLegend
PE-anti-mouse CD11b	0.25 μg	BioLegend
PE-anti mouse TLR2	0.50 μg	eBioscience (San Diego, CA)
PE-anti mouse CD 21/35	0.50 μg	BD Biosciences (San Jose, CA)
PE-Cy5.5 anti-mouse CD19	0.10 μg	Caltag Labs (Buckingham, UK)
PerCP anti-mouse CD5	0.25 μg	BioLegend
APC-anti-mouse CD11b	0.25 μg	BioLegend
APC-anti-mouse CD5	0.25 μg	eBioscience
Biotin anti-mouse CD5	0.50 μg	BioLegend
Biotin-anti-mouse CD9	0.50 μg	BD Biosciences
Purified Goat IgG	0.10 μg	Jackson ImmunoResearch
FITC-Rat IgG2a	0.25 μg	BioLegend
PE-Rat IgG2a/b	0.25/0.50 μg	eBioscience, BD Bioscience
PerCP Rat IgG2a	0.25 μg	BioLegend
PECy5.5 Rat IgG2a	0.10 μg	Caltag Labs, eBioscience
APC Rat IgG2a/b	0.25 μg	eBioscience, BD Biosciences
Biotin Rat IgG2a	0.50 μg	BioLegend

Results

Wild type peritoneal exudate cell B1 B cell surface marker expression in response to hypoxia

We tested the hypotheses that hypoxia activates B1 B cells and alters the surface phenotype by evaluating the surface phenotype of negatively selected PerC B cells. PerC B cells of C57Bl/6 mice were stained for CD19, CD11b, CD5, and TLR2 to identify the subsets of B1 B cells (Fig. 1A and B). The $CD19^{+}$ cells (Fig. 1A), were divided into $CD11b^{+}CD5^{+}$ (B1a) or $CD11b^{+}CD5^{-}$ (B1b) B cells (Fig. 1B), B1b cells predominated over the B1a subset (Fig. 1B). In wild type mice, we observed a ratio of B1a to B1b B cells which averaged 1:1.2. As stimulation of TLRs activates B1 B cells, we also established that both B1 B cell subsets express TLR2 in the basal state (Fig. 1C). We found that B1a B cells exhibited higher levels of surface TLR2 compared to B1b cells.

It was possible that hypoxia altered the expression of CD11b and CD5 on B1 B cells. Compared to normoxic cells, the hypoxic PerC B cells contained a similar percentage of CD19+ cells and an increased the number of $CD5^{+}CD11b^{+}$ double positive cells. Hypoxic B1 B cells expressed TLR2 similar to normoxic cells and overall, hypoxia exerted negligible effect on the B1 B cell phenotype and TLR2 expression.

PerC B1 B cell cytokine production in response to hypoxia

As hypoxia increases TNFα and IL-6 secretion in professional phagocytes [159, 223, 253], we hypothesized TNFα and IL-6 production by B1 B cells may increase in response to hypoxia as well. After two hours normoxia, PerC B1 B cells secreted approximately 50 pg/ml TNF and 500 pg/ml IL-6 (fig. 2-2 A, B open bars). Unexpectedly, cells subjected to hypoxia exhibited a significant decrease in the secretion of both TNFα and IL-6 (Fig. 2-2 A, B diagonal bars). Similar to pro-inflammatory cytokine levels, normoxic cells secreted 9 pg/mL IL-10 which significantly decreased following hypoxic treatment.

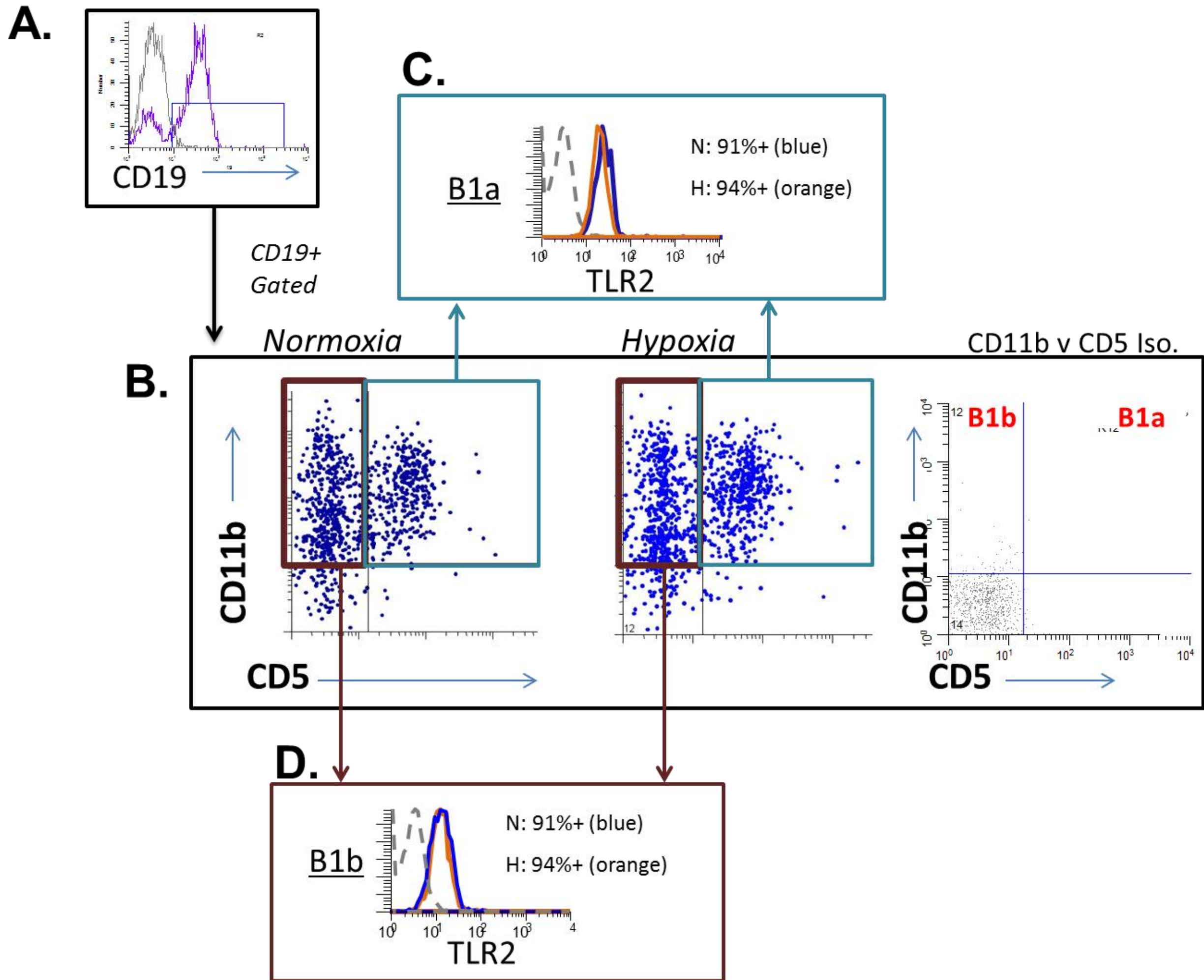


Figure 2-1. The effect of hypoxia on B1 B cell surface marker expression

After 2 hours of normoxia or hypoxia, wild type PerC B cells were labeled for CD19, CD11b, CD5, and TLR2 followed by flow cytometry analysis. After gating on CD19+ cells (A), B1 B cell subsets were identified based on the expression of CD11b with or without CD5 (B). The level of TLR2 on normoxic (blue line) and hypoxic (orange line) B1a B cells (C) or B1b B cells (D) was determined. Isotype controls are shown in gray. Histograms are representative of 3 independent experiments.

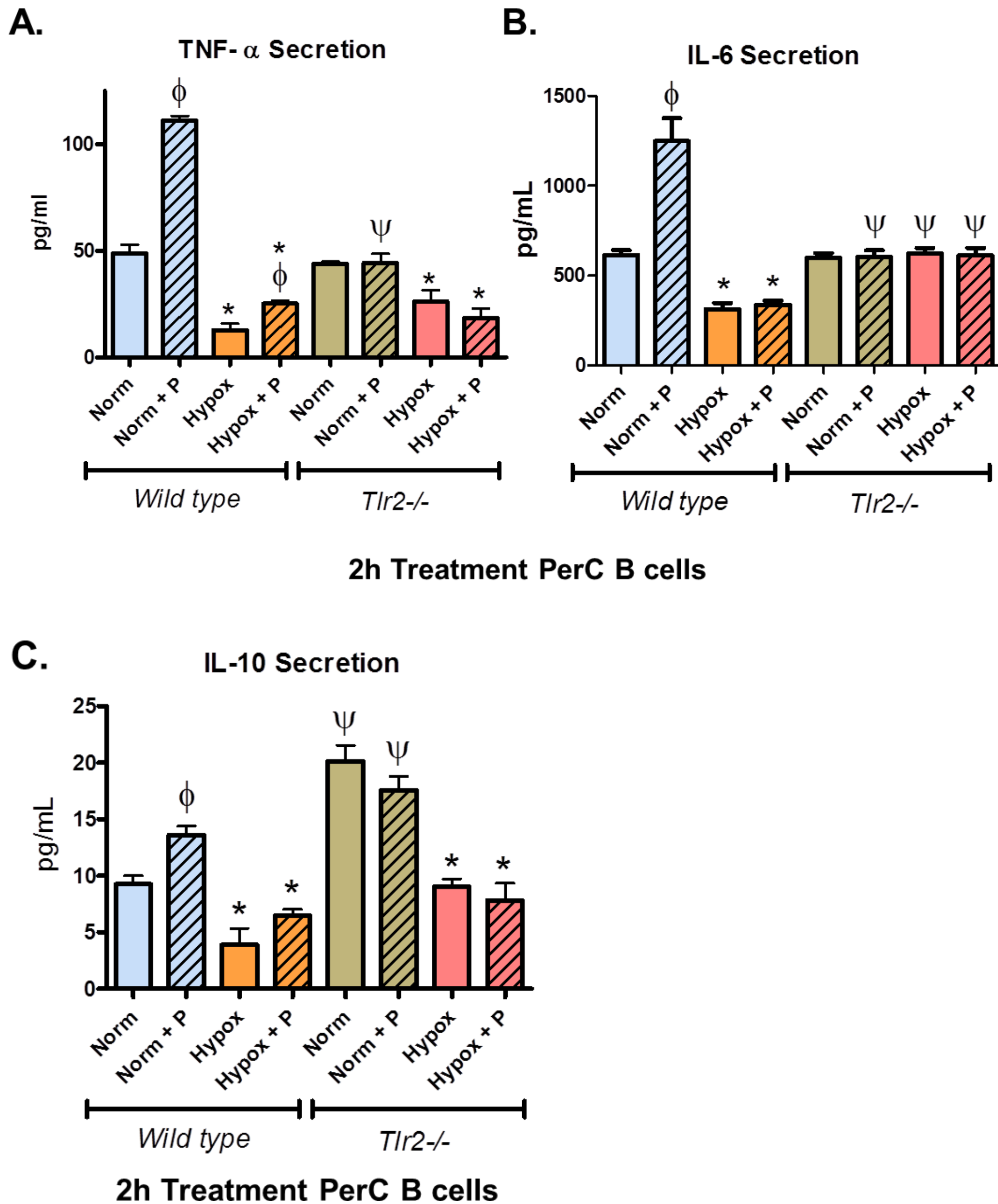


Figure 2-2. The effect of hypoxia and TLR2 expression on B1 B cell cytokine production

Supernatants were collected from wild type or $Tlr2^{-/-}$ PerC B cells after 2 hours of normoxic (Norm, blue and brown bars) or hypoxic (Hypox, orange and red bars) treatment with or without simultaneous administration of 1 µg/mL Pam_3CSK_4 (P, angled lines). The concentration (pg/mL) of TNFα (A) and IL-6 (B) were determined by ELISA and IL-10 (C) was determined with a multiplex assay. Data are from 3 – 4 independent experiments and results were significant compared to unstimulated controls (ϕ), normoxic controls (*), or wild type controls (ψ) with a p-value <0.05.

Upregulation of phagocytosis in response to hypoxia

Since hypoxia upregulates the phagocytic activity of macrophages [85], we hypothesized that hypoxia would enhance B1 B cell phagocytosis. Initial studies indicated that 29% of normoxic PerC B cells phagocytosed sera-opsonized beads with a PI of 70.3 (Fig. 2-3 C). Importantly, 45% of B1 B cells were phagocytic after hypoxia treatment. Additionally, each hypoxic B cell ingested a significantly increased number of particles compared to normoxic B cells.

To determine if opsonization affected the phagocytic activity of B1 B cells during hypoxia, B1 B cells were incubated with unopsonized beads. A similar percentage of normoxic B1 B cells phagocytosed uncoated and sera-opsonized microspheres, 22.7% and 26.7% respectively. However, hypoxia failed to upregulate phagocytosis of unopsonized beads by B1 B cells (Fig. 2-3 D). On average, 24.7% of hypoxic B1 B cells phagocytosed unopsonized particles compared to 45% observed with sera-opsonized microspheres.

Recently, Parra et al. [66] demonstrated that under normal culture conditions, B1 B cells may not phagocytose large particles (≥ 3 microns). In other cell types, hypoxia increases the amount of particles ingested, taking up more cytoplasmic space. We hypothesized the enhanced B1 B cell phagocytosis during hypoxia may allow for ingestion of larger particles. B1 B cells failed to phagocytose serum opsonized zymosan (~3 μm in size) under either normoxic or hypoxic (data not shown) [317]. These data suggest that despite increased phagocytic activity in response to hypoxia, particle size remains a limiting factor.

