Determining the role of hypoxia-induced autophagy in the survival of tumor-promoting macrophages

PhD Off-Topic Candidacy Examination Proposal
A. Specific Aims.

Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancers and is the leading cause of death among gynecological cancers. For years, it was thought that cancer progression was driven solely by cancer cells. However, abundant evidence has suggested that tumor cells are in constant contact with their surrounding microenvironment. The ovarian cancer tumor microenvironment (TME) has been linked to the ineffectiveness of standard chemotherapeutic treatments and the pathogenesis of ovarian cancer. The TME is a complex system of non-malignant cells, including mesenchymal cells, immune cells, fibroblasts, and endothelial cells that are in close communication with tumor cells. Hypoxia, commonly found in solid tumors, is a deficiency in the amount of oxygen reaching cells due to poor vascularization. It has been demonstrated that the transcription factor, hypoxia-inducible factor 1 alpha (HIF-1α), is rapidly activated in hypoxic cells. HIF-1α expression in tumor cells leads to the transcription of tumor promoting factors. These include chemottractants, which bring immune cells into the TME, that cause chronic inflammation, nurture immune suppression, tumor invasion and promote angiogenesis.

Circulating monocytes are attracted to the TME by tumor cells where they differentiate into tumor associated macrophages (TAMs). TAMs are highly plastic and can be either M1 or M2-polarized. However, M2-polarized macrophages are the most abundant immune cell observed in ovarian cancer. They function in wound healing, immune suppression and promote angiogenesis. Hypoxia entraps TAMs in the TME by decreasing their mobility. High levels of TAMs in the ovarian cancer TME are associated with low patient survival. TAMs as a potential therapeutic target in ovarian cancer is becoming more appealing. However, mechanisms of TAM survival in the tumor microenvironment remain poorly defined. HIF-dependent autophagy is a mechanism of survival utilized by multiple cells of the TME to persist in the oxygen deprived space. Autophagy is an evolutionary conserved catabolic process involved in the degradation of cellular components for energy or reuse. During times of cellular stress, autophagy can be induced to provide vital energy. Evidence shows that serum-deprived stromal cells provide increased tumor support through the activation of autophagy. HIF-dependent autophagy in lung cancer cells leads to increased survival during cisplatin-treatment. Additionally, autophagy is critical for monocyte differentiation into macrophages, suggesting that this pathway could potentially be essential to macrophages in response to hypoxia as well. The long-term goal of this study is to elucidate the significance of M2-polarized macrophages in the progression of ovarian cancer. It is my central hypothesis that hypoxia-induced autophagy mediates the survival of M2-polarized macrophages and aids in their promotion of tumor progression. The rationale for this study is that by revealing mechanisms of tumor support in ovarian cancer, this may aid in the development of novel therapeutic targets. I propose to test this hypothesis, by achieving the following aims:

Aim 1. Examine the role of hypoxia in the promotion of tumorigenesis through macrophage signaling. I hypothesize that M2-polarized macrophages will increase the secretion of tumor invasion and angiogenesis promoting factors in response to hypoxia. Human M2-polarized macrophages will be generated in vitro from fresh PBMCs. To confirm the M2-phenotype, CD68+ CD163+/CD206+ expression will be assessed by flow cytometry. 3D tumor cell invasion assays and transwell migration assays will determine if hypoxia affects the influence of M2-macrophages on tumor and endothelial cells. Human ovarian cancer cell lines, OVCAR3 and SKOV3, will be cultured with M2-polarized macrophages to form 3D spheroids. Hypoxia incubation chambers will mimic hypoxia in vitro. HIF-1α immunoblotting will determine their response to hypoxia. Additionally, HIF-1α knockout macrophages will be made using the CRISPR/cas9 system. Secretion of metastatic factors, VEGF and MMP-9, by M2-macrophages will be assessed by ELISA and immunoblotting following in hypoxia.

