Understanding CD8 T cell Function under the Tumour Environment Condition Hypoxia

by

Katelin N. Townsend
B.Sc., University of Victoria, 2009

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

Supervisory Committee

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Dr. Julian J. Lum (Department of Biochemistry and Microbiology)
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Dr. Juan Ausio (Department of Biochemistry and Microbiology)
Departmental Member

Dr. Robert Chow (Department of Biology)
Outside Member

Abstract

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As CD8 T cells migrate to tumour sites, they experience conditions of low oxygen or hypoxia, in the tumour environment. Hypoxia results due to the rapid proliferation of tumour cells which deplete essential nutrients such as oxygen as they expand beyond normal vasculature. Hypoxia can cause attenuated immune responses due to the resultant signalling events and metabolic changes initiated in CD8 T cells under these conditions. CD8 T cells are important mediators of anti-tumour activity as they directly kill tumour cells, and are associated with increased survival outcomes in cancer patients. Therefore, I sought to determine the impact of low oxygen on CD8 T cell function. In addition, I investigated the role for autophagy, a cell survival process induced by nutrient depletion, in T cells under hypoxia.

The first chapter of this thesis outlines the effects of the hypoxic tumour environment and the known roles for autophagy in T cells. In the second chapter, the role of hypoxia and hypoxia-induced autophagy will be explored in CD8 T cells and the impact on cell function assessed using a transgenic mouse model. The importance of hypoxia for T cell activity clinically will be examined in Chapter 3. High-grade serous

ovarian tumours will be evaluated for their oxygenation levels and immune status and correlations with patient survival will be assessed. These results are important for understanding how CD8 T cells function during pathophysiological oxygen conditions found in tumours and reveal hypoxia as a new relevant inducer of autophagy in T cells. Ultimately, these results highlight the need for further research discoveries which promote T cell function during conditions of low oxygen in tumours. Such future discoveries may be combined with therapies which boost or enhance immune responses, allowing more optimal tumour treatments to improve patient survival.

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Acknowledgments

I have a lot of people to thank for their support over the course of my master's degree. First, I would like to thank Dr. Julian Lum for his supervision during both my undergraduate degree and master's degree. Thank you for giving me the opportunity to explore the field of cancer and immunology. I have learnt more than I ever thought possible and really appreciate your guidance over the years.

I would also like to thank my co-supervisor Dr. Terry Pearson and the members of my committee: Dr. Juan Ausio and Dr. Robert Chow for their valuable input throughout my degree.

To the members of the Lum lab, past and present, thank you for making my time at the Deeley Research Centre (DRC) so enjoyable. To Dr. Katrin Schlie, Vincent Poon, and Luke Hughson, thank you for your scientific discussion and collaboration on the T cell autophagy project in Chapter 2, I have really appreciated your help. To Jill Brandon, Ashley Westerback and Dean Rysstad, thank you for your work in generating the ACTO mice. Thank you to Jaeline Spowart whom I collaborated with on the immunohistochemistry project in Chapter 3. In addition to your collaboration, you have contributed to my graduate experience in countless other ways, thank you for your support and understanding over the years, it has been invaluable.

To the members of the DRC, thank you for your willingness to share your knowledge with others and making the DRC a great place to be. I would like to thank my fellow graduate students Dr. Nathan West and Dr. Eric Tran for all of your help over the years, especially in my early days. Thank you to Dr. Peter Watson for sharing your pathology expertise for the IHC story in Chapter 3.

I did not realize that when I registered to do my master's degree that I would be signing up my family and friends with me. Thank you for your understanding of my long hours in the lab. I would like to thank my family, especially my grandparents Bill and Lindy Newman and Bernice Townsend for always supporting me. Thank you to my mom Maureen Newman for teaching me compassion and to care for others, my drive to improve cancer care is strongly driven by your example. Thank you to my dad Gary Townsend for teaching me that I could do anything I wanted and that there are no limits to my capabilities. To my sister Carley Townsend, thank you for always listening and being there for me. To my partner Brent Dallimore, thank you for being a constant source of calm and support, I could not have done my degree without you.

Last, I would like to thank the late Trev and Joyce Deeley for providing an endowment to start the DRC to increase our knowledge of the immune systems involvement in cancer and to allow students like myself to learn and explore science. Thank you to the women who donated their tumours for science and let us ask research questions. I hope that in your honour our research may go towards improving ovarian cancer patient outcomes in the coming years. Thank you to all the mice that sacrificed their lives to science, I did my best to make sure every experiment counted.

Dedication

To my great aunt Peggy, your passing from breast cancer inspired me at a young age to cure cancer. To Maureen, Gary, Carley and Brent, you have shaped who I am today and this work is a reflection of your love, support and belief in me.

Chapter 1: Introduction

1.1 Prologue

This thesis aims to understand CD8 T cell function under the tumour environment condition of low oxygen, or hypoxia, and to determine a role and relevance of a hypoxia-induced process called autophagy in these cells. The following sections are designed to provide context to the research discoveries made in Chapters 2 and 3 and to highlight outstanding questions in the immunology field which lead to the experiments described in these chapters. First, I introduce the anti-tumour immune system and where CD8 T cells fall within the vast array of cell types involved. Next, I examine the negative environmental feature of hypoxia which CD8 T cells face while carrying out their role as tumour eliminators. I also discuss the signalling events that are induced in T cells under hypoxic conditions. Finally, I outline the model systems I used to study the biological effects of hypoxia on CD8 T cells, including a mouse model system applied in Chapter 2, and the human ovarian cancer setting used to assess the clinical relevance of hypoxia on T cell function in Chapter 3.

1.2 Anti-tumour immunity

CD8 T cells are the focus of this thesis, however, their activity depends on a complex, cellular and humoral network which constitutes the anti-tumour immune response. Here I discuss principles of tumour recognition and how cancers arise in the face of the immune system. Next, I discuss CD8 T cell development and outline a number of immune cell types which are involved in anti-tumour immunity. These different immune cells can initiate or inhibit immune responses or directly kill tumours and are discussed in order to add context

to the role of CD8 T cells in tumour eradication. In addition, therapies which enhance or induce positive anti-tumour responses are introduced.

1.2.1 Immunosurveillance and immunoediting

During the 1900s it was first postulated by Paul Ehrlich that the immune system may protect long-lived hosts from cancer [1]. Although, it was not until 50 years later that the cancer immunosurveillance hypothesis, which implicates the adaptive immune system in preventing cancer, arose [1]. The majority of studies assessing this hypothesis were initiated in the 1990s with the development of mouse models to allow determination of the importance of the immune system in cancer development [2-6].

The immunosurveillance hypothesis states that the immune system is important for the elimination of cancerous cells. However, tumours can still form even though the body contains a battery of immune cells designed to naturally eliminate cancerous cells [7]. The term cancer immunosurveillance has since been encompassed by the term cancer immunoediting [7]. The concept of immunoediting outlines the protective and sculpting roles of the immune system in tumour growth. Overall, tumour growth in the presence of the immune system occurs through a series of steps consisting of elimination or immunosurveillance, equilibrium and escape phases [1]. Many cancerous cells may be eliminated by the immune system in the immunosurveillance phase, however, some cells may remain and are controlled by the immune system during the equilibrium phase. This phase may last a number of years, however, some cells may escape immune recognition by various means. This can include down-regulation of molecules required for T cell recognition called major histocompatibility complex (MHC) molecules [8], secretion of immunosuppressive factors such as transforming growth factor β (TGFβ) or indoleamine 2,3-dioxygenase [9,10]

and recruitment of suppressive immune subsets such as T regulatory cells (T regs) or myeloid derived suppressor cells (MDSCs) [11,12]. Tumours also proliferate rapidly, resulting in lactate secretion or hypoxia which can both dampen immune responses [13,14]. Interestingly, the ability of tumour cells to escape the immune system has recently been coined a hallmark of cancer generation [15].

The immune system is important for tumour control, even after tumours have escaped immune recognition and present clinically. This has been demonstrated by the improved survival of patients with tumour infiltrating immune cells, particularly cytotoxic CD8 T cells, in a variety of tumour types including breast, ovarian and melanoma, as compared to patients without tumour infiltrating immune cells [16-18]. Indeed, even commonly used cancer treatments such as chemotherapies have been shown to elicit beneficial anti-tumour immune responses [19].

1.2.2 CD8 T cell development and differentiation

The process of T cell development begins with committed lymphoid progenitor cells which arise in the bone marrow from hematopoietic stem cells [20]. These cells migrate to the thymus, a primary lymphoid organ, where they become T cell precursors called thymocytes. Thymocytes develop a T cell receptor and undergo selection in the thymus based on their recognition of self-peptide–MHC ligands expressed by cortical epithelial cells [20]. Highly self-reactive cells and completely non-reactive cells are deleted and those with intermediate levels of recognition are positively selected for and allowed to migrate to the peripheral secondary lymphoid tissues [20].

Once in the periphery, naïve CD8 T cells are activated in secondary lymphoid organs, including the spleen and lymph nodes, by antigen presenting cells [21]. The process of

activation requires engagement of the T cell receptor and costimulation *via* the molecule CD28 which is expressed on CD8 T cells [22]. Upon activation, a naïve T cell will take up to 24 hours before undergoing its first cell division; subsequently, very short cell cycle periods occur, with replication occurring every 4 to 6 hours resulting in rapid cellular expansion [23,24]. This rapid expansion is required for T cells to respond quickly to danger signals. Once activated, CD8 T cells, now called effector T cells, secrete cytotoxic molecules including perforin, granzyme B and T cell intracellular antigen-1 (TIA-1) [25,26]. These molecules are used to directly kill tumour cells. In addition CD8 T cells secrete cytokines, which are soluble proteins, such as interferon γ (IFNγ) and tumour necrosis factor α (TNFα) to promote anti-tumour activity [27]. Once activated CD8 T cells have undergone massive expansion and the antigen load has been vastly decreased their numbers contract, resulting in elimination of up to 95 % of the existing activated effector cells [27]. After the contraction phase, CD8 T cells differentiate into a memory population consisting of effector memory and central memory subsets [28]. These cells are capable of generating robust cellular responses when they re-encounter their antigen [28]. This thesis will focus on the function of activated CD8 T cells during the effector stage.

1.2.3 The players: cells involved in anti-tumour immunity

Dying tumour cells are phagocytosed by antigen presenting cells such as dendritic cells (DCs). These tumour cells can elicit a process called immunogenic cell death against other tumour cells through the release of soluble factors, resulting in the activation and maturation of DCs. DCs then migrate to the secondary lymphoid organs where they activate T cell responses against the tumour. Tumours also activate immune responses through the expression of tumour-associated antigens on MHC class I which are recognized as foreign

by the immune system. This can include mutated self-proteins, viral proteins such as those comprising human papilloma virus, or proteins that have distinct tissue distribution such as those expressed in the testis, collectively known as cancer testis antigens [29-31]. DCs display tumour antigens to the immune system on MHC class II molecules to activate CD4 T helper (Th) cells or cross-present antigen onto MHC class I to activate cytotoxic CD8 T cells. CD4 T helper cells are skewed by their cytokine microenvironment to a Th1, Th2, Th17 or regulatory phenotype and generate distinct immune responses through the secretion of various cytokines.

A CD4 Th1 response is generated by interleukin-12 (IL-12) and IL-18 [32] and Th1 CD4 cells directly promote the activation of CD8 T cells *via* production of the cytokine IL-2 [33]. CD8 T cells directly kill tumour cells through the secretion of cytotoxic molecules. Upon activation by DCs, CD8 T cells traffic to the tumour where they can contact tumour cells for a number of hours in the harsh tumour environment in order to mediate killing [34].

CD4 T helper 2 (Th2) responses are induced by the production of IL-2 and IL4 and activate B cells by the secretion of IL-4 [32,33]. This response may not be favorable in the cancer setting given that B cells have been described as immunosuppressive by inhibiting anti-tumour CD8 T cells [35-37]. However, B cells are also known to serve as antigen-presenting cells and may be important for optimal T cell activation and clonal expansion, which was demonstrated to be important also for anti-tumour immune responses[38-40]. B cells also produce antibodies to promote tumour killing and antibody dependent cellular cytotoxicity [41]. Indeed, tumour antigen-specific antibodies have been correlated with positive cancer patient outcomes [42].

CD4 T helper 17 (Th17) cells have been shown to play controversial roles in anti-tumour immunity and are generated by the cytokines TGFβ, IL-6, IL-23 and IL-21 [32,43].

Th17 cells secrete the cytokine IL-17 which can promote vasculature formation in some cancers, thus allowing tumour cells to support their growth [44,45]. However, adoptively transferred Th17 cells have also been used to eradicate tumours in an *in vivo* mouse model [46]. Whether Th17 cells inhibit or promote anti-tumour immunity may be dependent on the cancer type they are observed in [47].

Lastly, CD4 T regs can occur naturally in the thymus or are induced from naïve CD4 T cells by the cytokines TGFβ and IL-2 [32,43]. T regs suppress immune responses through the production of the immunosuppressive factors IL-10 and TGFβ [32]. T regs are characterized by a number of markers, the most common being the expression of the transcription factor forkhead box protein P3 (Foxp3) and the IL-2 receptor, CD25 [48]. T regs are critical in preventing self-reactive T cells from eliciting an autoimmune response [49]. In the context of cancer, T regs may have a detrimental effect by actively suppressing cytotoxic tumour specific T cells. Several studies demonstrate that depletion of T regs results in an enhanced anti-tumour effect [50-52]. In addition, poor patient prognosis has been correlated with increased T reg presence within tumours [48,53]. However, some studies report that T regs are associated with improved cancer patient survival and thus their activity may not be immunosuppressive in some contexts [54,55]. The discrepant role of T regs in the cancer setting may be attributed to the heterogeneity of cells which can express FoxP3, including, T regs and non-regulatory cell types such as effector CD4 T cells [56]. Indeed, patient studies which define T regs as cells which express FoxP3 in addition to a second defining marker, associate these cells with negligible prognostic significance or poor patient prognosis [57]. Thus, defining a T reg using multiple markers may allow the T reg population to be assessed more directly. The impact of T regs on patient survival has also been shown to be dependent on the tumour site as T regs in hepatocellular cancer are associated with

negative patient outcomes while T regs are most commonly associated with positive patient prognosis in colorectal cancers [57].

In addition to cellular subsets of the adaptive immune system, innate immune cells such as natural killer (NK) cells and macrophages also play important roles in anti-tumour activity. NK cells are professional killer cells, able to directly target and induce the death of tumour cells, which they can recognize by low expression levels of MHC class I molecules or by bound antibodies which are produced by B cells [41,58]. Macrophages are involved in tumour immune surveillance, but with disparate roles in promoting anti-tumour immunity and cancer progression. Macrophages normally function to phagocytose damaged cells. However, they have been described to play both positive and negative roles in the immune response to cancer depending on whether they are of inflammatory (M1), or anti-inflammatory (M2) phenotypes [59]. The determining factor for whether M1 versus M2 macrophages are induced appears to be associated with the cytokines IFNγ or IL-4 and IL-13 respectively [59]. In addition, a subset of cells known as MDSCs which resemble a heterogeneous population of myeloid cells, or non-lymphoid cells, can also be found at tumour sites. These cells are able to suppress NK and T cells and often have negative associations with cancer outcomes by decreasing patient survival [60,61].

Overall, numerous cell types are involved in the process of immunosurveillance and immunoediting and the ratio of immune effectors versus immune suppressors can dictate the tendency of the host immune system to clear tumours.

1.2.4 Immunotherapy

The importance of the immune system in cancer eradication has long promoted an interest in boosting immune responses to promote cancer elimination. Recently, therapies

eliciting immune responses have been approved by the United States Food and Drug Administration, including monoclonal antibodies such as Herceptin® (trastuzumab) to target cancer-associated proteins; Provenge® (sipuleucel-T), an autologous cellular immunotherapy for advanced prostate cancer; and ipilimumab, an antibody directed against the negative T cell regulator cytotoxic T lymphocyte antigen-4 (CTLA4) for treatment of metastatic melanoma [62].

Another form of immune enhancing therapy is called adoptive immunotherapy which is a potential cancer treatment of research interest. This therapy comprises a process whereby T cells are removed from a patient's tumour, cultured and expanded *in vitro*, and large numbers of these cells are infused back into the patient [62]. Adoptive immunotherapy is effective in metastatic melanoma patients given lymphodepletion to allow T cell engraftment, and results in durable responses [63,64]. One difficulty of this therapy is that tumour infiltrating T cells often do not expand *in vitro* or a tumour cannot be removed for T cell isolation. This has been overcome through T cell engineering, whereby retroviral vectors are used to elicit expression of chimeric antigen receptors made up of antibody binding domains tethered to domains which activate T cells [65,66]. Additional engineering techniques employ bispecific antibodies to engage the T cell receptor and antigen on the tumour surface [67] or include the transduction of T cells with high-affinity T cell receptors [68]. Despite these advances, T cell persistence in the patient remains an important consideration for adoptive immunotherapy. Research efforts have striven to improve the persistence of adoptively transferred T cells by determining the best T cell subset to transfer. These studies have found that generating CD8 memory T cells is an optimal approach to promote persistence [69]. It may be that conditions which dampen immune responses such as hypoxia also play a role in the failure of adoptively transferred T cells to persist. We

employ the use of adoptive transfer in Chapter 2 to study the effect of autophagy inhibition on anti-tumour activity in a hypoxic tumour environment.

1.3 Cellular consequences of the tumour environment condition hypoxia

While a number of suppressive features exist in tumours, this thesis will focus on the role of hypoxia, particularly 1.5 % oxygen for *in vitro* experiments, in supressing CD8 T cell activity. Ambient air is 21 % oxygen, however, T cells experience various oxygen tensions as they traffic throughout the body, indeed arterial blood is 13 % oxygen while the peripheral tissues are approximately 5 % [70]. On direct assessment of oxygen levels within the spleen, it was found that oxygen ranges from 0.5–4.5 % depending on the distance from blood vessels [71]. Tumour hypoxia, which has been shown to range from anoxic at 0 % oxygen up to 3 % oxygen, can occur through a number of mechanisms whereby tumour cell oxygen demand is not met by sufficient oxygen supply [72-74]. Oxygen delivery to tumours can be hindered by the development of abnormal tumour microvessels, a decrease in oxygen diffusion and tumour or therapy-induced anemia [73]. Moreover, conditions of hypoxia are often intermittent, resulting in cyclic conditions of nutrient delivery and deprivation due to structural defects in vasculature [75]. It is thought that hypoxia inhibits immune cells as a mechanism of tissue-protection. During an immune response against pathogen-infected cells, immune cells may cause damage to normal tissues as well, including microcirculatory structures, thus generating hypoxia [76]. Dampening the immune response during excessive inflammatory damage prevents damage to healthy tissues, however, this dampening mechanism is not optimal in the hypoxic tumour setting. Studies have identified that processes which dominate in T cells during hypoxic conditions, namely adenosine receptor

signalling and stabilization of the transcription factor hypoxia inducible factor-1(HIF-1), both play a role in dampening immune responses.

1.3.1 HIF-1 stabilization

Under low oxygen, the transcription factor HIF-1 is responsible for a number of key transcriptional changes which allows the cell to adapt to this condition. HIF-1 consists of a heterodimeric protein complex composed of HIF-1α and HIF-1β [77]. HIF-1α is regulated by cellular oxygen levels. Under normal oxygen concentrations, HIF-1α is hydroxylated on its oxygen-dependent degradation domain by prolyl-4-hydroxylase domain proteins (PHDs) [78]. This hydroxylation causes binding of the von Hippel-Lindau protein (VHL), a component of the ubiquitin ligase complex that targets HIF-1 for proteosomal degradation [79,80]. The action of PHDs requires oxygen, thus under low oxygen conditions the rate of hydroxylation is limited and HIF-1α is no longer degraded by the proteasome [78]. HIF-1α stabilization has been shown to occur at 6 % oxygen in cervical carcinoma HeLa cells with maximal expression at 0.5 % oxygen and half maximal expression between 1.5 and 2 % oxygen after 4 hours of culture [81]. The stabilization and degradation processes are readily responsive to changes in oxygen concentration, allowing cells to quickly adapt to their environment. HIF-1α stabilization under 5 % oxygen was shown to occur within 2 minutes [82] and degradation under normoxia occurs after 5 minutes on exposure to higher oxygen levels [83]. Interestingly, HIF-1α is also regulated by metabolites which can form under normoxia including reactive oxygen species (ROS) or succinate. These metabolites decrease PHD activity, thus stabilizing HIF-1α [84]. The importance of HIF-1 is demonstrated by HIF-1α and HIF-1β knockout mice which die during embryogenesis due to defects in the formation of vasculature [85,86]. This is based on the role of HIF-1 in angiogenesis, the

formation of blood vessels. In addition, HIF-1 targets genes that are involved in promoting cell survival, autophagy, and glycolysis [87,88].

1.3.2 HIF-1-mediated T cell metabolism

In the absence of oxygen, anaerobic glycolysis is the main adenosine triphosphate (ATP) producing pathway used by cells. During anaerobic glycolysis glucose is broken down through a series of steps to generate pyruvate, which is converted to lactate for secretion by the cell. When oxygen is present, the other major ATP-producing process is oxidative phosphorylation which occurs in the mitochondria where pyruvate or fatty acids are converted to acetyl coenzyme A and processed by the tricarboxylic acid (TCA) cycle. The reducing equivalents generated by this process donate electrons to the electron transport chain (ETC) which relies on oxygen as the terminal electron acceptor [89-91]. As electrons pass through the ETC, protons are pumped into the mitochondrial intermembrane space from the matrix to produce an electrochemical gradient that is used by ATP synthase to produce ATP [92].