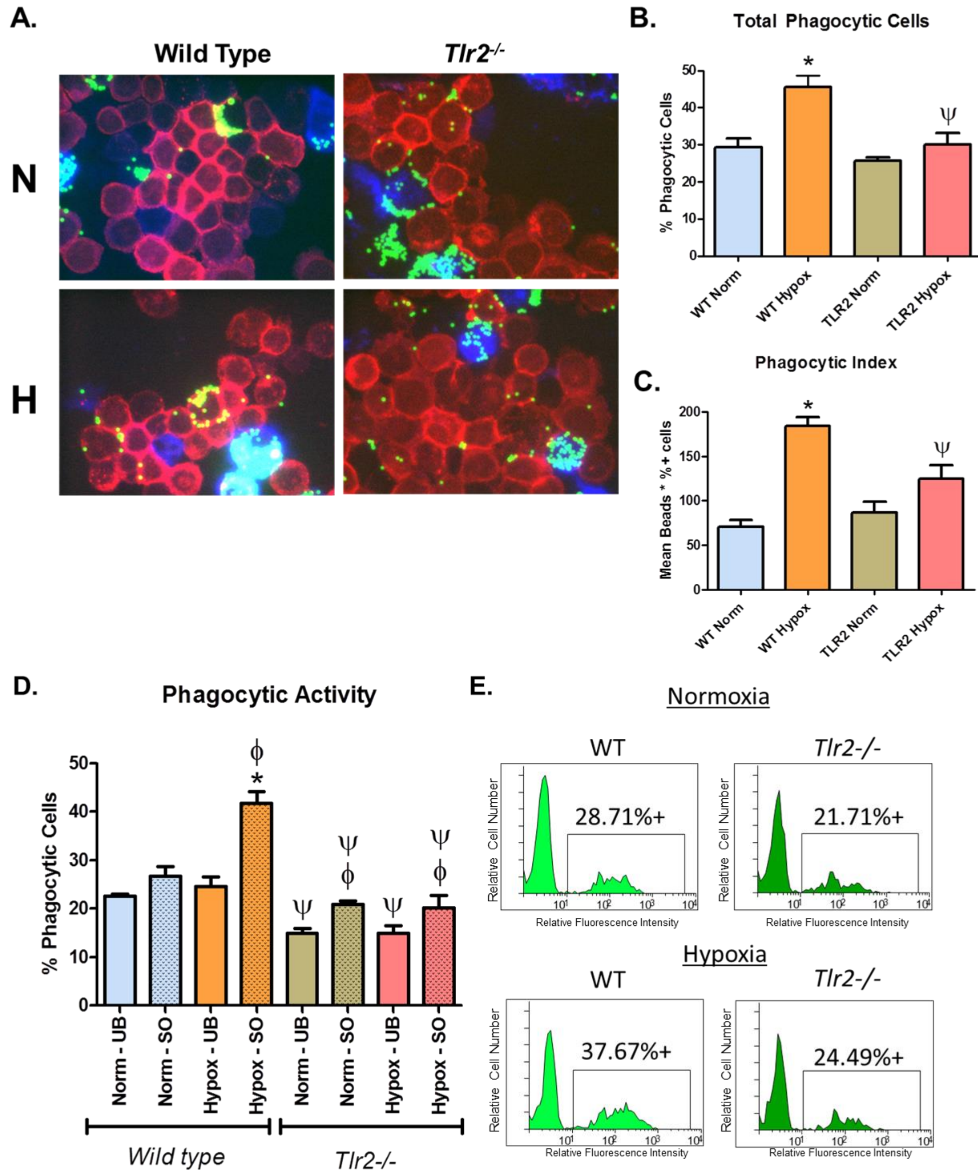


Figure 2-3. The effect of hypoxia and TLR2 expression on B1 phagocytosis

PerC B cells from B6 (WT) or *Tlr2*$^{-/-}$ (TLR2) mice were incubated with sera-opsonized microspheres under normoxic (blue, brown bars) or hypoxic (orange, red bars) conditions for two hours. Fluorescent microscopy (A) was used to evaluate the phagocytosis of fluorescently labeled microspheres (photo- green) by IgM+ (photo- red) B cells. The percent of phagocytic cells (B) and phagocytic index (C) for WT and *Tlr2*$^{-/-}$ IgM+ cells was determined based on photomicrographs. Photos are representative of data collected from 2 – 3 independent experiments. Based on flow cytometry analysis, we determined the percent of phagocytic wild type or Tlr2$^{-/-}$ PerC B cells ingesting uncoated beads (UB, open bars) or sera-opsonized beads (SO, thatched bars) with 2 hours of normoxic (blue, brown bars) or hypoxic (orange, red bars) treatment (D). Histograms of CD19+CD11b+ WT and Tlr2$^{-/-}$ B cells subjected to normoxia or hypoxia which phagocytosed SO beads are representative of 3 independent experiments (E) and gates were based on background fluorescence of unlabeled beads. Data are from 3 – 4 independent experiments and the results were considered significant compared to unopsonized controls (Φ), normoxic controls (*), or wild type controls (ψ) with a p-value <0.05.

Hypoxic cytokine response of TLR2 stimulated B1 B cells

As TLR2 signaling activates B1 B cells [123], we hypothesized that Pam_3CSK_4, a synthetic TLR2-1 agonist, may promote the secretion of TNFα, IL-6, and IL-10 in response to hypoxia. Normoxic PerC B1 B cells stimulated with Pam_3CSK_4 significantly upregulated production of all three cytokines (Fig. 2-2, striped bars) Similar to previous studies, Pam_3CSK_4 stimulation attenuated cytokine levels of hypoxic cells (Fig. 2-2). To verify that the cells were stimulated through TLR2, we assessed cytokine secretion by *Tlr2*$^{-/-}$ cells after stimulation with Pam_3CSK_4. Compared to unstimulated B cells from wild type mice, *Tlr2*$^{-/-}$ PerC B cells failed to respond to Pam_3CSK_4 stimulation indicating that signaling was only through TLR2 and excluding endotoxin contamination. Although normoxic wildtype and *Tlr2*$^{-/-}$ PerC B cells produced a similar level of TNFα and IL-6, the constitutive level of IL-10 produced by *Tlr2*$^{-/-}$ cells doubled to 20 pg/mL versus 9 pg/mL. Additionally, hypoxia reduced the amount of TNFα and IL-10 secreted by *Tlr2*$^{-/-}$ PECs but failed to decrease IL-6. These data suggest the hypoxia induced downregulation of IL-6 depends on TLR2 expression.

The role of TLR2 expression on B1 B cell phagocytosis

Since TLR2 stimulation upregulated B1 B cell cytokine production under normoxic conditions, we hypothesized that TLR2 may play a role in phagocytic activity. *Tlr2*$^{-/-}$ B1 B cells phagocytosed unopsonized microspheres less efficiently than wild type cells, with a significant decrease in the percent cells ingesting at least one particle (Fig. 2-3 D). Phagocytosis by *Tlr2*$^{-/-}$ B1 B cells increased with serum opsonization of the particles but the percent phagocytic cells remained lower than wild type cells (Fig. 2-3 D). Under normoxic conditions, *Tlr2*$^{-/-}$ B1 B cells exhibited a PI similar to that of wild type cells (Fig. 2-3 C). As TLR2 expression altered phagocytosis under normal culture conditions, we evaluated the phagocytic response of *Tlr2*$^{-/-}$ B1 B cells subjected to hypoxia. *Tlr2*$^{-/-}$ B1 B cells failed to upregulate phagocytic activity in response to hypoxia. The percent hypoxic, phagocytic *Tlr2*$^{-/-}$ cells, 24%, was not increased and remained similar to Tlr2$^{-/-}$ normoxic (Fig. 2-3 B, D). The phagocytic index of *Tlr2*$^{-/-}$ B1 B cells increased slightly with hypoxia but remained significantly lower than that of wild type cells (Fig. 2-3 C). These data indicate that B1 B cells require TLR2 expression for hypoxia-induced upregulation of phagocytosis.

TLR2 expression modulates the B1 compartment by shifting the B1a to B1b ratio

As B1 B cell subsets exhibit differential phagocytic activity, [63, 66], we hypothesized TLR2 deficiency may alter phagocytosis by altering the B1 B cell compartment. Following the same gating scheme as described above, we identified the *Tlr2*$^{-/-}$ CD19^{+} CD11b^{+} CD5^{+} B cell subsets after normoxia and hypoxia. A loss of TLR2 expression skewed the B1 compartment toward the B1a phenotype, with a 1.5:1 ratio of B1a to B1b cells (Fig. 2-4 A, B). However, the distribution remained unchanged with hypoxia suggesting the subset ratio does not account for the altered phagocytic activity of Tlr2$^{-/-}$ cells.

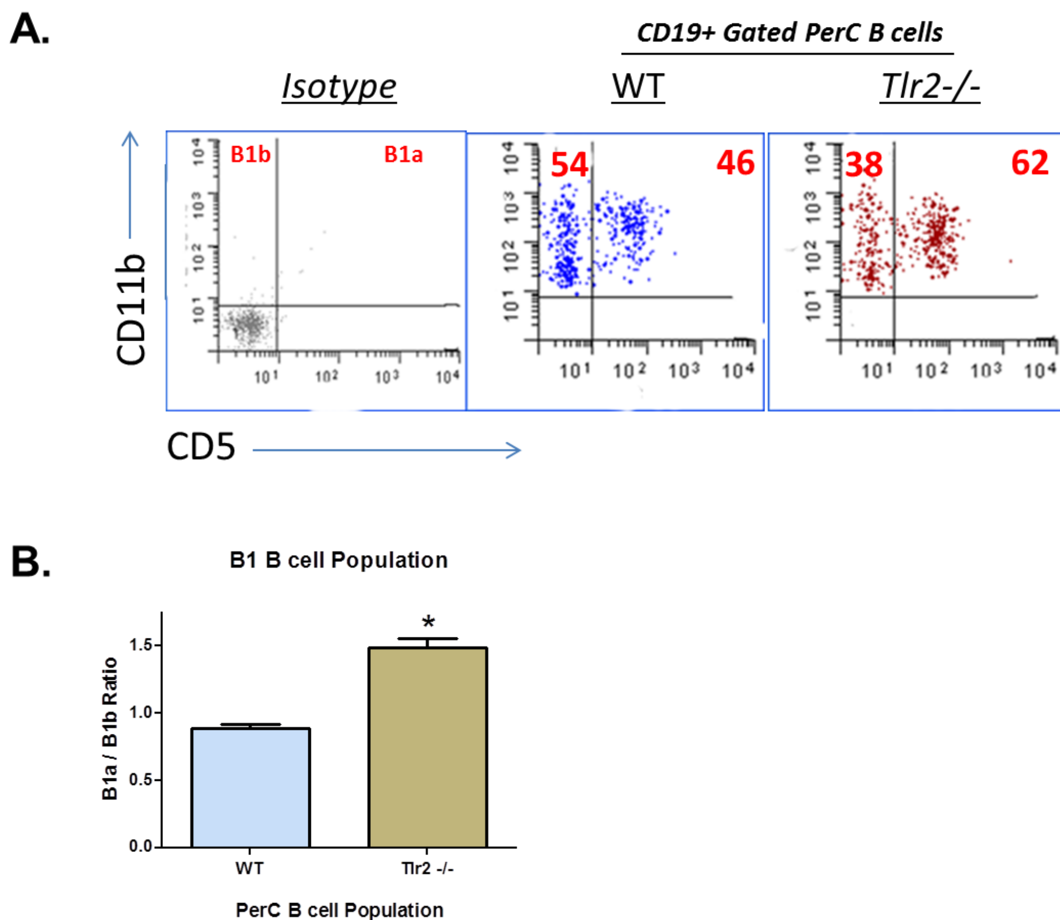
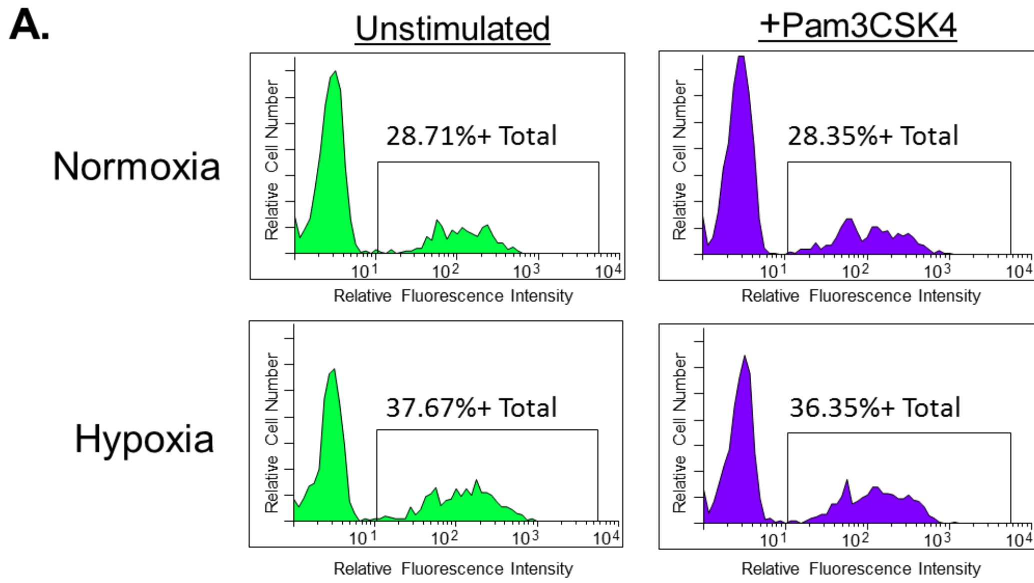


Figure 2-4. TLR2 expression alters the peritoneal B1 B cell compartment

The percent of CD19+ gated B1a B cells (CD11b+CD5+, upper right quadrants) and B1b B cells (CD11b+, CD5-, upper left quadrants) from 5 – 8 pooled normoxic wild type (WT, blue dot plot) or Tlr2$^{-/-}$ (red dot plot) mice was determined by flow cytometry (A). The ratio of B1a to B1b cells from the total PerC B cells of WT and Tlr2$^{-/-}$ mice was determined (B). Gates were based on isotype controls (gray dot plot). The numbers displayed in upper right and left quadrants are indicative of %+ cells. Data are representative of 3 – 5 independent experiments.

The effect of TLR2 stimulation on B1 B cell phagocytosis under normoxic or hypoxic conditions

TLR2 stimulation upregulates the phagocytic activity of mononuclear phagocytes (Reviewed in [318]). As TLR2 deficiency negatively affected phagocytosis under hypoxic conditions, we hypothesized that stimulation through TLR2 may play a role in B1 B cell phagocytosis and affect phagocytic activity in response to hypoxia. We evaluated phagocytosis of serum opsonized particles by Pam_3CSK_4 stimulated B1 B cells (Fig. 5A, B). Compared to unstimulated cells, Pam_3CSK_4 stimulation of Normoxic B1 B cells did not change the phagocytic activity. Although hypoxia upregulated phagocytosis by wildtype cells, Pam_3CSK_4 stimulation did not effect to the phagocytic response by either wild type or *Tlr2*$^{-/-}$ cells. These data suggest B1 B cells require TLR2 expression but stimulation through the TLR2/1 agonist, Pam_3CSK_4, fails to enhance phagocytosis under normoxic or hypoxic conditions.



