THE EFFECT OF HYPOXIA INDUCIBLE FACTOR-1 ON THE EXPRESSION OF THE COINHIBITORY LIGANDS B7-H3 AND B7-H1 IN CANCER: RELEVANCE TO CANCER IMMUNE ESCAPE

by

Chelsea Anne Smallwood

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Queen’s University
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Abstract

The interactions between tumour cells and cells of the immune system are important in the natural evolution of cancer, and the acquired immune system plays an integral role in cancer immune escape. B7-H3 and B7-H1 ligands provide coinhibitory signals to T cells resulting in T cell anergy or apoptosis and their expression has been shown to increase in cancer cells. Tumour hypoxia (oxygen concentration below physiological level) is a major contributor to the spread of cancer and resistance to radiation and chemotherapy. We proposed that hypoxia results in the upregulation of the B7 molecules B7-H3 and B7-H1. Furthermore, studies in our laboratory have shown that acquisition of malignant properties in tumour cells exposed to hypoxia can be inhibited by low concentrations of nitric oxide mimetic agents such as glycercyl trinitrate (GTN). Using cultured breast and prostate cancer cells, we investigated whether the hypoxia-inducible factor HIF-1α, would mediate an upregulation of these ligands. Using a mouse model, we investigated the effect of GTN on tumour growth in vivo. For the in vitro studies, we exposed MDA-MB-231 and MCF-7 breast cancer cells and DU-145 prostate cancer cells to standard culture conditions, hypoxic conditions, or 100 μM CoCl₂ (stabilizes HIF-1α) for 24 hours. Our findings indicate that B7-H3 mRNA was upregulated in hypoxia (P = 0.0101). Contrary to our hypothesis, B7-H3 protein was not upregulated in hypoxia. Interestingly, increased B7-H1 protein expression correlated with increased HIF-1α expression (r²=0.48, P<0.0001), and HIF-1α bound to the hypoxia response element (HRE) of B7-H1. These results indicate a role for HIF-1α in the upregulation of B7-H1 levels in MDA-MB-231 cells. While in vitro studies indicated no effect of GTN, a study using female BALB/c mice injected with 4T1 mammary
carcinoma cells resulted in a decrease in tumour volume in the GTN treated mice. Together, these results indicate a novel role for HIF-1α in the up-regulation of B7-H1 on cancer cells, thus potentially contributing to immune escape of cancer cells and additionally, a role for GTN as a possible breast cancer therapy.
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List of Abbreviations

2-OG (2-oxoglutarate)
APC (Antigen presenting cell)
B7-H (B7- homologue)
bHLH-PAS (b Helix-loop-helix-Per Arnt Sim)
ChIP (Chromatin Immuno-precipitation assay)
CBP (CREB binding protein)
CcO (Cytochrome c oxidase)
ccRCC (Clear cell renal cell carcinoma)
cGMP (Cyclic guanosine monophosphate)
CTL (Cytotoxic lymphocyte)
CTLA-4 (Cytotoxic T lymphocyte antigen-4)
DC (Dendritic cell)
eNOS (endothelial NOS)
FIH1 (Factor-inhibiting HIF-1)
GTN (Glyceryl trinitrate)
GTP (Guanosine tri-phosphate)
HIF-1 (Hypoxia inducible factor – 1)
HRE (Hypoxia response elements)
IFN-γ (Interferon –gamma)
ICOS (Inducible T cell COStimulator)
IL-2 (Interleukin-2)
IL-10 (Interleukin-10)
IL-12 (Interleukin-12)
MAPK (Mitogen-activated protein kinase)
mB7-H1 (murine B7-H1)
Mdm2 (Murine double minute)
MHC (Major histocompatibility complex)
MIC (MHC class I chain related molecules)
NK (Natural killer)
NKT (Natural killer T cell)
NO (Nitric oxide)
NOS (Nitric oxide synthase)
nNOS (neuronal NOS)
NSCLC (Non-small cell lung cancer)
ODD (Oxygen-dependent degradation domain)
PCR (Polymerase chain reaction)
PD-1 (Programmed death-1)
PD-L1 (Programmed death ligand-1)
PDE (Phosphodiesterase)
pfp (Perforin)
PHD (Prolyl hydroxylase)
PI3K (Phosphatidylinositol 3-kinase)
PKG (Protein kinase G)
PSA (Prostate specific antigen)
PTEN (Phosphatase and tensin homologue)
qPCR (quantitative PCR)
ROS (Reactive oxygen species)
sGC (soluble guanyl cyclase)
siRNA (Small interfering RNA)
TAA (Tumour Associated Antigen)
TAD (Transactivation domain)
TCA (Tricarboxylic acid)
Tm (melting temperature)
TNF-α (Tumour necrosis factor- alpha)
TRAIL (TNF-related apoptosis inducing ligand)
Treg (T regulatory cells)
TREM (Triggering receptor expressed on myeloid cells)
TLT-2 (TREM-like transcript 2)
VEGF (Vascular endothelial growth factor)
VHL (von Hippel-Lindau)
Chapter 1

Introduction

1.1 Cancer biology and immunology

Cancer comprises over 100 distinct diseases that may arise from essentially every type of human cell. According the Canadian Cancer Statistics 2012, every hour 21 new cases of cancer will be diagnosed, and 9 people will die from cancer in Canada (1). These statistics indicate an urgent necessity for novel and efficacious treatments.

A tumour is characterized as a mass of rapidly proliferating cells of host origin. The tumour comprises a heterogeneous population of cells, possessing a variety of mutations and epigenetic alterations that enable tumour cells to propagate, invade, and metastasize to distant tissues where they may form secondary tumours. This is the most dangerous aspect of cancer as the secondary tumours often lead to therapeutic failure (2). The steps leading to metastasis involve local invasion of host tissue followed by intravasation to nearby blood and lymphatic vessels, extravasation and the ability to engraft in the new environment (2, 3). These are intricate and complex processes, and are therefore being thoroughly investigated to determine modes of intervention. A challenge, though, is that the genetic and epigenetic alterations confer upon this population varying responsiveness to treatment. Since cancer cells are remarkably plastic – their genetic and epigenetic instability lends them the ability to adapt to environmental selective pressures and, as a result, develop resistance to therapies. Moreover, the phenotypic heterogeneity
of a tumour results in cells with varying degrees of susceptibility to elimination by the immune system.

### 1.1.1 Immunoediting: elimination, equilibrium, escape

For multiple advanced cancers, chemotherapy remains the route of treatment choice in terms of management, although it fails to result in a cure for many patients. Chemotherapeutic agents tackle cancer in unique ways, and in their course they have the potential to modulate the immune system (4). Because a tumour is a complex of interacting cells including tumour cells, the surrounding stroma, and infiltrating cells, the entirety of the tumour microenvironment must be considered when developing anti-cancer strategies, especially immunotherapy. Understanding how the immune system may be altered will greatly contribute to the manner in which therapies are administered to patients.