HIF-1 promotes a switch in cellular metabolism from oxidative phosphorylation to anaerobic glycolysis under low oxygen by promoting the transcription of genes encoding glucose transporters and glycolytic enzymes [90,93]. Furthermore, HIF-1 inhibits the conversion of pyruvate into acetyl coenzyme A, preventing pyruvate from entering the TCA cycle. The ability of HIF-1 to regulate pyruvate metabolism is achieved primarily through the induced expression of pyruvate dehydrogenase kinase 1 which phosphorylates and inhibits pyruvate dehydrogenase, a central regulator of oxidative metabolism [94,95].

Naïve T cells and memory T cells derive most of their energy from fatty acid oxidation, whereby fatty acids are used to generate acetyl coenzyme A through β-oxidation

[91,96]. However, once naïve T cells become activated they undergo increased glycolysis even under normoxic conditions, a process termed aerobic glycolysis [92]. Interestingly, many tumours are also heavily reliant on aerobic glycolysis, a process termed the "Warburg effect" [97]. Though anaerobic glycolysis is a less efficient form of metabolism compared to oxidative phosphorylation, it is capable of providing sufficient energy to sustain growth and proliferation and has the advantage of providing a platform of biosynthetic precursors necessary for nucleotide, amino acid, and glycerol synthesis [98]. In addition, a report demonstrated that high glycolytic flux can surpass oxidative phosphorylation in terms of ATP production [99].

HIF-1α has been shown to play a role in metabolism even under normoxic conditions. Growth factor-stimulated hematopoietic cells express HIF-1α to regulate glycolysis [100] and CD4 T cells express HIF-1α when cultured under normoxia in Th17 polarizing conditions [43]. It has been suggested that HIF-1-mediated glycolysis and conversion of pyruvate to lactate may allow for proliferating cells to match a rapid induction of glycolysis with the TCA and electron transport chain. This would allow cells to reduce oxidative stress which would result if all the pyruvate generated from glycolysis was used in the TCA which can occur simultaneously with aerobic glycolysis under normoxia [98].

Unlike activated T cells undergoing aerobic glycolysis, hypoxic activated T cells may be unable to effectively use oxidative phosphorylation to produce ATP. In support of this, one study found that activated CD4 T cells cultured for 6 hours in a sealed chamber to result in cumulative hypoxia had similar ATP levels compared to activated T cells cultured under normoxia. This indicates that hypoxia-induced glycolysis can produce similar ATP levels compared to aerobic glycolysis [101]. However, hypoxic T cells also had higher levels of glycolytic activity and lactate production than T cells cultured under normoxia, indicating the

normoxic T cells may have been generating ATP *via* oxidative phosphorylation in addition to aerobic glycolysis. In addition, another study found that CD4 and CD8 T cells cultured at 2 % oxygen for 24 hours had an altered distribution of naïve and memory T cells compared to activated normoxic samples which had increased memory pools [102]. This may be due to the fact that memory T cells are reliant on oxidative phosphorylation and this could not occur under hypoxia, thus hindering memory formation. A similar study found that glycolytic induction during oxidative phosphorylation inhibition under normoxia, thus mimicking anaerobic glycolysis, resulted in poorer function than in CD4 T cells without inhibited oxidative phosphorylation [103]. Therefore, given that under hypoxia cells must utilize only anaerobic glycolysis and not oxidative phosphorylation, cells may turn to alternative energy sources such as autophagy to support cellular function. However, this question has not been studied in CD8 T cells and is an objective to be addressed by this thesis.

1.3.3 HIF-1 and T cell function

The function of T cells under hypoxia is strongly linked to their metabolism, but the vast majority of reports have explored this in CD4 T cells only. Early studies have shown that T cell function including cytokine secretion and proliferation is decreased under low oxygen [71,104-106]. This may be due to the negative effect of hypoxia on calcium signalling in T cells which is required upon T cell receptor engagement and is important for signal transduction [107,108].

The most recent of reports are exciting because they explain why the conventional Th1 cytokines, which were assessed in earlier studies, are decreased under hypoxia. This is because hypoxia skews CD4 cells to the Th17 CD4 subset which produces the cytokine IL-

17 [109]. Reports have found that glycolytic or hypoxic T cells differentiated into the Th17 lineage [43,110] and inhibiting glycolysis, a process induced by hypoxia, promoted T reg formation [110]. Interestingly, HIF-1 was shown to initiate IL-17 production and promoted degradation of the T reg transcription factor Foxp3, thus selecting for the Th17 subset [43]. While most groups have found that hypoxia suppresses T reg cells and promotes Th17 generation, a contrasting study found that hypoxia promoted the generation of T reg cells [111]. In addition, environmental hypoxia in ovarian tumours promoted the recruitment of T reg cells [112]. These seemingly contradictory results may be explained by the intensity and duration of cellular exposure to hypoxia, or by differences in secondary signals converging with HIF1-α stabilization. This collective data provide strong evidence that T cells are under the regulation of the metabolites in their environment and oxygen-dependent activation of HIF-1 plays a key role in determining T cell effector function. This is relevant for an oxygen-deprived tumour and requires further exploration in CD8 T cells.

1.3.4 Adenosine signalling and T cell function

The production of adenosine occurs *via* the molecules CD39 and CD73 and acts to inhibit the immune system as a host safety mechanism during hypoxia [113]. CD39 is an ecto-ATP apyrase, which converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP). CD73 is an ecto-5'-nucleotidase, that converts AMP to adenosine [114]. Adenosine production is exploited by tumour cells which may over express CD39 and CD73 [115,116]. T regs have also been shown to produce adenosine in order inhibit immune cells [117]. HIF-1 promotes the formation of adenosine as it controls the expression of both CD39 and CD73 [117,118].

T cells are impacted by adenosine because they predominantly express the adenosine A2A receptor which when engaged has been shown to inhibit T cell function through an increase in immunosuppressive cyclic AMP [119,120]. Recent studies have also shown that adenosine signalling in T cells resulted in the suppression of the transcription factor Nuclear factor κβ (NF-κB) causing decreased cytokine production in CD4 T cells [121]. While the adenosine produced by tumour cells represents a significant inhibitor of T cell activity under hypoxia, we sought to understand the role of HIF-1-mediated energy production in impacting T cell function under hypoxia.

1.4 Autophagy in T cells

Under conditions of stress such as nutrient deprivation, cells activate a survival process called macroautophagy, herein referred to as autophagy [122]. Autophagy is used by multiple cell types including cancer cells and immune cells. T cells use autophagy for a number of processes including survival, differentiation, cytokine production, organelle turnover, and maintenance of energy homeostasis. The current literature on autophagy in T cells is summarized in this section. The mechanisms of hypoxia-induced autophagy are also outlined, however, this process has not yet been demonstrated in T cells.

1.4.1 An overview of the autophagy pathway

(The following section has been adapted from the review article: Townsend KN, Hughson LR, Schlie K, Poon VI, Westerback A and Lum JJ. Autophagy inhibition in cancer therapy: metabolic considerations for anti-tumour immunity. Immunol. Rev. 2012, accepted)

During autophagy, cellular constituents are engulfed by double-membraned vesicles called autophagosomes, which fuse with lysosomes for degradation. Degraded cellular components are then recycled for cellular use (Figure 1). Autophagy requires a number of

autophagy-related (Atg) proteins involved in the processes of: initiation, nucleation, elongation, closure, maturation and degradation of autophagosomes. The initiation of autophagy occurs through the unc-51-like kinase-1 (ULK1) complex along with 200 kDa focal adhesion kinase family-interacting protein (FIP200) and Atg13. During nutrient rich conditions, the mammalian target of rapamycin complex 1(mTORC1) inhibits autophagy by phosphorylating and inactivating ULK1 [123]. Autophagy-activating signals converge at the Beclin 1- vacuolar protein sorting 34 (Vps34) complex, resulting in autophagosome elongation and the recruitment of two ubiquitin-like conjugation systems [124]. The first conjugation system results in an Atg12-Atg5 complex reliant on Atg7 and Atg10. Atg5 then binds to Atg16L to form an Atg12-Atg5-Atg16L complex which causes nucleation of the autophagosome. The second conjugation system results in cleavage of pro-microtubule-associated protein light chain 3 (LC3) to LC3I by Atg4. LC3I is further processed to LC3II on conjugation to phosphatidylethanolamine (PE) for inclusion in the inner and outer leaflets of the autophagosome membrane by Atg7 and Atg3. LC3II can bind to molecules such as the p62 which targets ubiquitin-labelled proteins to the autophagosome [125]. The autophagosomal membrane then closes on itself and in the maturation phase, the autophagosome fuses with lysosomes whereby the autophagolysosomal components are degraded and subsequently efluxed for cellular use [126]. These degraded products can include biosynthetic precursors for processes such as metabolism [127].

Autophagy can be pharmacologically induced using rapamycin, an inhibitor of mTOR which alleviates negative regulation of ULK1. In addition, chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) can be used to inhibit autophagy. These drugs cause increases in lysosomal pH that inhibit degradative enzymes [128,129]. Consequently, cells treated with CQ and HCQ are unable to undergo lysosomal degradation and exhibit

vesicular organelle accumulation in the cytoplasm consistent with blocked autophagy [130]. A number of the proteins outlined here are required for the autophagic process to occur. Of particular interest for this thesis is the protein Atg5. Atg5 is required for the formation of the autophagosome, thus cells lacking Atg5 are unable to carry out autophagy [131].

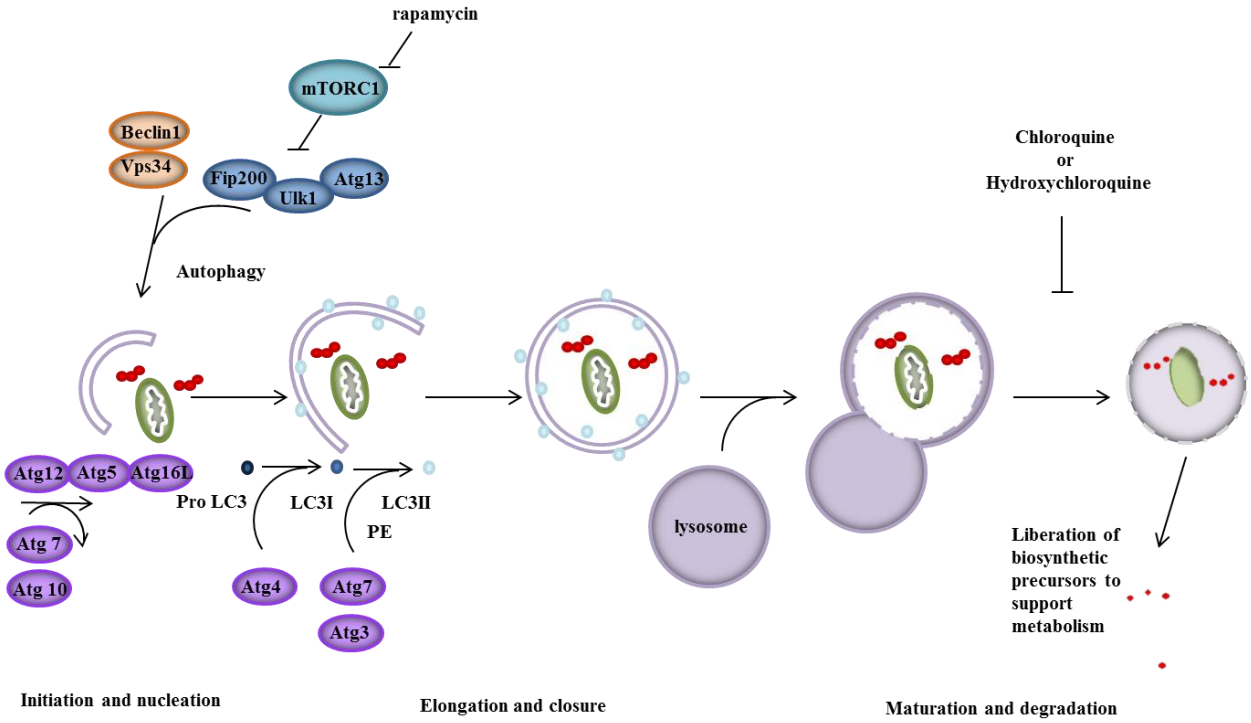


Figure 1: General features of the autophagy pathway.
During autophagy a series of proteins are involved in the initiation, nucleation, elongation, closure, maturation and degradation of autophagosomes. These processes are outlined in section 1.4.1 (Figure 1 is from Townsend *et al.* Immunol. Rev. 2012, accepted.)

1.4.2 The role of autophagy in T cells

In the early phases of T cell development, ablation of autophagy causes a minor reduction in thymocyte number [132-135]. Once T cells mature and traffic to the secondary lymphoid organs, autophagy is required for survival [132-134,136,137]. The role of autophagy in T cell survival has been attributed to autophagy causing degradation of certain

cell death machinery [137]. Autophagy also maintains regular cellular homeostasis by preventing the buildup of mitochondria and endoplasmic reticulum in T cells which may cause cell death [132-134,138]. For example, mitochondrial clearance allows a reduction of ROS build-up which can be detrimental to the cell [132-134].

Autophagy is induced on T cell receptor engagement and is required for cellular proliferation [132-134,136,137]. The requirement of autophagy for proliferation post-activation is likely due to the drastic increase in the metabolic demands of T cells, as they rely on glycolysis and most likely the liberation of nutrients by autophagy for this process. In one study, activated CD4 T cells produced less ATP, had reduced glycolytic activity and produced less cytokines during pharmacologic autophagy inhibition[139]. These findings support the notion that autophagy is required for cellular function by supporting metabolism through the liberation of biosynthetic precursors.

Interestingly, autophagy has recently been implicated in regulating the signalling of CD4 and CD8 T cells once activated. Autophagy degrades Bcl10 which is targeted to the autophagosome for degradation by p62 [140]. The degradation of the adaptor molecule Bcl10 causes a decrease in NF-κβ signalling which is needed for T cell proliferation and IL-2 production [141]. This finding is important as it is the first study to show that autophagy can control signalling in T cells and implicates autophagy in regulating T cell function.

Autophagy may also be more important in certain T cell subsets. Autophagy has been shown to be upregulated in CD4 Th2 cells compared to CD4 Th1 cells [142]. However, cells cultured under Th1-polarizing conditions rely more heavily on autophagy for survival compared to the Th17 subset [137]. These findings indicate that the role of autophagy is dependent on the cell type and stimuli and that blocking autophagy can skew the balance of immune subsets [137]. The differential requirement of autophagy by T cell subsets may also

translate to CD8 T cells. The mTOR inhibitor rapamycin, an autophagy inducer, has been shown to promote the formation of CD8 memory T cells [143]. However, whether autophagy induction supports CD8 memory development requires further study. Currently, the role of mTOR inhibition in promoting memory CD8 formation has been attributed to the increased expression of the transcription factor Eomesodermin [144] and reprogramming metabolism to include oxidative phosphorylation [145].

1.4.3 Hypoxia-induced autophagy

(The following section has been adapted from the review article: Schlie K, Spowart JE, Hughson LR, Townsend KN, and Lum JJ. When Cells Suffocate: Autophagy in Cancer and Immune Cells under Low Oxygen [105].)

Hypoxia can induce autophagy through several mechanisms (Figure 2), however, this induction has not been assessed in T cells. As mentioned earlier, HIF-1 causes a cellular switch from oxidative phosphorylation to oxygen-independent glycolysis and this, together with a shortage of nutrients in the environment, leads to a decrease of ATP in the cells and an increase in AMP [146]. This change in energy levels is sensed by the key metabolic enzyme AMP-activated protein kinase (AMPK). AMPK activates autophagy by two mechanisms: through the inhibition of mTOR and by directly phosphorylating ULK1 to cause activation [147]. A second mechanism of autophagy induction is directly mediated by HIF-1. HIF-1 induces Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) expression. BNIP3 and BNIP3L induce a process termed mitophagy, whereby mitochondria are selectively degraded to reduce the formation of ROS under hypoxia which is toxic to cells [148]. These proteins inhibit the interaction between Beclin1 and B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-xL) allowing Beclin1 to

induce autophagy [88]. Lastly, the unfolded protein response is upregulated during hypoxia and induces autophagy [149]. Oxygen is required for proper protein folding by promoting disulphide bond formation [150]. Resultant unfolded proteins under hypoxia activate PKR-like ER kinase (PERK) which induces the activity of the transcription factor activating transcription factor 4 (ATF4). This allows for transcription of genes encoding key autophagy proteins Atg5 and LC3 for employment in the autophagic process [149].

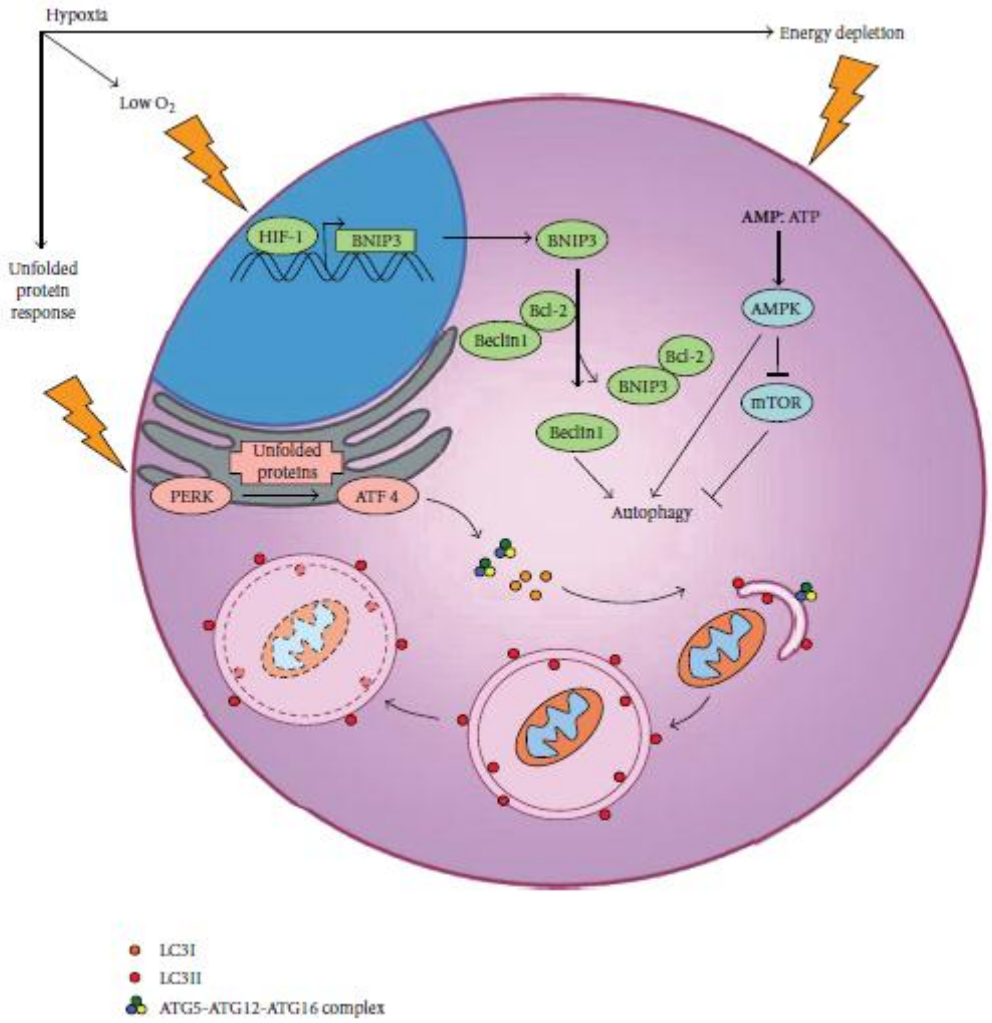


Figure 2: Mechanisms of hypoxia-induced autophagy.
Several events which occur under hypoxia induce autophagy including: energy depletion, HIF-1α stabilization and the unfolded protein response. These processes are outlined in section 1.4.3 (Figure 2 is from Schlie and Townsend *et al.* [105])

1.4.4 Immune-mediated killing of autophagic tumours

The importance for autophagy within tumour cells for immune-mediated killing has been studied in several different contexts in mouse models. One study found that inhibiting hypoxia-induced autophagy *in vitro* in lung carcinoma cells promoted T cell mediated killing and this was further demonstrated *in vivo* using B16 melanoma cells [151]. Inhibition of autophagy in B16 melanoma cells using the drug HCQ combined with vaccination to boost immune activity reduced tumour burden significantly [151]. Similarly, a second study found that autophagy inhibition in mammary carcinoma cells suppressed tumour growth *in vivo* due to the recruitment of CD8 T cells. These autophagy-deficient tumours had increased infiltrates due to the secretion of factors which recruit T cells called chemokines [152]. In contrast to these studies, Michaud *et al.* found that autophagy was required for immune-mediated killing on treatment with the chemotherapeutic agents oxaliplatin and mitoxantrone [153]. Mitoxantrone, an anthracycline chemotherapeutic can elicit immunogenic cell death [154]. Anthracycline-treated tumours release three signals important for this process, consisting of: calreticulin, high mobility group box 1 (HMGB1) and ATP which all bind to receptors on DCs allowing for presentation of antigen to the immune system to activate tumour killing [153]. Michaud *et al.* showed that autophagy was required to produce ATP for release from colorectal cancer cells treated with chemotherapy and this activated anti-tumour activity [153]. Overall, these studies highlight the importance of autophagy in tumours for T cell mediated killing. The disparate findings between the studies may be due to the different cancer cell types used as well as the methods of immune activation used, including anthracyline-based chemotherapy in the latter study. While these works assessed the role of autophagy in tumours for immune-mediated killing, there have

been no studies which assess autophagy's role in CD8 T cells during tumour killing and investigating this role is an objective of this thesis.