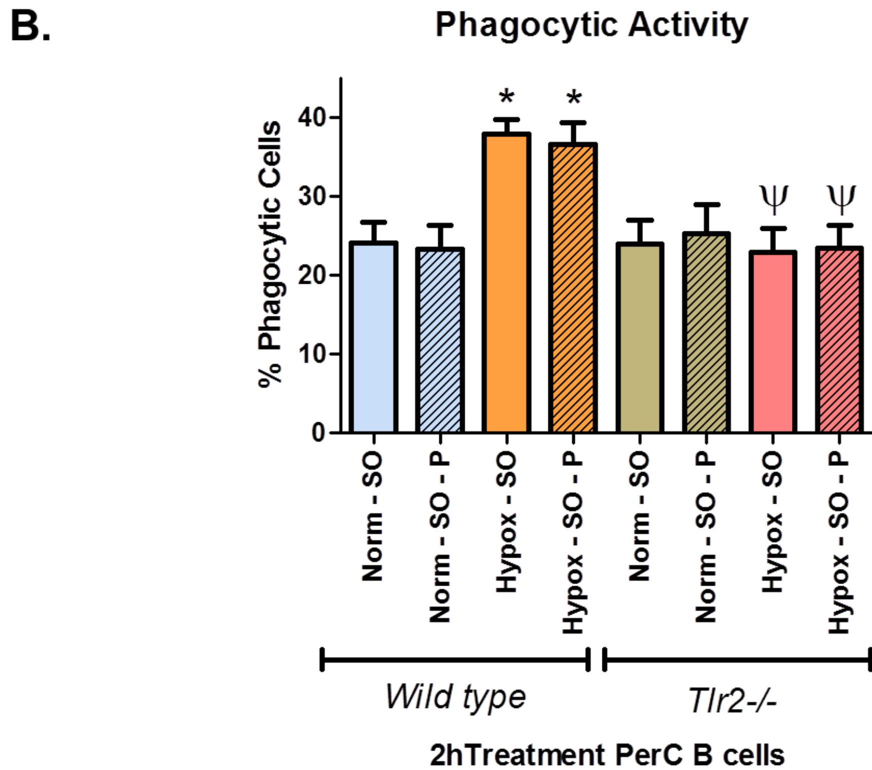


Figure 2-5. The effect of TLR2 stimulation on B1 B cell phagocytosis

B1 B cells from wild type or Tlr2$^{-/-}$ mice were subjected to 2 hours of normoxia (blue, brown bars) or hypoxia (orange, red bars) with sera-opsonized (SO) beads in the presence or absence of 1 ug/mL Pam_3CSK_4 (P, angled lines) and phagocytosis was evaluated by flow cytometry (A, B). Histograms are representative of at least 3 independent experiments. Results were considered significantly different compared to normoxic controls (*), or wild type controls (ψ) with a p-value <0.05.

Discussion

Toll-like receptor and hypoxic stimulation significantly alters the inflammatory response and effector function of professional phagocytes. Similarly, TLRs play a role in the B cell inflammatory response as TLR 2/1 or TLR 2/6 agonists activate B1 B cells to produce antibody and nitric oxide [60]. Hypoxia activates B1 B cells to express chemokine receptors and induce migration [165]. While some aspects of TLR and hypoxic activation of B1 B cells have been characterized, many components such as the effect on phagocytosis remain unclear. In our studies, we evaluated the role of TLR2 expression and stimulation on B1 B cell phagocytosis and cytokine production under normoxic and hypoxic conditions. To test the hypothesis, wild type and *Tlr2*$^{-/-}$ B1 B cells were subjected to normoxic or hypoxic treatment in the presence or absence of polystyrene beads and the synthetic TLR 2/1 agonist, Pam_3CSK_4.

We determined both B1 B cell subsets express TLR2 with the B1a B cell subset expressing slightly higher levels. The levels of surface TLR2 increase on macrophages and other phagocytes exposed to hypoxia [319]. However, in contrast to professional phagocytes, expression remained unchanged with hypoxia on B1 B cells. The lack of upregulation of TLR2 may be due to hypoxia inhibition of protein synthesis [320]. Many studies with professional phagocytes evaluated the effect of periods of priming and reoxygenation. Perhaps these additional conditions play a critical role in the B1 B cell response to hypoxia similar to that observed in other leukocytes.

TLR expression and opsonization also affect phagocytosis by professional phagocytes. A requirement for TLR expression has been described in neutrophils to allow for efficient phagocytosis and killing of unopsonized bacteria [321]. We found TLR2 expression was not required for B1 B cell phagocytosis of opsonized particles under normoxic conditions. However, *Tlr2*$^{-/-}$ B1 B cells exhibited diminished capacity to phagocytose unopsonized particles. These data suggest that TLR2 may play a role in non-opsonic phagocytosis by B1 B cells.

Hypoxia induced upregulation of phagocytosis in macrophages has been linked to p38 MAPK expression. Interestingly, downregulation of TLR2 by siRNA inhibits p38 phosphorylation [322]. The number of wild type B1 B cells increased with hypoxic treatment compared to normoxic controls. However, our data indicate the absence of TLR2 negatively affects hypoxia induced upregulation of phagocytosis by B1 B cells. It is possible hypoxic *Tlr2*$^{-/-}$ B1 B cells exhibit a defect in p38 MAPK

phosphorylation, preventing the hypoxia induced upregulation of phagocytosis. Future studies will be required to determine if TLR2 expression mediates B1 B cell p38 levels and if hypoxia induces MAPK phosphorylation.

While TLR2 expression plays an important role in the phagocytosis pathway of professional phagocytes and response to hypoxia, phagosome formation and maturation may be independent of TLR expression in B1 B cells [100, 314]. Studies with *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ macrophages demonstrated that these cells exhibit similar particle uptake and phagosome maturation compared to wild type cells [100]. Gao *et al*. demonstrated B1 B cells ingest *Staphylococcus aureus* or 1 micron polystyrene beads through phagosome formation and maturation similar to other phagocytes [65]. In our studies, we observed B1 B cells from *Tlr2*$^{-/-}$ mice ingested a similar average number of particles compared to wild type cells. These data suggest that TLR2 expression is not required for internalization of polystyrene beads. Studies with *Tlr2*$^{-/-}$ B1 B cells similar to Gao *et al*. are necessary to determine if TLR2 expression affects phagosome formation and maturation.

An unexpected observation with *Tlr2*$^{-/-}$ B1 B cells was the increased B1a compartment and enhanced production of IL-10. Signaling through TLRs, including TLR2, produces a profound effect on B1 B cell development, immunoglobulin class switching, and migration. It is difficult to explain why TLR2 deficiency resulted in an increased B1a B cell compartment. The mechanism of B1 B cell development remains unclear and it is possible a developmental defect arises in the absence of TLR2 signaling. Alternatively, TLR signals induce migration of B1 B cells [323] and a lack of TLR2 signaling may prevent the egress of B1a B cells from the peritoneal cavity. We observed enhanced IL-10 production from *Tlr2*$^{-/-}$ B1 B cells. As B1a B cells are known to constitutively secrete high levels of IL-10, the increased percentage of B1a B cells in the PerC of *Tlr2*$^{-/-}$ mice compared to wild type may account for the increased cytokine level. An alternative possibility is that the high level of IL-10, an autocrine growth factor for B1a B cells [278], enhances proliferation of this subset in *Tlr2*$^{-/-}$ mice.

Constitutive production of both TNFα and IL-6 by granulocytes and monocytes has been previously described [324, 325]. Both cytokines display autocrine and paracrine activity. In addition to IL-10, we observed that wild type and *Tlr2*$^{-/-}$ B1 B cells constitutively secreted low levels of TNFα and IL-6. B1 B cells use IL-10 as an autocrine cytokine to promote proliferation [278]. It is possible that an

autocrine function exists for TNFα and IL-6 in B1 B cells. Conflicting findings have been reported on the effect of hypoxia on cytokine production [86]. Both up- and down-regulation of TNFα and IL-6 have been described following periods of hypoxia [289] [223] [134]. The results vary widely based on the cell type and experimental conditions. We found that hypoxia significantly attenuated constitutive secretion of TNFα and IL-6 by B1 B cells. Unexpectedly, we found the downregulation of IL-6 in response to hypoxia was dependent on TLR2 expression. An interaction between the TLR2 signaling pathway and hypoxia may occur to regulate secretion of IL-6. TLR2 deficiency has been associated with inhibition of the PI3K/AKT pathway [326]. This pathway may be activated under hypoxic conditions and plays a role in regulating IL-6 production [327].

TLR2 expression affected B1 B cell phagocytosis with hypoxic stimulation. Therefore, we hypothesized an alternative stimulus through the TLR2 pathway may also enhance phagocytosis. Granulocytes stimulated with TLR2 agonists such as Pam_3CSK_4 significantly upregulate phagocytic activity [328]. However, we observed that stimulation with the TLR2-1 agonist Pam_3CSK_4 failed to enhance the number of phagocytic B1 B cells. In our experiments, Pam_3CSK_4 was administered as a soluble agonist. It is likely that Pam_3CSK_4 coupled to the polystyrene beads may result in enhanced phagocytosis. The use of polystyrene beads may be a reason in itself as Mae, et al. report TLR2-associated upregulation of phagocytosis in macrophages occurs with bacteria but not with artificial particulates [121]. Alternatively, B1 B cells may upregulate phagocytosis through TLR2/6 interactions. An appropriate agonist would need to be used to determine which heterodimer TLR2 forms (2/1 or 2/6) to play a role in B1 B cell phagocytic activity.

Chapter 3 - The effect of hypoxia and role of complement in B1 B cell effector function

Abstract

Professional phagocytes utilize opsonins, such as complement (C) fragments, and their cognate receptors, complement receptors (CR), to enhance phagocytic and inflammatory activity. Phagocytes also respond to environmental factors such as hypoxia to enhance their inflammatory and phagocytic response. B1 B cells exhibit similar phagocytic activity, although studied less extensively. Although B1 B cells depend on the presence of C for phagocytic activity, the specifc C components required remain unknown. B1 B cells express multiple CR, including CR2 which is not expressed on other phagocytes. Additionally, the effects of hypoxia on B1 B cells are poorly characterized despite the prevalence of B1 B cells in hypoxic environments. We tested the hypothesis that hypoxia and opsonization with C3d, the ligand for CR2, enhance B1 B cell phagocytosis and stimulate an inflammatory response similar to professional phagocytes. Using a B1 B cell-like cell line, Wehi 231, we evaluated phagocytic activity and inflammatory secretions in the presence and absence of C3d. Additionally, wild type and *Cr2*$^{-/-}$ peritoneal cavity (PerC) B1 B cells were evaluated to determine the role of CR2 as a phagocytic receptor. We determined that either hypoxia or C3d opsonization upregulated Wehi-231 phagocytosis. Hypoxia alone enhanced iNOS, NO, and Ab production by this cell line. Hypoxia upregulated the percent of phagocytic PerC B1 B cells in a CR2-dependent manner. In contrast, C3d opsonization with or without hypoxia upregulated phagocytic activity in a CR2-independent fashion. In contrast to augmented phagocytosis, hypoxia decreased inflammatory cytokine production by PerC B1 B cells. Incubation of B1 B cells with C3d-coated beads failed to act as a stimulus for production of pro-inflammatory cytokines. Taken together, the data suggest that hypoxia, as well as opsonization, enhance phagocytic activity and some effector functions of B1 B cells.

Introduction

Phagocytosis plays an important role in the immune response, allowing for antigen presentation and the clearance of immune complexes or antigens [83]. Professional phagocytes, generally belonging to the myeloid lineage, act as the major immune cells critical for this process. Non-opsonic phagocytosis occurs through ligation of pattern recognition receptors (PRR), some of which also enhance the activity of opsonin-mediated phagocytosis [98]. Depending on the ligand present, some receptors such as CR3, may act as either an opsonic or PRR to facilitate phagocytosis [206]. Opsonic phagocytosis, known as Type I, requires the expression of specific receptors, typically Fc receptors (FcR) and complement receptors (CR), on a professional phagocyte's cell membrane [75, 83, 177]. Type I phagoycptosis requires antibody (Ab) or C fragment opsonization of a particulate's surface. Opsonization acts to reduce charge-charge repulsion against a phagocyte's cell membrane and provides a high affinity ligands for opsonin receptors. The high affinity nature of opsonin-opsonin receptor interaction plays an important role in rapidly facilitating the process of phagocytosis [177].

Opsonization with C fragments occurs after cleavage of the central C pathway component, C3. Following C deposition on a particulate, C3 degrades into C3b, C3b to iC3b, and iC3b to C3d [173]. Both C3b and iC3b act as opsonins recognized by CR1 (CD35), CR3 (CD11b/CD18), and CR4 [206, 329, 330]. The majority of professional phagocytes express these CRs. While C3d acts as an opsonin, the fragment plays an unknown role in phagocytosis due to restricted expression of the cognate receptor, CR2 [170]. C3d binds to CR2, generally expressed only on B cell subsets, follicular dendritic cells, and eosinophils. CR2 acts as the high affinity receptor for C3d, although CR3 binds C3d with low affinity [188, 206]. The major function of C3d-mediated opsonization serves to reduce the threshold for B cell activation [11]. CR2, in a co-receptor complex, binds C3d on an opsonized antigen, with the antigen potentially recognized by a cognate B cell receptor (BCR). The crosslinking of C3d to CR2 and antigen to the BCR enhances the ligand-receptor interaction to augment signaling, resulting in increased antibody production [11]. Similarly, due to the high affinity interaction of C3d bound to CR2, antigen presenting cells display opsonized antigens for extended periods to enhance stimulation of phagocytes and T cells [170].

In addition to opsonins and CR expression, environmental factors play a key role in modulating the activity of phagocytes. Due to the nature of the sites where professional phagocytes migrate, they

often encounter localized areas of transient hypoxia, where the oxygen level decreases below 1% O_2. The effect of hypoxia on phagocyte function occurs through activation of transcription factors such as NFκB and HIF1 as well as through alterations in metabolism [319, 331, 332]. Hypoxia enhances the number of phagocytic cells and the average number of particulates ingested per cell [85]. Part of the enhanced activity occurs through autocrine stimulation with pro-inflammatory cytokines such as TNFα and IL-6 as well as with inflammatory mediators such as nitric oxide [222, 253, 298].

While excluded from the group of professional phagocytes, but a potent antigen presenting cell, a subset of B cells, B1 B cells, has recently been described as a mononuclear phagocyte [58]. As the major B cell population in the peritoneal and pleural cavity, B1 B cells secrete the majority of natural antibody, exhibit microbicidal activity, phagocytic activity, and produce both pro- and anti-inflammatory cytokines [31, 60, 65, 333]. The population of B1 B cells exhibit a unique surface phenotype and also express both CR2 and CR3 [31, 193, 197]. In contrast, professional phagocytes fail to express CR2 [193]. B1 B cell phagocytosis depends on the presence of C component C3, but a role for CR2 as a phagocytic or stimulatory receptor for C3 fragments on this subset is unknown [63]. Similar to professional phagocytes, B1 B cells are often found at sites of hypoxia where they contribute to limiting infection, wound healing, and tumor growth [165, 166, 334]. However, the direct effect of hypoxia on the B1 B cell inflammatory response remains unclear once B1 B cells arrive at these sites.

We tested the hypothesis that both hypoxia and CR2, recognizing C3d-opsonized particulates, alter B1 B cell phagocytosis and inflammatory responses. We subjected a B1 B cell-like cell line and negatively selected peritoneal cavity (PerC) B1 B cells from wild type and *Cr2*$^{-/-}$ mice to 2 hours of hypoxia to determine the effect on phagocytosis and requirement for CR2 expression. We demonstrate the Wehi 231 cell line resembles B1a B cells and exhibits effector functions similar to primary cells in addition to responding to hypoxic treatment. We also establish a role for hypoxia and C3d-opsonization in enhancing B1 B cell phagocytosis despite decreased cytokine production with treatment. Hypoxia induced upregulation depended on the presence of CR2 while C3d-opsonized upregulation remained independent of CR2 expression. Finally we present data indicating that C3d-opsonized particulates fail to act as a pro-inflammatory stimulus of B1 B cells. Taken together, our studies identified an *in vitro* model of

B1 B cells as well as established a role for C3d-opsonization and CR2 expression in PerC B cell phagocytosis under normoxic or hypoxic conditions.