Aim 2. Investigate the effect of hypoxia on the induction of autophagy in M2-polarized macrophages. I hypothesize that elevated levels of HIF-1α in macrophages in response to hypoxia, will lead to the activation of pro-autophagic pathways and the down-regulation of apoptotic activity leading to cell survival. Hypoxia chambers will be utilized to mimic hypoxia in vitro. WT and HIF-1α knockout macrophages will cultured in hypoxia. Following incubation, apoptotic activity will be assessed by protein expression of pro-apoptotic genes and annexin V staining. MTT cell viability assays will determine the effect of autophagic activity on macrophage survival. Additionally, the induction of autophagy will be assessed by western blot analysis of autophagosome surface proteins, LC3-I and LC3-II, and genes involved in autophagy upregulation. Fluorescently-labelled LC3 will serve...
as a maker for autophagosome formation in response to hypoxia and will be visualized by microscopy. 3-MA, an autophagy inhibitor will be used to determine if apoptotic activity can be revived in hypoxic macrophages.

**B. Background and Significance**

**The role of the tumor microenvironment in the pathogenesis of ovarian cancer**

Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancers and affects thousands of people each year [1-5]. The overall 5-year survival rate for EOC is about 40% [6]. However, a significant portion of patients present with late stage disease which has a considerably lower rate of survival. The majority of patients have an objective response to the current standard of care, which utilizes surgical tumor debulking followed by platinum-based chemotherapies. Despite the initial response, disease recurrence due to chemoresistance remains a major challenge. The tumor microenvironment (TME) is increasingly being recognized as one of the key factors in disease progression. The TME is a critical component of the multistep development of tumors and malignancy [7]. All cancers comprise a tumor microenvironment that varies in its make-up and ability to promote immune escape, resistance, and metastasis [7-9]. The stroma of the TME is a complex network of multiple cell types that interact with tumor cells [8, 10]. In EOC, it is thought that stromal cells of peritoneum and omentum aid in the early malignancy of ovarian cancer cells [2]. The main components of the ovarian cancer TME consists of non-malignant cells, including fibroblasts, adipocytes, immune and inflammatory cells, lymphatic endothelial cells, and the extracellular matrix [8, 9, 11]. Studies have shown that stromal cells form a mutualistic relationship with tumor cells early on in malignancy, leading to the co-evolution of the TME with cancer cells [11]. Stromal cells can be modified by cancer cells to synthesize a wide range of chemokines, cytokines, and growth factors [12, 13]. Cross-talk between cells within the TME and tumor cells have been shown to alter the responses of tumor cells to therapeutics, rendering them ineffective [10, 11, 14, 15]. These include, the production of factors that induce tolerance through increased DNA repair, and alterations of genes involved in the activation and regulation of cell death pathways [1, 16, 17]. The TME also provides a protective barrier against the effective delivery of anti-cancer treatments, allowing for the escape of the tumor cells from cytotoxicity triggered programed cell death [9]. It has been reported that there are an abundance of immune cells, including regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages, which promote a highly inflammatory and immunosuppressive TME [18, 19]. One of the key characteristics of the tumor microenvironment is hypoxia. Hypoxia occurs when tumor cell growth surpasses the formation of new blood vessels, needed to supply vital oxygen to the tumor mass. Hypoxia in the TME has been implemented in chemoresistance, stromal and cancer cell survival, and metastasis of cancer cells. Together, this underlines the importance of further illuminating the role of hypoxic TME in the support of ovarian cancer progression.