1.5 Avenues to study the immune response to cancer

I have employed two models in Chapters 2 and 3 to assess the role of hypoxia in CD8 T cell function. Hypoxia-induced autophagy will also be assessed in Chapter 2 using a transgenic mouse. This mouse has several introduced transgenes in order to inhibit autophagy, to track adoptively transferred T cells and to assess T cell function once they are activated with a known antigen. The second model used to study hypoxia's impact on T cell function included a cohort of high-grade serous ovarian carcinoma patients from whom tumour specimens and outcomes data had been gathered.

1.5.1 A Transgenic Mouse model

In order to study autophagy in CD8 T cells we have developed a transgenic mouse allowing inducible *Atg5* knockout. These mice contain a *LoxP*-flanked *Atg5* gene at exon 3 in the nucleus [131]. They also ubiquitously express a high affinity, mutated human estrogen receptor (ER) protein fused to Cre under control of the *Rosa26* promoter [155]. Heat shock proteins normally inactivate the Cre fusion protein, however, treatment with the synthetic ER agonist tamoxifen allows liberation of Cre from the complex [156], allowing Cre to recombine the *LoxP*-flanked region of the *Atg5* gene.

This transgenic mouse also expresses the glycoprotein thymocyte differentiation antigen 1.1 (Thy1.1) on thymocytes, T cells and neuronal cells and is used for tracking adoptively transferred donor T cells in the host mouse [157,158]. In addition, the CD8 T cells within this mouse express a transgenic T cell receptor specific for the amino acids 257–264 (SIINFEKL) from the ovalbumin (OVA) protein [159]. OVA is a protein expressed

naturally in avian egg whites [160] and it provides us with a model system to assess the impact of the tumour environment on T cell function using a known T cell antigen. To study the effect of the hypoxic environment on T cell function in Chapter 2 we will use E.G7 cells, a mouse thymoma tumour cell line which forms solid tumours and expresses OVA [161]. Thus, when co-cultured with T cells expressing the SIINFEKL-specific T cell receptor, E.G7 cells are killed by the T cells.

1.5.2 Ovarian carcinoma

Epithelial ovarian carcinoma is the most lethal of the gynecological malignancies and is classified into five main types including high-grade serous, endometrioid, clear cell, mucinous and low-grade serous [162]. The 5 year survival rate for women with ovarian cancer is approximately 45 % [163]. The subsets are named based on their cell type of origin and present in the vicinity of the ovary [162]. Each ovarian subset is genetically distinct and responds differently to therapy. The survival rate of ovarian carcinoma patients has not changed over the past 30 years since the introduction of platinum-based chemotherapy treatment [164]. Therefore, alternative methods varying from the standard treatment used today of surgery followed by chemotherapy are required.

A number of studies have assessed the role of the immune system in various ovarian carcinoma subtypes, however, few differentiate between the subtypes. Patients with tumours infiltrated by CD3 or CD8 expressing T cells have drastically improved survival in various ovarian cancer subsets [17,54,165-167]. Although, some studies have found that cytotoxic T cells do not improve survival [168,169]. This is potentially due to the combination of ovarian subtypes used for analyses. Indeed, studies have found that while endometrioid and clear cell ovarian carcinoma subtypes are infiltrated by cytotoxic CD8 T cells, their presence does not

improve patient survival [54,165]. In contrast, high-grade serous carcinoma patients had improved survival outcomes when their tumours were infiltrated by cytotoxic CD8 T cells [54,165].

T regs have also been identified in various ovarian subsets and have been shown to be a predictor of poor [48,170] and beneficial survival outcomes [54]. Overall, these findings indicate that ovarian carcinomas are immunogenic, providing a beneficial setting to study the immune response. Additionally, ovarian carcinomas have been identified as hypoxic *via* expression of HIF-1α [171,172]. Thus, ovarian carcinoma offers a relevant context in which to correlate the impact of hypoxia with T cell function.

This thesis focuses on the most prevalent form of ovarian carcinoma, the high-grade serous subtype which accounts for 68-71 % of ovarian carcinoma cases [173]. Given that approximately 80 % of high-grade serous patients present at advanced stage when tumour eradication by surgery and chemotherapy is difficult [162], understanding immune parameters in this subtype may be particularly beneficial for future immunotherapy treatments. The role of hypoxia and the immune system in this subtype will be analysed in Chapter 3.

1.6 Chapter 1 summary and hypotheses

This chapter has outlined the importance of T cells for tumour eradication and the harsh environmental features these cells face while eliminating tumours. Hypoxia results in a number of signalling events within T cells causing both metabolic and functional changes, however, these events have not been studied in great detail in CD8 T cells. I have also introduced the role of autophagy in T cells, however, its role under hypoxia has never been assessed in T cells. Therefore, we sought to assess the role of hypoxia and hypoxia-induced

autophagy in CD8 T cell function. *Our hypothesis was that CD8 T cell function would be negatively impacted by the tumour environmental condition hypoxia, and that autophagy would play a role in maintaining CD8 T cell function during this condition.* We next assessed the clinical relevance of our data from Chapter 2 using a human ovarian cancer patient cohort in Chapter 3. High-grade serous carcinoma has been recognized as an immune-infiltrated cancer type and these tumours have been shown to be hypoxic; however, correlations of hypoxia and markers of cytotoxic T cell activity with patient survival have not been explored. We sought to compare the survival outcomes of women with immune infiltrates in hypoxic tumours to those of women with infiltrates in non-hypoxic tumours. *We hypothesized that given the negative role of hypoxia on T cell function, women with T cell infiltrates in non-vascularized, hypoxic tumours would have poorer survival than women with T cells in vascularized, oxygenated tumours.*

Chapter 2: Hypoxia induces autophagy in CD8 T cells and negatively impacts their effector function

Katelin N. Townsend[1,2], Vincent I. Poon[1,2], Katrin Schlie[1], Jill Brandon[1], Ashley Westerback[1], Mary Elrick[1], Noboru Mizushima[3], Julian J. Lum[1,2] (Manuscript in preparation)

[1]Deeley Research Centre, BC Cancer Agency, Victoria, BC, Canada

[2]Department of Biochemistry and Microbiology, University of Victoria, BC, Canada

[3]Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan

KNT and JJL designed the study. KNT, VIP, KS, JB, AW, ME, NM and JJL were involved in acquisition of the data. KNT, VIP, KS, and JJL were involved in the analysis/interpretation of data. KNT wrote the manuscript.

2.1 Abstract

Tumour cells depend on a large supply of essential nutrients to support rapid cell growth and proliferation. This excessive utilization of nutrients can lead to the depletion of metabolites such as oxygen or glucose within the tumour environment. As lymphocytes migrate to tumour sites to carry out tumour cell killing, these unfavorable nutrient conditions may result in attenuated immune responses. We investigated how cytotoxic CD8 T cells are impacted by the metabolic condition hypoxia and whether they upregulate the cellular stress response, autophagy, to overcome the challenges of the tumour environment. We found that under hypoxia, the killing activity of T cells and the production of the functional cytokines, IFNγ and TNFα, were decreased. CD8 T cells were also found to upregulate autophagy in response to low oxygen. To elucidate the impact of autophagy on the functionality of T cells we developed a transgenic mouse which enabled us to induce the deletion of the essential autophagy-related gene, *Atg5*, upon treatment with the drug tamoxifen. After 72 hours of treatment to induce knockout we detected a drastic reduction of Atg5 on the genomic deoxyribonucleic acid (DNA), messenger ribonucleic acid (mRNA) and protein levels. The inhibition of autophagy during low oxygen did not largely affect *in vitro* T cell cytotoxicity and cytokine production or tumour regression *in vivo*. However, it did lead to a significant reduction in expansion *in vivo*. Our results suggest that in the tumour environment, hypoxia may have dramatic effects on infiltrating T cell anti-tumour activity while autophagy plays a role in CD8 T cell expansion rather than cytotoxic activity during hypoxia. These findings are important for the understanding of T cell function in the tumour environment.

2.2 Introduction

The immune system plays a vital role in the host response against many tumours [1,15]. CD8 T cells directly eliminate tumour cells and are correlated with improved patient outcomes in a variety of cancer types [16-18]. However, once CD8 T cells have trafficked to the tumour site, they must adapt to varying oxygen concentrations as tumour cells proliferate and expand beyond nutrient-providing vasculature [72-74]. In response to low oxygen, CD8 T cells stabilize the alpha subunit of the transcription factor HIF-1. This allows T cells to reprogram their metabolism to maintain cellular function under low oxygen, by regulating genes that are involved in promoting glycolysis and autophagy [87,88,102].

Autophagy is a cell survival process which occurs under stress-inducing conditions such as nutrient depletion. During autophagy, intracellular constituents are engulfed by autophagosomes which fuse with lysosomes to induce degradation of the constituents. In the context of T cells, studies have highlighted the importance of autophagy for processes such as organelle turnover [133,134,138], proliferation upon activation [132,133,136] and energy production [139]. Autophagy-deficient CD4 T cells have been described to produce less cytokines and ATP than autophagy-proficient cells [139]. These processes were rescued upon addition of the metabolite methyl pyruvate, an intermediate of glucose metabolism, indicating that autophagy liberates metabolites required for T cell cellular function [139].

Given that hypoxia has been shown to induce autophagy in other cell types [88], we wanted to investigate its induction and role in CD8 T cells under hypoxia. Under low oxygen, T cells undergo glycolysis to produce ATP while limiting oxidative phosphorylation [102,174]. Therefore, we hypothesize that hypoxia induces autophagy in T cells in addition to causing a metabolic switch to anaerobic glycolysis and that autophagy is required to provide metabolites during hypoxia. Production of cytokines and anti-tumour killing is an

energetically costly process [91], thus autophagy inhibition under hypoxia may negatively impact CD8 T cell effector function.

We sought to determine how hypoxia impacted CD8 T cell function and whether autophagy was induced under 1.5 % oxygen by using a mouse model. We developed this transgenic mouse to allow for inducible deletion of the essential autophagy gene *Atg5*. To determine the importance of autophagy during hypoxia for effector function we induced autophagy knockout in transgenic T cells and measured cytokine production and anti-tumour cell activity. We found that hypoxia dampened effector activity by decreasing cytokine production and anti-tumour killing and that autophagy was indeed induced at 1.5 % oxygen. *In vitro*, autophagy-deficiency did not decrease the defect in cytokine secretion and killing activity observed under low oxygen. Lastly, we carried out an *in vivo* adoptive transfer experiment to determine the relevance of autophagy in effector function during pathophysiological oxygen concentrations in the tumour environment. Our *in vivo* results indicated that autophagy is important for T cell expansion in the tumour environment. Our findings enhance the understanding of the role of hypoxia in CD8 T cell function and highlight hypoxia-induced autophagy as a process that occurs in CD8 T cells.

2.3 Materials and Methods

2.3.1 Cell culture and hypoxic conditions

A thymoma tumour cell line known as EL4 and its derivative cell line E.G7, which was transfected with an ovalbumin (OVA) and geneticin (G418) resistance expressing construct [161], were purchased from ATCC (Manassas, VA, USA). Primary mouse T cells, EL4 and E.G7 cells were cultured in cRP10 media consisting of 1640 RPMI media (Fisher

Scientific, Nepean, ON, CA) containing the following supplements: 1 mM sodium pyruvate, 1 mM Hepes, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM L-glutamine, 10 % fetal bovine serum (FBS) (Fisher Scientific), and 50 μM 2-mercaptoethanol (Sigma-Aldrich, Oakville, ON, CA). For E.G7 cell culture, cRP10 media was supplemented with 400 μg/ml G418 (Fisher Scientific) and glucose (Sigma-Aldrich) to reach a final concentration of 4.5 mg/ml glucose in the media. All cells were incubated at 37 ºC, 21 % O_2 and 5 % CO_2 in a Water Jacketed Forma incubator (Fisher Scientific). For all hypoxia experiments, cells were placed in a humidified hypoxia chamber (Coy Laboratories, Grass Lake, MI, USA) at 37 ºC with 1.5 % oxygen, 5 % CO_2, and 93.5 % nitrogen and cultured for varying time periods as indicated.

2.3.2 Mice

Wild type C57BL/J6, Thy1.1 (T), and OT-I (O) mice which contain a transgenic T cell receptor against SIINFEKL, were purchased from Jackson Laboratories (Bar Harbor, ME, USA). CreERT2 (C) mice were purchased from Taconic (Hudson, NY, USA). *Atg5*$^{fl/fl}$ mice (A) on a mixed B6.129 background were provided by N. Mizushima [131]. *Atg5*$^{fl/fl}$ mice were crossed with C57BL/J6 mice to generate an *Atg5*$^{fl/fl}$ mouse on a C57BL/J6 background. A breeding strategy was designed where *Atg5*$^{fl/fl}$ mice were crossed with CreERT2 mice, Thy1.1 and OT-I mice to generate the CO and ACO mice used for the *in vitro* experiments and the CTO and ACTO transgenic mice were used for *in vivo* experiments. All mice used for experiments were heterozygous for the OT-I transgene, depicted by a single "O". The Thy1.1 and Cre status varied amongst experiments thus, mice containing either a heterozygous or homozygous Thy1.1 or Cre transgene are indicated by a single "T" and "C" respectively. The heterozygous and homozygous state of the floxed *Atg5* transgene are

indicated in the respective experiments. Mice which are heterozygous for the floxed *Atg5* transgene are designated Aa and mice which are homozygous are designated AA. Animal studies were approved by the University of Victoria Animal Care Committee.

2.3.3 T cell activation and autophagy knockout in vitro

Mouse spleens were harvested from the specified transgenic mice after euthanasia. The splenocytes were passed through a 40 μm strainer in phosphate-buffered saline (PBS) to generate a single cell suspension. Red blood cells were lysed using ACK lysis buffer (0.15 M ammonium chloride, 0.1 mM ethylenediamine tetracetic acid (EDTA) salt, 0.1 M hydrogen carbonate, and ddH_2O, pH 7.2). The cells were then passed through a 100 μm strainer and washed once with PBS and resuspended in cRP10 medium for activation. OT-I expressing splenocytes were seeded at 1e6 cells per ml and activated with 2 μg/ml SIINFEKL peptide (Anaspec, Fremont, CA, USA) for 2 hours prior to removal of medium and replenishment with fresh cRP10. 100 U/ml IL-2 (Peprotech, Dollard des Ormeaux, QC, CA) was added on day 1 of culture and added to the media with every passage following. AACO splenocytes and control CO splenocytes were treated with 3 μM tamoxifen (Sigma-Aldrich) on day 3 post-activation for 72 hours to initiate Cre-induced recombination of the floxed *Atg5* gene. Every 24 hours the cells were given fresh medium containing 3 μM tamoxifen.

2.3.4 Polymerase chain reaction (PCR)

T cell pellets were flash frozen and stored at -80 ºC until DNA extraction. For DNA extraction, cells were lysed in lysis buffer (0.2 % SDS, 100 mM tris pH 8.5, 5 mM EDTA, 500 μg/ml proteinase K, 200 mM NaCl) at 55 ºC with shaking at 1500 rpm (Eppendorf thermomixer, Mississauga, ON, CA) for 1 hour. The cells were centrifuged at 17000 x g (Eppendorf) for 10 minutes, the supernatant removed and 1 ml ethanol was added to the

supernatant. The samples were incubated at -80 ºC for over 30 minutes, centrifuged as above and the supernatant removed. Samples were dried using a SpeedVac concentrator (Fisher Scientific) and resuspended in dH_2O. PCR reactions were prepared with a volume of 20 µl containing the following components at the indicated final concentrations from Invitrogen unless otherwise noted (Invitrogen, Burlington, ON, CA): 1x PCR buffer, 1x PCR enhancer, 1x sucrose/cresol red (2 % sucrose (Fisher Scientific), 0.1 mM cresol red (Sigma-Aldrich)), 1.75 mM $MgCl_2$, 0.2 mM dNTP mix, 0.05 U/µl *Taq* DNA polymerase, and 1- 4 µl of extracted DNA per sample. Samples were run on a thermocycler (Biorad, Hercules, CA, USA). The following primer sequences which were previously published by Hara *et al.* [131], were used to determine the floxed status of the *Atg5* gene in splenocytes treated with or without tamoxifen at a final concentration of 0.25 µM (5' to 3'): short2 - GTACTGCATAATGGTTTAACTCTTGC, check2 - ACAACGTCGAG CACAGCTGCGCAAGG, 5L2 – CAGGGAATGGTGTCTCCCAC.

2.3.5 Real-time quantitative PCR (qPCR)

T cell pellets were flash frozen and stored at -80 ºC until ribonucleic acid (RNA) extraction. RNA was extracted using an RNeasy mini kit (Qiagen, Mississauga, ON, CA). 1 µg RNA was then reverse transcribed with a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). qPCR reactions were carried out using a Perfecta SYBR Green supermix (Quanta Biosciences). An iCycler thermal cycler with a MyIQ real time detection system was used (Biorad). Each sample was run in triplicate. *Atg5* expression was normalized to *β-actin*. The following primer sequences were used (5' to 3'): *Atg5* forward – TGCCAAGAGTCAGCTATTTGACGTT, *Atg5* reverse –

TGAAAGGCCGCTCCGTCGTG, *β-actin* forward - CTAAGGCCAACCGTG AAA AG, *β-actin* reverse - ACCAGAGGCATACAGGGACA.

2.3.6 Measuring autophagic flux and Western blotting

To monitor autophagic flux, the indicated T cells were cultured under 1.5 % oxygen and treated with or without 50 μM CQ (Sigma-Aldrich). As a positive control for HIF-1α protein detection, cells were treated with cobalt chloride ($CoCl_2$) at 100 μM (Sigma-Aldrich). To make protein lysates cells were centrifuged at 650 x g with a Sorvall Legend RT centrifuge (Mandel Scientific, Guelph, ON, CA) and resuspended in PBS to wash the cells for 1 minute at 1000 x g (Eppendorf). The pellet was resuspended in lysis buffer to generate protein lysates (2 % SDS, 0.1 M DTT, 0.06 M Tris pH 6.8, 10 % glycerol, 1 EDTA-free protease inhibitor cocktail tablet (Roche, Mississauga, ON, CA)) and boiled at 99 ºC for 10 minutes with shaking at 1500 rpm (Eppendorf thermomixer). Protein lysates were run on 4-12 % Bis-Tris or 3-8 % Tris-Acetate polyacrylamide gels (Invitrogen), transferred onto nitrocellulose membranes (Life Sciences, Pensacola, FL, USA) and blocked for 60 minutes at room temperature in Tris-buffered saline (TBS) with 5 % milk powder (Saputo Inc., Montreal, QC, CA). Blots were probed with the following primary antibodies at the specified concentrations overnight: anti-Atg5 at 1:1000 (Novus Biologicals, Oakville, ON, CA; rabbit polyclonal), anti-HIF-1α at 1:1000 (Cayman, Ann Arbor, MI, USA; rabbit polyclonal), anti-LC3 at 1:2000 (MBL, Des Plaines, IL, USA; rabbit polyclonal), anti-p62 at 1:2000 (Sigma-Aldrich; rabbit polyclonal) and anti-β-actin at 1:10,000 (Sigma-Aldrich; clone AC-15, mouse monoclonal) in TBST with 5 % milk powder. Secondary antibodies including goat anti-rabbit IgG (H&L) IRDye®800 conjugated (Rockland, Gilbertsville, PA, USA) and Alexa Fluor 680 goat anti-mouse IgG (Invitrogen) were used at a 1:10,000 dilution in TBST

with 5 % milk powder for 1 hour at room temperature. Immunoblots were developed using a LI-COR (Lincoln, NE, USA) and quantified using the Odyssey program version 3.0. All quantified protein band intensities were normalized to β-actin.