Materials and Methods

Mice

Experiments were conducted with 6 – 12 week old adult C57Bl/6 (B6) mice obtained from Jackson Labs (Bar Harbor, MA) and *Cr2*$^{-/-}$ mice originally obtained from Dr. V.M. Holers [190]. Mice were bred within the Division of Biology at Kansas State University in a 12-hour light to dark, temperature controlled, specific pathogen free facility *(Helicobacter sp.*,mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus*, Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, and endo- and ecto- parasites). Food and water were provided *ad libitum.* All research was approved by Kansas State University's Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals.

Cell culture, peritoneal exudate cell isolation, and B1 B cell enrichment

Wehi 231 cells (a generous gift from Dr. James R. Hagman, Integrated Department of Immunology, National Jewish Health) and PerC B cells were cultured in standard medium consisting of DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10% Opti-MEM (Gibco), and 50 μM 2-mercaptoethanol (Fisher Scientific, Waltham, MA). Cell lines were passaged regularly in antibiotic free medium and maintained at 37°C in a humidified incubator with 5% CO_2. For phagocytosis experiments, cells were incubated in serum free standard medium. PEC were collected from male and female C57Bl/6 or *Cr2*$^{-/-}$ mice by peritoneal lavage with ice cold phosphate buffered saline containing KCl and KH_2PO_4 (PBS) and pooled by strain. Cells were prepared as described in Chapter 2 to obtain negatively selected PerC B cells. PerC B cells were resuspended in standard medium without FBS and used in further experiments. Population purity, determined by flow cytometry analysis by gating on CD19, was >90% for all experiments.

Hypoxia

Cells were subjected to hypoxia as previously described [316]. Standard medium was deoxygenated for 15 minutes in a sealed vacuum flask at >15 inHg prior to hypoxia. For hypoxic treatment, cells were cultured in deoxygenated standard medium and placed in a hypoxia chamber

(Billups-Rothenburg, Del Mar, CA), purged with 1% O_2 gas mixture, and incubated at 37°C for 2 hours as an *in vitro* model of oxygen deprivation. Normal culture conditions with fresh standard media served as a normoxic control for all experiments.

Mitochondrial Reductase

The relative level of mitochondrial reductase was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis as described previously [335]. Briefly, 100 uL of $5x10^5$ cells/mL was added to wells of 96-well plates and incubated for 24 hours under normoxic or hypoxic conditions. After 24 hours, the plates were centrifuged at 150 g and 10 uL of 250 μg/mL MTT was added to each well of normoxic or hypoxic treated cells and incubated under normal culture conditions for 4 hours at 37°C. The plates were centrifuged again and the supernatants were discarded. Formazan crystals were dissolved by adding 200 μL of 3% HCl 27% isopropyl alcohol to each well and shaking for 1 hour at room temperature. The absorbance was determined using a Model 680 microplate reader (BioRad, Hercules, CA) with Microplate Manager 5.2.1 software (BioRad) at 450 nM. Results were compared to 100 μL normoxic cells, 10^6 cells/mL, serially diluted seven times at a 1:2 dilution in standard medium 24 hours prior to use to determine the relative level of reductase under normoxic growth conditions.

ATP Assay

The total cellular and secreted relative ATP level was quantified with the Cell Titer Glo Luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's protocol. Wehi-231 cells, 75 μL of $1x10^5$ cells/mL, were seeded into 96-well plates. Cells were incubated under normoxic or hypoxic conditions for 2, 6, 12, and 24 hours. After each time point of normoxic or hypoxic treatment, 75 μL of room temperature Cell Titer Glo reagent was added to each sample and the plate was incubated for 10 minutes at room temperature. From each sample, 25 μL aliquots were transferred to a 384-well white flat bottom microtiter plate in quadruplicate. The plate was read on a Victor 3 chemiluminometer (PerkinElmer, Waltham, MA) and analyzed with Wallac 1420 Workstation software (PerkinElmer) within 30 minutes of adding the reagent. Results were compared to a known concentration of normoxic cells, 75

μL of 6.67×10^5 cells/mL stock, serially diluted six times 1:2 in standard medium 24 hours prior to determining relative ATP levels.

Flow Cytometry

Flow cytometry was performed as described above in Chapter 2 Materials and Methods with the addition of anti-mouse CD21/35. Antibodies are listed in Table 1.

Preparation of FITC-labeled, C57Bl/6 sera- or purified C3d-opsonized microspheres

Preparation of microspheres was performed under sterile conditions. FITC-labeled, uncoated and sera-opsonized microspheres were prepared as described above. For covalently bound C3d coated particles, 100 μL of amino microspheres (0.97 μm) were washed in PBS, incubated in 8% glutaraldehyde in PBS (pH 7.4) with rotation overnight at room temperature, washed, and incubated with 40 μg/mL purified C3d (Fitzgerald, Acton, MA) for 4 hours at room temperature with rotation. The beads were centrifuged and resuspended in 0.5 M ethanolamine in PBS for 30 minutes at room temperature, followed by three washes with PBS. After coating with C3d, the microspheres were FITC-labeled as described above and finally incubated in 0.1 M glycine in PBS for 30 minutes at room temperature to block non-specific binding. After the incubation, the beads were centrifuged and resuspended in 100 μL of the same buffer.

Supernatant Generation

Wehi 231 cells or PerC B cells from B6 or *Cr2*$^{-/-}$ mice were aliquoted into 24-well tissue culture plates at 2×10^6 cells/mL. Normoxic and hypoxic cells were unstimulated or stimulated with uncoated, B6 sera- or C3d-opsonized microspheres for 2 or 24 hours where indicated. Supernatants were collected and stored at -80°C until analyzed for IgM, IL-6, TNFα, and nitric oxide production. Secreted IgM was detected by capture ELISA with a mouse IgM quantitation set (Bethyl Labs, Montgomery, TX) following the manufacturer's protocol. Levels of secreted IL-6 and TNFα were determined using mouse IL-6 or TNFα ELISA MAX Standard kits (BioLegend) according to the manufacturer's protocol. Nitric oxide was detected using a method previously described by Ding et al., using Griess reagent and acidified sulfanilamide with *N*-1-napthylethylenediamine dihydrochloride [64]. When required, the absorbance of

ELISAs or NO assay plates was measured on a Model 680 microplate reader (BioRad) at and compared to a standard curve of known concentration.

Western Blot

Cell lysates were prepared by mechanical shearing through a 24 gauge needled fitted on a 1.0 mL syringe in 1% Triton X-100 and Tris-ethylenediaminetetraacetic acid (EDTA) buffer containing protease inhibitors. The protein concentration of each sample was determined with a BCA assay (Pierce, Rockford, IL) and a total of 50 μg protein was incubated for 5 minutes in 4X Laemmli buffer at 95°C prior to use. Samples were run on a SDS-PAGE gel and transferred to Immobilon PVDF membrane (Millipore, Billerica, MA). After transfer, non-specific binding was blocked by incubating the blot in 5% non-fat milk in Tris buffered saline (TBS) for 1 hour. The blot was incubated overnight at 4° C with purified rat anti-mouse inducible nitric oxide synthase (iNOS, NOS II) antibody (BD Biosciences) diluted 1:1000 in blocking solution containing 0.1% Tween-20 (Fischer Scientific). The blot was washed with 0.1% Tween-20 in TBS three times for 5 minutes each and incubated with 1:10,000 diluted secondary antibody, anti-rat IgG-HRP (Jackson ImmunoResearch) for 1 hour at room temperature. The membrane was washed an additional three times and then submerged in SuperSignal West Pico chemiluminescent substrate (ThermoScientific, Waltham, MA) for 1 minute and proteins were detected using X-ray film development.

Phagocytosis Assay

A modified version of the phagocytosis assay described by Nakashima, et al. [63] was used to evaluate Wehi 231 cell or PerC B1 B cell phagocytic activity under normoxic or hypoxic conditions. PerC B1 B cells were pooled from three to eight B6 or *Cr2*$^{-/-}$ mice and the assay was performed as described in Chapter 2. Wehi 231 cells were subjected to the same procedure as PerC B1 B cells with the following modifications: Cells were collected, washed in serum free DMEM, counted by trypan blue exclusion and resuspended at a concentration of 2.0×10^6 cells/mL. For the assay, 0.5 mL of cell suspension was used with an appropriate number of microspheres per well of a 24-well plate.

Immunocytochemistry

Samples were prepared by centrifuging 1.5×10^5 cells from B6 or *Cr2*$^{-/-}$ mice onto glass slides (Fisher) using a Cytospin200 following the procedure described above. The percent phagocytic B1 B cells

(B cells ingesting at least one FITC-labeled particle/total B cells X 100) and Phagocytic Index (PI; Mean particles per phagocytic cells X percent phagocytic cells) were determined by counting 100 – 200 IgM^+, $CD11b^+$ total cells in multiple fields of view in merged microphotographs.

RNA isolation, cDNA synthesis, and RT-quantitative PCR analysis

Total RNA was isolated by collecting normoxic or hypoxic treated Wehi 231 cells in TRIzol reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized with a qScript first strand cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol using random primers. The cDNA was used for real time quantitative PCR (qPCR) analysis. Reverse transcriptase qPCR was performed on a MJ MiniOpticon thermocycler (BioRad) using PerfeCTa SYBR Green supermix (Quanta Biosciences) to detect product amplification with the indicated primer pairs (Integrated DNA Technologies, Coralville, IA). 18S RNA levels were used as a housekeeping gene to normalize data for variation in cDNA synthesis. Changes in mRNA expression were determined with the delta-delta-Ct method compared to normoxic, untreated cells. Primers, 18S Fwd: GGTTGATCCTGCCAGTAGC, Rev: GCGACCAAAGGAACCATAAC, Tm 58°C; iNOS Fwd: CAC CTT GGA GTT CAC CCA GT, Rev: ACC ACT CGT ACT TGG GAT GC, Tm 55°C.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Data were analyzed by a t-test. Data are presented as the SEM with significant differences considered with a p-value <0.05

Results

The effect of hypoxia on B1 B cell surface marker expression in Wehi 231 cells

Used extensively in studies on B cell apoptosis, Wehi 231 cells originated from the spleen of a Balb/c mouse following mineral oil injection. Since their original deposit into the ATCC in 1981, multiple subclones of Wehi 231 cells have been described [336, 337]. The expression of membrane Ig, CD11b, CD5, Siglec-G, CD43, and CD9 were analyzed by flow cytometry to determine if Wehi 231 resembled B1 B cells, for in vitro studies of a homogenous population (Fig. 3-1). Figure 1 demonstrates that Wehi 231 cells highly expressed the B1a markers, IgM and CD5. The cells also expressed low levels of CD11b. Together these data indicate that Wehi 231 cells resemble B1a B cells. In addition, the cells expressed CD9 and Siglec-G suggesting that they resemble PerC B1 B cells (Fig. 3-1). Importantly, we found that Wehi 231 cells subjected to 2 hours of hypoxic treatment expressed a similar surface phenotype compared to normoxic cells.

As B1 B cell phagocytosis requires C [63], we hypothesized complement receptor 2 (CR2) may play a role in Wehi 231 phagocytosis. Wehi 231 cells were $CR2^{lo}$ under normoxic conditions (Fig. 3-1). Similar to the B1 B cell surface markers examined, CR2 expression remained unchanged with hypoxic treatment (Fig. 3-1). These data suggest hypoxia does not alter B1 B cell or CR2 surface expression.

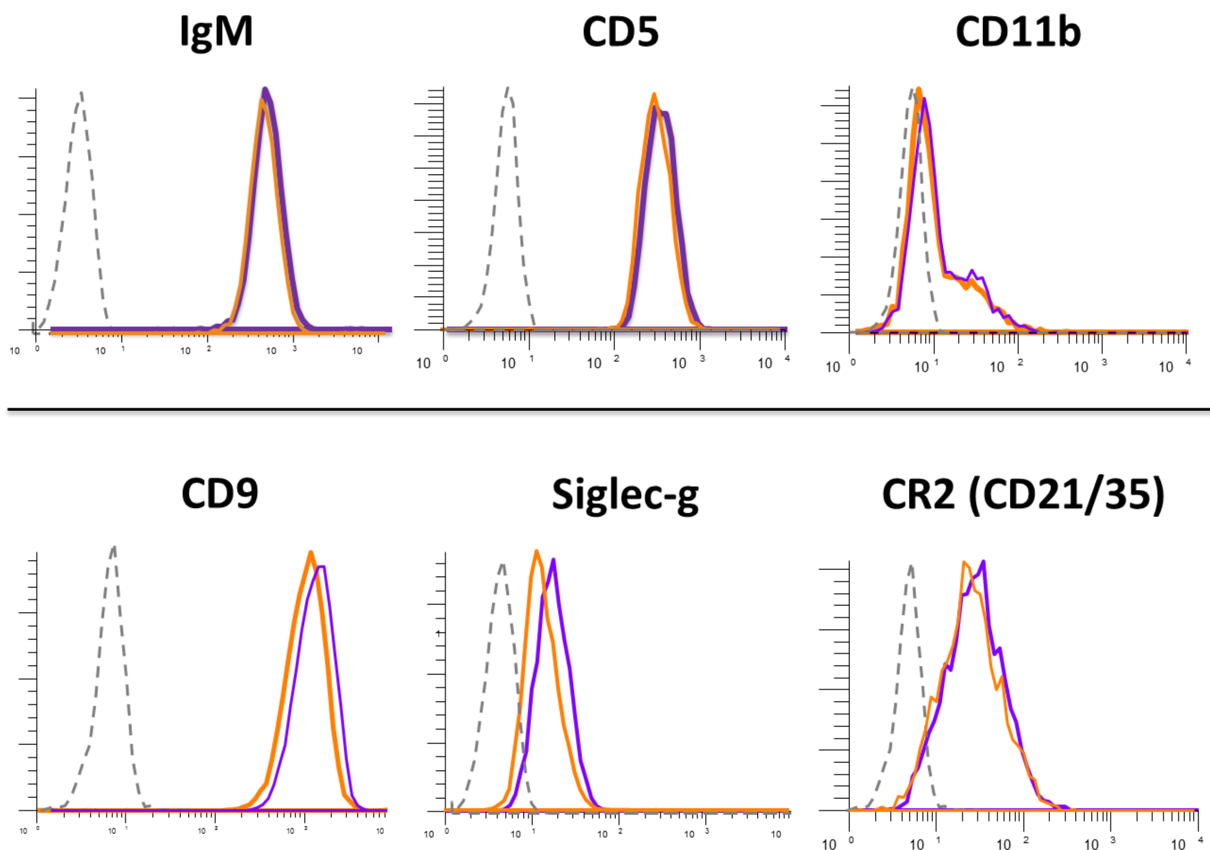


Figure 3-1. B1 B cell surface phenotype of Wehi 231 cells in response to hypoxia

Wehi-231 cells were subjected to 2 hours of normoxia (purple) or hypoxia (orange) and stained for flow cytometry analysis. Isotype controls (dashed gray) are displayed. Histrograms are representative experiment of 3 replicates.