In 1909, Paul Erlich proposed the concept of immunosurveillance of tumours, imparting upon the immune system a role in preventing the proliferation of tumour cells (5). Recent advances in the field of cancer research have provided insight that immunosurveillance represents only one aspect of a process of complex interactions between tumour cell populations and immune cells. This process is referred to as cancer immunoediting (6). A growing tumour is indicative that cancer cells have managed to evade the immune system, but by what means? The answer may lie in cancer immunoediting, which is comprised of the three phases: elimination (immunosurveillance), equilibrium, and escape (Figure 1.1.1).
Figure 1.1.1  Cancer immunoediting
The process of immunoediting consists of three phases: elimination, equilibrium and escape. The elimination phase is characterized by detection and elimination of cancer cells (orange) by the immune system. Should this fail, cancer cells may exist in equilibrium with the immune system, whereby the tumour remains dormant. During this stage, cancer cells may acquire new mutations (yellow) that confer an increased ability to resist elimination. Finally, cells with immune suppressive or that have lost immunogenicity persist and escape the immune system (orange + yellow).
1.1.1.1 Elimination

The elimination or ‘surveillance’ stage of cancer immunoediting involves both the innate and adaptive arms of the immune system. This is the phase during which the immune system scans for transformed cells displaying danger signals and destroys such cells. The most convincing evidence for the elimination phase is the observed spontaneous regression of tumours that occurs simultaneously with an expansion of T cells, suggesting a role of T cells in tumour eradication (7). Tumour elimination has been described as a four-stage process involving the innate and adaptive components of the immune system (8). The first stage occurs at the onset of the requirement for vasculature and remodeling of the surrounding stroma by the growing tumour. The tumour at this stage secretes cytokines - allowing for the infiltration of inflammatory cells. The innate immune cells such as natural killer (NK) cells and natural killer T cells (NKT) (which bridge the innate and adaptive immune systems) are recruited by inflammatory cytokines produced by tumour cells, surrounding macrophages and stromal cells. The second stage is characterized by the production of cytokines such as interleukin-12 (IL-12) and interferon-γ (IFN-γ) by the innate NK and NKT cells, in turn activating cytotoxic mechanisms. These cells then exhibit their cytotoxicity through perforin (pfp) and tumour necrosis factor TNF-related apoptosis inducing ligand (TRAIL), in effect killing the tumour cells (8). The destruction of tumours cells leads to the release of tumour-associated antigens (TAA), which are then ingested by dendritic cells (DC). This marks the initiation of the third stage of elimination that involves cross talk between the innate and adaptive immune systems, promoting DC maturation. The DCs then migrate to
tumour draining lymph nodes where they present TAA to CD4$^+$ T cells (8). This step is followed by a subsequent maturation of the CD4$^+$ T cells to specific CD8$^+$ T cells capable of travelling to the tumour site and execute targeted killing of tumour cells both directly and through the production of IFN-γ, which denotes the fourth and final stage (8).

### 1.1.1.2 Equilibrium

The second phase of immunoediting, equilibrium, manifests when the elimination of cancer cells by the immune system is unsuccessful. While in equilibrium with the immune system, though, the tumour’s expansion remains controlled (7); the tumour cells are being continuously eliminated, during which time less immunogenic cells are selected for and survive. This phase is characterized as a period of latency and “Darwinian selection” (8). At this stage in the process, clinical symptoms may not have emerged. A tumour consists of a heterogeneous population of cells. During the equilibrium phase it is thought that new alterations leading to more robust phenotypes (i.e. greater fitness) contribute to the modified interaction between tumour and immune system. As outlined previously, cancer genomes are variable due to the genetic instability and this may give rise to cells with reduced immunogenicity (5). Essentially, as the immune system eliminates cells with high immunogenicity, cells that have acquired low immunogenicity will persist. Chemically induced tumours in nude mice had higher immunogenicity than those grown in immunocompetent mice, lending evidence for the immune selection of resistant tumour variants (9). The equilibrium phase is the longest of the three processes.
as it has been postulated that the possible outcomes include eventual elimination, a state of permanent equilibrium, or immune escape (5).

1.1.1.3 Escape

The tumour that “ultimately emerges from the equilibrium phase is instructively shaped by the repertoire of immune ‘editors’ in its local environment” (5); this process has thus selected for these cells to transition to the escape phase of immunoediting. As a result of either loss of immunogenicity or gain of immunosuppressive features, the tumour has acquired the ability to evade both innate and adaptive immune systems. The escape phase of immunoediting involves the proliferation of tumour cells that have escaped recognition and destruction by the immune system. At this stage the tumour grows in an unrestricted manner and as such becomes clinically detectable. There are various potential mechanisms of immune escape, including lack of tumour antigen recognition by immune effector cells, loss of vulnerability to cytotoxic lymphocyte induced cell death, and gain of immune suppressive factors by the tumour cells. These phenomena may result from genetic/epigenetic changes as well as microenvironmental pressures. Mechanisms involved in downregulating major histocompatibility complex (MHC), the molecules responsible for presenting antigens to the immune cells, and the expression of soluble suppressive factors, among others, contribute to the ability of tumours to escape the immune system (7). For example, NKG2D on NK cells is involved in tumour cell lysis upon ligation to NKG2D ligands. Tumours have the ability to escape NK cell mediated killing through shedding of the soluble NKG2D ligand, MHC
class I chain (MIC) related molecules. Upon shedding of MIC molecules, there is an apparent down-regulation of NKG2D on NK cells (10). Furthermore, in the absence of appropriate costimulation, T cells undergo anergy -which is the inability of the T cell to perform its effector functions. Tumours may also express various T cell-inhibitory molecules including members of the B7 family of immune regulators, which may also lead to T cell anergy and subsequent escape from acquired immunity.

1.2 Acquired immunity

1.2.1 T cells and antigen presenting cells

The journey upon which cancer cells must embark to reach clinical cancer status involves escaping critical steps including the equilibrium in which they co-exist with the immune system. Both the innate and adaptive immune systems are involved in eliminating cancer cells, although CD8⁺ cytotoxic T-lymphocytes (CTL) are the most effective anti-cancer immune cells (11). In order for the anti-tumour response by T cells to be effective, there are certain milestones that must be realized. Firstly, tumour antigens must be present and accessed by antigen-presenting cells (APCs), for example dendritic cells (DC), in the draining lymph nodes. APCs have a crucial role in regulating the adaptive immune response as they manage all T-effector cell subsets (12). Once engaged, the APC must prime T cells and the T cells must then respond by proliferating. Circulating T cells will then enter the tumour and, upon surviving local
immunosuppressive molecules, destroy the tumour cells. Lastly, memory cells should be generated to produce a sustained anti-tumour response (4).

MHC molecules are necessary to present antigen on the cell surface. While all nucleated cells express MHC class I and thus have the ability to present antigens, only professional APCs have the additional costimulation needed to activate naïve T cells (11). Endogenous antigen is transported to the endoplasmic reticulum and subsequently sent to the plasma membrane to be presented on MHC class I molecules. Conversely, exogenous antigens such as those from tumours must be taken up by APCs in endosomes and transported to the plasma membrane to be presented by MHC class II molecules (13). Dendritic cells, which are a type of professional APC, may also “cross-present” exogenous antigens on MHC class I molecules to CD8⁺ CTLs (11).

The DC is important in determining the destiny of the T cell it binds and the state of maturation of the DC plays a significant role. Whereas activated DCs have the ability to cross-prime T cells, inactivated DCs may cross-tolerize T cells (13). Mature DCs result in the upregulation of MHC, increased antigen uptake and most importantly, in the context of T cells, express the costimulatory B7-1 and B7-2 molecules (11, 14). These molecules provide a second stimulation to T cells, ensuring their activation.

Should there be inadequate T cell costimulation during the priming phase the T cell could become anergic. Anergic T cells may not undergo expansion or produce memory cells. Anergy may also occur after the initiation phase during clonal expansion (11). Moreover, in the case of a growing tumour, T cells will be chronically exposed to antigen and this may also have an ensuing tolerizing/anergic effect. Of note, any or all of
the aforementioned aspects of acquired immunity may be modulated within a tumour to ensure the escape and survival of cancer cells.