**Hypoxia in the tumor microenvironment induces cell survival**

Hypoxia initially arises from a lack of oxygen in primary tumors or metastases, stemming from poor vascularity [13, 20]. The severity and incidence of hypoxia in the tumor microenvironment is variable from patient to patient [20]. However, one commonality is that it remains a negative prognostic factor. Hypoxia contributes to chemoresistance, angiogenesis, invasiveness, vasculogenesis, and resistance to cell death [20]. The response to hypoxia in tumor cells is well defined. It is known that in response to the hypoxic TME, tumor biology is altered [20, 21]. This serves as a good foundation to look further into how hypoxia effects the behavior of other cells within the tumor microenvironment. Additionally, understanding how hypoxia might affect the cross-talk between tumor cells and cells of the TME through alterations in molecular signaling pathways, this might aid in the identification of novel therapeutic targets. One important cellular pathway affected by hypoxia is apoptosis. Apoptosis is a form of programmed cell death that can be induced in response to cellular stress. The tumor suppressor gene, P53, can be upregulated in cells as a response to stress. P53 can transcribe genes that encode for proteins associated with the activation of apoptosis [22]. However, there is a complex interplay between apoptosis, and another pathway called, autophagy. Autophagy is an evolutionarily conserved catabolic process that occurs in all cells as means to recycle and reused damaged organelles, unused, and misfolded proteins [23-25]. There is a basal level of autophagy that occurs cells as a form of housekeeping and to maintain cellular homeostasis [23]. Autophagy can serve as pro-survival mechanism in tumor cells though providing vital nutrients under hypoxia [26-28]. Although over-activation of autophagy can lead to cell death. There are three main forms of autophagy, macroautophagy, microautophagy, chaperone-mediated autophagy [24, 29]. However, macroautophagy (autophagy) is main form of autophagy and can be stimulated in times of cellular starvation.
Autophagy has four important steps, the initiation, nucleation, elongation, and degradation of cellular components. In response to stimuli, the initiation of autophagy begins with signaling through the PI3K pathway and mTOR. Essential autophagy related genes (ATgs) interacting with the gene ULK1 start the formation of autophagosomes. The elongation of the phagophore, includes additional atgs and the surface protein LC3-II, which leads to a double-membrane organelle that engulfs protein aggregates. Cytosolic LC3-I is converted into LC3-II. The abundance of LC3-I and LC3-II are often used as a marker for the induction of autophagy. Once an autophagosome is completed, it fuses with lysosomes for the degradation of the entrapped contents. Lysosomes release amino acids and macromolecules for energy and protein synthesis [24, 30].

In the TME, both tumor cells and stromal cells exploit the protective role that autophagy plays. Autophagy protects against genome instability and is able to prevent the induction of apoptosis, where cells would have otherwise been susceptible to death. It has been demonstrated that apoptosis and autophagy share common proteins. Some pro-apoptotic proteins can block the activation of autophagy and vice versa. In response to hypoxia, tumor cells show enhanced expression of pro-survival genes that suppress apoptosis, and support autophagy. The major regulator of the cells response to hypoxia is the transcription factor, hypoxia-inducible factor 1 alpha (HIF-1α). It is lowly expressed in cells under normal oxygen conditions, and is degraded in proteasome dependent matter. However, in hypoxia the degradation of HIF-1α is blocked. Pro-apoptotic protein, B-cell lymphoma 2 (BCL-2), is the master regulator of apoptotic cell death. In normal oxygen conditions, BCL-2 binds to the mediator of autophagy, Beclin-1, preventing the induction of autophagy [22, 31]. However, in response to hypoxia, previous studies have shown that HIF-1α is accumulated. It binds to a region on DNA in the nucleus, known as hypoxia response elements (HREs). When HIF-1α binds to HREs it transcribes the pro-apoptotic gene, BCL2 interacting protein 3 (BNIP3), that encodes for the protein of the same name. BNIP3 competitively binds with BCL-2, preventing Beclin-1 from binding [32]. When Beclin-1 is displaced from BCL-2, it is free to form a protein complex with other proteins involved in the initiation of the autophagosome formation, as shown in Figure 1.

The induction of autophagy in lung and ovarian cancers mediates cisplatin resistance [8, 33, 34]. In vitro experiments with lung cancer cells showed, that hypoxia enhanced cisplatin resistance through blocking apoptotic cell death. Lung cancer cells showed an increase in BNIP-3 following hypoxia incubation and cisplatin treatment. However, an increase in autophagosome formation and phosphorylated Beclin-1 was seen as HIF-1α levels increased. Blocking of autophagy resulted in the death of lung cancer cells by cisplatin. Mesenchymal stem cells (MSCs) provide essential stromal support for tumor cells through the production growth factors. In vitro culture of serum-deprived mesenchymal stem cells with a serum-deprived breast cancer cell line, showed that the MSCs significantly increased breast cancer cell viability. In comparison to serum-deprived breast cancer cells cultured with normal MSCs. Staining showed a significant decrease in apoptotic activity. An increase in Beclin-1 and autophagosome formation was observed in both serum-deprived MSCs and breast cancer cells. These studies provide evidence that hypoxia-induced autophagy can be induced as mechanism of cell survival and support for tumor cell progression. Cross-talk between cells of the TME and tumor cells is also enhanced. This suggests that any cell of the hypoxic TME could be critical to tumor support. Macrophages are the most abundant immune cell found in the TME and are to foster an immunosuppressive microenvironment. They also function in the promotion of angiogenesis, wound healing, and tumor invasion. Hypoxia-induced autophagy may be essential to macrophage support of tumor cells with in the TME.