Hypoxia induces metabolic but not proliferative changes in Wehi-231 cells

Hypoxia induces significant changes in cell metabolism and growth, potentially leading to cell death [338]. The mitochondrial reductase and ATP levels of normoxic (purple) or hypoxic (orange) Wehi 231 cells were used as indirect measures of metabolic activity (Fig. 3-2). When we evaluated ATP and mitochondrial reductases at 2 hours (data not shown), the same time frame allotted for phagocytosis in our studies, metabolic activity was not affected. However, when compared to the relative basal level after 24 hours, hypoxic Wehi 231 cells contained an approximate 50% reduction in reductases (Fig. 3-2, orange, open bar) and ATP (Fig. 3-2, orange, striped bar) suggesting an extended period (24h) of hypoxia downregulates metabolic activity. As cell death may account for the decreases observed above, we analyzed cell viability immediately. following 24 hours of normoxic (Fig. 3-3, ■) or hypoxic treatment

(Fig. 3-3, ▼) and in subsequent 24 hour increments. As the normoxic and hypoxic treated cells grew at the same rate, Cell death did not account for the decreases in metabolism.

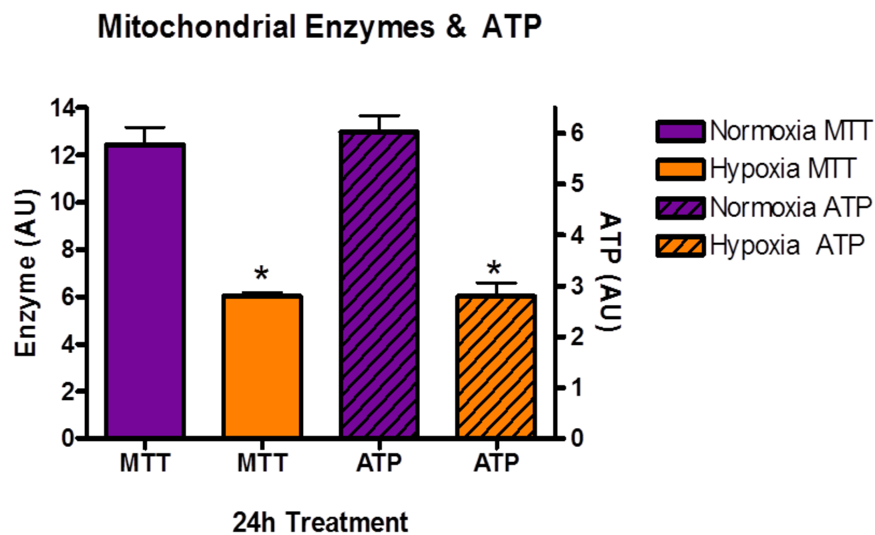


Figure 3-2. Extended (24h) hypoxia decreases Wehi 231 metabolic activity.

Wehi 231 cells were subjected to 24 hours of normoxia (purple) or hypoxia (orange). Following treatment, Cell Titer Glo reagent or MTT was added to cell samples to determine relative ATP (striped bars) and mitochondrial enzyme (open bars) levels respectively. Mean ±SEM is shown for 6 – 8 samples per treatment. *$p<0.01$ indicates significance from normoxic controls.

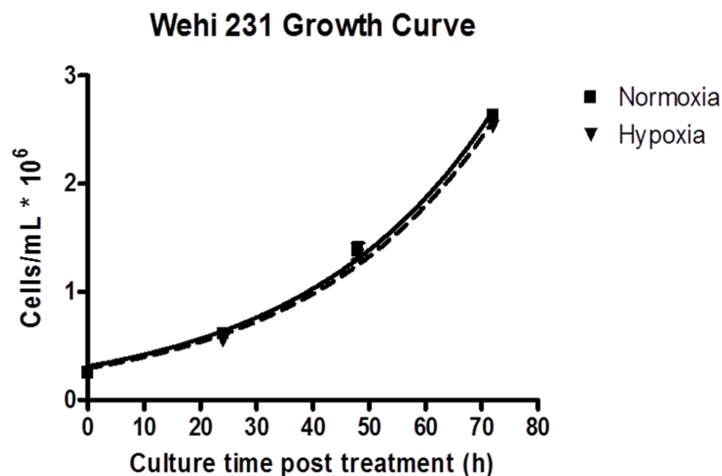


Figure 3-3. Wehi 231 cell growth following hypoxia.

After 24h of either normoxia (■) or hypoxia (▼), cells were maintained in culture for an additional 72 hr. Cell concentration was determined immediately after treatment and every subsequent 24 hours. Mean ±SEM is shown for 6-9 samples per treatment.

B1 B cell secretions by Wehi 231 cells in response to hypoxia

As Wehi 231 cells phenotypically resemble B1a B cells and remain viable with hypoxic treatment, we evaluated the cells for B1 B cell associated secretion. We tested the hypothesis that hypoxia enhances B cell release of nitric oxide, antibody production and cytokine secretion, by Wehi 231 cells.

As B1 B cells release nitric oxide when stimulated [60], iNOS mRNA (Fig. 3-4) and protein levels (Fig. 3-5) were evaluated in Wehi 231 cells. Compared to normoxia, 2 h of hypoxia upregulated iNOS mRNA and remained elevated with 24 hours of hypoxia (Fig. 3-4, orange bars). Wehi 231 cells constitutively expressed low level of iNOS protein, and similar to mRNA levels, iNOS visibly increased when detected by Western blotting (Fig. 3-5). After 2 h of hypoxia (Fig. 3-6, orange bars), the cell supernatants contained increased quantities of NO compared to normoxic controls (Fig. 3-6, blue bars) (Fig. 3-6 A) but NO was significantly decreased by24 hours of hypoxia (Fig. 3-6 B). These data suggest hypoxia upregulates iNOS and NO release with short periods of hypoxia.

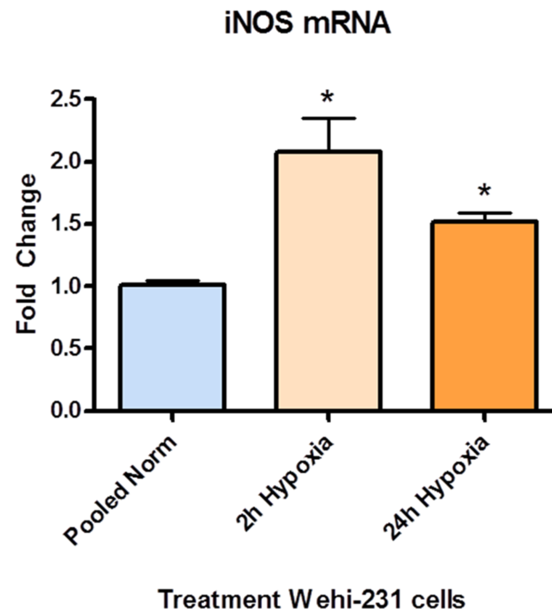


Figure 3-4. Wehi 231 iNOS mRNA expression with hypoxic treatment

Following 2 or 24h of normoxic (blue) or hypoxic (orange) treatment, the level of iNOS mRNA in Wehi 231 cells was determined by quantitative RT-PCR. Fold change in mRNA was based on normoxic levels of iNOS after treatment groups were normalized to 18s levels. Treatments were considered significant (*) when the p-value was <0.05 with 3 – 6 independent samples per group.

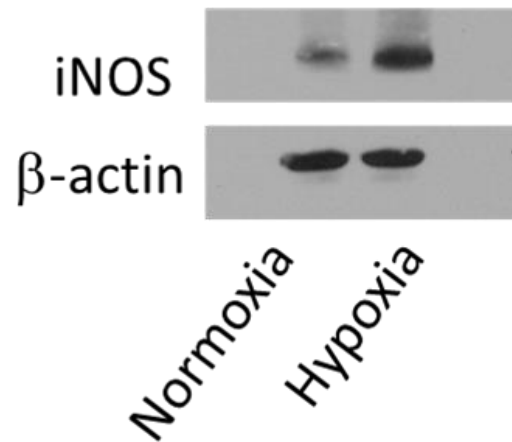


Figure 3-5. Hypoxia upregulates iNOS protein in Wehi 231 cells

Wehi 231 cells were subjected to 2h of normoxic and hypoxic treatment followed by preparation of whole cell lysates. The protein concentration of the lysates was determined and an equal amount was analyzed by western blot analysis under non-reducing conditions for iNOS. β-actin was used as a loading control. Blots are representative of 3 independent experiments.

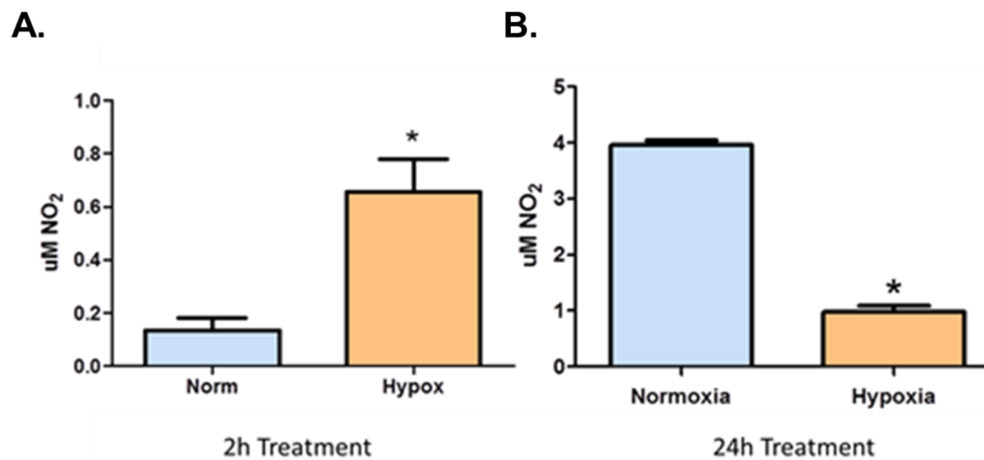


Figure 3-6. Hypoxia alters Wehi 231 NO production in a time dependent manner

Wehi-231 cells were subjected to 2 or 24 hours of normoxia (blue bars) or hypoxia (orange bars) and supernatants were harvested. μM nitrite was determined by Griess assay. A t-test was used to determine significance. *$p<0.05$ indicates significance from normoxic controls.

As B1 B cells spontaneously produce IgM which may increase in response to hypoxia [339], we examined antibody production by hypoxia treated Wehi 231 cells. Under normal culture conditions, Wehi 231 cells constitutively secreted detectable levels of IgM (Fig. 3-7 A). IgM secretion by Wehi 231 cells significantly increased after 2 h hypoxia (Fig. 3-7 A, orange bar), In contrast, after 24 hours, hypoxia reduced the constitutive antibody secretion by Wehi 231 cells (Fig. 3-7 B). The data demonstrate that extended oxygen stress reduces spontaneous IgM secretion by Wehi-231 cells despite maintaining a constant level of surface IgM (Fig. 3-1).

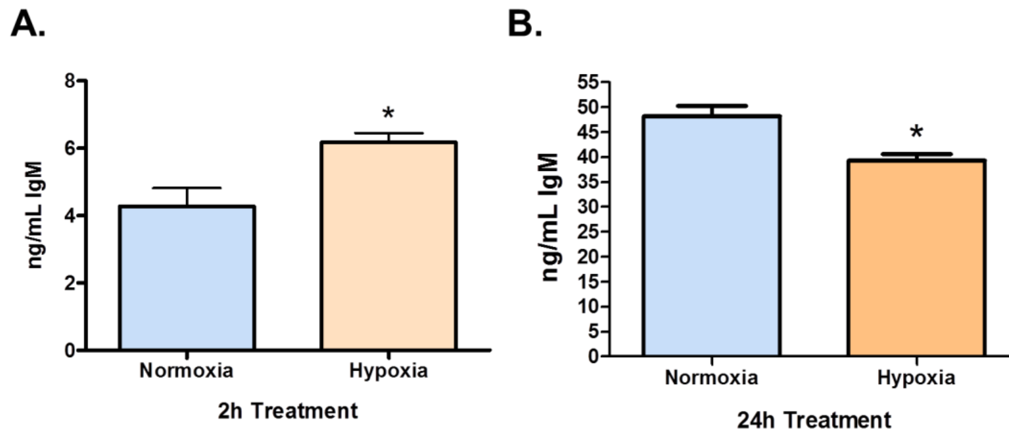


Figure 3-7. Hypoxia alters Wehi 231 IgM production in a time dependent manner

The supernatants of Wehi-231 cells subjected to 2 (A) or 24 (B) hours of normoxia (blue bars) or hypoxia (orange bars) were evaluated for IgM production by ELISA. . Mean ±SEM is shown for 6 – 8 samples per treatment. *p<0.01 indicates significance from normoxic controls.

B1 B cells may produce IL-10, TNFα, and IL-6 constitutively or when stimulated [59]. Similar to NO and Ab, we expected that TNFα, IL-6, and IL-10 secretion by Wehi 231 cells would increase in response to hypoxia (Fig. 3-8, 3-9). Although Wehi 231 cell supernatants contained no detectable TNFα of IL-6 after 2 hours of normal culture, constitutive levels of TNFα (Fig. 3-8 A) and IL-6 (Fig. 3-8 B) were present after 24 hours under normoxic treatment (blue bars). However, 24 h of hypoxic treatment significantly reduced the amount of TNFα and IL-6 produced (Fig. 3-8 A, B, orange bars).

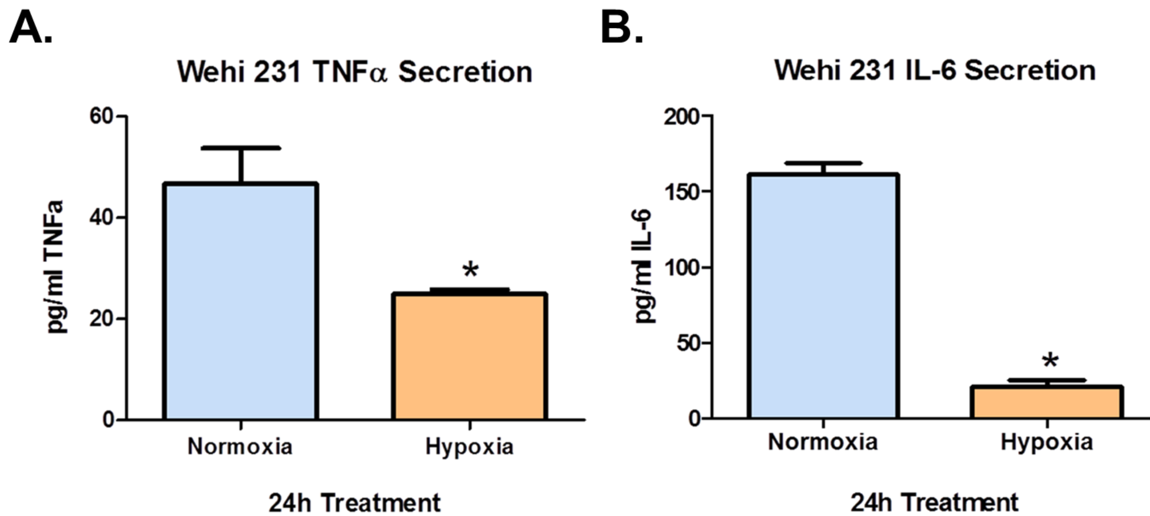


Figure 3-8. Hypoxia attenuates pro-inflammatory cytokine production by Wehi 231 cells

Wehi-231 cells were subjected to 24 hours of normoxia (blue bars) or hypoxia (orange bars) and supernatants were harvested. The amount of TNFα (A) and IL-6 (B) was determined with an ELISA. Mean ±SEM is shown for 4 – 8 samples per treatment. A t-test was used to determine significance. *p<0.05 indicates significance from normoxic controls.