2.3.7 Cytotoxicity assay

The indicated splenocytes were activated and cultured for 3 days, treated with or without 3 μM tamoxifen for the following 3 days and primed under 1.5 % oxygen for 16 hours or left under 21 % oxygen. On day 7 post-activation, splenocytes were counted using Accucount fluorescent particles according to manufacturer's instructions (Spherotech, Lake Forest, IL, USA) and viability was assessed using propidium iodide stain (Invitrogen) with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Samples with greater than 15 % cell death were magnetically sorted with the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA, USA), washed twice and co-cultured with target cells. EL4 and E.G7 cells were washed with PBS twice, labeled with 0.75 μM carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich) for 15 minutes at 37 ºC, centrifuged, and the CFSE dye was quenched with cRP10 medium at 37 ºC for 30 minutes. The cells were then washed 3 times with PBS. CD8 T cells were co-cultured with labeled EL4 or E.G7 cells at the indicated ratios for 4 hours under normoxia or hypoxia. To ensure hypoxia was maintained in the co-culture during the 4 hour assay preconditioned hypoxic medium was used when combining the hypoxic preconditioned T cells with the tumour targets or control cells. 7-amino-actinomycin D (7-AAD, Sigma-Aldrich) was added to the cells at 10 μg/ml 5 minutes prior to cell flow cytometric analysis by Guava EasyCyte (Millipore, Billerica, MA, USA). Acquisition and analysis was completed using the cytosoft 5.3 cell toxicity program (Guava Technologies Inc. Hayward, CA, USA). Percent killing was corrected using the formula

((sample death - spontaneous death) / (maximum death - spontaneous death)) * 100 %. Maximal death was determined by heat killing target cells for a minimum of 1 hour at 55 ºC. Spontaneous death was determined by the amount of death in target cultures alone.

2.3.8 Intracellular cytokine staining

The indicated splenocytes were activated and cultured for 3 days, treated with or without tamoxifen for the following 3 days and primed under 1.5 % oxygen or normal conditions for 15 hours. On day 7 the cells were restimulated using 10 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) and 0.5 μg/ml ionomycin (Sigma-Aldrich) in the presence of 4 μl per 6 ml golgistop (BD Biosciences) and 5 μg/ml brefeldin A (Sigma-Aldrich) for 5 hours under 1.5 % oxygen or under normal conditions. Intracellular cytokines were detected using a FoxP3 intracellular staining kit (ebioscience, San Diego, CA, USA) following the manufacturer's instructions. Briefly, cells were stained with anti-CD8 allophycocyanin-H7 (APC H7, BD Bioscience; clone 53-6.7, rat monoclonal) at 1:200 for 30 minutes at 4 ºC and washed with staining buffer (PBS, 3 % FBS, 0.09 % sodium azide) in 96 well round bottom plates (Corning Costar, Tewksbury, MA, USA). The cells were fixed with fixation/permeabilization working solution for 30 minutes to 18 hours. The cells were washed twice with permeabilization buffer and incubated for 15 minutes with 0.5 μg Fcblock (BD Bioscience) to prevent non-specific antibody binding. Antibodies specific for intracellular cytokines were incubated with the cells in the presence of 0.5 μg Fc block for 30 minutes at 4 ºC. The following antibodies were used at the indicated concentrations: anti-IFNγ phyco-erythrin (Pe, BD Biosciences; clone XMG1.2, rat monoclonal) at 1:100, anti-granzyme B Allophycocyanin (APC, Invitrogen; clone GB11, mouse monoclonal) at 1:1000 and anti-TNFα fluorescein isothiocyanate (FITC, ebioscience; clone MP6-XT22, rat

monoclonal) at 1:100. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences). Data analysis was completed using FlowJo 7.6.5 software (Tree Star, Ashland, OR, USA). Mean fluorescence intensities were assessed by the geometric means.

2.3.9 Tumour establishment in vivo

E.G7 tumour cells were thawed 7 days prior to the day of injection and cultured immediately in G418. Prior to the injection, the cells were washed 3 times with PBS and left on ice during transport to the animal facility. Mice were injected subcutaneously with 3e6 E.G7 cells in 300 µl PBS into the right flank. Tumour size was measured with digital calipers (Fisher Scientific). Tumour area is reported as length by width in mm^2. Mice bearing established tumours which had reached 10 mm in at least one dimension by 15 days post-injection were treated with adoptive transfer. Mice with tumours which were naturally regressing or had failed to reach 10 mm were removed from the study as endogenous responses often cleared tumours which had not reached 10 mm by day 15.

2.3.10 T cell adoptive transfer and autophagy knockout in vivo:

Splenocytes from CTO, AaCTO and AACTO mice were activated with SIINFEKL peptide as outlined above. 4 days post-activation CTO, AaCTO and AACTO cells were sorted with the Dead Cell Removal Kit (Miltenyi Biotec) and washed 3 times in PBS. The cells were injected at the indicated doses (1e5, 5e5 or 1e6) in 200 µl PBS intravenously. To induce recombination in the AACTO or AaCTO T cells, mice were injected intraperitoneally with 1.5 mg tamoxifen (Sigma-Aldrich) in 100 µl peanut oil (Sigma-Aldrich) for 4 consecutive days starting the day after adoptive transfer.

2.3.11 Monitoring T cell expansion in vivo

T cells were monitored on day 1, 4, 7 and 11 post-adoptive transfer by peripheral blood draw. Blood samples were collected in heparin-coated capillary tubes (Scientific Glass Inc., Rockwood TN, USA), combined with additional heparin (Sandoz Canada Inc., Boucherville, QC, CA) and stained for adoptively transferred T cells with the following antibodies at the indicated concentrations: anti-Thy1.1 Pe-Cy-7 (ebioscience; clone H1S51, mouse monoclonal) at 1:800, anti-CD8 FITC (BD Bioscience; clone 53-6.7, rat monoclonal) at 1:100, and anti-CD44 APC (ebioscience; clone IM7, rat monoclonal) at 1:500. Antibodies were incubated with 80 µl mouse blood and heparin at 4 ºC for 30 minutes and lysed with 1 ml ACK lysis buffer (as above) for 10 minutes at room temperature. Cells were centrifuged and the supernatant aspirated prior to the addition of 200 µl stain buffer (as above). Flow cytometry was carried out on a FACSCalibur flow cytometer (BD Biosicence) and analysis completed using FlowJo software (Tree Star).

2.3.12 Pimonidazole administration and immunohistochemistry (IHC)

Mice were injected intraperitoneally with 100 mg/kg pimonidazole (HPI Inc. Burlington, MA, USA) from a 20 mg/ml stock solution in 0.9 % saline. Mice were euthanized 1 hour post-injection, the tumour harvested, fixed in 10 % neutral-buffered formalin (Sigma-Aldrich) and embedded in paraffin (Fisher Scientific). Tumour tissues were sectioned at 5µm onto Superfrost plus slides (Fisher Scientific) and incubated overnight at 37 °C. The slides were deparaffinized in xylene and graded alcohols and a Ventana Discovery XT autostainer (Ventana, Tucson, AZ, USA) was used for IHC staining. Ventana's standard CC1 protocol was used for antigen retrieval. All blocking steps were included as part of the DABMap kit used for detection (Ventana). The primary antibody

anti-pimonidazole (HPI Inc.; clone 2627, rabbit polyclonal) was manually added to the slides at a dilution of 1:200 in Ventana's Antibody Diluent and incubated for 60 minutes. A cross-adsorbed biotinylated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was manually applied at a dilution of 1:500 for 32 minutes. Bound antibodies were detected using the DABMap kit (Ventana), counterstained with hematoxylin (Ventana), and coverslipped manually with Cytoseal-60 (Richard Allan, Kalamazoo, MI, USA).

2.3.13 Statistics

Statistical analysis was carried out using GraphPad Prism software version 5.04 (GraphPad Software, La Jolla, USA). Statistically significant *p* values were considered to be less than 0.05. Kaplan-Meier curve analysis was carried out using Log rank tests. Unpaired T-tests were used to compare significant differences between the mean values observed for treatment groups.

2.4 Results

2.4.1 Hypoxia negatively impacts CD8 T cell effector function

To assess the impact of hypoxia on the cytokine production of cytotoxic T cells we isolated CD8 T cells from mice which contained a transgenic T cell receptor that is specific for the model OVA peptide SIINFEKL. T cells were activated and cultured for 6 days, following this, the cells were left under normal conditions or primed under 1.5 % oxygen for 15 hours to ensure hypoxic tensions were reached in the cell medium. On day 7, the cells were maintained under normal or low oxygen conditions and were restimulated with PMA and ionomycin to induce the production of effector cytokines which were measured by flow

cytometry. We found that CD8 T cells which were cultured under hypoxia during the period of priming and restimulation produced significantly less IFNγ ($p = 0.0313$) and trended towards decreased TNFα production ($p = 0.1721$) but increased granzyme B production ($p = 0.1793$) compared to cells restimulated under normoxia (Figure 3A).

Next, we used an additional method to assess T cell function under low oxygen by measuring the ability of SIINFEKL-recognizing T cells to kill antigen-expressing targets. Unlike the PMA and ionomycin restimulation method used to assess cytokine production, this method of restimulation activates the T cell receptor which binds to SIINFEKL in the context of the MHC class I molecule on target cells. For this assay, CD8 T cells were cultured as above, with a subset of cells cultured under normoxia or primed under hypoxia overnight for 16 hours to ensure hypoxic conditions were reached. The CD8 T cells were then co-cultured with CFSE labeled E.G7 target cells, or labeled EL4 control cells for 4 hours continuing culture under normoxia or hypoxia. After 4 hours of co-culture, the percentage of dead labeled target or control tumour cells was assessed by flow cytometry. T cells cultured under 1.5 % oxygen with E.G7 tumour targets showed reduced killing ability. This trend was consistently observed over 5 independent experiments and the separation in killing under hypoxia was most observable at effector to target ratios above 1:1 (Figure 3B). The differences between killing under normoxia and hypoxia trended towards significance at ratios of 1:1 ($p = 0.0803$), 2:1 ($p = 0.0617$), 5:1 ($p = 0.0505$) and 10:1 ($p = 0.0695$). When T cells were cultured with control EL4 cells, little unspecific lysis was observed.

Overall, these results show that hypoxia dampens the function of CD8 T cells by decreasing the production of IFNγ and TNFα and reducing T cell killing of antigen specific targets.

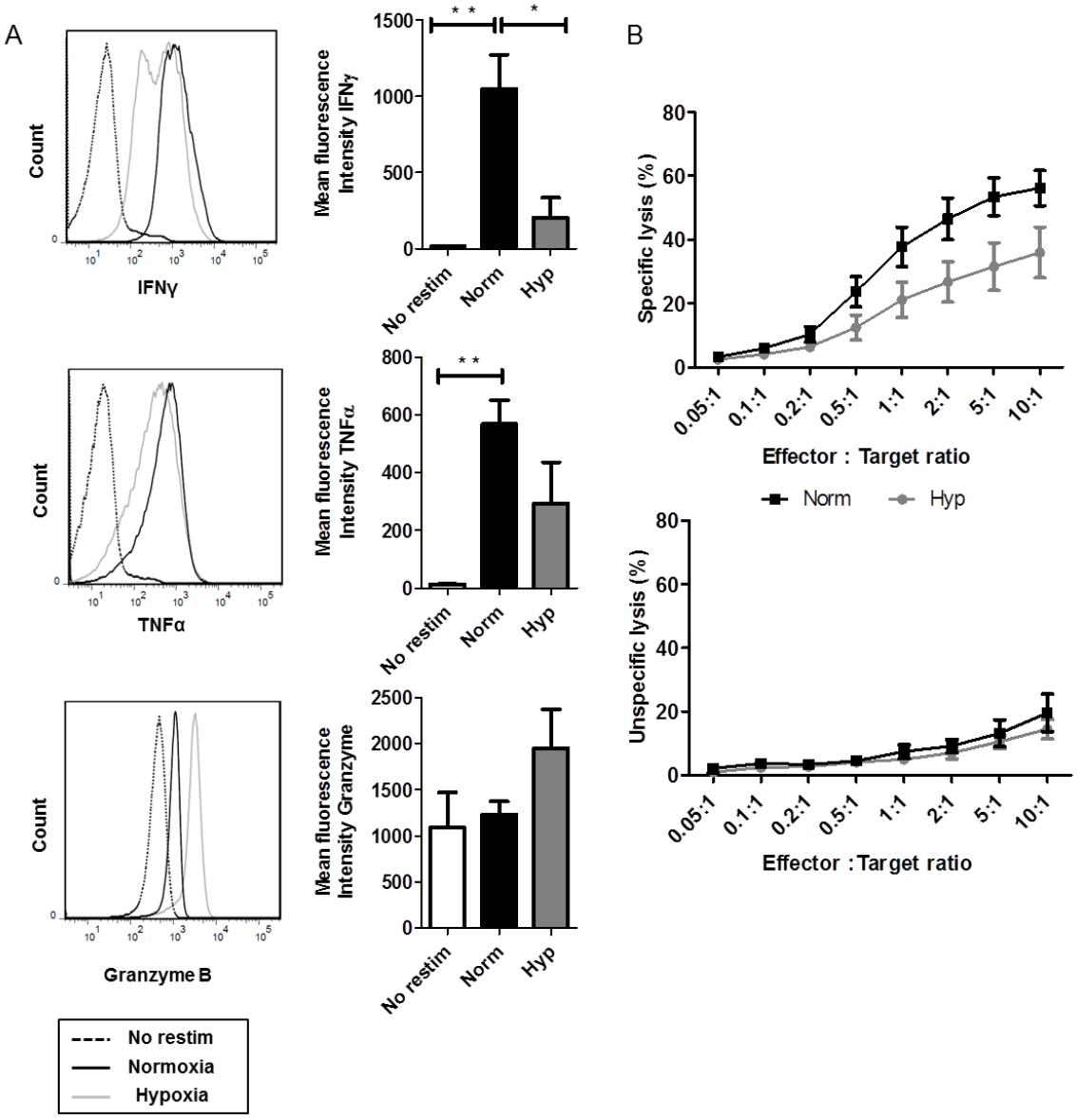


Figure 3: CD8 T cell effector function is decreased under 1.5 % oxygen.

(**A**) Left panel: Representative histogram plots for the mean fluorescence intensity of IFNγ, TNFα, and granzyme B produced by CD8 T cells cultured under normoxia or 1.5 % oxygen. Right panel: The average geometric mean fluorescence and SEM for 3 independent experiments is shown. (**B**) Top panel: OT-I T cells were co-cultured with E.G7 target cells at various effector: target ratios for 4 hours under normoxia (norm) or 1.5 % oxygen (hyp). Bottom panel: OT-I cells were cultured with control EL4 tumour cells to show non-specific killing. The mean and SEM of 5 independent cytotoxicity experiments is shown. ** $p < 0.01$, * $p < 0.05$.

2.4.2 Hypoxia Induces Autophagy in CD8 T cells

We next wanted to assess whether CD8 T cells upregulate autophagy under low oxygen. This information was required to establish if autophagy was important for CD8 T cell function under hypoxia. To determine whether hypoxia induces autophagy in CD8 T cells at 1.5 % oxygen we carried out an autophagic flux assay. On day 6 post-SIINFEKL activation, CD8 T cells were cultured under normoxia or hypoxia for 20 hours in the presence of CQ to inhibit degradation of autophagosomes. We then assessed the stabilization of HIF-1α to ensure hypoxia was achieved, and the buildup of the autophagosomal proteins LC3-II and p62. T cells cultured under 1.5 % oxygen showed an increase in HIF-1α protein (Figure 4A). In addition, T cells cultured under normoxic and hypoxic conditions showed a buildup of LC3II and p62 when treated with CQ, indicating autophagic flux was occurring (Figure 4A). Upon quantification, LC3-II and p62 levels were found to be increased in the hypoxia-treated cells over the normoxia-treated cells by 2 and 1.5 fold respectively (Figure 4B). This indicates that CD8 T cells upregulate autophagy under hypoxia.

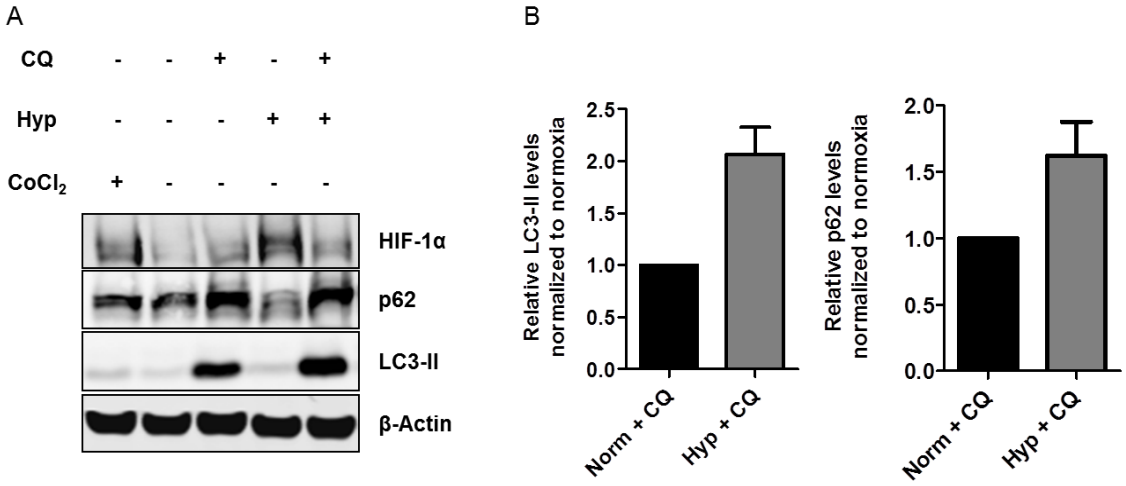


Figure 4: Hypoxia induces the upregulation of autophagy in CD8 T cells cultured under 1.5 % oxygen.

(Figure 4 continued) (A) A representative Western blot indicating protein expression of HIF-1α, p62, LC3-II and β-actin is shown for OT-I CD8 T cells cultured under 1.5 % oxygen. $CoCl_2$ treatment was used as a positive control for HIF-1 α protein expression. **(B)** Autophagic flux is shown by quantification of LC3-II and p62 accumulation with CQ treatment under normoxia (norm) or 1.5 % oxygen (hyp). Protein levels were normalized to β-Actin then the fold change over normoxia plus CQ was determined. The mean and SEM for the fold change of 3 independent experiments is reported.

2.4.3 The autophagy protein Atg5 is knocked out in transgenic CD8 T cells treated with tamoxifen

Having determined that autophagy is induced in T cells under low oxygen, we wanted to assess autophagy's role in T cell function under this condition. In order to do this, we developed a transgenic mouse model to inhibit autophagy in CD8 T cells, allowing us to monitor defects in cytokine production or killing activity under hypoxia. This mouse, termed ACO, contains floxed *Atg5*, expresses Cre fused to the estrogen receptor (ER) to induce *Atg5* deletion and has a transgenic T cell receptor which recognizes the SIINFEKL epitope from the OVA protein. It has been shown that the Atg5 protein is essential for autophagosome formation, thus deletion of *Atg5* inhibits autophagy [131].

Because this model had not been used before, we sought to characterize the knockout of Atg5 on the genomic, mRNA and protein levels. After activation with SIINFEKL, the CD8 T cells were cultured for 3 days to allow for T cell proliferation and expansion. The cells were then treated with 3 μM tamoxifen for 24, 48 and 72 hours and lysates were collected on day 7 post-activation corresponding to 96 hours post-initial treatment with tamoxifen (Figure 5A). On the genomic DNA level, *Atg5* was almost completely recombined 48 hours post-treatment with tamoxifen and was completely recombined 72 hours post-treatment. This was visualized by the disappearance of the floxed product at 600 base pairs (bp) and presence of a PCR product at 300 bp in the genomic DNA (Figure 5B). This

deletion required tamoxifen treatment and did not occur in the absence of tamoxifen, indicated by the untreated T cells which did not show any recombined DNA product. By RT-PCR, *Atg5* mRNA was drastically reduced at 48 hours and had completely disappeared by 72 hours post-tamoxifen treatment in AACO T cells. The mRNA level of 72 hour tamoxifen treated AACO T cells was 150 times less than untreated AACO T cells and 1000 times less than untreated control CO T cells (Figure 5C). This was also reflected on the protein level with almost undetectable levels of Atg5 protein by 48 and 72 hours. The protein levels of 72 hour tamoxifen-treated AACO T cells was 10 times less than untreated AACO T cells and 36 times less than untreated control CO T cells (Figure 5D). The Atg5 protein and mRNA levels of untreated AACO T cells was less than control CO T cells which did not contain the floxed *Atg5* gene. This is not because of leaky recombination in the absence of tamoxifen as confirmed by assessing recombination in untreated AACO T cells in Figure 5A. The decrease was caused by a neomycin-resistance cassette that had previously been inserted into the DNA, which resulted in a decrease in *Atg5* transcription levels [131].

Overall, our system allows deletion of Atg5 on the genomic, mRNA and protein levels in an inducible manner upon treatment with tamoxifen.