**TAMs are mediators of angiogenesis and tumor invasion in the tumor microenvironment**

Macrophages, derived from circulating monocytes, are the major effector cells of both the innate and adaptive immune responses [35]. The function of macrophages consists of surveillance of the body for the elimination of foreign pathogens, but they also produce factors that recruit and activate lymphatic cells of the
adaptive immune response [19]. Macrophages are heterogeneous in nature can be divided into two subgroups. Classically activated macrophages, which are also known as M1-polarized, produce both pro-inflammatory and immunostimulatory cytokines in response to infection [36]. Alternatively activated macrophages, or M2-polarized, play a role in the upregulation of immunosuppressive factors, during tissue repair [36]. Extensive studies of the EOC tumor microenvironment has shown that M2-polarized macrophages are the most abundant immune cell type found. Macrophages located in the TME are referred to as tumor associated macrophages (TAMs). Circulating monocytes are recruited by ovarian tumors through the expression of a chemoattractant, CCL2. The differentiation factor, colony stimulating factor 1 (CSF1) is secreted by tumor cells of the TME, and helps to differentiate monocytes into M2-polarized macrophages. Autophagy is also critical for monocyte differentiation into macrophages [36]. In vitro experiments showed that monocytes stimulated by the differentiation factor, CSF-1, activated autophagy through the activation of the JNK pathway [36]. JNK mediates the dissociation of Beclin-1 from BCL-2. When autophagy is blocked during differentiation, monocytes do not survive [36]. It has also been demonstrated that serum starved monocytes, also upregulated autophagosome formation. This suggest that autophagy might aid in more than just the differentiation of macrophages, but might be critical for cell survival. 

TAMs in the tumor microenvironment play a critical role in tumor support. It has been demonstrated that hypoxia entraps TAMs in the TME, but suppressing their homing abilities. This immobilizes them in the TME, where they can produce multiple cytokines and growth factors that influence the behavior of both tumor cells and the surrounding cells of the TME. Tumor growth and metastasis is reliant upon the formation of new blood vessels. The upregulation of angiogenesis is a key factor involved in the metastasis of cancers. Studies have demonstrated that tumor vascularization correlates with the growth of tumors, while the absence of vascularity leads to tumor necrosis or apoptosis [2, 7, 37]. Due to stress caused by the lack of oxygen and nutrients found in the hypoxic TME, angiogenesis promoting chemokines and cytokines can be upregulated as a mechanism of survival [2, 10, 34]. Endothelial cells are the most common source of new blood vessel formation [6]. Studies have demonstrated that TAMs are in close contact with endothelial cells in the tumor microenvironment. In EOC growth factors that promote angiogenesis such as, transforming growth factor-β (TGF-β), IL-8 and vascular endothelial growth factor (VEGF) are upregulated by TAMs through the influence of tumor cells.

Tumor cells produce tumor-necrosis factor alpha (TNF-a), which activates the NF-κB pathway in macrophages, leading to increased IL-8 production. In vitro co-culture studies of macrophages with ovarian cancer cells, show that the production of IL-8 by TAMs increased the mobility of endothelial cells. M2-polarized macrophages are also responsible for the secretion of enzymes known as matrix metallopeptidases (MMP). Specifically, MMP-9 produced by M2-polarized macrophages is involved in the degradation of the basement membrane and extracellular matrix, which aids in tumor cell invasion of neighboring tissue [38]. Serum-deprivation autophagy has recently been linked to the production of MMP-9 by liver cancer cells. Serum-deprivation induced autophagy through the TGF-b/Smad3 signaling pathway and stimulated tumor invasion [39]. Potentially, HIF-dependent autophagy in TAMs could be a mechanism of cell survival, an inducer of pro-angiogenic factors and enzymes that support tumor invasion. Therefore, it is my central hypothesis that hypoxia prolongs the lifespan of M2-polarized TAMs and leads to increased activation of tumor promoting factors in the tumor microenvironment.