Wehi 231 Phagocytosis

B1 B cells demonstrate phagocytic activity in the presence of particles less than 3 μm [66]. Wehi 231 cells phagocytosis of fluorescent uncoated (UB) or sera-opsonized (SO) beads was determined by flow cytometry. Wehi 231 cells equally phagocytosed uncoated (Fig. 3-9, UB, open blue bar) and sera opsonized microspheres (Fig. 3-9, SO, striped blue bar). We found that hypoxic treatment (Fig. 3-9, orange bars) of Wehi 231 cells significantly increased the percent of cells ingesting uncoated beads. Approximately 25 % of normoxic cells ingested particles compared to 35 and 40% of hypoxia treated cells (Fig. 3-9, orange open bar) .Similar increases were observed for sera-opsonized beads (Fig. 3-9, angled orange bar) (Fig. 3-9, blue bars). We also evaluated the number of beads per Wehi231 cells (phagocytic index) and found a 2 – 3 fold increase with hypoxia (Fig. 3-10, open bars, diagonal bars). The data indicate that hypoxia enhances the phagocytic activity of the B1a-like B cell line, Wehi 231.

We tested the hypothesis that opsonization with C3d would enhance phagocytosis by the CR2+ Wehi 231 cell. While 20% of normoxic Wehi 231 cells phagocytosed uncoated beads (Fig. 3-9, blue open bar), 45% of cells phagocytosed C3d-opsonized beads (Fig. 3-9, C3d, blue horizontal stripes). Similar to

the data above, hypoxia significantly increased the number of phagocytic cells ingesting C3d-coated beads (Fig. 3-9, C3d, orange horizontal stripes) as well as upregulating the PI (Fig. 3-10, C3d, orange horizontal stripes). The data suggest that opsonization with C3d significantly enhances phagocytosis under normoxic and hypoxic conditions and may proceed through CR2.

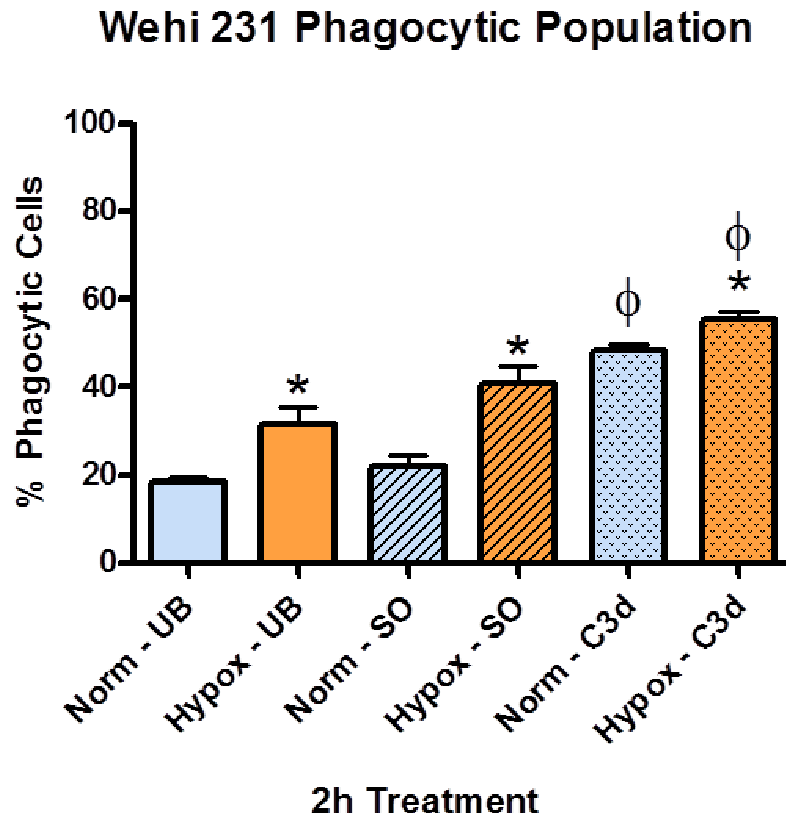


Figure 3-9. Hypoxia upregulates the number of phagocytic Wehi 231 cells

Wehi 231 cells were incubated with uncoated (UB, open bars), sera-opsonized (SO, angled stripes), or C3d-opsonized beads (C3d, horizontal stripes) for 2h under normoxic (blue bars) or hypoxic (orange bars) conditions followed by trypsinization to remove extracellular beads. The percent mean ±SEM of phagocytic cells ingesting at least one particle for 3 independent experiments is shown. A t-test was used to determine significance. *$p<0.05$ compared to normoxic controls and Φ$p<0.05$ compared to uncoated beads (UB).

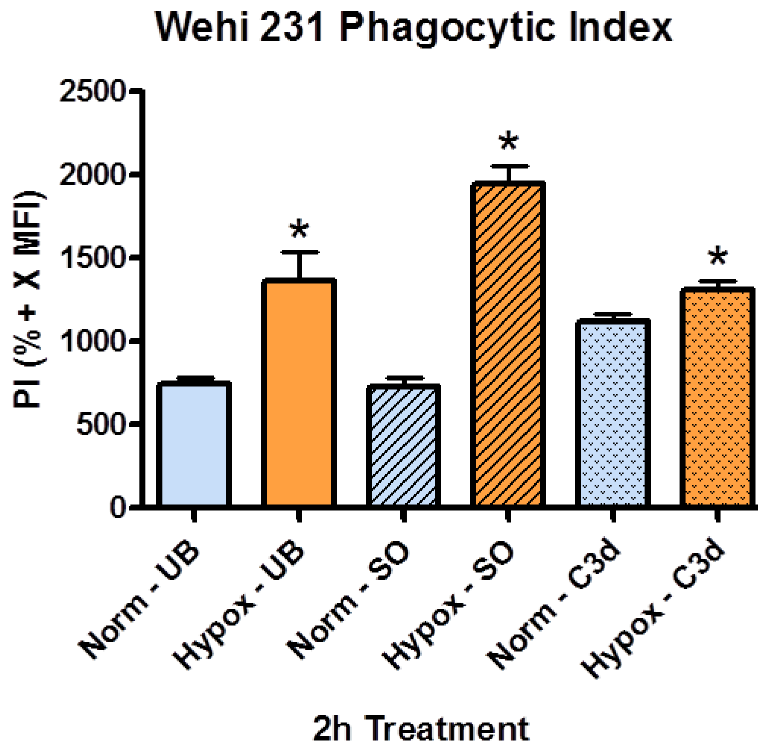


Figure 3-10. Hypoxia augments the phagocytic index of Wehi 231 cells

Wehi 231 cells were incubated with uncoated (UB, open bars), sera-opsonized (SO, angled stripes), or C3d-opsonized beads (C3d, horizontal stripes) for 2h under normoxic (blue bars) or hypoxic (orange bars) conditions followed by trypsinization to remove extracellular beads. The phagocytic index ±SEM for 3 independent experiments is shown. Results were considered significant compared to normoxic controls with a *p-value <0.05.

The role of CR2 in B1 B cell phagocytosis in response to hypoxia

Using wild type or *Cr2*$^{-/-}$ B1 B cells, we tested the hypothesis that CR2 acts as the cognate receptor for phagocytosis of C3d-opsonized beads. The percent of PerC B cells phagocytosing uncoated (UB) and sera-opsonized (SO) beads was evaluated by flow cytometry analysis (Fig. 3-11). Consistent with the findings in Wehi 231 cells, approximately 25% of wild type (Fig. 3-11 blue bars) and *Cr2*$^{-/-}$ (Fig. 3-11 green bars) B1 B cells ingested uncoated (UB) or sera-opsonized (SO) beads under normoxic conditions. In addition, the PI was similar between the two cell types as determined by either fluorescent microscopy (Fig. 3-12 A) or flow cytometry (Fig. 3-12 B, angled blue bar, angled green bar). The data suggest that CR2 is not required for phagocytosis of uncoated and sera-opsonized beads by PerC B cells under normoxic conditions.

Hypoxia increased the PI and the percent phagocytic Wehi 231 cells (Fig. 3-9, 3-10). Similarly, the percent of hypoxic wild type PerC B cells ingesting sera-opsonized (SO) particles increased by 15% (Fig. 3-11, orange angled bar) with a doubling in the PI (Fig. 3-12, A, B orange angled bar) when compared to normoxic cells (Fig. 3-11, 12 blue bars). However, we found that CR2 was required for the hypoxia-induced upregulation of phagocytosis as a similar percent of normoxic (Fig. 3-11 green angled bar) and hypoxic (Fig. 3-11 purple angled bar) *Cr2*$^{-/-}$ B1 B cells ingested sera-opsonized beads. Similarly, CR2 expression was also required for the hypoxia induced increase in the PI as hypoxia failed to increase the PI of *Cr2*$^{-/-}$ B1 B cells.

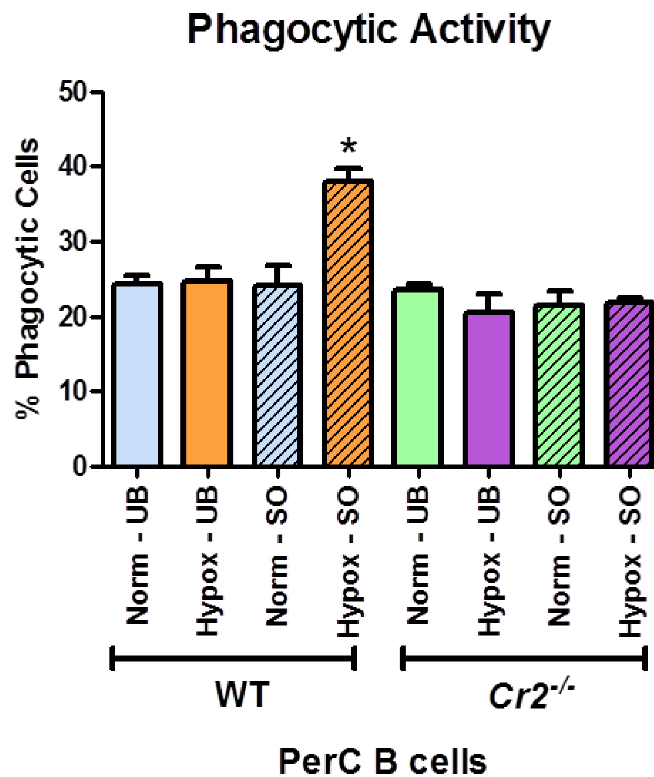


Figure 3-11. Hypoxia upregulates PerC B cell phagocytosis in a CR2 dependent manner

Negatively selected PerC B cells from B6 (WT) or *Cr2*$^{-/-}$ mice were subjected to 2h of normoxia (Norm, blue bars, green bars) or hypoxia (Hypox, orange bars, purple bars) with uncoated (UB) or sera-opsonized (SO) beads. The percent mean ±SEM of phagocytic cells ingesting at least one particle was determined by flow cytometry analysis. Data are from 4 independent experiments. Results were considered significant compared to normoxic controls with a *p-value <0.05.

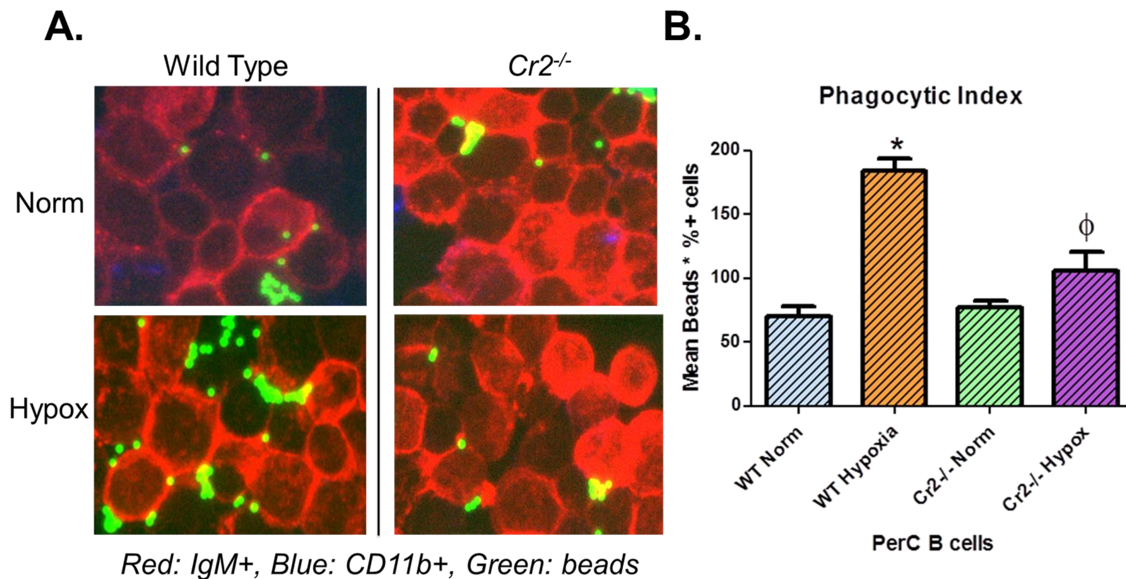


Figure 3-12. Hypoxia enhances the phagocytic index of WT and CR2$^{-/-}$ PerC B cells

Negatively selected PerC B cells from B6 (WT) or *Cr2*$^{-/-}$ mice were subjected to 2h of normoxia or hypoxia with serum opsonized beads (green) and then stained for IgM (red) and CD11b (blue) to identify phagocytic cells by fluorescent microscopy (A). Flow cytometry analysis was used (B) to determine the phagocytic index of normoxic (blue angled bar, green angled bar) or hypoxic (orange angled bar, purple angled bar) wild type and Cr2$^{-/-}$ cells when incubated with serum opsonized beads. Photomicrographs are representative of 2 – 3 independent experiments. The relative median fluorescence intensity multiplied by the percent mean of phagocytic cells ±SEM ingesting at least one particle for 3 independent experiments is shown. A t-test was used to determine significance. *$p<0.05$ compared to normoxic controls, Φ$p<0.05$ compared to wild type controls.