1.2.2 B7 family of immune regulators

The ligands that comprise the B7-family of cell surface proteins regulate the immune response upon interaction with their cognate receptors on the surface of various immune cells such as T cells. T cell activation is initiated by an engagement with an antigen, although a secondary costimulatory signal is required. The costimulatory ligands are responsible for enhancing the secretion of cytokines, as well as promoting T cell proliferation and survival. Of the costimulatory molecules, the CD28 receptor/ B7-1 and B7-2 interaction has been identified as a chief player in the costimulatory pathway. Costimulation by the binding of B7-1 or B7-2 to CD28 results in both the differentiation of T cells as well an overall enrichment of the immune response (15). On the other hand, in the absence of a costimulatory signal, T cell tolerance occurs, whereby the T cell undergoes anergy (16). To further complicate the matter, while costimulation is important for the activation of T cells, more so for CD4+ T cells, there also exist coinhibitory molecules, which may signal to the T cell after the initial activation.

Coinhibitory signals result in the inactivation of T cells with the potential for the induction of apoptosis. Hence, an elaborate network of costimulatory and coinhibitory signaling is required to ensure specific and well-controlled T cell responses. While signaling through CD28 by B7-1 and B7-2 provide costimulation, these molecules bind to a second receptor, cytotoxic T lymphocyte antigen-4 (CTLA-4), with a higher affinity
The CTLA-4/B7 interaction provided the framework for understanding costimulatory signals and also coinhibitory signals as CTLA-4 interacts with B7-1 and B7-2 in an inhibitory fashion. The CTLA-4 ligation results in an attenuation of the T cell activation and a resulting decrease in T cell activity, including the production of the pro-inflammatory cytokine interleukin-2 (IL-2) and a decrease in cell cycle progression (12).

As previously discussed, many tumours lack the ability to induce an effective immune response due to a loss of immunogenicity, and therein a lack of costimulatory signals. It follows that the introduction of B7-1 and B7-2 to tumour cells may immunosensitize the tumours through the activation of the immune system. Tumours expressing B7-1 and B7-2 prior to their implantation in vivo lead to spontaneous tumour rejection (18). Thus, enhancing the tumour’s immunogenicity leads to its destruction by the immune system. The same result, though, was not found to be true using these B7 molecules for pre-established tumour therapy in vivo (19). Indeed, when introduced into the clinical setting, B7-1 therapies have been met with limited success in cancer patients, stimulating a further investigation into the mechanisms of immune escape of cancer cells (20).

The B7-1 and B7-2 ligands belong to Group I of three groups of B7 family members, including B7 homologues (B7-H). This first group includes the aforementioned B7-1 and B7-2, as well as B7-H2, which is responsible for CD4+ T cell activation (16). The group II B7 molecules comprise the ligands of programmed death – 1 receptor (PD-1), B7-H1 and B7-DC. The primary function of group II B7 molecules is immune tolerance. Lastly, the group III B7 molecules B7-H3 and B7-H4 have unidentified receptors (16), and therefore their role in immunology remains, in part, elusive.
B7-H1 is one of the more established of the T cell co-inhibitors and interacts with PD-1. PD-1 is a molecule located on the surface of activated T cells and B cells. Its ligand was first identified as programmed death ligand-1 (PD-L1), which was later found to be B7-H1. Importantly, PD-1 is expressed several times higher on TIL effectors compared to intratumoural Tregs (21). While less is known about B7-H3, its expression in many human cancers suggests a role for this ligand in cancer immune escape.

1.2.2.1 The B7 family: B7-H3

B7-H3 is a transmembrane protein that is expressed across a broad range of tissue types; high mRNA levels were detected in heart, liver, placenta, prostate, testis, uterus, pancreas, small intestine and colon with low expression in other (including lymphoid) organs (22). B7-H3 has been found in many cancer types, and due to its expansive expression it has been postulated that the role of B7-H3 may extend beyond its immunological functions. Thus far, its role in both immunology and cancer has been a topic of debate. Immunologically speaking, the first functional studies demonstrated a stimulatory effect of B7-H3. Indeed, the in vitro work by Chapoval et al. indicates that B7-H3 induction increases the proliferation and growth of CD4+ and CD8+ T cells, stimulates production of IFN-γ, and enhances CTL activity (22). In the context of the in vivo role of B7-H3 in cancer, tumours that were injected with B7-H3 completely regressed in half the mice, with the remaining treated tumours significantly slowed in their growth rate (23). In this study, it was determined that the effect of B7-H3 depended largely on the CD4+ T cells, CD8+ T cells, and NK cell subsets. Furthermore, syngeneic
mouse tumour cells were transfected with B7-H3 prior to inoculation to mice - leading to complete regression in half the transfected tumours and an increase in tumour specific CD8$^+$ CTLs (24). The stimulatory function of B7-H3 via CD8$^+$ T cells has been illustrated in various other in vivo models (25).

In contrast, there is both in vitro and in vivo evidence for the inhibitory role of B7-H3. Human B7-H3 can exist as one of two isoforms, 2IgB7-H3 or 4IgB7-H3. While comparing the two isoforms, it was found that functionality of B7-H3 contradicted what had been previously established. B7-H3, both 2Ig and 4Ig, were shown to inhibit CD4$^+$ T cell activation by interfering with both proliferation and cytokine production, including tumour necrosis factor-α (TNF-α) and IFN-γ (26). Moreover, the results of in vitro and in vivo studies by Suh et al. demonstrated that B7-H3 protein could inhibit CD4$^+$ and CD8$^+$ T cell proliferation, and that B7-H3 deficient B lymphoblasts had a greater capacity to stimulate T cell activity (27). Murine studies subsequently indicated that B7-H3 interferes with T cell activation and proliferation via inhibition of IL-2 production (28). In fact, B7-H3 has the capacity to impair T cell stimulatory activity when induced on DCs by Tregs (CD4$^+$CD25$^+$) (29).

Retrospective analyses have illustrated the dual-nature of B7-H3 expression in human cancers – having both beneficial and adverse effects. For example, B7-H3 may have a putative stimulatory role in pancreatic and gastric cancers, as it has been correlated with prolonged survival in these cancer types (25). In contrast, in non-small cell lung cancer (NSCLC), clear cell renal cell carcinoma (ccRCC), prostate, colorectal and ovarian cancers, B7-H3 expression has been correlated with increased metastasis, high stage disease, increased risk of death and cancer progression (25).
Due to its implication as a negative immune regulator in prostate cancer, various groups have been investigating the role of B7-H3 as a diagnostic and therapeutic target (30). To fully elucidate the immunologic and potential non-immunologic functions of B7-H3 it will be necessary to resolve its receptor(s). When B7-H3 was first discovered, it was established that it was not a ligand for the established B7 receptors: CD28, CTLA-4, Inducible T cell COStimulator (ICOS) or PD-1 (22). To date, there has been one receptor identified - Triggering receptor expressed on myeloid cells (TREM)-like Transcript 2 (TLT-2) - which is expressed constitutively on CD8^+ T cells and induced on CD4^+ T cells upon activation (31). Notably, Leitner et al. found no interaction between B7-H3 and TLT-2 (32). As such, there remains much work to be done in establishing well-defined receptors for B7-H3. The role of B7-H3 in cancer is important, and thus elucidating its mechanisms of regulation and downstream effects will provide insight to its role in cancer.