C. Research Design

Aim 1. Examine the role of hypoxia in the promotion of tumorigenesis through macrophage signaling. A key feature of the tumor microenvironment is hypoxia [20, 34, 40]. Studies have shown that tumors in hypoxia, recruit circulating monocytes into the TME. Tumor cells produce chemoattractants, such as CCL2 and endothelin-2 [41]. Monocytes are differentiated into M2-polarized macrophages by tumor cells through secretion of colony stimulating factor 1 (CSF-1). Additionally, the cytokines IL-13, IL-4, TGF-β, and IL-10 aid in the activation of M2-macrophages. M2-polarized macrophages are found in high numbers in hypoxic areas of ovarian cancer, and promote immune suppression, angiogenesis and tumor invasion. Tumor associated macrophages have been shown to be in close contact with tumor cells and endothelial cells within the tumor-microenvironment [42]. Co-culture of M2-macrophages with tumor cells increases the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and IL-8, which can induce the formation of new blood vessels mediated by endothelial cells. Additionally, extracellular matrix remodeling occurs through M2-polarized macrophage secretion of matrix metalloproteinatease-9 (MMP-9). This allows for the invasion of tumor cells into neighboring tissues, leading to metastasis. The transcription factor, HIF-1α is upregulated in
macrophages exposed to hypoxia, \textit{in vitro} and in TAMs found in hypoxic areas of human tumors [38]. In tumor cells, the accumulation of HIF-1\(\alpha\) leads to the expression of genes involved in glucose metabolism, cell proliferation, and migration. Stem cells and fibroblasts of HIF-1\(\alpha\)-deficient mice are unable to up-regulate genes that are normally seen to increase during hypoxia, suggesting an important role of HIF-1\(\alpha\) in the hypoxic response of cells. \textit{I hypothesize} that elevated levels of HIF-1\(\alpha\) in M2-polarized macrophages in response to hypoxia, will upregulate the production of factors that support tumor cell invasion, and endothelial cell migration.

Aim 1a. \textbf{Determine if hypoxia leads to increased production of ovarian cancer promoting factors, VEGF and MMP-9 by M2-polarized macrophages.} In order to study the effect of hypoxia on M2-polarized macrophages in the ovarian cancer, monocyte-derived macrophages (MDMs) will be generated first from fresh donor PBMCs. Monocytes isolated from human PBMCs will be cultured in medium containing M2-polarized cytokines. These will include IL-10 and TGF-\(\beta\). After a 10 day incubation, M2-phenotype will be assessed by flow cytometry. Mature macrophages will be stained for expression of CD68+/CD63+/CD206 phenotype, also referred to as the M2-phenotype. Ovarian cancer cell lines, SKOV3 and OVCAR3, will be cultured with M2-polarized macrophages in ultra-low attachment 96 well plates that aid in the formation of 3D spheroids. After spheroids are formed, they will be cultured in hypoxia incubation chambers at 1\% oxygen for 0, 2hrs, 12hrs, and 24hrs. These time points will serve to show the difference in protein expression of HIF-1\(\alpha\). As a control, 3D cultures will also be incubated at 21\% oxygen (normoxia). Additional controls will include, macrophages alone and ovarian cancer cells in hypoxia and normoxia. Following incubation, the cells will be harvested and HIF-1\(\alpha\) expression will assessed. ELISA will determine levels of VEGF and MMP-9 in solution. A One-way ANOVA test will be performed to determine significant differences in secreted factors. \textbf{Expected results:} If HIF-1\(\alpha\) accumulation occurs in response to hypoxia in M2-polarized macrophages, then I would observe an increase in HIF-1\(\alpha\) protein expression in cells cultured in hypoxia. Macrophages cultured alone will still produce these tumor promoting factors, but I expect to see an increase in VEGF and MMP-9 in 3D culture models. In addition, macrophages cultured in normoxia with ovarian cancer cells should have lower levels of VEGF and MMP-9. This is supported by previous \textit{in vitro} studies that demonstrated increases in growth factors that activate macrophages, are seen in response to hypoxia by tumor cells.