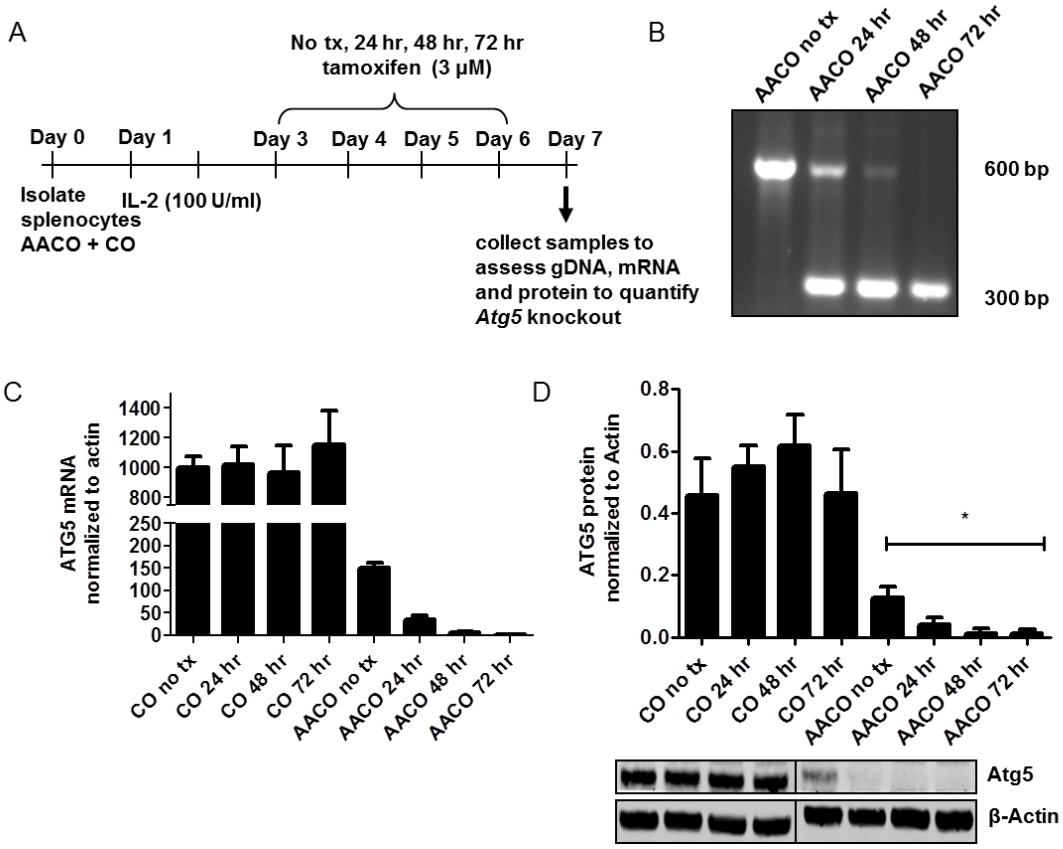


Figure 5: Validation of Atg5 deletion in CD8 T cells from AACO transgenic mice. **(A)** The methodology for CD8 T cell activation and tamoxifen (tam) treatment to recombine AACO T cells is indicated. No tx indicates no tamoxifen treatment. **(B)** A representative image of PCR results for 3 independent experiments showing recombination of AACO CD8 T cells after culture with tamoxifen for the indicated time periods. **(C)** The mean and SEM of 3 independent experiments for A*tg5* mRNA expression normalized to *Actin* as assessed by qPCR. The fold change over *Atg5* mRNA levels at 72 hours tamoxifen treatment was determined for all samples. **(D)** Top panel: Quantified Atg5 protein levels and the mean and SEM of 4 independent experiments. Bottom panel: Representative Western blot for Atg5 and β-Actin protein expression. * $p < 0.05$.

2.4.4 Autophagy inhibition causes a slight decrease in effector function in CD8 T cells in vitro

To determine whether autophagy is important for CD8 T cell function under low oxygen, we measured the production of effector cytokines and tumour target killing by flow cytometry. AACO T cells and control CO T cells and were activated with SIINFEKL and

cultured for 3 days prior to treatment with or without tamoxifen for an additional 3 days. The T cells were then primed under 1.5 % oxygen or normoxia and on day 7 the T cells were restimulated with PMA and ionomycin for 5 hours or were cultured with tumour and control target cells in a cytotoxicity assay for 4 hours (Figure 6A).

We first assessed the percentage of T cells producing one, two or three of the following cytotoxic molecules: IFNγ, TNFα and granzyme B (Figure 6B). T cells capable of producing multiple cytokines, also known as polyfunctional T cells, are an indicator of a more functional cell [175] and have been shown to be beneficial in the anti-tumour response [176,177]. Thus we wanted to assess the impact of autophagy-deficiency during hypoxia on the polyfunctional ability of CD8 T cells.

We observed there was an increase in T cells secreting only a single cytokine under hypoxia compared to normoxia, especially when autophagy was deficient. The proportion of CD8 T cells producing three cytokines under hypoxia was significantly reduced ($p = 0.0482$) and the proportion of CD8 T cells producing only a single cytokine was significantly increased ($p = 0.0482$) in autophagy-deficient T cells compared to autophagy-proficient CO T cells cultured under normoxia (Figure 6B). An increased percentage of AACO, CO and CO tamoxifen-treated T cells produced all three cytokines under normoxia compared to cells cultured under hypoxia. Cells which were autophagy-deficient (AACO plus tamoxifen) appeared to produce more of a single cytokine and less triple cytokines under normoxia compared to other T cell subsets restimulated under normoxia, however this was not statistically significant.

The killing of antigen-specific targets by all T cells was reduced under hypoxia as in Figure 3 and this trend was observed in 3 independent experiments for both CO and AACO T cells (Figure 6C and D). At the effector: target ratio 20:1, CO T cells cultured under

normoxia had maximal killing at 74 % while under hypoxia their killing was 54 % (Figure 6C). This was similarly observed for AACO T cells, which had maximal killing at 66 % under normoxia and this was reduced to 46 % under hypoxia (Figure 6D). When autophagy was inhibited in the tamoxifen-treated AACO T cells, the maximal amount of killing was 79 % under normoxia and this was reduced to 53 % under hypoxia (Figure 6D). Autophagy deficiency did not cause a statistically significant reduction in killing activity under normoxia or 1.5 % oxygen. Low levels of unspecific lysis were observed when T cells were cultured with non-antigen expressing control tumour cells, however, higher than expected lysis did occur in CO and CO plus tamoxifen cultures reaching up to 41 % in cells cultured under normoxia with control targets (Figure 6C). However, this lysis was reduced compared to CO and CO plus tamoxifen cells cultured with SIINFEKL-expressing targets by up to 33 % at the highest effector: target ratio. This increase in unspecific lysis may be due to carry over of the SIINFEKL peptide from activated T cell cultures into the co-culture. This peptide may have been taken up by the control cells, causing T cell recognition and lysis. The rest of the T cell cultures had little unspecific lysis averaging at low levels of 20 % (Figure 6C and D).

Overall, autophagy did not play a large role in reducing cytokine production or killing activity under 1.5 % oxygen, however, hypoxia consistently dampened these functions.

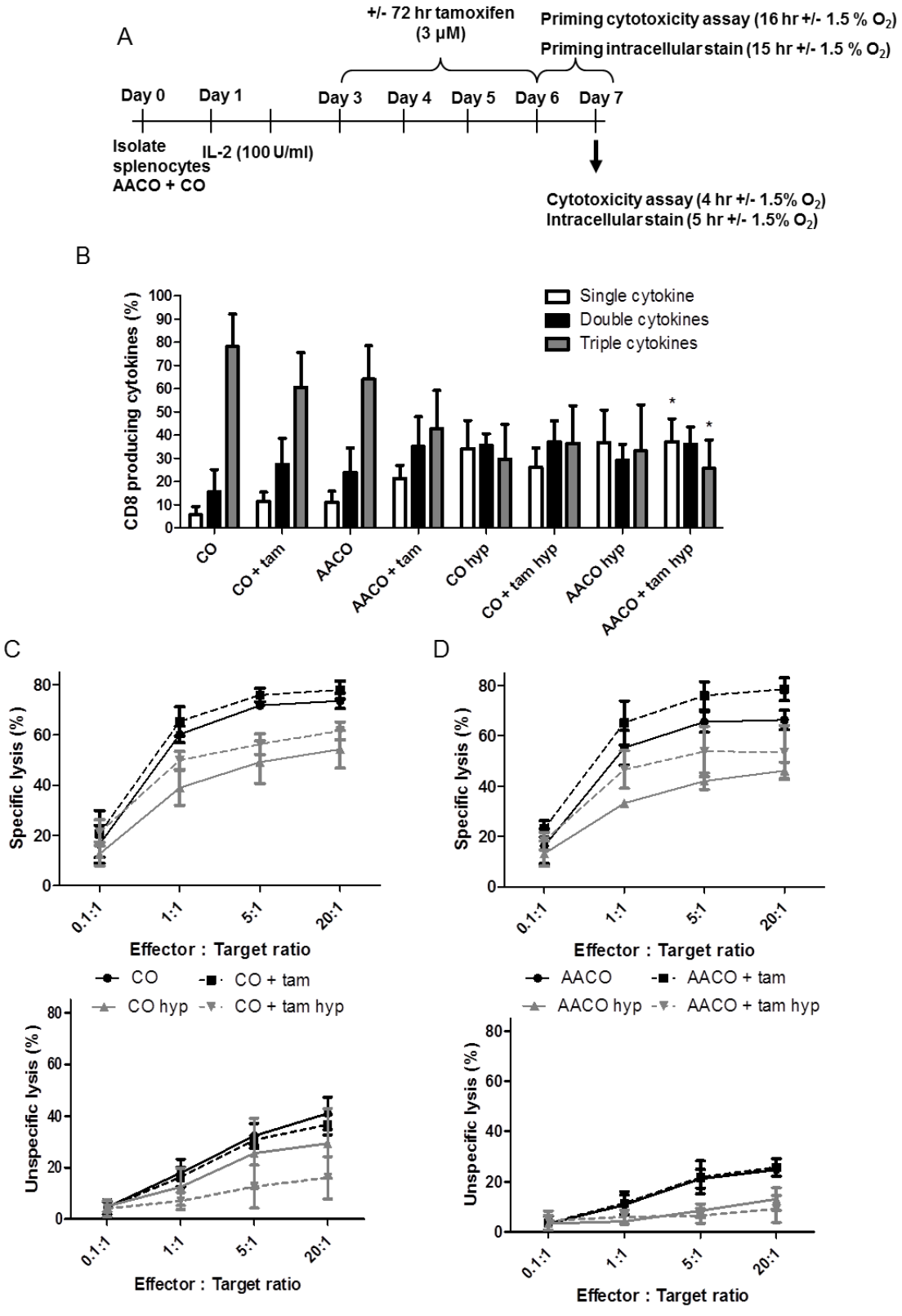


Figure 6: Autophagy deficiency causes a slight defect in effector function under 1.5 % oxygen.

(Figure 6 continued) (A) The methodology for CD8 T cell activation, tamoxifen (tam) treatment and hypoxic conditioning is indicated. **(B)** Percent cytokine production is plotted for CD8 T cells secreting one, two or three of the following cytokines: IFNγ, TNFα, and granzyme B. The mean percentage of positively staining cells and SEM is indicated for 3 independent experiments. (**C**) Top panel: CO T cells treated with or without tamoxifen were co-cultured with E.G7 target cells at various effector: target ratios for 4 hours under normoxia or hypoxia (hyp). Bottom panel: CO T cells were cultured with control EL4 tumour cells to show unspecific killing. **(D)** Top and bottom panel: AACO T cells treated with or without tamoxifen were cultured with E.G7 or EL4 cells as in (C). The mean and SEM of 3 independent cytotoxicity experiments is shown. * $p < 0.05$.

2.4.5 E.G7 tumours are hypoxic and are controlled by adoptively transferred T cells in vivo

To assess the role of autophagy for effector activity in CD8 T cells *in vivo* we used an E.G7 thymoma model to monitor the ability of T cells to control tumour growth. While *in vitro* conditions did not show a substantial defect in the activity of autophagy-deficient T cells, we wanted to ascertain whether pathophysiological oxygen concentrations found within tumours would require that T cells could undergo autophagy to control tumours. To assess the oxygenation status of E.G7 tumours we used the drug pimonidazole to measure hypoxic regions. Pimonidazole can detect hypoxia below 1.3 % oxygen [70]. This drug is reduced in hypoxic tissues and forms adducts which can be detected by IHC using an antibody directed against pimonidazole. We detected regions of pimonidazole staining throughout E.G7 tumours (Figure 7A). Thus, we concluded that E.G7 tumours have regions of hypoxia and would therefore serve as a suitable model to assess the role of the hostile tumour environment, including hypoxia and nutrient depletion, on T cell function.

To elucidate whether autophagy-deficient T cells have a killing defect *in vivo* we endeavored to set-up a system where we could monitor the recurrence of E.G7 tumours post-adoptive transfer by measuring the time to experimental endpoint. In the literature,

various numbers of T cells have been adoptively transferred into mice bearing E.G7 tumours to result in suboptimal cure [178,179]. To determine the suboptimal dose of T cells to adoptively transfer in our model we carried out a titration experiment. Mice were separated into 4 groups, including mice given no treatment (N=2), and three additional treatment groups which were given adoptive transfers with autophagy-competent T cells expressing Cre, the SIINFEKL-specific T cell receptor and Thy1.1 in order to track the adoptively transferred cells (CTO). Tumour-bearing mice were treated with 1e5 (N=4), 5e5 (N=5) or 1e6 CD8 T cells (N=5). Tumour areas were monitored as well as T cell expansion in the peripheral blood. Average tumour areas were plotted up until day 6 as all mice were still in the study at this point (Figure 7B).

Mice treated with 5e5 or 1e6 T cells responded to treatment and showed tumour regression for several days (Figure 7B). Mice receiving 1e6 and 5e5 T cells had smaller tumours ($p = 0.0437$ and $p = 0.0676$ respectively) than mice treated with 1e5 T cells. The tumour regression was reflected in the survival of mice post-adoptive transfer as those treated with 5e5 and 1e6 T cells were able to significantly control their tumours prior to the experimental endpoint by up to 10 days more than mice treated with 1e5 T cells with p values of 0.0195 and 0.0351 respectively (Figure 7C). The tumour regression and survival of mice treated with 5e5 and 1e6 T cells was not statistically improved over mice which were not treated, however, this is likely due to the small sample size of the no treatment group (N=2). Mice given 1e5 T cells had no tumour regression and closely matched the tumour growth and survival outcomes of untreated controls (Figure 7B and C). This correlates with the observation that very few adoptively transferred T cells were detected in the peripheral blood of these mice compared to mice given 5e5 and 1e6 T cells, indicating that T cell expansion did not occur after adoptive transfer of 1e5 T cells and thus T cells did not

infiltrate the tumour to mediate regression (Figure 7D). Due to the fact that 5e5 and 1e6 T cells caused similar regression and recurrence of E.G7 tumours, the treatment dose of 1e6 T cells was chosen for future adoptive transfer experiments to allow a margin of counting and injection error as there was a sharp contrast in tumour regression between mice given 1e5 and 5e5 T cells.

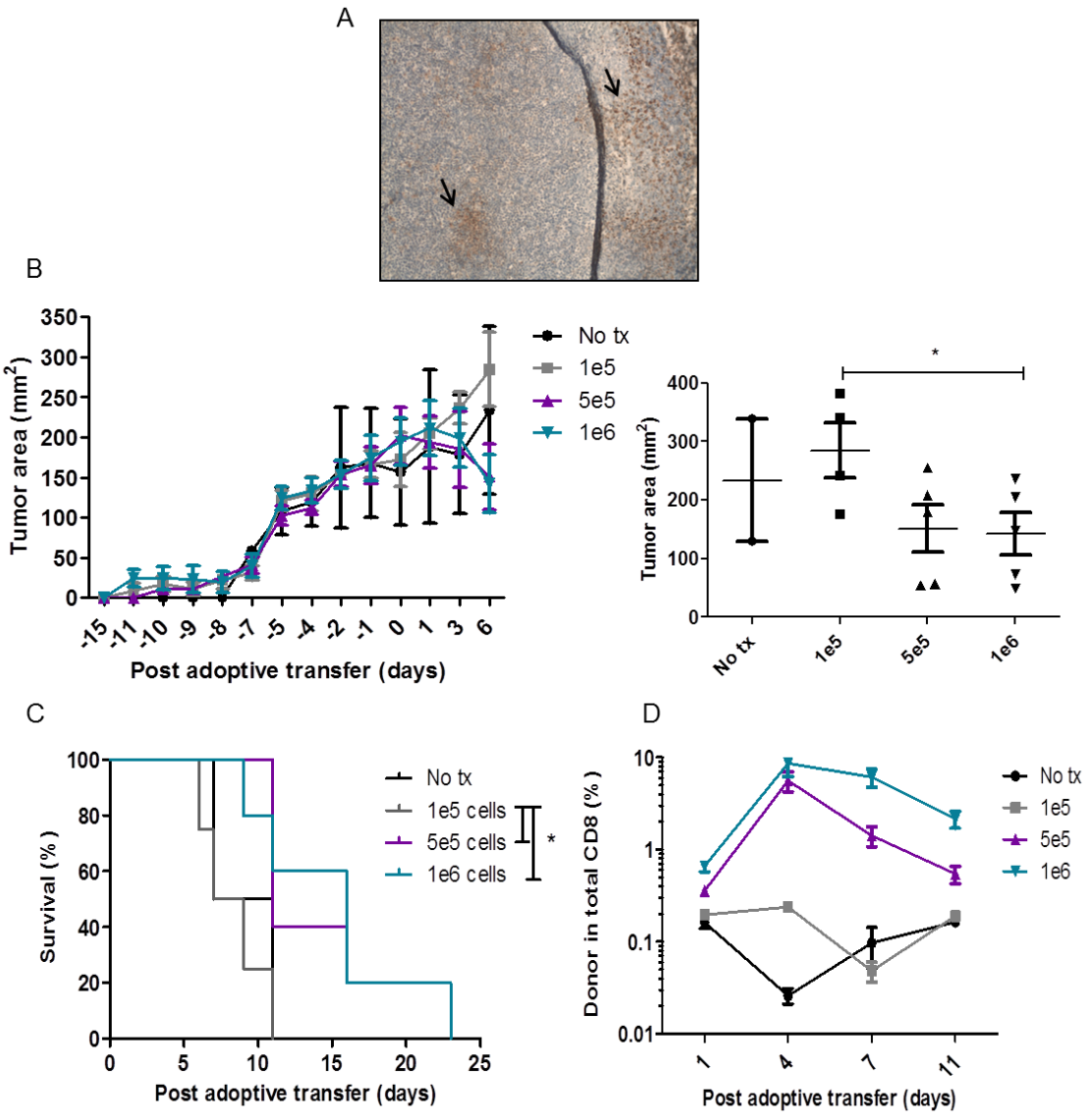


Figure 7: E.G7 tumours respond to adoptive transfer with CD8 T cells in a dose-dependent manner and are hypoxic.

(Figure 7 continued) (A) A representative IHC image of an E.G7 tumour stained for pimonidazole and imaged at 100x magnification. This staining has been repeated in 3 different E.G7 tumours. Arrows indicate regions of staining. **(B)** Left panel: Average tumour areas per treatment group and SEM were plotted up until day 6 post-adoptive transfer. Right panel: Average tumour growth on day 6 post-adoptive transfer. **(C)** Kaplan-Meier curves showing time to experimental endpoint. (**D)** Donor T cell expansion in the peripheral blood was tracked over time. * $p < 0.05$.

2.4.6 Autophagy-deficient T cells expand less than autophagy-competent T cells in vivo but are not impaired in their ability to control E.G7 tumour growth

To assess the role of autophagy for effector function *in vivo* during pathophysiological tumour oxygen concentrations, mice bearing E.G7 tumours were treated with *Atg5* homozygously floxed T cells termed AACTO, or control T cells (Figure 8A). The control T cells termed AaCTO, contained heterozygously floxed *Atg*5 with one functional copy of *Atg5* allowing for autophagy to occur, however, these cells also expressed Cre so that Cre-mediated toxicity could be monitored.

E.G7 tumour-bearing mice were separated into 6 treatment groups, including mice given no treatment (N=4), tamoxifen injection alone (N=3), AACTO T cells (N=4), AACTO T cells plus tamoxifen (N=6), AaCTO T cells (N=5) and AaCTO T cells plus tamoxifen (N=7). The T cells used for the adoptive transfer had been activated with SIINFEKL peptide *in vitro* for 4 days prior to injection thus, these T cells were reactivated when they trafficked to the tumour. Mice were given tamoxifen injections the day following adoptive transfer, therefore autophagy was inhibited in the T cells starting day 5 after their initial activation. Tumour area was monitored throughout the experiment and T cell expansion in the peripheral blood was monitored as indicated in the schematic in Figure 8A. The average tumour areas of mice are plotted up until day 7 post-adoptive transfer as all mice in the study were alive up until this point (Figure 8B).

We observed that control mice which were not given adoptive transfers and were treated with or without tamoxifen alone, did not have regression of established E.G7 tumours (Figure 8B). Mice treated with autophagy-competent AaCTO and AACTO T cells had significant or approaching significant, tumour regression by day 7 compared to untreated mice (AaCTO $p = 0.0153$, AACTO $p = 0.0522$) (Figure 8B). AaCTO and AACTO T cell-treated mice also had significantly increased survival compared to untreated mice with p values of 0.0135 and 0.0285 respectively (Figure 8C). Autophagy-competent AaCTO and AACTO adoptively transferred T cells also showed expansion in the peripheral blood (Figure 8D). Interestingly one mouse in the autophagy-competent AACTO treatment group mediated long term regression of her tumour compared to all other treatment groups (Figure 8C).

In mice treated with AACTO T cells plus tamoxifen, where the T cells were deficient in autophagy, mediated tumour regression by day 7 ($p = 0.0332$) (Figure 8B), had increased survival ($p = 0.0103$) (Figure 8C) and had increased AACTO T cell expansion in the peripheral blood (Figure 8D) compared to mice given no adoptive transfer. However, this expansion was reduced on day 4 ($p = 0.0017$) and 7 ($p = 0.0227$) compared to the expansion of AACTO T cells which were not treated with tamoxifen.