1.2.2.2 The B7 family: B7-H1/PD-L1

B7-H1 is a transmembrane glycoprotein with its mRNA expressed in many tissue types including heart, skeletal muscle, placenta, and lung (17). The protein, though, is found predominantly in activated immune cells such as activated T and B cells. In addition, high levels of B7-H1 have been found in most human cancers (33, 34). B7-H1, a homologue of the B7 costimulators B7-1 and B7-2, was discovered in 1999 as a costimulator of T cells leading to the secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) (17). Dong et al. found that when B7-H1 was immobilized on
tissue culture plates, T cell proliferation was enhanced and that this effect was dependent on IL-2 (17).

Upon evaluation of murine B7-H1 (mB7-H1), it was determined that this molecule enhances CD4+ T-helper responses in vivo rather than inducing CTLs to respond to MHC antigens (35). In addition, this group found that mB7-H1 costimulates IL-10 and IFN-γ secretion, confirming their previous human in vitro results. While these early studies define a costimulatory role for B7-H1, there appears to be a dual immunologic function for this ligand.

At the time of the discovery of B7-H1 as an immunomodulator, its receptor was unknown, though Dong et al. had concluded that the potential receptor(s) excluded CD28, CTLA-4 and ICOS (17). Soon after it was determined that the programmed death-1 (PD-1), found on mature T and B cells post-activation (36) and involved in programmed cell death (37), was a receptor for B7-H1 (38). Indeed, B7-H1 delivered a pronounced inhibitory signal to CD3 induced T cell proliferation and cytokine secretion. Due to conflicting results, Freeman et al. postulated that there might exist multiple receptors for B7-H1 leading to the differential responses in vitro (38-40). Furthermore, while signaling through PD-1 by B7-H1 results in apoptosis of T cells, it should be noted that B7-H1 was able to deliver apoptotic signals in the absence of PD-1 on T cells (41), indicating that B7-H1 engagement with other yet to be discovered receptors may convey inhibitory signals.

In addition to the findings that B7-H1Ig stimulated IL-10 production, it was also determined that anti-B7-H1 antibody could interfere with DC induced Treg IL-10 production (42), which is involved in memory CD4+ anergy and Treg induction (43).
These combined findings lead to the concept that B7-H1 may be involved in negative regulation of immune responses.

Tumour cell lines, including lung, colon, breast, placenta, melanoma and glioma, express B7-H1 (34), and those cell lines that express limited levels may be stimulated with IFN-γ to increase B7-H1 protein expression (41, 44). B7-H1 works through a number of mechanisms to allow immune evasion by expressing cells. While B7-H1 signaling through PD-1 or other receptors results in T cell apoptosis, immune suppression induced by B7-H1 also occurs via T cell anergy, increased secretion of the immune-inhibitory cytokine IL-10 as well as promotion of Tregs (41). Moreover, B7-H1 expression reduced the susceptibility of tumour cells to T cell mediated lysis in vitro, and increased their invasiveness and tumourigenicity in vivo (45). Thus, B7-H1 may play a role in cancer immune evasion, and hence represents an attractive target for immune-based therapies.

As outlined previously, B7-H1 mRNA transcript is widely expressed in many tissue types, though the protein expression is limited. In cancers, though, B7-H1 protein expression is high – indicating a post-translation regulatory mechanism during oncogenesis or malignant progression (46). In recent years, multiple explanations for the regulation of B7-H1 expression have been proposed, though a full mechanism of induction has yet to be elucidated (46-48). Thus, a more broad investigation of the regulation of B7-H1, including microenvironmental factors, will provide useful insight into the mechanisms of cancer immune escape.
1.3 Tumour microenvironment

1.3.1 Hypoxia and the metastatic phenotype

The tumour microenvironment has significant implications in cancer as both the cancer cells themselves, as well as the supporting stroma, contribute to the metastatic potential of the disease. Cancer cells proliferate at abnormal rates and as such, the tumour vasculature is unable to maintain an adequate supply of oxygen and nutrients to the highly metabolically active malignant cells. Tumour vessels also often lack structural integrity, which results in defective microcirculation and increased diffusion distances, thereby further limiting oxygen and nutrient delivery. In effect there is an imbalance between oxygen supply and the requirements of the cells. Tumour hypoxia is defined as oxygen tensions below physiological levels \((O_2 \leq 10 \text{ mmHg})\) and is a hallmark of many solid tumours (49). Hypoxia occurs at a diffusion distance greater than 70 µm (generally 100-200 µm) from blood vessels (Figure 1.3.1) (50, 51). There have been various methods described to measure tumour hypoxia including imaging by blood oxygen level-dependent magnetic resonance, using scanning agents that are hypoxia-activated, the use of a polarographic oxygen electrode, as well as analysis of hypoxia induced genes by immunohistochemistry (52). Tumour hypoxia has been described in many cancers including cancer of the breast (53), cervix (54), prostate (55), brain (56), and pancreas (57), and has been correlated with poor patient prognosis.

Hypoxia contributes to an increased aggressiveness of a tumour primarily through the activity of the Hypoxia Inducible Factor-1 (HIF-1), a transcriptional activator of many hypoxia-inducible genes. Tumours with hypoxic areas also have increased genetic
instability, metastatic phenotypes and invasive properties (58). Moreover, hypoxia leads to tumour resistance to chemotherapy and radiation therapy (59, 60), lending to an overall more advanced malignant progression (61).

The effect of hypoxia is multifactorial, for it does not have one single role in promoting tumour growth. Tumour hypoxia also contributes to cell demise and death, for example through severe hypoxia (or anoxia) in conjunction with acidosis, the apoptotic molecule p53 becomes stabilized through HIF-1α accumulation (50, 62, 63). Advances in the area have provided further insight to the dual nature of hypoxia, suggesting a continuum from cellular adaptations to therapeutic resistance and on to apoptosis. A deeper exploration of the many functions of HIF-1 will shed light on the matter.
Figure 1.3.1  Tumour hypoxia
Due to the inherent nature of tumours, the vasculature becomes chaotic as a result of rapidly proliferating cells. This leaves hypoxic regions of cells, at diffusion distances greater than 70 µm from blood supply (generally 100-200 µm). The gradient from orange to navy represents decreasing oxygen concentrations.
1.3.2 HIF-1α

HIF-1 consists of two subunits, the constitutively expressed oxygen independent HIF-1β subunit and the oxygen-sensitive HIF-1α subunit. When the two subunits dimerize, they form an active complex that is translocated to the nucleus where it acts as a transcription factor. The HIF-1 complex may bind any DNA sequence with hypoxia response elements (HRE) and induce transcription of its target genes (49). Due to the oxygen-sensitive nature of HIF-1α, this molecule serves as an oxygen sensing mechanism.

HIF-1α may be regulated at the level of mRNA expression, protein translation, protein degradation, in addition to its ability to bind DNA and its transcriptional activity. The primary mode of HIF-1α accumulation is through the oxygen-dependent protein degradation pathway.