Aim 1b. \textbf{Elucidate the effect of hypoxia on M2-polarized macrophage promotion of ovarian cancer metastasis.} In order to determine if increased production of tumor promoting factors by macrophages has an effect on ovarian cell metastasis. 3D tumor cell invasion assays and transwell migration assays will be conducted. Utilizing the same experimental design from Aim 1a, basement membrane matrigel will be added to 3D cell cultures incubating in hypoxia. Matrigel contains essential fibers and connective tissues that mimic the extracellular membrane found \textit{in vivo}. Ovarian tumor cell invasion will be determined by imaging using Celigo cytometer. This cytometer allows for the visualization and quantification of tumor cell spread on the matrigel membrane. In parallel, supernatants will be collected from 3D cultures and transferred to HUVEC cells in transwell plates. These are a human umbilical cord derived endothelial cell line. Migration of endothelial cells will be assessed by microscopy and quantified to determine the number of migrated cells. A student’s t-test will be performed to determine significant differences migrated cells. All experiments will be repeated in triplicates. \textbf{Expected results:} Migration of both ovarian tumor cells and endothelial cells should be significantly increased in comparison to control cells cultured in normoxia. Due to the elevated levels of VEGF and MMP-9 that should be secreted into the media by macrophages in response to hypoxia.

Aim 1c. \textbf{Determine if inhibition of HIF-1\(\alpha\) diminishes the effect of hypoxia on M2-polarized macrophage signaling.} HIF-1\(\alpha\) knockout macrophages will be made using the CRISPR/cas9 system to determine if blocking hypoxia induced accumulation of HIF-1\(\alpha\) will alter the tumor promoting phenotype observed. HIF-1\(\alpha\)-/- will be confirmed through immunoblotting. WT and HIF-1\(\alpha\)-/- knockout macrophages will be cultured in hypoxia and normoxia for 0 and 24 hrs. The effect of HIF-1\(\alpha\)-/- on tumor-promoting factors, such as VEGF and MMP-9, will be assessed by ELISA. 3D tumor cell invasion assays will be conducted to determine if ovarian tumor cell invasion is lessened when cultured with HIF-knockout macrophages. \textbf{Expected results:} I expect to see no difference in HIF-1\(\alpha\) expression in knockout macrophages in hypoxia and in normoxia. While similar to aim 1a, WT macrophages should have increased levels of HIF-1\(\alpha\) after incubation
in normoxia. HIF-knockout macrophages should produce less tumor promoting factors and this should be reflected in both the ELISAs and in the tumor invasion assay.

**Aim 1 potential pitfalls and alternative approaches:** Although, HIF-1α is considered to be the most critical regulator of cells in response to hypoxia, there are alternative transcription factors activated during hypoxia that might have similar roles. This includes, NF-κB, which is involved in the production of cytokines and cell survival in response to stress. Alternatively, this could be used as a target if results from HIF-1α experiments don’t yield the expected results. Although the majority of macrophages found in the ovarian cancer tumor microenvironment are M2-polarized. It would be of interest to use M1-polarized macrophages as an alternative or even in comparison to observe how hypoxia affects these pro-inflammatory cells.