AaCTO T cells were used to serve as a control for Cre-mediated toxicity, however mice given AaCTO T cells plus tamoxifen showed minimal tumour regression (Figure 8B) and survival comparable to mice which were not treated with adoptive transfer (Figure 8C). This was likely due to very low expansion of these T cells in the peripheral blood (Figure 8D).

We did not expect tamoxifen treatment to cause a decrease in the expansion of the control AaCTO cells. A difference between the AaCTO and AACTO T cells was their Cre

status. AaCTO T cells were homozygous Cre while the AACTO T cells were heterozygous for Cre expression. It is likely that the homozygous expression of Cre caused death within the AaCTO T cells when they were treated with tamoxifen and this could account for the decline in cell numbers observed over the course of tamoxifen treatment. While it is possible that heterozygous Cre expression was toxic in the tamoxifen treated AACTO cells and could account for the decrease in T cell expansion observed, this may not be the case. Other experimental results from our laboratory suggest that heterozygous Cre expression does not cause a decrease in AaCTO T cell expansion (data not shown).

Overall these results suggest that autophagy deficiency has a negative impact on a T cell's ability to expand *in vivo*, however the T cells which do proliferate are functionally able to control tumour growth and improve survival at a similar level as autophagy-competent T cells.

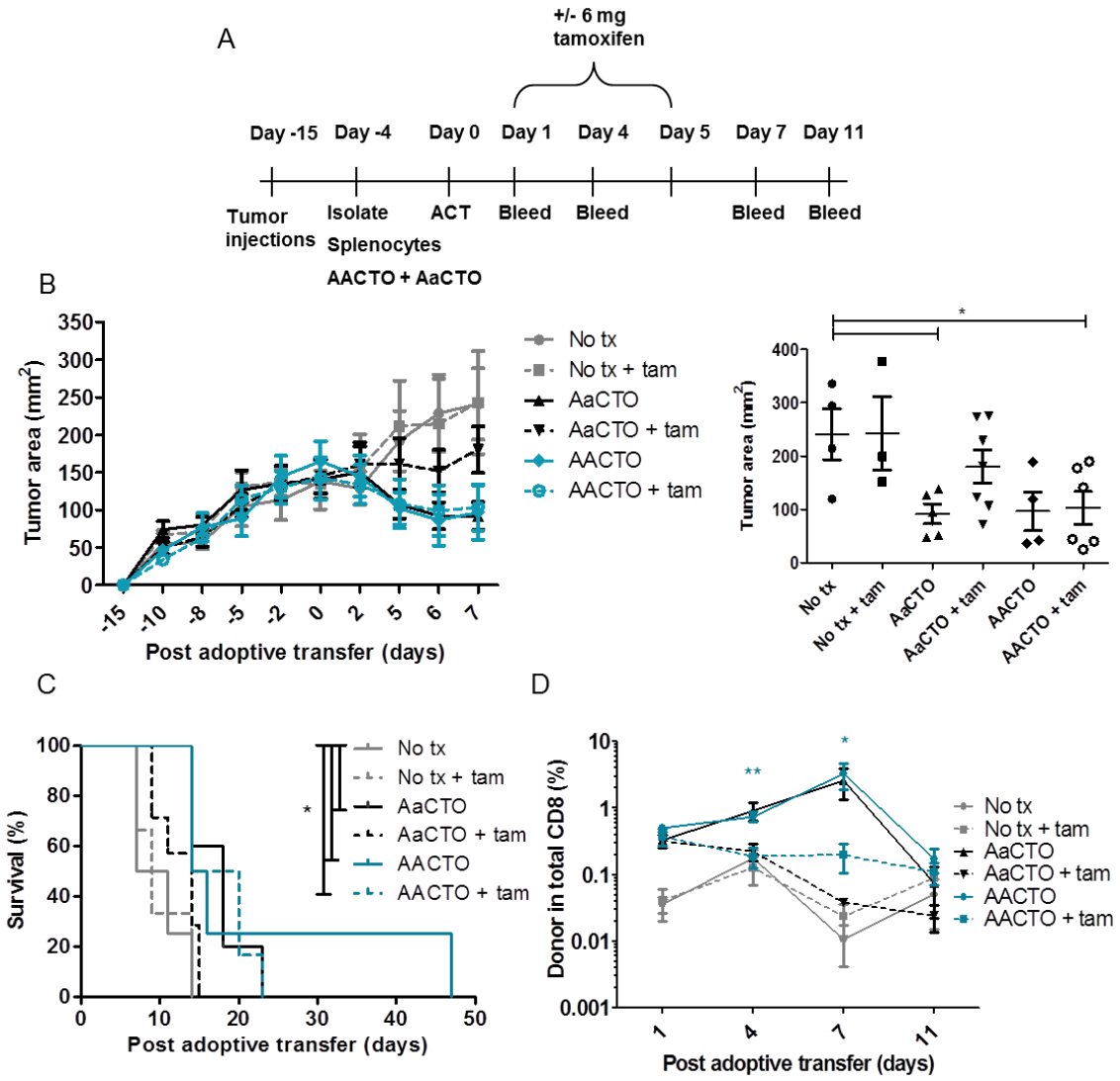


Figure 8: Autophagy-deficient T cells control tumour burden but expand less *in vivo*. **(A)** The methodology for tumour injections, adoptive cell transfer (ACT), tamoxifen (tam) treatment and peripheral blood draws are indicated. **(B)** Left panel: Average tumour areas per treatment group and SEM were plotted up until day 7 post-adoptive transfer. Right panel: Average tumour growth on day 7 post-adoptive transfer. **(C)** Kaplan-Meier curves showing time to experimental endpoint. **(D)** Donor T cell expansion in the peripheral blood was tracked over time. ** $p < 0.01$, * $p < 0.05$.

2.5 Discussion

Previous reports have shown that autophagy is important for maintaining T cell processes including proliferation, cytokine production, organelle turnover and maintenance

of energy homeostasis [132-134,137-139]. We wanted to determine whether CD8 T cells use autophagy under hypoxia and whether autophagy is important for providing energy during anaerobic glycolysis, which could be used to fuel energetic processes such as production of cytokines or promote T cell killing activity. We found that at 1.5 % oxygen, an oxygen concentration relevant in the tumour setting [72-74], CD8 T cells upregulated autophagy. Autophagy inhibition did not decrease T cell killing activity or cytokine production during low oxygen conditions. In addition, *in vivo*, autophagy-deficient cells controlled tumour burden. However, these cells expanded less than cells which were competent in autophagy. These results indicate that autophagy may be most crucial to drive the energy-demanding process of proliferation in the tumour environment.

This study is the first to our knowledge to demonstrate that T cells induce autophagy under low oxygen conditions. To assess the impact of autophagy inhibition during hypoxia in CD8 T cells, we developed a transgenic mouse with a ubiquitously inducible *Atg5* knockout system. Given that $Atg5^{-/-}$ mice die within 24 hours after birth [180], various mouse models have been developed to study the importance of *Atg5* knockout in T cells. *Atg5* depletion in T cell development has previously been studied using $Atg5^{-/-}$ fetal liver chimeric mice which contain *Atg5*-deficient reconstituted immune systems [136] and $Atg5^{fl/fl}$ Lck-*Cre* mice which express Cre during early thymocyte development under control of the lymphocyte protein tyrosine kinase promoter (Lck) [132]. Our system is unique in that we can control the timing of *Atg5* gene deletion using the tamoxifen-induced CreERT2 system, and carry out adoptive transfers using Thy1.1 labeled cells specific for the known OVA-antigen SIINFEKL.

Upon restimulation with PMA and ionomycin, CD8 T cells secreted substantial amounts of the cytotoxic molecule granzyme B which was increased under hypoxia. Despite

their ability to produce granzyme B, both autophagy-competent and deficient T cells had reduced killing of tumour targets *in vitro* under hypoxia. This may be due to the decrease in IFNγ production under hypoxia which promotes the expression of MHC class I and thus antigen presentation by the tumour cell for recognition by T cells [181]. Granzyme B production may not have been affected under hypoxia because it is made following primary activation and stored in cytoplasmic granules which CD8 T cells release upon target cell contact [182,183]. In contrast, IFNγ and TNFα are newly transcribed upon restimulation [182,184] thus hypoxia may prevent this process. Indeed, hypoxia has been implicated in decreasing IFNγ and IL-2 cytokine production in the literature [71,104]. Other reports suggest that hypoxia may actually promote CD4 T cell IFNγ production at 1 % oxygen [185] and anti-tumour killing at 2.5 oxygen [71]. These reports are conflicting regarding the impact of hypoxia on T cell function, however, this may be due to the different experimental conditions used.

More recent reports suggest that hypoxia's impact on cytokine production and T cell activity is due to the role of hypoxia in skewing CD4 T cell differentiation. The transcription factor HIF-1 which is stabilized under low oxygen, has been shown to cause increased [111] or decreased [43] expression of the regulatory transcription factor, FoxP3 which can result in a concurrent decrease or increase in Th17 CD4 development. In addition, the expression of HIF-1 has been shown to be sufficient to induce naïve CD4+ T cells to differentiate into Th17 cells in the presence of physiological oxygen [186]. The promotion of transcription factor expression by hypoxia would be interesting to study in the context of CD8 T cells as they have been shown to vary in naïve and memory cell distribution under 2 % oxygen [102].

A report by Hubbard *et al.*, found that both IFNγ and IL-2 production were decreased in autophagy-deficient T cells under normoxia [139]. This is in contrast to our finding

whereby the production of cytokines under normoxia was not significantly different between cells which were able to undergo autophagy or were inhibited in their ability to undergo autophagy. This is likely due to several differences between the studies including the difference in T cell subsets used. We evaluated CD8 T cells while the study by Hubbard *et al.* assessed CD4 T cells. Hubbard *et al.* also used different methods of autophagy inhibition including pharmacological inhibition and an *Atg7*$^{fl/fl}$*- Lck-Cre* model, whereby autophagy is inhibited during primary T cell activation. In contrast, we inhibited autophagy after primary activation and on restimulation of day 7 activated CD8 T cells. Thus, autophagy may be most important for cytokine production during primary activation.

We found that autophagy deficiency did not cause a drastically reduced ability of T cells to kill tumour targets under hypoxic conditions *in vitro* or *in vivo*. Although overall cytokine production and killing activity was dampened under hypoxia compared to normoxia in all T cell types, autophagy-deficient T cells cultured under hypoxia secreted similar levels of cytokines compared to control cells. In addition, inhibiting autophagy during the effector phase in *vivo* did not cause a drastically reduced ability to control tumour burden, however, it did result in a decrease in T cell expansion. This decrease in expansion was not enough to affect tumour killing as transferred T cells remained above background levels detected in control untreated mice. Autophagy deficiency has been shown to impact T cell expansion in several other mouse models upon T cell receptor engagement [132,133,136] however, this is the first study to report an autophagy-dependent defect in T cell expansion in the tumour-bearing mice.

While our *in vivo* experiment did not show a drastic difference in killing activity with autophagy inhibition, one mouse in the autophagy-competent group had substantial survival benefit with adoptive transfer while no mice within the autophagy-deficient group survived

as long. Future experiments should include an increased number of mice in each group to determine if this is a repeatable observation.

To account for Cre recombinase-induced toxicity in our model we included a group of tumour-bearing mice treated with heterozygously floxed *Atg5* T cells (AaCTO), thus the T cells transferred would not have complete autophagy knockout on Cre induction by tamoxifen treatment. To cause recombination of adoptively transferred cells, mice were treated with tamoxifen at a dose consistent with that used by other groups to cause recombination [155,187-189]. Surprisingly, in our AaCTO control group, all tamoxifen-treated T cells disappeared after the first day of treatment *in vivo*. It has been shown that through expression or integration into the cell genome, Cre recombinase can have an effect on cell function [190]. However, Cre recombinase is integrated into a well-characterized region, the Rosa26 locus, in CreERT2 mice and integration is likely not the cause of toxicity [156]. Therefore, the higher expression of Cre is likely the cause of T cell disappearance as this has been reported in other cell types whereby cell survival was impacted [156,191]. Future *in vivo* studies should include a control containing Cre in the heterozygous state to ensure Cre expression does not impact T cell expansion *in vivo* and to validate the finding that autophagy deficiency impacts effector T cell expansion. Tamoxifen-induced toxicity has also been reported [192], therefore an additional control to include in future adoptive transfer experiments would be T cells lacking Cre expression to account for toxicity from tamoxifen alone.

We hypothesized that T cells would rely on autophagy during hypoxia in order to provide metabolites during conditions of anaerobic glycolysis. We found that processes such as cytokine production and killing are not reliant on autophagy during conditions of restimulation at 1.5 % oxygen. However, we found expansion was negatively impacted.

These findings support the fact that autophagy is important for T cells during expansion under low oxygen conditions potentially *via* provision of energy supply. Indeed, proliferation is an energetically costly process [91]. Autophagy may also be important for the clearance of ROS during low oxygen which has also been shown to cause cell death when levels are not reduced [148]. These findings offer a new assessment of the role of autophagy in T cells compared to the majority of studies as we were able to inhibit autophagy after naïve T cell activation during the effector phase. Based on our results and others in the literature, autophagy may be most important for cytokine production during initial activation. Our results also implicate a new autophagic inducer, that is hypoxia, in T cells which is especially relevant as T cells traffic to tumour sites or locations of inflammation during immune responses, both of which may contain hypoxic environments.

Overall, these findings are an important consideration for adoptive immunotherapy or treatments which aim to improve the CD8 T cell anti-tumour response. By determining how certain tumour conditions such as hypoxia dampen immune responses, we can learn how to augment CD8 T cells so that they are able to better function under negative tumour environmental conditions.

2.6 Acknowledgments

The authors thank Dean Rysstad for his technical assistance with mouse experiments. Thank you to Luke Hughson for his helpful scientific discussions and to Jaeline Spowart for aiding with *in vivo* tumour injections and for reviewing the manuscript.

Chapter 3: T cell infiltration and markers of T cell function are associated with vascularized tumours and improve patient outcome in high-grade serous ovarian carcinoma

Katelin N. Townsend[1,2], Jaeline E. Spowart[1,2], Hassan Huwait[3], Sima Eshragh[4], Nathan R. West[1,2], Steve Kalloger[4], Michael Anglesio[4], Peter H. Watson[1,2,4], C. Blake Gilks[4], David G. Huntsman[5], Julian J. Lum[1,2] (Manuscript in preparation)

[1]Deeley Research Centre, BC Cancer Agency, Victoria, BC, Canada

[2]Department of Biochemistry and Microbiology, University of Victoria, BC, Canada,

[3]Anatomical Pathology, Vancouver General Hospital, BC, Canada

[4]Department of Pathology and Laboratory Medicine, University of British Columbia, BC, Canada

[5]Centre for the Translational & Applied Genomics, BC Cancer Agency, Vancouver, BC, Canada

KNT, JES and JJL designed the study. KNT, JES, HH, SE, SK, MA, CBG, DGH, and JJL were involved in acquisition of data. KNT, JES, NRW, PHW and JJL were involved in the analysis and interpretation of data. KNT wrote the manuscript.

3.1 Abstract

On migration into the tumour milieu, T cells encounter metabolic stress due to the exhaustion of nutrients caused by rapidly proliferating tumours. Activated T cells must overcome this suppressive tumour environment in order to mount an effective anti-tumour response. Our goal was to determine the effect of hypoxia on T cells and to correlate this with high-grade serous ovarian cancer patient survival. Using IHC we stained and scored a tissue-microarray (TMA) for markers indicating vasculature (CD31), T cells (CD8 and CD4), the T reg transcription factor FoxP3, and CD8 T cell functional markers (granzyme B and TIA-1). In high-grade serous ovarian patients our results show that all immune markers analysed are associated with vascularised tumours. We found that non-vascularized tumours were less likely to contain CD8 T cells or cells expressing FoxP3, granzyme B, or TIA-1. To analyze the functional impact of hypoxia on T cells we carried out dual survival analysis combining CD31 with CD8, CD4, FoxP3, granzyme B or TIA-1. Patients trended towards, or had significantly worse survival, when CD8 ($p = 0.0818$), TIA-1 ($p = 0.0601$) or FoxP3 ($p = 0.0377$) expressing T cells were located in non-vascularized, hypoxic tumours compared to patients with immune infiltrates in vascularized, oxygenated tumours. Overall, the non-vascularized, hypoxic tumour environment was inversely correlated with the degree of T cell infiltration in high-grade serous ovarian tumours. Additionally, patients with T cells located in non-vascularized tumours trend towards poorer survival outcomes.

3.2 Introduction

Ovarian cancer is the most lethal of gynecological malignancies, largely due to the fact that the majority of cases are detected in advanced stage [193]. These advanced cases are

treated with cytoreductive surgery followed by chemotherapy using a combination of platinum and taxane-based compounds [194]. However, after treatment, ovarian cancer often recurs and results in patient mortality within 2-5 years [195]. The poor outcomes associated with ovarian cancer highlight the need for improved therapies and a better understanding of the disease. One factor that has been shown to contribute positively to the outcomes of ovarian cancer patients is the immune system. In particular, the presence of CD3 and CD8 tumour-infiltrating T cells is associated with a significant survival advantage in epithelial ovarian cancer patients [17,54,165,166,169]. This finding has led to an interest in further determining the roles of immune cells in ovarian cancer patients and potentially developing immunotherapeutic treatments for this disease; indeed, groups have started to assess the feasibility of immune-based therapies in ovarian cancer [196-201].

Ovarian tumours have been shown to exhibit features of hypoxia [171,172]. Thus, on migration to an ovarian tumour, lymphocytes may encounter low oxygen due to the increased usage of oxygen by rapidly proliferating tumour cells. Hypoxic tumour cells often induce angiogenesis (i.e., the formation of new blood vessels) *via* the production of vascular endothelial growth factor [202]. The resulting formation of vasculature may provide nutrients to tumour cells in addition to tumour-infiltrating immune cells. The role of tumour vasculature is highlighted by studies which show treatments using anti-angiogenesis agents can promote tumour hypoxia [203,204]; recruited vasculature can thus promote tumour oxygenation.

Hypoxic conditions have been shown to skew T cell responses and dampen T cell effector function [43,102,111]. Therefore, we wanted to determine whether the oxygenation status of tumours affects the ability of T cells to improve patient survival. We assessed 200 tumours of the most common epithelial ovarian cancer subtype, high-grade serous. We

stained the tumours for CD31, a marker found on the endothelial cells which form blood vessels, and therefore indicates tumour vascularity. We also stained for markers of an adaptive immune response including CD8, CD4, and the cytotoxic T cell effector molecules, granzyme B and TIA-1 which are used to directly kill tumour targets [25,26]. The T reg transcription factor, FoxP3, was also assessed as T regs have recently been shown to traffic to hypoxic ovarian tumours [112].We hypothesize that ovarian carcinoma patients with T cells in non-vascularized, hypoxic tumours as assessed by IHC will have poorer survival than those with T cells in vascularized tumours.

3.3 Materials and Methods

3.3.1 Patient Population

The retrospective patient cohort used for this study has been described elsewhere [205]. Briefly, the Cheryl Brown Ovarian Cancer Outcomes Unit collected ovarian tumour specimens from 1984-2000 from women located in British Columbia. Patients were treated with surgery resulting in no macroscopic residual disease and had platinum-based chemotherapy. Tumours were collected at primary surgery prior to treatment with chemotherapy, fixed with 10 % neutral-buffered formalin, processed and embedded in paraffin. A representative area of each tumour was selected and 0.6 mm cores in duplicate were used to construct a TMA. For this study, 200 high-grade serous ovarian tumours were analysed. Patient characteristics are shown in Table 1. For Kaplan-Meier analysis, overall survival was defined as survival without death due to any cause, progression-free survival was survival without evidence of disease recurrence and disease-specific survival was survival without death due to ovarian cancer. Study approval was provided by the University of British Columbia, British Columbia Cancer Agency Research Ethics Board (H02-61375).

Table 1: Patient characteristics, follow-up time and survival characteristics for high-grade serous ovarian carcinoma cases.
FIGO = Federation of Gynecology and Obstetrics.