Although *Cr2*$^{-/-}$ B1 B cells (Fig. 3-11 green bars) phagocytosed uncoated and sera-opsonized particles similar to that of wild type cells (Fig. 3-11 blue bars), we hypothesized that enhanced phagocytosis of C3d-opsonized beads requires CR2 expression. As expected based on the Wehi 231 data, (Fig. 3-9) the number of phagocytic wild type cells which ingested at least one C3d-coated bead, 75% (Fig. 3-13 A, blue horizontal bar), significantly exceeded the number of cells phagocytosing unopsonized beads, 25% (Fig. 3-13 A, blue open bar). Surprisingly, phagocytosis of C3d-opsonized beads by *Cr2*$^{-/-}$ B1 B cells (Fig. 3-13 A, green horizontal bar) significantly increased compared to uncoated beads (Fig. 3-13 A, green open bar) from 25% to 65% of cells respectively. However, when compared to the 75% of wild type cells which ingested C3d-coated particles (Fig. 3-13 A, blue horizontal bar), a significant decrease was observed in the number of phagocytic *Cr2*$^{-/-}$ cells, 65% (Fig. 3-13 A, green horizontal bar). The data suggest that while B1 B cells do not require CR2 expression for phagocytosis of C3d-opsonized particles, a lack of CR2 significantly impacts the number of phagocytic

cells. As hypoxia and CR2 expression alter the phagocytic activity of B1 B cells when treated with uncoated and sera-opsonized particles, we tested the hypothesis that hypoxia induced upregulation of C3d-opsonized particles depends on CR2 expression (Fig. 3-13 B). Hypoxia significantly increased the percent of wild type cells ingesting C3d-opsonized particles by 10% (Fig. 3-13 B). Based on the data presented above, we expected no increase in the number of C3d-phagocytosing *Cr2*$^{-/-}$ B1 B cells. However, in contrast to phagocytosis of uncoated and sera-opsonized particles, hypoxia significantly increased the number of *Cr2*$^{-/-}$ B1 B cells which ingested C3d-opsonized beads.

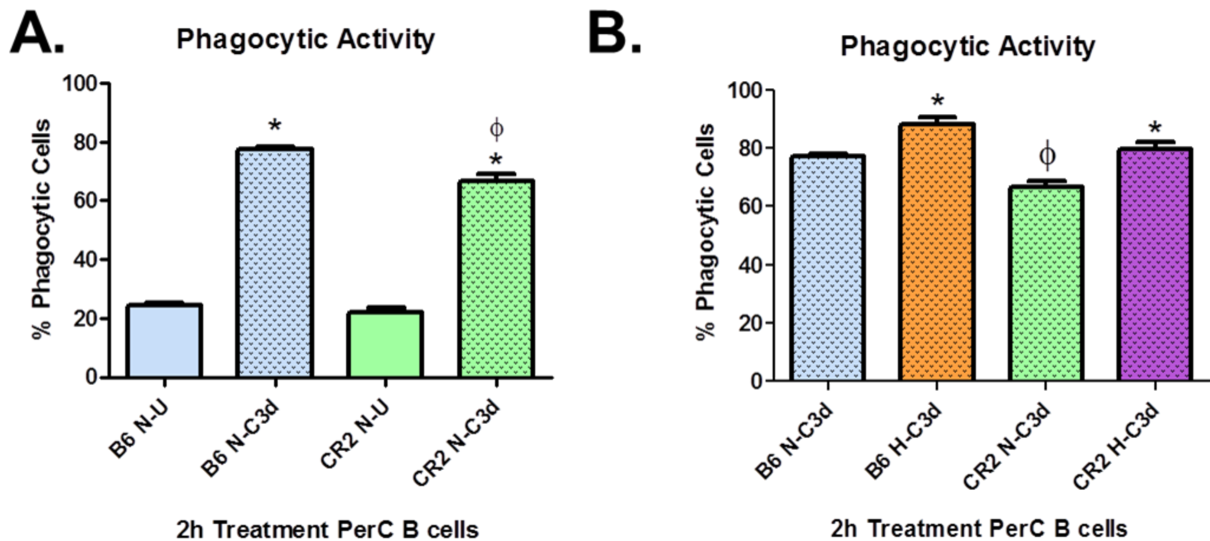


Figure 3-13. C3d-opsonization and hypoxia enhance PerC B cell phagocytosis

After 2 hours of normoxia or hypoxia with uncoated (UB) or C3d-opsonized (C3d) beads, phagocytosis by wild type (B6) and Cr2$^{-/-}$ (CR2) PerC B cells was determined by flow cytometry. The percent mean ±SEM of phagocytic cells ingesting at least one particle for 3 – 4 independent experiments is shown. Results were considered significant compared to normoxic controls (*) or wild type controls (Φ) with a p-value <0.05.

The effect of hypoxia on PerC B1 B cell CR2 expression

Multiple groups have presented convincing, yet opposing data suggesting that one of the two B1 B cell subsets exhibit higher phagocytic activity. Additionally, Molina, et al. demonstrated that *Cr2*$^{-/-}$ mice exhibit a normal number of B1a B cells in the PerC of C57Bl/6 mice but did not report the number of CD11b+ B cells [190]. Based on our data indicating CR2 plays a role in hypoxia-induced upregulation of phagocytosis, we tested the hypothesis that *Cr2*$^{-/-}$ mice contain an altered B1b B cell pool. Of the CD19+ PerC B cells, the B1 B cell subsets were determined in wild type and *Cr2*$^{-/-}$ mice. When comparing the

ratio of B1a:B1b B cells based on flow cytometry analysis, both wild type and *Cr2*$^{-/-}$ cells exhibited a 1 to 1 distribution under normoxic conditions (Fig. 3-14, blue and green bar). Both cell types exhibited a similar subset ratio following hypoxic treatment suggesting CR2 expression or hypoxia does not alter the B1b B cell compartment (Fig. 3-14 dot plots).

As *Cr2*$^{-/-}$ mice contained a normal subset distribution, the level of CR2 present during hypoxia may play a role in mediating phagocytosis through upregulated surface expression Of the wild type CD19+ cells, flow cytometry analysis revealed two distinct populations, CR2^{lo} B1a B cells and CR2^{int} B1b B cells, under normoxic conditions (Fig. 3-15 blue histogram). We determined that the level of CR2 expression does not affect B1 B cell phagocytosis as hypoxic cells (Fig. 3-15 orange histogram) expressed a similar level of CR2 compared to normoxic cells.

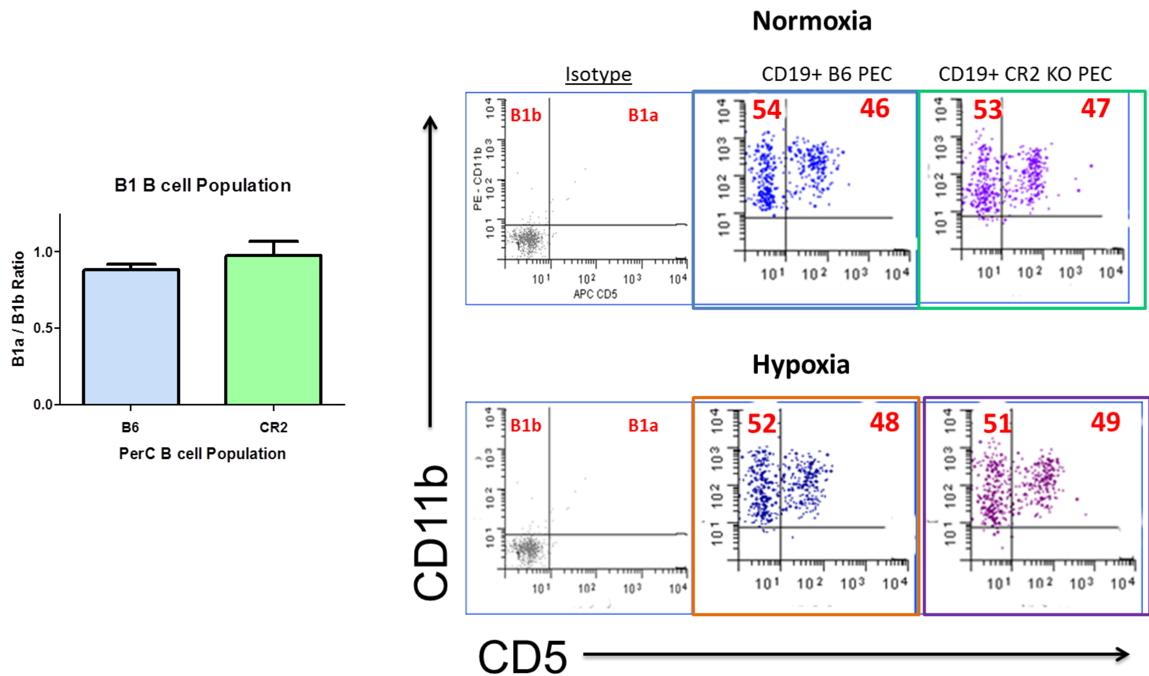


Figure 3-14. CR2 mice exhibit normal B1a and B1b B cell numbers

Negatively selected PerC B cells from wild type (B6, blue bar) or *Cr2*$^{-/-}$ (CR2, green bar) mice were subjected to 2h of normoxia or hypoxia and the ratio of CD19+ B1a B cells(upper right quadrants) to B1b (upper left quadrants) cells was determined by flow cytometry. Dot plots are representative of the data presented from three independent experiments.

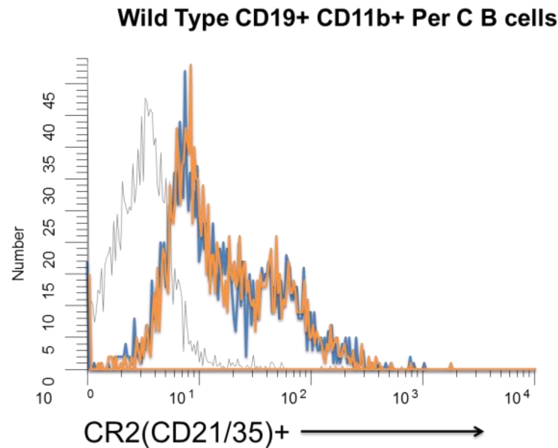


Figure 3-15. Hypoxia does not alter CR2 expression on WT PerC B cells

After 2 hours of normoxia (blue line) or hypoxia (orange line) CR2 expression was evaluated on CD19+CD11b+ PerC B cells from wild type mice. Isotype controls (grey line) were used to determine background fluorescence. Histograms are representative of 3 independent experiments.

The effect of C3d stimulation and CR2 on cytokine production

Cytokines such as TNFα and IL-6, which may increase in response to hypoxia, play an important role in modulating phagocytic activity. We tested the hypothesis that CR2 expression alters the production of pro-inflammatory cytokines by B1 B cells. B1 B cells from wild type mice constitutively produced low levels of TNFα (Fig. 3-16 A, open blue bar) and IL-6 (Fig. 3-16 B, open blue bar) under normal culture conditions (Fig. 3-16 A). We found when compared to wild type cells (blue bars) the level of constitutive TNFα and IL-6 produced was 2- and 3- fold less respectively (Fig. 3-16 A, B, open green bars). The data demonstrate that CR2 expression alters the natural production of cytokines by B1 B cells.

Since CR2 expression affected the production of cytokines by B1 B cells, we then tested the hypothesis that stimulation with a CR2 ligand, C3d, may enhance cytokine secretion. B1 B cells were incubated with C3d-coated beads similar to the phagocytosis assay. After the incubation, the particles were removed and supernatants were evaluated for TNFα and IL-6. The presence of C3d-coated particles failed to enhance production of the cytokines by wild type cells under normal conditions (Fig. 3-16 A, B, stippled blue bars). C3d failed to alter the production of TNFα and IL-6 by *Cr2*$^{-/-}$ B1 B cells as well (Fig. 3-16 A, B stippled green bars). Both groups exhibited levels similar to unstimulated cells. Based on these data, C3d fails to act as a stimulus for B1 B cells to alter the production of TNFα and IL-6.

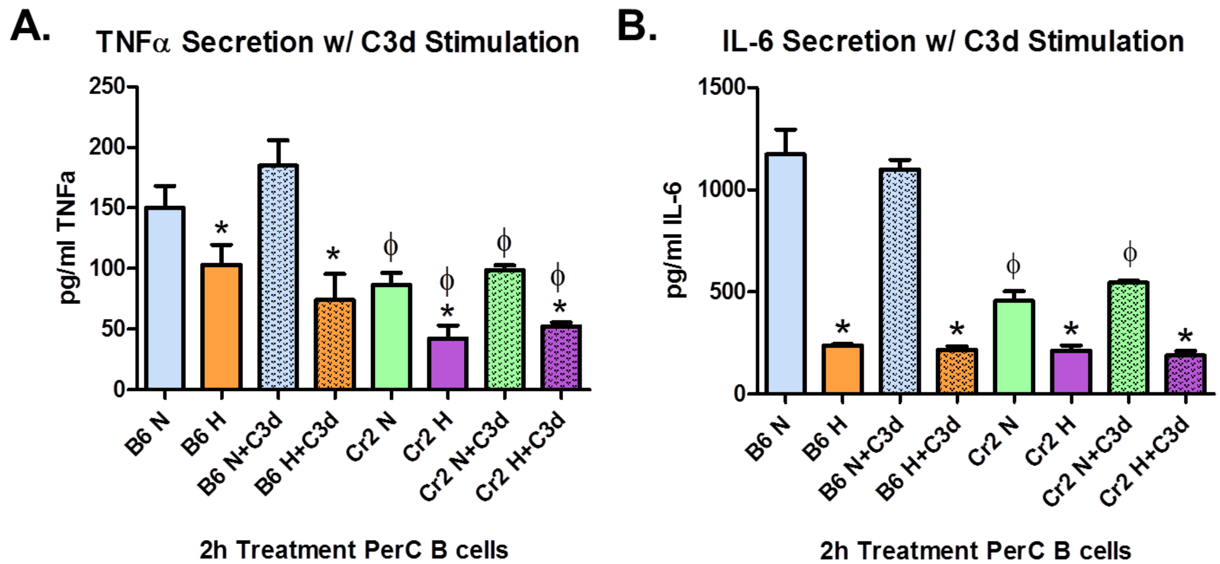


Figure 3-16. C3d stimulation fails to enhance PerC B cell cytokine production

Wild type (B6) or $Cr2^{-/-}$ (Cr2) PerC B cells were subjected to 2 hours of normoxic (N, blue, green) or hypoxic (H, orange, purple) treatment with C3d-opsonized beads (stippled bars) or without beads (open bars). Supernatants were analyzed for TNFα and IL-6 by ELISA. Data are from 3 – 4 independent experiments and considered significant compared to normoxic controls (*) or wild type controls (Φ) with a p-value <0.05.

Finally, we evaluated if an additional stimulus, hypoxia, in conjunction with C3d stimulation enhances cytokine production. Similar to the results described in Chapter 2, hypoxia alone reduced cytokine production by wild type B1 B cells (Fig. 3-16, A, B, open orange bars). Additionally, cytokine production was reduced under hypoxic conditions by *Cr2*$^{-/-}$ B1 B cells for TNFα and IL-6 (Fig. 3-16 A, B ,open purple bars). Compared to normoxic *Cr2*$^{-/-}$ B1 B cells (open green bars), a 50% reduction in cytokine secretion occurred with hypoxia (open purple bars). While C3d alone failed to upregulate production, a secondary stimulus may allow for activation. Wild type cells stimulated with C3d and hypoxia (Fig. 3-16 striped orange bars) produced a similar level of cytokines compared to hypoxia alone (Fig. 3-16 open orange bars). The same trend occurred for *Cr2*$^{-/-}$ B1 B cells, which exhibited similar levels of cytokine production with one (Fig. 3-16 open purple bars) or both stimuli (striped purple bars). Taken together, C3d fails to act as a stimulus or priming agent under hypoxic conditions for B1 B cell production of pro-inflammatory cytokines. Additionally, hypoxia-induced downregulation of cytokines occurs independently of CR2 expression, although CR2 expression alters constitutive production of TNFα and IL-6 by B1 B cells.