**Aim 2. Investigate the effect of hypoxia on the induction of autophagy in M2 polarized macrophages.**
Autophagy is normally activated in primary cells to maintain homeostasis for short-term durations. In prolonged serum and nutrient deprivation, both tumor cells and mesenchymal cells have demonstrated an increase in the activation of autophagy. Mechanistic studies of both normal cells and tumor cells have revealed that hypoxia mediates autophagy through increased transcription of the gene, BNIP3 that competes with Beclin-1 for binding to BCL-2. The binding of Beclin-1 to BCL-2 in normoxia stops beclin-1 from activating autophagy. It has been shown that this is a mechanism for survival in cells as an alternative to apoptotic cell death. Previous studies have shown that increased levels of autophagy have been detected in human pancreatic cancer cells and in patient tumors [43]. In these cells, autophagy enabled tumor growth by maintaining ATP production [44]. Inhibition of autophagy in these cells in xenograft mouse models led to tumor regression and prolonged mice survival [44]. Under normoxia, monocytes require autophagy to differentiate into macrophages. Inhibition of autophagy in differentiating monocytes leads to their death. The activation of autophagy as a means of survival in response to hypoxia has not been explored in tumor associated macrophages. However, it has been shown that in serum deprivation, as short as 2hrs, autophagy is activated in monocytes. This suggest that the pathway may be activated in mature macrophages in TME during hypoxia. I hypothesize that elevated levels of HIF-1α in macrophages in response to hypoxia, will lead to the activation of autophagy and the down-regulation of apoptotic activity leading to cell survival.

**Aim 2a. Identify if autophagosome formation is increased in M2-polarized macrophages in a HIF-dependent matter.** The first step in the initiation of autophagy is the formation of autophagosomes. LC3 is the major protein that incorporates into the surface membrane of phagosomes during their formation. There are two isoforms of LC3-I and LC3-II. LC3-I is converted into LC3-II when it binds to the surface of autophagosomes during formation. Anti-LC3 antibodies can be used to track the abundance of autophagosomes. Using my generated WT and HIF-knockout M2-polarized macrophages, I will culture cells for 0, 2, and 24 hrs in hypoxic chambers. HIF-1α expression will be assessed by immunoblotting. Cells will also be cultured in normoxia. Autophagosome formation will be assessed by two methods. Antibodies specific for LC3-I and LC3-II proteins will be used to measure the abundance of autophagosome formation in macrophages cultured under hypoxic conditions by western blot. Additionally, I will perform intracellular staining to visualize the abundance of autophagosome formation with fluorescence-tagged LC3 antibodies. Puncta abundance will be used to measure LC3 for statistical analysis and a t-test will be done to analyze significance.

**Expected Results:** I expect to observe increases in autophagy related protein expression and autophagosome formation in response to HIF-1α upregulation. Macrophages cultured normoxia should show no autophagy induction. I also expect that autophagy in HIF-1α-/- macrophages will look similar to WT cells cultured in normoxia.

**Aim 2b. Determine if HIF-dependent autophagy reduces apoptotic activity.**
If HIF-1α mediates the induction of autophagy in macrophages, then apoptotic activity should decline over time. In order to determine if HIF-induced autophagy leads to diminished apoptosis, will find the threshold for apoptotic cell death in macrophages due to hypoxia. A time-course experiment with HIF-1α knockout macrophages in normoxia and in hypoxia will be performed. Several different time points will be set-up in culture 0 minutes, which will serve as baseline, 30 minutes, 2hr, 4hrs, 6hrs, 12hrs, 18hrs, 24hrs, 36hrs, 48hrs, and 72hrs. At each time point the cells will be harvested and co-immunoprecipitation of BCL-2 will be conducted with anti-Beclin-1 and anti-BNIP3 will determine the level of apoptotic activity. Apoptosis should start to decline as less expression of BCL-2/Beclin-1 protein interactions is seen. If this is HIF-dependent, then HIF-1α knockout lines will have higher
levels of BCL-2/Beclin-1 expression as time persists. Additionally, apoptotic cell death will be assessed by annexin V staining and analyzed with flow cytometry. WT-macrophages will serve as a control.

**Expected results:** Since the dissociation of Beclin-1 from BCL-2 is known to be important for the initiation of autophagy. I expect to see little to no change in BCL-2/Beclin-1 eluted through Co-IP in HIF-1α knockout macrophages cultured in hypoxia. Since BNIP3 is transcribe in response to hypoxia. I expect to see more expression of BNIP3 as time persist in HIF-WT cells. While HIF-1α<sup>−/−</sup> knockout should show no increase in BNIP3. This experiment will also reveal the time-point where apoptotic activity begins to diminish in WT-macrophages in hypoxia, as autophagic pathways are activated. Also what I am referring to as the apoptotic threshold.