HIF-1 exerts its effects as a result of stabilization under low oxygen conditions. Under normal tissue oxygenation, HIF-1α is rapidly degraded; HIF-1α has a half-life of less than five minutes in oxygenated tissue (58). The HIF-α subunit contains two oxygen-dependent degradation domains (ODD), one N-terminal, the other C-terminal (64). With oxygen and 2-oxoglutarate (2-OG), an intermediate of the tricarboxylic acid (TCA) cycle, as its cosubstrates and ferrous iron and ascorbate as cofactors, prolyl hydroxylase domain proteins (PHD) hydroxylate proline residues at position 402 and 564 in the HIF-α ODDs (63). Once hydroxylated, HIF-α becomes susceptible to poly-ubiquitination by von Hippel-Lindau protein (VHL)/E3 ubiquitin ligase complex. Upon poly-ubiquitination, HIF-α is degraded by the 26S proteasome (Figure 1.3.2).
Figure 1.3.2  HIF-α degradation pathway
Under conditions where oxygen is not limiting, HIF-α DNA undergoes hydroxylation in the oxygen-dependent degradation domain (ODD) by prolyl hydroxylases (PHDs), which require oxygen and 2-oxoglutarate (2-OG) as substrates. This renders HIF-α susceptible to polyubiquitination by von Hippel Lindau protein and subsequent 26 S proteasomal degradation.
Since oxygen is a limiting factor for the PHD-mediated hydroxylation of HIF-α, under hypoxic conditions HIF-α cannot be degraded.

During hypoxia, the stabilized HIF-1α is localized to the nucleus, where it forms a heterodimeric complex with HIF-1β before binding to HREs. The HIF-1α protein contains a bHLH-PAS domain in its N-terminus and this directs dimerization to the HRE in the target DNA. Once bound, transcription must be activated through the formation of a complex at a transactivation domain (TAD). HIF-1α contains two TADs, one that is oxygen sensitive at the C-terminus (C-TAD) and another at the oxygen independent N-terminus (N-TAD). The transcription-initiation complex may be formed at either TAD, though requires the cofactors CREB-binding protein (CBP), p300 and others. These two particular cofactors are important in the regulation of HIF transcriptional activity.

A second mechanism of HIF-1α stabilization is through nitric oxide mediated S-nitrosylation. Higher (micromolar) concentrations of nitric oxide (NO) under normoxic conditions can lead to S-nitrosylation of the ODDs of HIF-1α, consequently preventing the binding of PHDs and hydroxylation (65). There have been a number of other mechanisms implicated in HIF-1α stabilization. These include stabilization via reactive oxygen species (ROS) from the mitochondrial electron transport chain and limited availability of the PHD co-substrate 2-OG from inefficient mitochondrial respiration (58). In addition, altered metabolism (characteristic of cancer) can lead to perturbed TCA cycle function and increased TCA intermediates such as succinate and fumarate, which are structurally similar to 2-OG and may compete for PHD interaction (66). HIF-1 transcriptional activity can also be regulated via hydroxylation of an asparagine residue in the C-TAD of HIF-1α. This reaction is catalysed by the asparaginyl hydroxylase Factor-
inhibiting HIF-1 (FIH-1), also in an oxygen-dependent manner. Hydroxylation of the C-
TAD asparagine residue of HIF-1α prevents the recruitment of the previously described
transcriptional co-activators CBP and p300, thereby preventing HIF-α transcriptional
activity (67).

HIF-1α synthesis is another aspect that must be considered in terms of expression
of HIF-1. HIF-1α synthesis is mediated through growth factor signaling via
phosphatidylinositol 3-kinase (PI3K) or mitogen activated protein kinase (MAPK) and as
such, growth factors influence HIF-1 levels in a cell-type dependent manner (68). In
addition to the downstream effects of HIF-1 in promoting cancer progression, it should be
noted that mutations resulting from the genetic instability of tumours such as alterations
in VHL and others can in turn lead to HIF-1α stabilization (63). Studies have
demonstrated that uncontrolled signaling of stem cell factor through c-kit (leading to
malignancy) also stabilizes HIF-1 at normoxia through PI3K and Ras/MEK/Erk based
mechanisms (69). Furthermore, it should be noted that culture conditions of cancer cell
lines could result in HIF-1 stabilization. Previous work from the Graham lab has
indicated that cell density positively correlated with levels of HIF-1α and transcriptional
activity of HIF-1 (70).

It is apparent that HIF-1 may be regulated by numerous means, and that its
downstream targets may contribute to cancer progression. Work in our lab has
demonstrated the ability of hypoxia, through HIF-1 activity, to induce the shedding of the
aforementioned MIC molecule (here specifically MIC-A) leading to resistance to innate
immunity in prostate cancer cells (71, 72). These hypoxic effects mediated through HIF-
1 were abrogated by treatment with the nitric oxide (NO) mimetic GTN.
1.4 Nitric oxide signaling

Nitric oxide (NO) is an inorganic, uncharged molecule that has traditionally been known for its role in regulating cardiovascular and respiratory responses. These effects are a result of the classical NO-cGMP signaling pathway in vascular smooth muscle cells (65). Additionally NO has been characterized as having a role in preventing platelet aggregation, as a cytotoxic molecule involved in pathogen killing (73), as well as pathological implications (74). The production of NO occurs via a reaction catalysed by nitric oxide synthase (NOS) enzymes. There are three NOS enzymes – neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). These enzymes catalyze the production of NO and L-citrulline from L-arginine and oxygen as substrates, with NADPH as a reducing agent and calmodulin present (75, 76). The NOS enzymes may be distinguished based on localization, their inducible versus constitutive status, and calcium dependence (77).

There are three major signaling pathways in which NO partakes. The most well known is the classical NO signaling pathway through soluble guanyl cyclase [sGC], a heterodimeric hemoprotein that is involved in numerous signal transduction pathways (78). The binding of NO to the heme moiety of sGC catalyzes the conversion of GTP to cyclic guanosine monophosphate (cGMP). Cellular cGMP subsequently activates protein kinase G (PKG) leading to phosphorylation of PKG downstream targets in addition to gating ion channels and regulating phosphodiesterases (PDE) which in turn alter cyclic nucleotide concentrations (79). Two alternative NO signaling pathways exist. A second pathway involves an interaction between NO and mitochondrial Cytochrome c Oxidase
(CcO) (80-82) and finally a non-classical pathway involving posttranslational modification of target proteins through S-nitrosylation, S-glutathionylation, and tyrosine nitration (83).

1.4.1 NO mimetics as a cancer therapy

The role of nitric oxide in tumour pathophysiology has been controversial, and as such so has its potential to be used as a therapeutic intervention. Nitric oxide was first identified through its release from nitroglycerine (glyceryl trinitrate - GTN), which was discovered in 1847 by Ascanio Sobrero (as cited in 83). The vasodilatory properties of GTN were elucidated shortly following his discovery and GTN was first used therapeutically to treat angina in 1867; remaining to this day the primary treatment for angina pectoris (as cited in 84). GTN is an organic nitrate, a prodrug that must be denitrated to release NO, though the mechanisms of denitration are not fully understood (85). Due to its safety, minimal side effects, and use in clinical practice for angina, GTN as a cancer therapy is attractive.

How, then, might nitric oxide mimetics such as GTN be used in the treatment of cancer? As outlined previously, poor tumour vasculature results in not only hypoxic regions, but regions that are inaccessible to chemotherapeutics and radiation therapy. Thus, it has been postulated that multiple avenues exist through which NO and NO mimetics may function as therapeutic interventions, some of which include using NO as a provascular treatment to modulate blood flow and thus improve drug delivery (86), NO
delivery as an inhibitor of mitochondrial respiration (87), as well as sensitizing tumours to radiation therapy (88), among others.