Clinical characteristics	
Age at surgery (years)	
Median	60.87
Range	37.59-85.96
Silverberg grade	
1	0 (0 %)
2	56 (28 %)
3	143 (71.5 %)
Not graded	1 (0.5 %)
FIGO stage	
I	49 (24.5 %)
II	86 (43 %)
III	65 (32.5 %)
Total number patients	200
Follow-up time	
Median follow-up (range), years	5.35 (0.4-23.6)
Survival characteristics	
Disease progressions	102 (51 %)
Ovarian cancer deaths	92 (46 %)
Total number of deaths	124 (62 %)

3.3.2 Immunohistochemistry Staining

The ovarian tumour TMA was stained for the following markers: CD8, CD4, FoxP3, granzyme B, TIA-1 and CD31. CD8, CD4 and granzyme B staining was performed by Clarke *et al.* [165] using the following antibodies specific for CD8 (Dako, Burlington, ON, CA; clone C8/144B, mouse monoclonal, 1:50), CD4 (Novocastra, Concord, ON, CA; clone 4B12, mouse monoclonal, 1:50) and granzyme B (Dako; clone GrB-7, mouse monoclonal, 1:25). TIA-1 and FoxP3 staining was performed by Milne *et al.* [54] using the following antibodies specific for TIA-1 (Abcam, Cambridge, MA, USA; clone TIA-1, mouse monoclonal, 1:50), Foxp3 (eBioscience, San Diego, CA, USA; clone Ebio7979, mouse

monoclonal, 1:50). CD31 staining was carried out as described by Milne *et al.* [54]. Briefly, tumour tissues were sectioned at 4 μm onto Superfrost plus slides (Fisher Scientific, Nepean, ON, CA) and incubated overnight at 37°C. The slides were deparaffinized in xylene and graded alcohols and a Ventana Discovery XT autostainer (Ventana, Tucson, AZ, USA) was used for IHC staining. Ventana's standard CC1 protocol was used for antigen retrieval. The primary antibody anti-CD31 (Cell Marque, Rocklin, CA, USA; clone JC70, mouse monoclonal) was added to the slides at a dilution of 1:100 in Ventana's Antibody Diluent and incubated for 60 minutes. A cross-adsorbed biotinylated goat anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was manually applied at a dilution of 1:250 for 32 minutes. Bound antibodies were detected using the DABMap kit (Ventana), counterstained with hematoxylin (Ventana), and coverslipped manually with Cytoseal-60 (Richard Allan, Kalamazoo, MI, USA).

3.3.3 Marker scoring and analyses

CD8, CD4 and granzyme B were scored according to the following criteria: cores containing no positive cells were scored as 0, cores containing positive staining cells within the stroma were scored as 1, cores containing positive staining cells in the epithelium were scored as 2 and cores containing positive cells in both the epithelium and stoma were scored as 3. The scores were then binarized and patients were separated into two groups for analyses where patients were considered positive for the respective marker if the tumour cores had IHC scores of 2 or 3, and were considered negative if the tumour cores had IHC scores of 0 or 1. TIA-1 and FoxP3 were assigned scores based on cells located within the tumour epithelium only as follows: 0 (no cells), 1 (1-5 cells), 2 (6-19 cells), or 3 (≥20 cells). Results were binarized into groups of positive (IHC score 1, 2, 3) or negative (IHC score 0).

CD31 was scored at 100x magnification using a Chalkley grid consisting of 8 horizontal and 6 vertical cross hairs. Each core was scored for the number of cross hairs which intersected positively staining vasculature (V), tumour epithelium (T), or stroma (S). Samples were then assigned a vascular density score. This was assigned by first determining the proportion of stroma (PS) in each core by the formula (S+V)/(S+V+T) = PS. Vascular density was then calculated using the formula V/PS. The highest vascular density score within a set of duplicate cores was used for our analysis. For Kaplan-Meier curve analysis vascular density scores were separated into groups of low and high based on the 33rd percentile.

3.3.4 Statistics

Statistical analysis was carried out using GraphPad Prism software version 5.04 (GraphPad Software, La Jolla, CA, USA). Statistically significant *p* values were considered to be those less than 0.05. Kaplan-Meier curve analyses was carried out using Log rank tests. Mann-Whitney tests were used compare differences between the CD31 IHC vascular density scores observed for patients with positive or negative immune parameters. Fisher's exact tests were used to carry out contingency analyses.

3.4 Results

3.4.1 High-grade serous ovarian tumours are vascularized and are infiltrated by T cells

Ovarian cancers often show evidence of immune infiltration and it has been reported that such infiltration corresponds with improved patient prognosis [17]. The latter finding has also been reported for the high-grade serous patient cohort employed in this study [54,165]. We wanted to assess whether the tumour microenvironment, specifically hypoxia,

impacts the survival outcome of patients with immune infiltrates. To do this we first assessed vascular density in 200 high-grade serous ovarian tumours using the vasculature marker CD31 (Figure 9A). To assess whether the adaptive immune component was affected by the level of tumour oxygenation we also assessed the tumours for the immune markers CD8 (Figure 9B), CD4 (Figure 9C), FoxP3 (Figure 9D), granzyme B (Figure 9E), and TIA-1 (Figure 9F). We found that there was evidence of vascularization, as well as immune cell infiltration and markers of activation in a proportion of the tumour samples, indicating the high-grade serous tumours may be vascularized, and/or contain functional immune infiltrates.

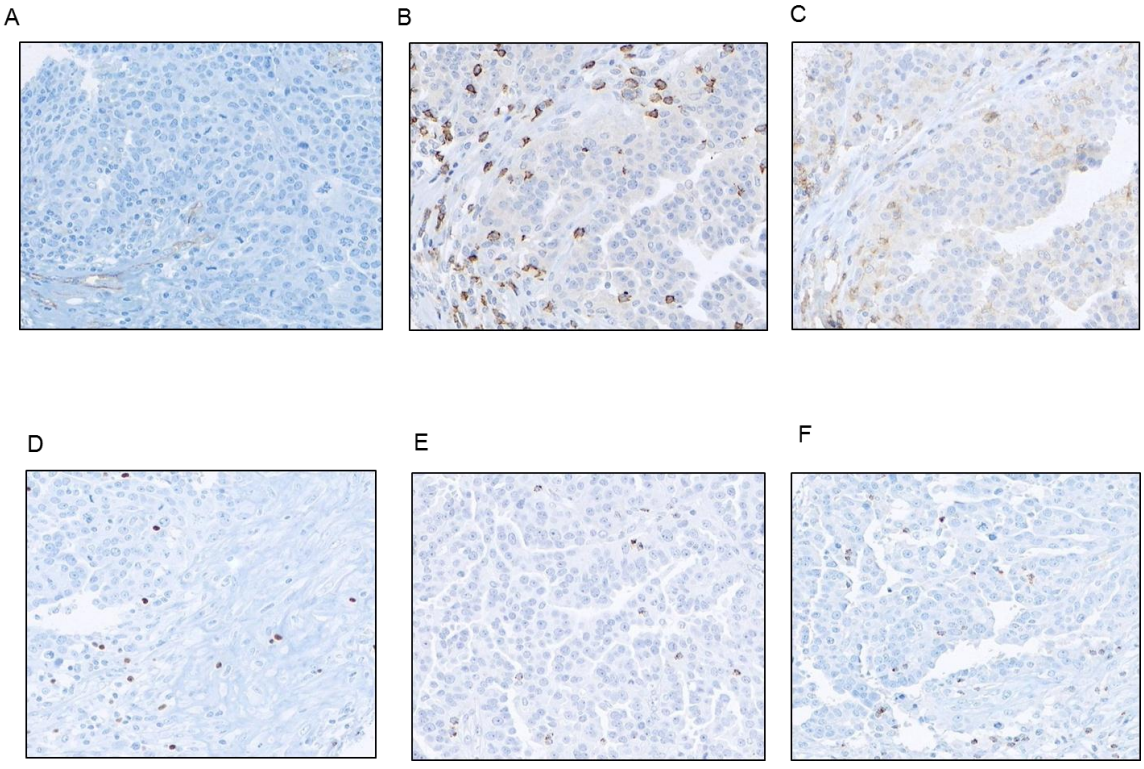


Figure 9: IHC images of high-grade serous ovarian carcinoma.
Images show staining for: **(A)** CD31, **(B)** CD8 T cells, **(C)** CD4 T cells**, (D)** FoxP3, **(E)** granzyme B and **(F)** TIA-1 at 200x magnification.

3.4.2 Vascularized tumours are more likely to contain immune infiltrates

We wanted to determine whether patients with well-vascularized tumours were more likely to contain immune infiltrates. Patients were first separated into two groups corresponding to those containing T cells within their tumours and those lacking immune infiltrates. These groups were further separated into those with a high or low vascular density in their tumours. Using a Fisher's exact test we found that patients with non-vascularized, hypoxic tumours were less likely to contain CD8 (p =0.0037), FoxP3 (p =0.0267), granzyme B (p =0.0412) or TIA-1 (p =0.0079) expressing cells in their tumours (Table 2). CD4 T cells were generally more abundant in vascularized tumours, but this did not reach statistical significance (p =0.0842).

Table 2: Patients with immune infiltrates most often have vascularized tumours. Contingency analyses comparing the number of patients in each category: CD31 low, CD31 high and infiltrate positive or negative. CD8, CD4, FoxP3, granzyme B, and TIA-1 (+) indicates > 0 positive cells stained positive in the epithelial compartment of the tumour and (-) indicates no positive cells within the tumour epithelium. The indicated p values were determined using a Fisher's exact test.

	CD31 low	**CD31 high**
CD8 -	21	19
CD8 +	40	111
p value	0.0037	
CD4 -	39	66
CD4 +	21	64
p value	0.0842	
FoxP3 -	35	52
FoxP3 +	24	75
p value	0.0267	
Granzyme B -	42	68
Granzyme B +	19	61
p value	0.0412	
TIA-1 -	28	34
TIA-1 +	32	94
p value	0.0079	

To further validate the positive relationship between immune cell infiltration and tumour vascularisation, we also found that tumours positive for immune cell markers had higher vascular density scores than tumours negative for immune cell markers. This relationship was statistically significant for all markers analyzed: CD8 (p =0.0017, Figure 10A), CD4 (p =0.0112, Figure 10 B), FoxP3 (p =0.0147, Figure 10C), granzyme B (p =0.0040, Figure 10D) and TIA-1 (p =0.0003, Figure 10E). These data indicate that tumours containing immune infiltrates are most often highly vascularized.

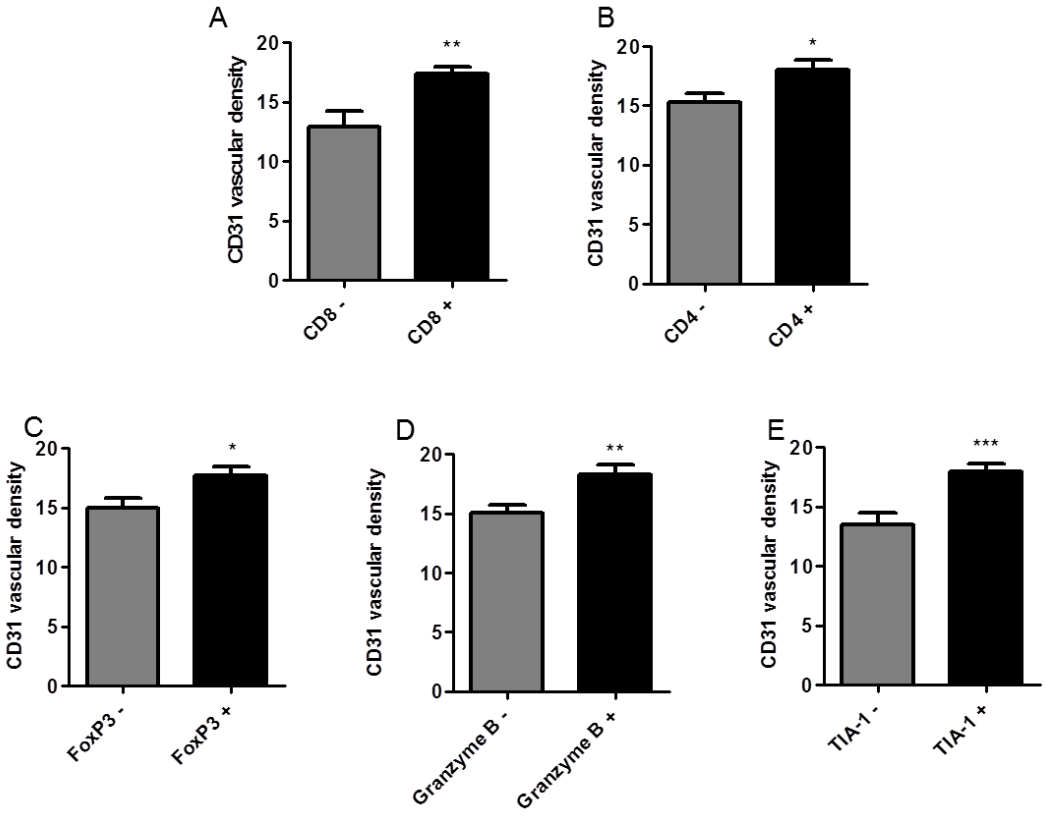


Figure 10: Tumours containing immune infiltrates have higher vascular density scores than tumours that do not contain immune infiltrates.

CD31 vascular density scores were compared between tumours with positive and negative expression for: **(A)** CD8, **(B)** CD4, **(C)** FoxP3, **(D)** granzyme B and **(E)** TIA-1 tumour infiltrates in high-grade serous tumours. CD8, CD4, FoxP3, granzyme B, and TIA-1 (+) indicates > 0 positive cells stained positive in the epithelial compartment of the tumour and (-) indicates no positive cells within the tumour epithelium. Error bars indicate SEM. p values were calculated using Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4.3 Immune infiltrates in non-vascularized tumours correlate with poorer patient survival outcomes.

To determine whether poorly vascularized, and thus hypoxic tumours were associated with dampened function of immune cells, patients were separated into immune marker positive or negative categories. The immune marker positive tumours were further separated into groups based on low or high vascular density, and disease-specific survival was compared between the patient groups. Patients with CD8 T cells (Figure 11A) and TIA-1 expression (Figure 11E) in vascularized tumours trended towards better survival compared to patients with expression of these markers within non-vascularized tumours with p values of 0.0818 and 0.0601 respectively. Patients with CD4 T cells (Figure 11B) and granzyme B expression (Figure 11D) in vascularized tumours weakly trended towards better survival compared to patients with these markers in non-vascularized tumours with p values of 0.1752 and 0.1785 respectively. Patients with FoxP3 positive cells (Figure 11C) within vascularized tumours had significantly better survival than patients with FoxP3 positive cells in non-vascularized tumours ($p = 0.0377$). Patients with high levels of FoxP3 positive cells in non-vascularized, hypoxic tumours had similar survival to patients without any FoxP3 infiltrates ($p = 0.9669$), indicating FoxP3 is associated with negative outcomes when in a hypoxic environment, but positive outcomes when in a normoxic environment.

In addition, we found patients lacking CD8 T cell infiltrates had significantly poorer survival than patients with CD8 T cell infiltrates regardless of whether or not their tumours were vascularized (Figure 11A). There was no significant difference between patients with immune infiltrates in vascularized tumours versus patients with immune infiltrates in non-vascularized tumours in terms of progression-free survival and overall survival for any of the immune markers assessed (data not shown).

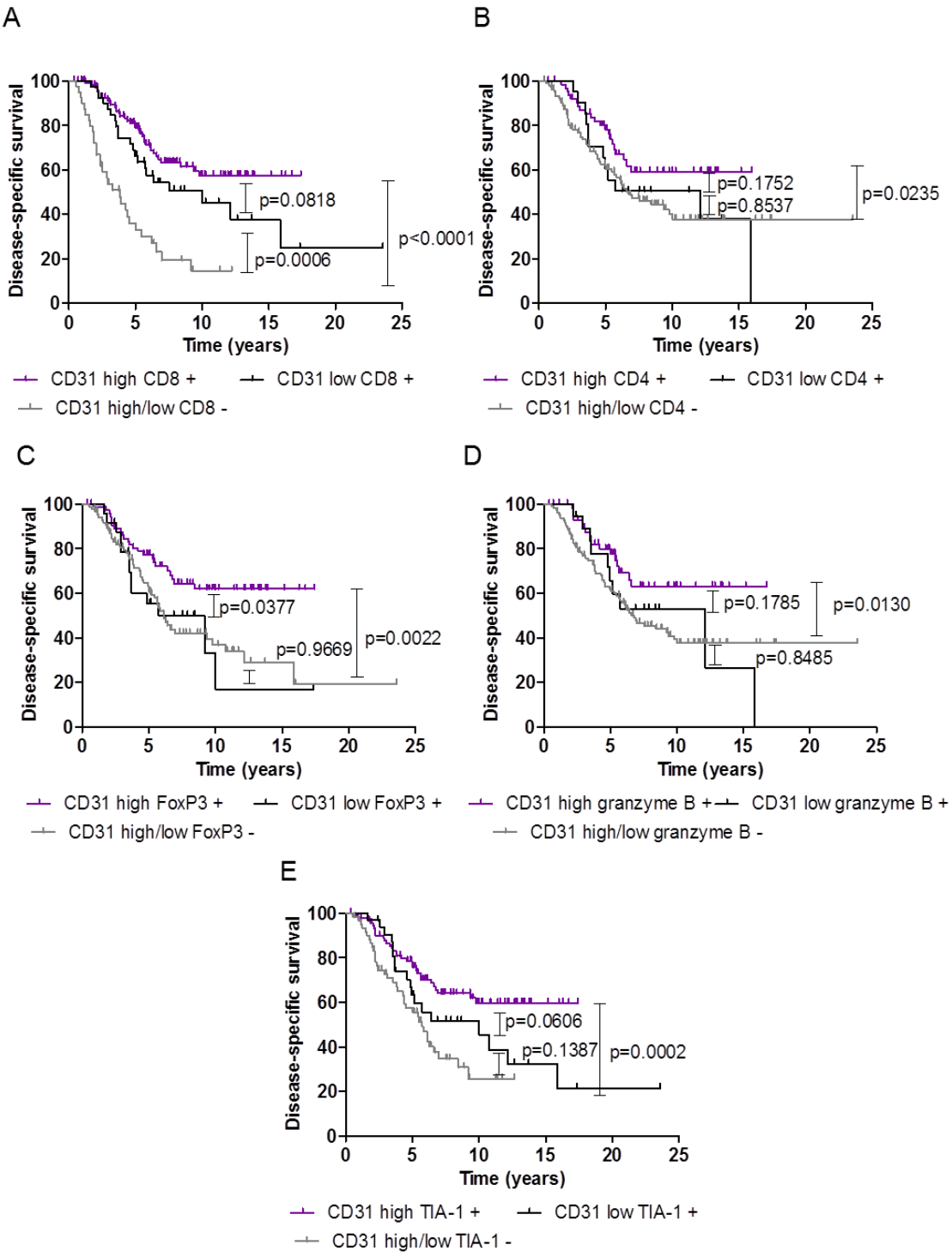


Figure 11: Immune infiltrates in vascularized tumours are associated with improved patient survival compared to immune infiltrates in non-vascularized tumours.
Kaplan-Meier analysis of disease-specific survival of high-grade serous ovarian patients separated by CD31 and the indicated immune markers.

(Figure 11 continued) (A) CD31-high, CD8 + (N = 111); CD31-low, CD8 + (N = 40) and CD31 high/low, CD8 – (N = 40). **(B)** CD31-high, CD4 + (N = 64); CD31-low, CD4 + (N = 21) and CD31 high/low, CD4 – (N = 105). **(C)** CD31-high, FoxP3 + (N = 75); CD31-low, FoxP3 + (N = 24) and CD31 high/low, FoxP3 – (N = 87). **(D)** CD31-high, granzyme B + (N = 61); CD31-low, granzyme B + (N = 19) and CD31 high/low granzyme B – (N = 110). **(E)** CD31-high, TIA-1 + (N = 94); CD31-low TIA-1 + (N = 32) and CD31 high/low, TIA-1 – (N = 62). CD8, CD4, FoxP3, granzyme B, and TIA-1 (+) indicates > 0 cells stained positive in the epithelial compartment of the tumour and (-) indicates no positive cells within the tumour epithelium. *p* values were determined using a Log rank test.

Overall, patients with CD8 T cells, TIA-1 and FoxP3 expression in vascularized tumours tended to have better survival outcomes compared to patients with these infiltrates in non-vascularized, hypoxic tumours.

3.5 Discussion

We sought to determine whether the level of vascular density, an indicator of the oxygen level within tumours, impacted the survival of high-grade serous ovarian carcinoma patients with tumours rich in immune infiltrates. The particular cohort of patients we analysed had previously been highlighted as an immune rich subset and patient survival was reported to be significantly increased in the presence of CD8, FoxP3 and TIA-1 positive infiltrating cells [54]. This cohort of patients had been optimally debulked, which is also associated with a high presence of immune infiltrates [168]. We further analyzed this cohort and compared the level of vascularization with immune infiltration and survival outcomes.

We found that tumours with immune infiltrates in the epithelium were more likely to have a higher vascular density score and that a high percentage of patients had tumours that were both vascularized and contained immune cells. This indicates that it is rare to find an immune infiltrate in a more hypoxic tumour, potentially because these cells do not infiltrate or survive under these conditions. In support of this hypothesis, one study found that tumour-infiltrating T cells were less likely to be localized in regions of hypoxia [206]. We

found that there was a trend towards decreased survival in patients with immune cells infiltrating non-vascularized, hypoxic environments compared to patients with immune infiltrates in vascularized tumours. To determine whether markers associated with immune function correlated with poor survival outcomes under hypoxia, we assessed the markers granzyme B and TIA-1. These molecules are also produced by natural killer NK cells which elicit anti-tumour activity. However, Milne *et al.* found that very few or no NK cells were located within the tumour epithelium, and we thus concluded that granzyme B and TIA-1 indicated CD8 T cell function alone [54]. We found that patients with CD8, granzyme B and TIA-1 expressing cells in non-vascularized tumours did not do significantly worse than women with vascularized tumours although a trend was observed, particularly for TIA-1. Studies assessing the function of human T cells under hypoxia *in vitro* show mixed results, with some stating T cell function is maintained under hypoxia [101,103] while others stated that function is decreased [207] or increased [208]. Contrasting reports may be due to the duration of hypoxic treatment and concentration of oxygen being tested across experiments. Further research is therefore needed to definitively determine the impact of hypoxia on T cell anti-tumour function and the specific contexts in which the impact(s) occur.