Aim 2c. Determine if HIF-dependent autophagy affects cell viability.

If HIF-dependent autophagy leads to prolonged cell survival, then WT HIF-1α cells should be alive in culture longer than HIF-1α knockout cells. Once a threshold for apoptotic-cell death is established, I will next determine if cell survival is increased in macrophages due to HIF-dependent autophagy. Similar to aim 2.2, HIF-α knockout and HIF-1α WT macrophages will be cultured in hypoxia for several time points. However, observations of cell viability will begin at the apoptotic threshold and precede for 48 hours after. Cell viability will be assessed by an MTT assay. This colorimetric assay can quantify live cells in culture. Fluorescent-LC3 punctate abundance will be used to determine the significance of differences between control and experimental groups. Significant differences will be measured by one-way ANOVA.

**Expected results:** If hypoxia-induced autophagy does lead to increased cell viability, then these results will confirm this. WT-macrophages should persist longer in culture than HIF-knockout macrophages. Autophagosome formation indicated by LC3 expression will be higher in HIF-1α WT cells as well.

Aim 2d. Determine if inhibition of autophagy leads to revived apoptotic activity in M2-polarized macrophages.

If the induction of autophagy leads to prolonged cell survival, then the inhibition of autophagy should lead apoptotic death. 3-MA, is a selective inhibitor of PI3K Vsp34, a key component of autophagosome formation. I will utilize 3-MA to block autophagic activity. A 24hr dose-dependent experiment will be conducted, where 3-MA will be titrated at 0, 1, 5, and 10 µM and incubated with WT and HIF 1-α<sup>−/−</sup> macrophages. Cells will be grown in normoxia as a control. Immunoblotting of LC3 will determine the level of autophagy activity. Immunoblotting of caspase-3 and caspase-9, indicators of apoptotic activity will be performed.

**Expected results:** I expect to see higher levels of caspases as the dose of 3-MA increases in WT cells cultured in hypoxia. I don’t expect to see much of any difference in HIF-knockout cells treated with 3-MA, as these already would have low to no activation of autophagy.

**Aim 2. Alternative approaches and pitfalls:**

Although knocking out HIF-1α might be enough to show the inactivation of autophagic activity in cells cultured in hypoxia. An additional confirmation experiment would be to transfec HIF-1α into HIF-1 into deficient cells and see if this rescues the phenotype seen in WT intact HIF cells cultured hypoxia. Another experiment that could confirm that HIF-1a is important for the induction of autophagy and decrease of apoptosis, would be to over express HIF-1α in macrophages and observe these pathways outside of hypoxia.

If M2-polarized macrophages in the TME are mediators of tumor promoting factors in response to hypoxia. The next steps would be to look at in vivo models to study M2 polarized macrophages. One way to go about this is to use the mouse model MISTRG, which possess human cytokines for the growth of human macrophages. Subcutaneous ovarian tumors could be grown in these mice with human ovarian cancer cells. Since hypoxia occurs when tumors have a low oxygen supply. Two groups of mice can be allowed to grow different size tumors, one group will serve as a control and tumors can be under .5 cm. Another group can grow up 1.5 cm. Tumors can be harvested from the mice. IHC staining for abundance of M2 polarized macrophages can be conducted. Additionally, HIF-1α and LC3 expression in M2 macrophages could be completed. If M2-polarized macrophages have high expression of HIF-1α and LC3 in the larger tumors, it might indicate that this phenomena is hypoxia induced. This would support my in vitro data, by showing that even in vivo macrophages have elevated HIF-1α and activation of autophagy.

This aim heavily depends on autophagy being upregulated in macrophages in response to hypoxia. While this may potentially be a means of survival, it also may not. As an alternative to looking at autophagy as the
singular mechanism of survival, I could look at how macrophages down-regulate surface markers to escape death by immune cells. Natural killer T cells have been shown to specifically target M2-polarized macrophages in a CD1d, dependent matter in the neuroblastoma tumor microenvironment. It would be interesting to address whether a similar behavior is seen ovarian cancer in response to hypoxia.

D. Bibliography