NO production requires oxygen as a substrate and as such, in regions of hypoxia where oxygen is limited, one may infer that NO production, and thus signaling, would be affected. Our previous studies have demonstrated that low concentrations of NO mimetics may interfere with hypoxia-mediated drug resistance and that this is, in part, due to activation of NO signaling through cGMP (89-91). Moreover, Nagai et al. found the use of NO donors such as GTN enhanced chemosensitivity of lung adenocarcinomas in a cGMP-dependent manner (92). Most relevant, though, are our recent findings indicating a potential for NO mimetics to inhibit HIF-1α accumulation in response to hypoxia and subsequent immune escape due to MICA shedding (72).

1.5 Prostate and breast cancer

The four most common types of cancer include cancers of the lung, colon, breast and prostate. Among these, breast and prostate cancers are the most common to be diagnosed in women and men, respectively. According to the Canadian Cancer Society, 1 in 7 men will develop prostate cancer in his lifetime and 1 in 28 men will die of it. Furthermore, 1 in 9 Canadian women will develop breast cancer in her lifetime and 1 in 29 women will die of it. It is clear that while there has been incredible progress, these cancers are difficult to treat and further advances are necessary to combat cancers of the prostate and breast. (http://www.cancer.ca/Canada-wide/About cancer/Cancer statistics/Stats at a glance.aspx?sc_lang=en).
1.5.1 Prostate cancer

The prostate is a male organ located directly below the bladder that wraps around the urethra and contributes to the volume of semen, providing nutrients to sperm. It is approximately the size of a walnut and may be palpated through a rectal examination. Prostate cancer is detected either by rectal palpation, or by measuring prostate specific antigen (PSA) levels in the blood. Prostate cancer may spread beyond the prostate directly to adjacent tissue or metastasize through local lymphatics and blood to distant sites (most commonly the ureters, bladder, urethra, rectum and bone). Treatment options, once cancer is detected, include surgery (radical prostatectomy) or radiation therapy for early stages, and hormone (to reduce testosterone) and chemotherapy for later stages. ([http://info.cancer.ca/cce-ecc/default.aspx](http://info.cancer.ca/cce-ecc/default.aspx), search: prostate cancer).

1.5.2 Breast cancer

Most cancers of the breast begin in the ducts or lobules (part of the milk producing mammary glands). Breast cancer is detected by a clinical breast examination involving palpation of the breast to check for lumps, diagnostic mammography and ultrasound technology. Breast cancers have the ability to metastasize to adjacent tissue and also through lymphatics and blood to distant sites such as lung, liver and brain. Treatment options depend on the stage of the cancer and the hormone receptor status (estrogen and progesterone) and they include surgery (lumpectomy – breast conserving, or radical mastectomy), radiation therapy, chemotherapy, and hormone therapy (for

### 1.6 Cell lines

One of the most important aspects of cancer progression is metastasis, as this is what leads to eventual demise. Hence, understanding the mechanisms that are involved in the progression from primary tumour to metastasis is critical. These aspects (such as aberrant levels of oncogenic/ lack of tumour suppressor proteins, for example) should be considered when developing an *in vitro* model. Cell lines are important tools in evaluating functions of proteins and in elucidating signaling networks. They are limited, though, in their applicability since many features that are present *in vivo* are lacking and may be difficult to simulate *in vitro*. As an important note, the degree of aggressiveness of a cancer cell line should be considered when choosing which cell lines are most appropriate for a study.

#### 1.6.1 Prostate cancer cell lines

Previous work in our lab concerning cancer immune escape has been accomplished using the prostate cancer cell line DU-145. This cell line was the first prostate cancer cell line to be established in cell culture (93) and one of the first three prostate cancer lines along with LNCap and PC-3 cell lines. DU-145 cells continue to be
amongst the most commonly used and are characterized as being androgen receptor negative and prostate specific antigen (PSA) negative (94). The androgen receptor and PSA status are important to be aware of when choosing cell lines as these are critical players in the efficacy of current therapies and have important implications in cell signaling. DU-145 is an epithelial line derived from a brain metastasis with a doubling time in cell culture of 34 hours (95). Furthermore, DU-145 cells are desirable to work with in cell culture as they form \textit{in vivo} tumours in nude mice – facilitating the transition from \textit{in vitro} to \textit{in vivo} work. Thus, DU-145 cells were chosen as the prostate line of choice for characterization of B7-H3 expression.

\subsection*{1.6.2 Breast cancer cell lines}

The sequence of breast cancer progression involves changes from atypical hyperproliferation of cells to \textit{in situ} carcinoma, invasive carcinoma and eventually metastatic disease. The breast cancer cell lines that are available are numerous, and can be classified into three groups based on markers and phenotype (96). The first group includes weakly invasive cell lines with an epithelial phenotype that express the estrogen receptor and E-cadherin (97, 96). Another group is made up of cell lines that are highly invasive, do not express the estrogen receptor, and have a mesenchymal phenotype (97, 96). A third group may be considered as an intermediate between these two groups (96). MDA-MB-231 cells belonging to the highly invasive group were chosen to represent metastatic disease. For some experiments, a weakly invasive cell line belonging to the first group, MCF-7, were chosen for comparative purposes. These two breast cancer cell
lines are derived from pleural effusions and are among the most commonly used of the breast cancer cell lines.

1.7 Murine syngeneic model of metastatic breast cancer

*In vivo* mouse models are important tools in evaluating the application of *in vitro* work. The 4T1 mammary carcinoma syngeneic xenograft model was chosen for these studies. This is an attractive model due to its capacity to form rapid metastases in an immunocompetent BALB/c mouse (98). The 4T1 cells may be injected into the mammary fat pad and palpable tumours grow within 1-2 weeks, forming metastases by week 3-6 (99). The 4T1 cells are resistant to 6-thioguanine, which allows for quantification of metastasis (99). Furthermore, luciferase expressing 4T1 cells are available, which makes this model useful for imaging studies (100).

1.8 Hypothesis

The hypoxic upregulation of the coinhibitory ligands B7-H3 and B7-H1 is HIF-1 dependent and the administration of a nitric oxide mimetic such as glyceryl trinitrate will interfere with the hypoxic upregulation of these ligands (Figure 1.8.1).
Figure 1.8.1  Working model of hypoxia induced immune escape through HIF-1
The above schematic represents our overall proposed model of acquired immune escape. The present thesis seeks to determine the relationship between HIF-1α and B7-H3 and B7-H1 expression, as well whether NO has a role.
1.9 Objectives

1. To characterize the role of hypoxia, more specifically HIF-1α, in the upregulation of B7-H3 and B7-H1 in cancer in vitro. This was evaluated using the prostate cancer cell line DU-145, and the mammary adenocarcinoma cell lines MDA MB 231 and MCF-7.

2. To determine the role of NO signaling in the regulation of B7-H3 and B7-H1. This was evaluated by determining the effect of NO mimetics such as GTN on their expression in vitro.

3. To determine the effect of GTN on breast tumour growth in vivo. This was be evaluated using a BALB/c syngeneic 4T1 mammary carcinoma model. If GTN attenuates tumour growth in vivo, this would confirm previous findings that demonstrated this effect in a prostate xenograft mouse model.
Chapter 2

Materials and Methods

2.1 Cell culture

Human MDA-MB-231 mammary adenocarcinoma cells derived from a pleural effusion and DU145 prostatic adenocarcinoma cells derived from a brain metastasis were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% and 5% FBS, respectively (Invitrogen, Burlington, ON, Canada). Mouse 4T1 mammary carcinoma cells, also obtained from ATCC, were cultured in RPMI 1640 medium supplemented with 10% FBS. All cells were maintained in monolayer culture in a Thermo Forma CO\textsubscript{2} incubator (20% O\textsubscript{2}, 5% CO\textsubscript{2}, 37°C).