Discussion

An understanding of the full role of phagocytosis by B1 B cells and the mechanisms behind this function remains incomplete. In addition to environmental factors, recent studies demonstrated a critical role for C3 and the BCR in B1 B cell phagocytosis of bacteria [11, 63] [65]. As the BCR typically associates with CD19, CD81, and CR2, it was likely that CR2 [11], and the frequent hypoxic environment of B1 B cells [165, 166, 334] influence phagocytosis and the inflammatory response. Thus, we sought to determine the role of CR2 expression and hypoxia on B1 B cell effector function. We demonstrated that opsonization, CR2 expression, and hypoxia all regulate B1 B cell phagocytosis using the B1a-like B cell line, Wehi 231, and PerC B cells from wild type and $Cr2^{-/-}$ mice.

We evaluated factors which may alter B1 B cell effector function, specifically factors which may activate phagocytes to induce CR-mediated phagocytosis such as hypoxia. Hypoxia may affect B1 B cells and their phagocytic activity given their role in wound healing and tumor development, both locations where phagocytosis and hypoxic conditions occur [166, 334]. Additional mounting evidence supports a role for hypoxia in the B cell inflammatory response. B1 B cells migrate to hypoxic sites, in part due to upregulation of CXCR4, a migratory and adhesion receptor, to infiltrate tumors [165, 334]. While some aspects of B cell hypoxic stimulation have been elucidated, the whole picture remains unclear. We determined hypoxia significantly upregulated the phagocytic activity of wild type PerC B1 B cells. Unexpectedly, the response depended on the conditional expression of CR2. Hypoxic stimulation of $Cr2^{-/-}$ B1 B cells failed to upregulate phagocytosis of uncoated- or sera opsonized-microspheres. Future studies will need to evaluate the mechanism of hypoxia induced activation of B1 B cells and determine how it relates to CR expression.

We found that B1 B cells from wild type mice exhibited phagocytic activity for sera-opsonized beads similar to previous studies [63, 66]. Surprisingly, uncoated beads were phagocytosed at a similar level in these studies. The ability of B1 B cells to phagocytose uncoated beads may be due to multiple factors. Previous studies have indicated that fluorescent labeling, specifically with FITC, influences neutrophil phagocytosis of bacteria [340]. A similar effect may occur with B1 B cells as our beads were labeled with FITC. Similarly, the beads used in these studies also express amino groups, a property known to encourage phagocytosis due to charge-charge interactions [341]. However, the latter possibility

remains unlikely as our beads were blocked with glycine to deplete free amino groups. Interestingly, hypoxia failed to upregulate the phagocytosis of uncoated beads. The mechanism of hypoxia-induced upregulation of phagocytosis may depend on the presence of serum components as we observed increases with both sera- and C3d-opsonized beads.

B1 B cells express CR2, the cognate receptor for C3d [342], whereas professional phagocytes do not. Given the dependence of B1 B cell phagocytosis on C3 [63] and the fact these cells express CR2, it was likely one or both components contribute to B1 B cell phagocytic activity. As expected, C3d opsonization significantly enhanced B1 B cell phagocytosis compared to that of uncoated- or whole sera-opsonized beads. However, *Cr2*$^{-/-}$ cells also exhibited a high level of phagocytosis of C3d-coated beads suggesting CR2 does not play a role in B1 B cell phagocytosis. As CR3 exhibits a weak affinity for C3d, it is possible that *Cr2*$^{-/-}$ B1 B cells compensate, utilizing CR3 to internalize C3d-opsonized particles. A similar mechanism occurs with monocytes, which do not express CR2 and utilize CR3 to internalize C3d-opsonized particulates [343]. As we cannot rule out our microspheres were overly-saturated with C3d in these studies, a C3d concentration gradient or CR blocking antibodies will be required to definitively determine if CR2 plays a role in B1 B cell phagocytosis. Thus, it is currently not possible to rule out CR2 as a phagocytic receptor on B1 B cells despite the data presented here.

In addition to acting as an opsonin via CR2, we evaluated if C3d stimulated B1 B cells to secrete inflammatory cytokines. As part of the BCR co-receptor complex, CR2 alters the B cell activation threshold [9, 11]. While generally not considered to exhibit signaling properties due to a short cytoplasmic tail, Barrault and Knight determined the cytoplasmic domain of CR2 contains tyrosine residues critical for antigen internalization through this receptor [344] suggesting signaling may occur. Surprisingly, neither C3d-opsonized particles or hypoxia, nor the combination, had any effect on the level of TNFα and IL-6 produced by B1 B cells. These data suggest that while CR2 may play a critical role in antigen internalization, C3d does not act as a stimulus for cytokine production under normoxic or hypoxic conditions. Similarly, if the alternative hypothesis that CR3 acts as the phagocytic receptor for B1 B cells, this receptor exhibits no cytokine-inducing signaling activity, and thus remains consistent with our data.

Multiple CD5+ B lymphoma cell lines have been used in research to study BCR-induced apoptosis to T cell antigen presentation. Previous studies indicate Wehi 231 cells respond to TLR

agonists to produce NO, as well as undergo apoptosis following BCR-crosslinking, similar to primary B1 B cells. However, to our knowledge, no studies to date have demonstrated C3d-mediated phagocytic activity by a murine CD5+ B cell line [51, 60]. Taken as a whole, the data suggest Wehi 231 cells provide an appropriate model for *in vitro* study of B1 B cells based on surface phenotype and effector function, which includes nitric oxide release, phagocytosis, spontaneous IgM production, and secretion of inflammatory cytokines. Like other phagocyte responses to hypoxia, we found Wehi 231 cells were resistant to hypoxia induced apoptosis and exhibited enhanced effector function in the form of NO production and phagocytosis. Although short term periods of hypoxia only induced a 2-fold increase in iNOS mRNA expression in Wehi 231 cells, the upregulation was sufficient to increases the level of iNOS protein.

Augmented phaogocytosis may seem paradoxical as we observed decreases in ATP and mitochondrial activity following 24 hours of hypoxia. However, when we evaluated ATP and mitochondrial reductases at 2 hours (data not shown), the same time frame allotted for phagocytosis, metabolic activity had not yet been affected. Additionally, preliminary data indicates hypoxia induced upregulation of phagocytosis may occur as early as 30 minutes of treatment, well before we observed metabolic activity decreases. While hypoxia inevitably leads to a decrease in aerobic respiration, the process of phagocytosis stimulates phagocytes to upregulate production of lactate and induce glycogenolysis to maintain sufficient ATP levels for phagocytic activity [345, 346]. These processes likely support the continued production of ATP even in hypoxic conditions.

While data from our lab and others support the use of Wehi 231 cells as an appropriate *in vitro* model of B1 B cells, findings with this cell line must be interpreted carefully. The original Wehi 231 clone was isolated from the spleen, not peritoneal cavity, of a mineral oil injected Balb/c mouse [336]. It is possible the i.p. injection of oil led to an activation of peritoneal B1 B cells, inducing egress from the PerC to spleen. Our findings support the hypothesis that Wehi 231 cells originated from the PerC, as they lack surface CD43 (data not shown), a marker of splenic B1 B cells [31]. However, i.p. mineral oil injection has been shown to activate splenic cells. Additionally, it remains difficult to determine the original source of Wehi 231 cells as B2 B cells may also express CD5 when activated for extended periods of time [347,

348]. Therefore, despite the resemblance to B1 B cells, findings obtained from Wehi 231 cells may not accurately represent B1 B cell function and should be confirmed with *ex vivo* studies.

Chapter 4 - Concluding Remarks

The overarching component of our studies evaluated the effect of hypoxia on B1 B cells. Despite the recruitment and high prevalence of B1 B cells at hypoxic sites, which include tumors and wounds, few studies have directly demonstrated the effect of hypoxia on this B cell subset [165, 166, 334]. Since the effect of hypoxia radically differs between cells (Reviewed in [349, 350]), it remains difficult to compare the B1 B cell response to any specific, well characterized cell type. Overall, our studies demonstrated that short periods of hypoxia, 2 hours of 1% O_2, diminished the inflammatory and humoral secretory response of PerC B1 B cells. Hypoxia inhibits protein synthesis and metabolic activity in some immune cells but not in others. These data suggest B1 B cells likely require reoxygenation to restore protein synthesis and metabolic function to enhance an inflammatory response induced by hypoxia. Evaluating secretions with periods of reoxygenation following hypoxic treatment will address this pitfall in our studies. While secretions were downregulated with hypoxic treatment, the percent of phagocytic cells and average number of particles ingested by each cell significantly increased compared to normoxic controls. These data resemble findings with professional phagocytes [85, 86]. Preliminary data indicate hypoxia rapidly affects B1 B cell phagocytic activity, as 30 minutes of 1% O_2 was sufficient to upregulate the percent of phagocytic cells compared to normoxic controls (data not shown). Time course studies would determine the minimum amount of hypoxic stimulation necessary to enhance B1 B cell phagocytic activity. Finally, the effect of priming with TLR agonists or BCR engagement prior to hypoxic treatment may provide insight on the requirement for co-stimulatory signals in the B1 B cell response to hypoxia.

Our data presented in Chapter 2 demonstrated a critical role for TLR2 in B1 B cell function. The PerC of *Tlr2*$^{-/-}$ mice contained a higher percentage of B1a to B1b B cells relative to wild type controls. *Tlr2*$^{-/-}$ B1 B cells also secrete significantly higher levels of IL-10. The effect of hypoxia on B1 B cell effector function was linked to TLR2 expression. Specifically, hypoxia induced upregulation of phagocytosis by PerC B1 B cells and hypoxia-mediated downregulation of IL-6 secretion was dependent on TLR2. Multiple questions remain to determine the full extent of hypoxic stimulation and TLR2 expression on B1 B cell function. We found that stimulation with the TLR2/1 ligand, Pam_3CSK_4, failed to enhance phagocytosis by B1 B cells. Multiple, structurally distinct, ligands bind TLR2/1 and it is possible

that an alternative ligand will play a role in modulating phagocytosis. As TLR2 also functions as a heterodimer with TLR6, we tested the ability of B1 B cells to phagocytose the TLR2/6 ligand zymosan (data not shown) and found no stimulatory effect or phagocytic activity, likely due to particle size. However, stimulation with alternative, smaller TLR2/6 ligands such as Pam_2CSK_4, structurally related to Pam_3CSK_4, may provide more insight into the role of TLR2 in B1 B cell function. The availability of both $Tlr1^{-/-}$ and $Tlr6^{-/-}$ mice provide an opportunity to evaluate if the effect of TLR2 depends on heterodimer formation. However, a solely TLR2-dependent effect would support the hypothesis that p38 MAPK, linked to TLR2 expression as well as hypoxia and phagocytosis in professional phagocytes, may affect the B1 B cell response. The availability of phospho-specific antibodies and p38 inhibitors allow for testing this hypothesis.

Our data presented in Chapter 3 demonstrated the utility of the Wehi 231 cell line to study B cell phagocytosis and response to hypoxia. Additionally, we determined opsonization, specifically with C3d, as well as hypoxia, and CR2 expression play a role in both the inflammatory and phagocytic activity of peritoneal B1 B cells. However, additional studies will be required to determine the mechanism and involvement of these components in altering the B1 B cell response. The CD43+CD5+CR2+, splenic B1 B cell-like line, LK 35.2 ([351] and data not shown) may act as a point of comparison to Wehi 231 cells to study differences between peritoneal and splenic B1 B cell-like populations. Similarly, the CH series of B cell lymphomas [352], also known to express CD5 and class switch from IgM to IgA *in vitro* [353, 354], resembling mucosal B cells, may expand the role of CRs in B cell phagocytosis as CH12.LX cells express CR3 but not CR2 (data not shown).

The effect of opsonization was evaluated in both Chapter 2 and 3. We found PerC B1 B cells from wild type mice phagocytosed FITC-labeled, unopsonized polystyrene microspheres. To address fluorescent label conjugation, such as FITC, as a component altering phagocytosis studies, internally fluorescent polystyrene microspheres are commercially available. Interestingly, the percentage of phagocytic $Tlr2^{-/-}$, but not $Cr2^{-/-}$, B1 B cells remained significantly lower than wild type controls. These data suggest TLR2 may play a role in B1 B cell non-opsonic phagocytosis. Studies involving TLR2 blocking antibodies or knockdown with wild type B1 B cells and incubation with truly unopsonized particles will address these problems. Opsonization with whole sera from wild type mice significantly

increased the percent of phagocytic B1 B cells, with this activity further enhanced by opsonization with purified C3d. Due to the possibility of C3d saturation in our studies, B1 B cell phagocytosis with a concentration gradient of C3d-coated beads will be required to determine if the high affinity C3d-receptor, CR2, or low affinity, CR3, act as a phagocytic receptor under physiological C3d concentrations. Similarly, competitive studies utilizing blocking antibodies against CR2 and/or CR3 on wild type PerC B1 B cells will clarify our results. The availability of CD11b deficient, *Itgam*$^{-/-}$, mice allow for testing the hypothesis that CR3, not CR2, acts as the B1 B cell phagocytic receptor.

Understanding the role of B1 B cells in response to hypoxia and other factors which influence effector function may provide insight into several physiologically relevant conditions. A role exists for B1 B cells in wound healing [166]. Enhancing the phagocytic activity and stimulating production of IL-10 by B1 B cells involved in this process may improve the ability to facilitate wound healing. As B1 B cells potentiate melanoma metastasis under hypoxic conditions [334], determining the effector function of B1 B cells and the cytokine milieu at these sites may provide a target for new treatments to counter this activity. The role of TLR2 and interaction with the complement system provides an important link to B1 B cell microbicidal activity as both TLRs and CRs are important for clearance of Gram positive bacterial infection. CD5+ B1 B cells serve as a model for studying lymphocytic leukemia. Similar to B1a B cells, many B lymphocytic leukemia cells express CD5, which correlates to poor prognosis due to increased resistance to anti-B cell depletion therapies [355] [356] [357]. As C3d may be used as an adjuvant [358], determining the cognate receptor and cell types recognizing this opsonin may allow for improved vaccine development. Finally, elucidation of B1 B cell function and the factors which influence that activity may expand our understanding of the evolutionary link between innate and adaptive immunity.

Many questions remain about the effect of hypoxia as well as the role of toll-like receptors and complement receptors on B1 B cell function. However, the studies included herein demonstrate novel findings for several of these components. In summary, we demonstrated the following points: Hypoxia significantly alters B1 B cell inflammatory secretions and phagocytic activity; TLR2 expression influences the distribution of PerC B1 B cell subsets and significantly alters the B1 B cell response to hypoxia; CR2 expression and opsonization, especially with C3d, significantly impact the phagocytic response of B1 B

cells. Future studies may build on these findings to enhance our understanding of B1 B cell function, including the components and mechanisms driving the response.

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