Interestingly, we found that FoxP3 infiltrates were beneficial for patient survival in vascularized tumours compared to FoxP3 infiltrates in non-vascularized tumours. FoxP3 positive cells have been shown to be significantly associated with favorable patient survival in ovarian carcinoma [54,209], however, they have also been associated with negative patient outcomes in ovarian carcinoma due to their role in actively suppressing T cells [48,170]. We have shown that FoxP3 positive cells are only associated with good outcomes when they are located in an oxygenated environment. A recent report by Facciabene *et al.* using an epithelial murine ovarian cancer model showed that hypoxic tumours recruit FoxP3 expressing T reg

cells to the tumour and these cells suppress anti-tumour immune cell subsets [112]. However, we did not observe that within patient tumours containing FoxP3 T cells that a higher number of FoxP3 T cells were located in non-vascularized, hypoxic tumours (data not shown). Thus a higher percentage of FoxP3 T cells may not account for the decrease in patient survival observed when FoxP3 expressing cells are located in hypoxic environments. One report in ovarian carcinoma found that a high ratio of CD8 T cells to T regs was positively associated with patient survival [169]. We were not able to assess the ratio of FoxP3 to CD8 cells in our cohort given the scoring methods used. However, this may account for the differences in survival observed when patients contained FoxP3 expressing cells in vascularized versus non-vascularized environments.

In order to confirm that patient survival is increased when T cells are located within vascularized, oxygenated environments, future studies using dual IHC staining would be beneficial. Although we carried out dual survival analysis with CD31 and the various indicated immune markers, we cannot say whether the immune cells were located in tumour epithelium close to vasculature or within hypoxic regions at a distance from vasculature. Dual IHC staining would allow a more direct assessment of T cell oxygenation to be made. In addition, assessment of ovarian cancer cases whereby patients have remaining residual macroscopic disease may allow further correlations to be made regarding patient survival when T cells are located in conditions of hypoxia. Indeed, it has been shown that patients with immune infiltrates in sub optimally debulked tumours do worse than those which are optimally debulked [17]. It would be interesting to determine if debulking status correlates with tumour hypoxia and whether the immune infiltrates in this environment confer better or worse patient survival.

Given that high-grade serous ovarian tumours are infiltrated by immune cells, causing a positive association with survival based on previous reports [54,165], we sought to determine how the ovarian tumour environment impacts immune cell function. Overall, we found that patients had significantly worse or trended toward worse survival outcomes when immune infiltrates were in a non-vascularized, hypoxic environment. Our results indicate that the tumour environment is an important consideration when assessing immune function. Understanding how T cells function under conditions such as hypoxia will allow for the ability to improve T cell responses during immune-based therapies or potentially other cancer therapies such as chemotherapies which elicit immune responses, and may help improve the outcomes of ovarian cancer patients.

3.6 Acknowledgments

The authors thank Christine Chow and Katy Milne for their roles in preparing tissue samples and carrying out the CD31 IHC staining respectively. The Cheryl Brown Ovarian Cancer Outcomes Unit of the British Columbia Cancer Agency supported this work.

Chapter 4: Concluding remarks

4.1 Chapter Summaries

The main goal of this thesis was to determine whether CD8 T cells are functionally impacted by low oxygen, a common feature of solid tumours and to assess the role of autophagy in a T cell's ability to cope with hypoxia. I set out to address these questions by employing two methods. The first method, outlined in Chapter 2, used a mouse model system whereby the oxygen levels of *in vitro* cell cultures could be controlled. I also used an *in vivo* E.G7 thymoma tumour model which exhibited hypoxic features. Using a newly designed transgenic mouse model, we could inhibit autophagy in T cells to specifically determine the consequences of the loss of autophagy under low oxygen and therefore elucidate the function of autophagy in this context. The second method, outlined in Chapter 3, was employed in an effort to understand the relevance of hypoxia in a human tumour setting. I analyzed a retrospective ovarian cancer patient cohort and assessed the levels of oxygenation and T cell infiltrates within tumour samples comprising a TMA. This form of analysis allowed correlations between the level of tumour hypoxia and presence of T cell functional markers to be made using patient survival as a final read out. Several conclusions can be made from these two methods of analysis and these are outlined below.

4.1.1 Chapter 2 and 3 Summaries

In Chapter 2 we showed that T cells displayed a defect in cytokine production and killing activity under low oxygen. To determine how T cells adapt to hypoxic conditions we looked to the process of autophagy as an energy-generating mechanism to support cellular function. We demonstrated that CD8 T cells undergo autophagy during hypoxia. This process did not cause a dramatic impact on cytokine production or killing activity under

hypoxic conditions, suggesting that the cells are not wholly reliant on autophagy to provide energy under hypoxia to support these facets of T cell function. Under *in vivo* pathophysiological oxygen concentrations we found that autophagy-deficient T cells were able to control tumour burden. However, autophagy-deficient T cells exhibited a significant defect in expansion indicating that autophagy is important for this process. These expansion studies must be corroborated using appropriate validation with Cre-mediated toxicity controls.

In Chapter 3 we showed that high-grade serous ovarian tumours exhibited vascularity as indicated by CD31 staining. These tumours, taken from an optimally debulked patient cohort, had a relatively high proportion of immune infiltrates. Vascularized tumours were more likely to contain immune infiltrates than non-vascularized, hypoxic tumours. This suggests that T cells do not infiltrate, survive or expand within hypoxic settings. The relevance of these results was further explored by assessing the survival outcomes of patients with vascularized or non-vascularized tumours containing immune infiltrates. There was a trend towards reduced survival outcomes when CD8 and TIA-1 expressing cells were located in non-vascularized tumours compared to vascularized tumours. Patient survival was most negatively impacted when FoxP3 expressing cells were located within a non-vascularized, hypoxic environment compared to when FoxP3 expressing cells were found within vascularized tumours. These results further highlight the importance of the tumour environment in immune activity.

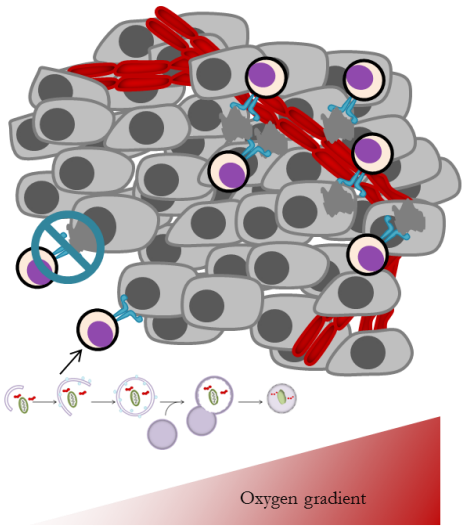
4.1.2 Integrating concepts from Chapters 2 and 3

Both Chapter 2 and 3 demonstrate the relevance of hypoxia in solid tumours and that T cells are impacted by the hypoxic environment. Interestingly, in the *in vitro* mouse cell

setting we found that the production of granzyme was not impacted by hypoxia and this was also true within our human study whereby patient survival was not decreased when granzyme B expression occurred within non-vascularized tumours. However, T cells were less likely to be found within non-vascularized ovarian tumours and similarly, T cells unable to perform autophagy expanded less in our mouse model. While these findings may not be directly related, it can be speculated that expansion defects may occur in T cells under pathophysiological oxygen tensions and that T cell expansion under these conditions is supported by autophagy. Overall, these studies have shown that hypoxia plays a negative role in T cell function in both the human and mouse tumour settings (Figure 12) and that hypoxia is a relevant feature to consider when characterizing immune function *in vitro* in order to recapitulate *in vivo* tumour conditions.

Chapter 2 (*in vitro* / *in vivo*, mouse, E.G7 thymoma)

- T cell killing of target cells was decreased under 1.5% oxygen
- T cells produced less IFNγ and TNFα and slightly more granzyme B under 1.5 % oxygen
- T cells increased autophagy induction at 1.5% oxygen
- Autophagy deficiency did not impact the killing or cytokine defect observed at 1.5 % oxygen
- Autophagy deficient T cells had decreased expansion *in vivo*.



Chapter 3 (*in vivo*, human, high-grade serous carcinoma)

- T cells were most commonly found in oxygenated tumours with a higher vascular density score
- Patient survival was increased when FoxP3 was expressed in vascularized tumours compared to hypoxic, non-vascularized tumours.

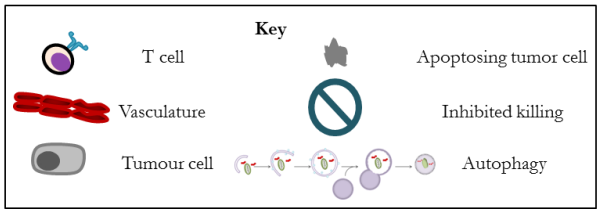


Figure 12: Main findings from Chapters 2 and 3.

4.2 Discussion and future directions tailored to unanswered questions

A number of scientific questions still remain following the completion of this thesis and may be used to direct future research. In Chapter 2 we identified a negative role for hypoxia in T cell function and found that autophagy is induced under this condition. We hypothesized that autophagy may be an energy source under hypoxia, allowing for processes such as cytokine secretion and killing activity to occur. Our data showed that cytokine production and killing activity were not drastically impacted *in vitro* when autophagy was inhibited in T cells, although we did identify an *in vivo* defect in T cell expansion during autophagy inhibition. It may be that if these *in vitro* studies were repeated at lower oxygen tensions or for extended time periods a defect in cytokine production or anti-tumour activity would become apparent. This is relevant given that pathophysiological oxygen levels can fall as low as 0 % in tumours [72-74]. A key remaining question is *"by what mechanism does hypoxia dampen CD8 T cell anti-tumour responses?"* Most recent reports suggest that HIF-1 mediates the differentiation of CD4 T cells, thus the transcription factors expressed during hypoxia skew cytokine profiles and this could potentially affect the ensuing anti-tumour response [43,110,111]. CD8 T cells are under strong regulation by their environment, thus cells experiencing hypoxia change their metabolism to induce glycolysis and reduce oxidative phosphorylation [101,102,145] potentially altering differentiation profiles. Indeed, CD8 memory formation is controlled by the induction of fatty acid oxidation [210], therefore changes in metabolism to glycolysis under hypoxia could alter memory T cell formation. As outlined in Chapter 1, a drastic reduction in T cell function occurs under low oxygen which is due to adenosine receptor signalling and HIF-1 stabilization in T cells, however, further work is required to determine the cause of dampened anti-tumour function in CD8 T cells. This thesis also implicates autophagy as a compensatory mechanism utilized by CD8 T cells

to function during conditions of low oxygenation. Future studies are required to determine the necessity of this mechanism.

A second remaining question from Chapter 2 is "*what is the role of hypoxia-induced autophagy in CD8 T cells?"* Based on the literature, I propose two mechanisms by which it may be required: 1) for the degradation of mitochondria which produce the toxic by-product ROS; and 2) for the provision of metabolites required for the energy-demanding process of cell division. While future studies will need to address these hypotheses in CD8 T cells, a study in mouse embryonic fibroblasts supports the first proposed mechanism finding that hypoxia-induced autophagy was important for ROS reduction [148]. In order to determine whether autophagy provides metabolites during conditions of hypoxia, the metabolic profile of effector CD8 T cells will need to be assessed. This baseline understanding of metabolism during hypoxia is required in order to assess the additional energy which is provided by autophagy. This thesis has demonstrated an energy deficit does exist as manifested by decreased T cell expansion in the absence of autophagy. This hypothesis is further supported by a study which found that cytokine production and ATP levels were decreased in autophagy-deficient T cells during primary activation, indicating that autophagy does indeed play a role in energy production [139]. However future studies assessing how ATP and ROS levels change in autophagy-deficient cells under hypoxia compared to autophagy-proficient cells will aid to further answer this question.

A clinical question which arises from Chapter 2 is *"should autophagy inhibitors be used in an immune cell-rich cancer setting during cancer treatment?"* The use of autophagy inhibitors in cancer has been gaining interest in recent years given the role of autophagy in promoting cancer cell survival during various clinical treatments such anti-angiogenesis therapy [204] and chemotherapy [211]. Indeed, the autophagy inhibitor CQ is currently being used in

numerous clinical trials for a variety of cancer types (Townsend, *et al.* Immunol. Rev. 2012 accepted). T cells are likely impacted by the clinical agents administered to cancer patients, thus they along with tumour cells, may use autophagy to survive these treatments. Whether an autophagy inhibitor should be used in an initially immune cell-rich cancer setting likely will depend on how harsh the clinical agent being used is. For example, certain chemotherapeutics such as high dose cyclophosphamide completely deplete all T cell subsets [212], thus autophagy inhibition will have no impact on immune cells as they are no longer present. However, agents such as anti-angiogenesis drugs which have been shown to induce hypoxia [203] may promote T cell hypoxia and thus autophagy inhibition in this context may be detrimental to the T cells given the results of Chapter 2. Overall this question likely requires different answers depending on the clinical agents administered to patients. Future studies which further determine the importance of autophagy in T cells during hypoxia may begin to answer whether autophagy should be inhibited during anti-angiogenesis treatments if a strong immune component is required to achieve a clinical response in the cancer patients. Ultimately, a decision may have to be made whether the risk of reducing T cell activity by inhibiting autophagy is outweighed by the potential enhancement of anti-tumour cytotoxicity of certain treatments.

In Chapter 3 we found that high-grade serous ovarian tumours expressing CD8, CD4, FoxP3, granzyme B or TIA-1 were most likely to be oxygenated as well. A remaining question from Chapter 3 is *"do T cells infiltrate hypoxic tumours and then die or do they specifically avoid, or proliferate less, in these areas?"* This question is difficult to answer in the human setting as IHC staining of tumours at a single time point is most often the only mode of analysis possible given the material available. Therefore, tracking T cells within patients to determine whether they do not infiltrate a hypoxic tumour at all or whether they die in this setting is

difficult to assess. Studies in mice have shown that T cells are less likely to be located in hypoxic tumour regions [206]. A second study showed that T cells were likely to be located in intermediate oxygen levels in lymphoid organs while avoiding more hypoxic regions [70]. *In vitro* human studies have also shown that T cells are able to survive periods of hypoxia, however, they do not proliferate under such conditions [101,213]. These cumulative results suggest that T cells avoid, or expand less in regions of hypoxia given that they are able to survive hypoxic conditions, however, the mechanism for this requires further study.

The survival outcomes for ovarian cancer patients are poor, thus alternative cancer treatments are required. Recent advances in immunotherapies including adoptive immunotherapy and anti-CTLA-4 treatment may serve to benefit ovarian patients given the evidence of a strong immune component in ovarian tumours [62,214]. Based on the findings from Chapter 3 a remaining question is *"is immune-boosting therapy a feasible option for ovarian cancers which are hypoxic?"* While the results of Chapter 3 are specific for an optimally debulked cohort of high-grade serous ovarian cancer patients and require further assessment in other ovarian cancer subsets, they suggest that immune-modulating therapy is a feasible option for ovarian cancer patients with hypoxic tumours. Non-vascularized tumours were less likely to contain immune infiltrates. However, patients with non-vascularized tumours and CD8-associated immune infiltrates did not have a statistically significant difference in their survival although they trended towards worse survival than patients with immune infiltrates in vascularized tumours. We did find that FoxP3-expressing cells were associated with the most significant survival outcomes when located in vascularized tumours, thus limiting FoxP3 expansion in non-vascularized, hypoxic tumours is ideal. The use of immune-boosting therapy in ovarian cancer is supported by studies which have assessed the feasibility of anti-CTLA-4 therapy [196,197] and adoptive immunotherapy [198-201] in ovarian cancer

patients. Given that we observed a trend towards decreased patient survival when immune cells infiltrated non-vascularized tumours, combination therapies aimed to reverse the negative effects of the tumour environment may add to the benefit provided by immune-modulating therapies as discussed below.

We found that hypoxia dampened immune function in Chapter 2 and that T cells are associated with an oxygenated tumour environment in Chapter 3, therefore a final question remains: *"how can we improve T cell function under hypoxia?"* The hypoxic tumour environment may limit clinical strategies aimed to induce beneficial immune responses. This suggests that combination treatments may be required in order to combat the immunosuppressive effects of hypoxia. Thus, several groups have suggested reversing the effects of hypoxia through novel mechanisms. One group indicated that inhibiting adenosine signalling in T cells by genetic inhibition of adenosine receptors and adenosine receptor agonists would allow for improvement of T cell responses in the tumour environment [120]. In addition, inhibition of molecules on T regs or tumours which mediate the conversion of adenosine such as CD73 may be beneficial in reducing the immunosuppressive effects of adenosine on T cells for optimal tumour killing [215]. Another study engineered a lentiviral vector to express IL-2 under the control of a HIF-1 response element in order to improve adoptively transferred CD8 T cell survival and proliferation during low oxygen [216]. This strategy could be used to promote the expression of other protective transgenes in T cells during hypoxia [216]. Given that genetic ablation of HIF-1 promotes T cell function, HIF-1 may serve as a beneficial target for molecular therapy [104,217,218]. In agreement, HIF-1 has been identified as a beneficial target in the cancer setting [219,220] thus HIF-1 inhibition may promote T cell activity while repressing tumour cells. HIF-1 inhibition in T cells would require further validation under hypoxia as T cells may require HIF-1 for regulation of important processes

such as glycolysis. Future studies such as those carried out in Chapter 2 and 3, will highlight the relevance of hypoxia on immune function and may promote the use of combination treatments in addition to immunotherapy treatments.

4.3 Outlook

In summary, the research performed and described in this thesis aimed to understand how the hypoxic features of tumours impact CD8 T cell function and to determine a role for autophagy in T cells under this condition. These findings have furthered the immunology field by considering a novel, relevant, autophagic inducer in T cells and the roles for its induction. This information adds to our understanding of how T cells function during conditions of low oxygen. Our results also suggest that boosting the anti-tumour T cell subset in high-grade serous ovarian cancer patients while limiting the impact of hypoxia would be beneficial for patient survival. Overall, these findings demonstrate that the CD8 T cell response is a crucial component of anti-tumour immunity and thus conditions such as hypoxia, which dampen their activity, should be considered when assessing the feasibility of cancer treatments for optimal patient responses.

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Appendix: Abbreviations

7-AAD – 7-aminoactinomycin D

Aa – Heterozygous floxed *Atg5*

AA – Homozygous floxed *Atg5*

ACT – Adoptive cell transfer

ACTO – Atg5, Cre, Thy1.1, OT-I

ADP – Adenosine diphosphate

AMP – Adenosine monophosphate

AMPK - AMP-activated protein kinase

APC - Allophycocyanin

ATF4 - Activating transcription factor 4

Atg – Autophagy-related

ATP – Adenosine triphosphate

Bcl-2 - B-cell lymphoma 2

Bcl-xL - B-cell lymphoma extra-large

BNIP3 - Bcl-2/adenovirus E1B 19-kDa interacting protein 3

BNIP3L - BNIP3-like

CD – Cluster of differentiation

CFSE – Carboxyfluorescein

CoCl2 – Cobalt chloride

CQ – Chloroquine

CTLA4 - Cytotoxic T lymphocyte antigen-4

DC – Dendritic cell

dH2O – Distilled water

DNA – Deoxyribonucleic acid

EDTA – Ethylenediamine tetraacetic acid

ER – Estrogen receptor

ETC – Electron transport chain

FBS – Fetal bovine serum

FIGO – International Federation of Gynecology and Obstetrics

FIP200 - 200 kDa focal adhesion kinase family-interacting protein

FITC – Flurorescien Isothiocyanate

fl - Flox

FoxP3 – Forkhead box P3

G418 - Geneticin

HCQ – Hydroxychloroquine

HIF-1 - Hypoxia inducible factor – 1

HMGB1 - High mobility group box 1

Hr – Hour

Hyp – Hypoxia

IFNγ - Interferon γ

Ig - Immunoglobulin

IHC - Immunohistochemistry

IL – Interleukin

LC3 - Microtubule-associated protein light chain 3

MDSC – Myeloid derived suppressor cell

MHC – Major Histocompatibility Complex

mRNA – Messenger ribonucleic acid

mTOR – Mammalian target of rapamycin

mTORC1 - Mammalian target of rapamycin complex 1

NFκB - Nuclear factor κβ

NK - Natural Killer

Norm – Normoxia

OVA – Ovalbumin

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PE - Phosphatidylethanolamine

Pe - Phyco-erythrin

PERK - PKR-like ER kinase

PHDs – Prolyl1-4-hydroxylase domain proteins

PMA - Phorbal myristate acetate

qPCR – Real-time quantitative polymerase chain reaction

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulphate

SEM – Standard error of the mean

SIINFEKL – serine, isoleucine, isoleucine, asparagine, phenylalanine, glutamate, leucine, lysine

Tam - Tamoxifen

TBST – Tris buffered saline tween – 20

TCA - Tricarboxylic acid

TGFβ – Transforming growth factor β

Th – T helper cell

TIA-1 - T cell intracellular antigen-1

TMA – Tissue-microarray

TNFα - Tumour necrosis factor α

T reg - T regulatory cell

Tx – Treatment

ULK1 - Unc-51-like kinase-1

VHL – von Hippel-Lindau protein

Vps - Vacuolar protein sorting