2.1.1 Exposure to hypoxic conditions

After a 24-hour incubation in standard culture conditions, the medium was replaced with fresh medium and the cultures were placed in either hypoxic conditions of 0.5% O\textsubscript{2} or standard conditions of 20% O\textsubscript{2}. Hypoxic conditions were established by placing the cells in culture plates within an air-tight chamber. This chamber was subsequently flushed with 95% N\textsubscript{2}/ 5% CO\textsubscript{2} (Boc Gases, Kingston, ON, Canada). The experimental oxygen level of 0.5% was chosen as it is within the hypoxic range.
(<10mmHg/ 1.3% O₂) and this was maintained using the Pro-Ox Model 110 O₂ regulators (Biospherix, Redfield, NY, USA).

2.1.2 Administration of nitroglycerin

For experiments including the use NO mimetics, 10 µL of nitroglycerin (5 mg/mL, Sandoz, Boucherville, QC, Canada) was added to 1 mL of media and subsequently diluted 1/20 000 (v/v) to obtain a final concentration of 1µM or 10 nM. Nitroglycerin was added just prior to the 24-hour incubation in 20% or 0.5% O₂.

2.1.3 Flow cytometry

Cells were plated in 6-cm dishes and incubated under standard culture conditions (20% O₂) or under hypoxic conditions (0.5% O₂) for 24 hours. The cells were subsequently harvested with 5 mM EDTA. The cells were washed in a PBS-BSA (PBS + 0.5% BSA w/v) solution and incubated in primary goat anti-human B7-H3 antibody (in PBS-BSA) or PBS-BSA alone as a negative control for 30 minutes on ice. The cells were washed in PBS-BSA and incubated in 2° donkey anti-goat Alexa Fluor 488 antibody (A11055, Invitrogen, Burlington, ON, Canada) for 30 minutes at 4°C. The cells were washed three times in PBS-BSA, fixed in 3% paraformaldehyde for 15 minutes at room temperature and analyzed by flow cytometry. Cell-surface protein expression was
analyzed with a Beckman Coulter EPICS Altra HSS flow cytometer (Beckman-Coulter). Approximately 10 000 events per sample were analyzed.

2.1.4 Immuno-blotting

Cells (~ 100 000 to 150 000) were seeded in 6-well tissue culture plates or 6-cm dishes and grown under standard culture conditions (20% O₂) or under hypoxic conditions (0.5% O₂) for 24 hours. Cells were plated such that the final confluence did not exceed 60%. The culture plates were then removed from the incubators, the medium discarded, and the cells rinsed in PBS and snap frozen on a liquid nitrogen bath. The cells were lysed and harvested using 100-200 μL of a protein extraction buffer (2% SDS pH 7.5, 10 mM Tris, 0.15M NaCl) with Protease Inhibitor Cocktail (Cat # 11836153001, Roche Diagnostics Canada, Laval, QC, Canada). The lysates were sonicated for 10 seconds at 40 Hz and then centrifuged (10 minutes at 10 000 x g). After transferring the supernatant to a new centrifuge tube, the protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). The samples were then normalized (to equal concentrations) and diluted in a 2x SDS sample buffer with 3% β-mercaptoethanol (Fisher Scientific, Ottawa, ON, Canada). Protein samples of 10 μg were resolved on 8-10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) using the semi-dry transfer technique.

After transfer, the membranes were blocked for 1 hour at room temperature in blotto (5% milk, and 0.1% Tween-20 in PBS) to prevent non-specific binding of the antibody. The membranes were subsequently probed with a primary antibody diluted in
blotto overnight at 4°C. Antibodies used were a goat anti-human B7-H3 polyclonal [R&D Systems AF1027, Minneapolis, MN, USA) primary antibody at 1/250, a mouse anti-human B7-H1 monoclonal antibody (R&D Systems MAB156, Minneapolis, MN, USA) at a 1/250 dilution, and a mouse anti-human HIF-1 monoclonal antibody (BD Biosciences 610958, San Jose, CA, USA) at a 1/250 dilution.

The membranes were washed three times in blotto (10 min per wash). The membranes were subsequently incubated in a corresponding secondary antibody - a horseradish peroxidase-conjugated anti-goat or anti-mouse antibody, at 1/5000 (Vector Laboratories, Burlington, ON, Canada), for 1 hour at room temperature. The membranes were washed once in blotto and three times in PBS-Tween and the secondary antibodies were detected by enhanced chemiluminescence (Amersham Biosciences, Baie D’Urfe, QC, Canada) and exposed to Kodak X-Omat Blue Film. The membranes were then probed for β-actin or α-tubulin in order to control for differences in the amounts of protein loaded onto the gel.

2.1.4.1 HIF-1α small-interfering RNA (siRNA) transfection

Knockdown of HIF-1α expression was achieved using Silencer® validated siRNA targeted against exon 5 of the human HIF-1α gene (ID #42840; Ambion Inc., Austin, TX, USA). Silencer® Negative Control siRNA #2 (Ambion Inc., Austin, TX, USA) was used as a control. The siRNA (25 nM final concentration) was introduced into cells by transfection using siPORT NeoFx reagent (Ambion) according to the manufacturer’s
instructions. The siRNA treatments were carried out for 48 hours under standard culture conditions prior to incubation in hypoxia.

2.1.5 Quantitative Real-Time Polymerase Chain Reaction (PCR)

Quantitative Real-Time PCR (qPCR) was used to determine changes in the levels of RNA. Cells (~ 100 000 to 150 000) were seeded in 6-well tissue culture plates or 6-cm dishes and grown under standard culture conditions (20% O₂) or under hypoxic conditions (0.5% O₂) for 24 hours. Cells were plated such that the final confluence did not exceed 60%. The culture plates were then removed from the incubators, the medium was discarded, and the cells were rinsed in PBS and snap frozen on a liquid nitrogen bath. A Qiagen kit was used to isolate total RNA from the cells (RNeasy minikit, Qiagen Inc., Toronto, ON, Canada). Following this step, 1 μg of RNA was transcribed to cDNA using random hexamers –PolyN (6mer) 5’-NNNNNN-3’ (Cortec DNA Service Laboratories Inc., Kingston, ON, Canada) at 100 µM, and an omniscript reverse transcription kit (Qiagen Omniscript RT kit, Qiagen Inc., Toronto, ON, Canada); this mix was incubated for 2 hours at 37°C. The qPCR reaction included the primers for B7-H3 (designed by Dr. Ivraym Barsoum) that span exons 3 and 4 (5’AGCTTCACCTGCTTCGTGAGCAT 3’ → Tm = 57°C, 5’AGGGTCATGCTGGGCTTCGAGTA 3’ → Tm = 59°C) (Eurofins mwg/operon, Huntsville, AL, USA) and SYBR Green (Fast Start SYBR Green Master, Roche Diagnostics Canada, Laval, QC, Canada). Samples (0.5 µL) were added to a 96-well plate in triplicate, and primer mixes were subsequently added (9.5 µL); β-actin primers were used to account for pipetting accuracy. Relative standard curves for the
target gene (B7-H3) and the housekeeping gene (β-actin) were constructed from a mix of equal volumes of each cDNA and serially diluted. Light Cycler 480 SW (Roche Diagnostics Canada, Laval, QC, Canada) was used for qPCR, the following program was used - 95°C for 3 min, then 50 cycles: 95°C for 20 seconds, melting temp (Tm) 60°C for 20 seconds, 72°C for 20 seconds.

2.1.6 Immunofluorescence

Cells (~100,000) were seeded in 6-well tissue culture plates on autoclaved glass coverslips and grown under standard culture conditions (20% O₂) or under hypoxic conditions (0.5% O₂) for 24 hours. Cells were plated such that the final confluence did not exceed 60%. The culture plates were then removed from the incubators, the medium was discarded, and the cells were rinsed two times in warm PBS (37°C). The cells were then fixed in either cold acetone (-20°C) for B7-H1 or 4% paraformaldehyde (37°C) diluted in PBS for HIF-1α for 10 minutes. The cells were then rinsed three times 5 minutes in PBS and permeabilized in 0.4% Triton X-100 with 10% serum in PBS for 30 minutes. The cells were then incubated in either mouse anti-human B7-H1 monoclonal antibody 1/50 dilution (M1H1 eBioscience, San Diego, CA, USA) and mouse anti-human HIF-1 monoclonal antibody 1/100 dilution (BD Biosciences 610958, San Jose, CA, USA) in an antibody solution of 0.04% Triton X-100 with 5% serum in PBS overnight at 4°C. This was followed by three 5 minute washes in PBS, an incubation with the secondary antibody, goat anti-mouse Alexa Fluor 568 (A11004, Invitrogen,
Burlington, ON, Canada) at a 1/200 dilution. Slides were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlington, ON, Canada)

2.1.7 Chromatin Immuno-Precipitation assay (ChIP)

The protocol followed was according to the ExactaChIP Human/Mouse HIF-1α Chromatin Immunoprecipitation Kit (R&D Systems, Minneapolis, MN, USA). DNA-protein complexes were cross-linked with 1% formaldehyde for 15 minutes at room temperature in MDA-MB-231 breast cancer cells. The formaldehyde was quenched using 125 mM glycine for 5 minutes, and the cells were lysed on ice for 10 minutes. The samples were then sonicated to shear the chromatin for 5 minutes. The anti-HIF-1α antibody or Normal Goat IgG was added (provided by the kit) overnight at 4°C on a rotator. This involved quenching, lysing and sonicating samples followed by incubation with a biotinylated antibody. Subsequently, Streptavidin beads were added to the samples for 30 minutes at 4°C and the beads were collected by centrifugation at 12 000 x g for 1 minute. The beads were washed and Chelating Resin Solution was added to the beads and boiled for 10 minutes, followed by centrifugation at 12 000 x g for 1 minute. The supernatant was collected and the DNA cleaned with a DNA purification kit (DNeasy blood and tissue kit, 69504) and amplified by PCR: 94°C for 3 min, then 50 cycles: 94°C for 20 seconds, melting temp (Tm) 60°C for 20 seconds, 72°C for 20 seconds. The products were run on an agarose gel.
2.2 Animal Procedures

2.2.1 Syngenic 4T1 mammary carcinoma injection

Six- to eight-week-old wild-type female BALB/c mice (JAX -Jackson Laboratories, Bar Harbor, ME, USA) were inoculated orthotopically in the mammary fat pad with $3.5 \times 10^3$ syngeneic 4T1 mammary carcinoma cells suspended in 100 µL of phosphate buffered saline (GIBCO Life Technologies, Burlington, ON, Canada). Approximately 5-7 days post injection, the tumours became palpable at which point treatment was initiated (Figure 2.3.1).

2.2.2 Treatment with Glyceryl Trinitrate (GTN)

Minitran 0.4 mg/h GTN patches (3M Pharmaceuticals, St. Paul, MN) and placebo patches were cut to a size of 0.25 cm². The GTN patches at this size deliver GTN at a rate of approximately 1.8 µg/hour. To ensure that the patches remained in place, mice were housed separately. Using clippers, the mice were shaven at the napes and the patches were secured using a thin layer of New Skin Liquid Bandage (Medtech Products, Irvington, NY). Mice were treated with either a GTN or placebo patch based upon random group assignment at the beginning of the experiment. For the duration of the trial, patches were changed daily to ensure continuous delivery of GTN. We have previously shown that such a delivery strategy yields detectable levels of GTN and its metabolites in the plasma of treated mice.
2.2.3 Measurement of Tumours

Tumours were measured using electronic callipers. In general, tumours were roughly oval in shape. To calculate the volume, the long axis (length) and the short axis (width) of the “oval” were measured and the values were entered into the following equation: \[ \text{length} \times (\text{width}^2)/2 \]. To minimize the potential for unintentional measurement bias, multiple individuals were involved in tumour measurement.

2.2.4 Sacrifice and Necropsy

One hour before sacrifice, mice were given an intraperitoneal injection of 60 mg/kg of pimonidazole hydrochloride (Hypoxyprobe-1; Millipore, Billerica, MA) in saline. Pimonidazole hydrochloride is a small molecule that selectively binds to proteins in hypoxic cells and can be detected immunohistochemically using a monoclonal antibody included with the kit (for future analyses beyond the scope of this project). Mice were euthanized using 0.1 mL Euthanyl (Bimed-MTC, Animal Health Inc., Cambridge, ON, Canada). The tumour was assessed \textit{in situ} for evidence of invasion and degree of adherence to the peritoneum. The tumour was then removed, weighed and cut in two halves. One half was snap frozen in liquid nitrogen and the other half was fixed in 4% paraformaldehyde. Following tumour dissection, the lungs, liver, heart, kidneys, and spleen were removed and fixed in 4% paraformaldehyde for later histological analysis. A standardized necropsy sheet was completed for each mouse noting the organs that were
removed, the mouse weight at sacrifice, the final tumour volume/weight, the relevant tumour characteristics and presence of any metastatic lesions.

### 2.3 Calculations and Statistical Analysis

For flow cytometry, approximately 10,000 cells per sample were analyzed. Membrane-bound B7-H3 expression was presented as the median fluorescence intensity (X-Median 50) of the cells. The means of the X-Median 50 values were calculated in Excel and plotted in GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, US).

A one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison test for significant differences, was used to analyze experiments comparing three or more categories with GraphPad Prism. Differences were considered to be statistically significant at P < 0.05.

For Western blot analysis, densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, Maryland, USA) or Image Processing and Analysis in Java (ImageJ, National Institute of Mental Health, Bethesda, MA, USA). The relative means were calculated (compared to α-tubulin to account for loading differences) in Excel and plotted in GraphPad Prism. A one-way ANOVA followed by Bonferroni’s Multiple comparison test for significant differences was used.

Tumour volume measurements were collected as described. The date of first measurement was described as Day 1 and subsequent measurements were calculated relative to the volume on Day 1. The data were entered in GraphPad Prism and a two-
way ANOVA, non-repeated measures, was performed followed by Bonferroni’s Multiple comparison test for significant differences was (consultation with Wilma Hopman, Research Facilitator).
Figure 2.3.1  Syngeneic mouse model
Pictured above is the mouse model employed in the experiment described. Female BALB/c mice were injected with 4T1 mammary carcinoma cells. Upon detection of a palpable tumour, the mice were randomly divided into two groups: GTN treated and placebo. The experiment occurred over a period of approximately four weeks.
Chapter 3

Results

3.1 Effect of hypoxia and GTN on B7-H3 expression in a prostate cancer cell line

3.1.1 Effect of hypoxia and GTN treatment on B7-H3 protein expression in DU-145 cells

Western blot analysis was used to determine the levels of B7-H3 protein in DU-145 cells exposed for 24 hours to standard culture conditions of 20% O₂ or hypoxic conditions of 0.5% O₂ in the absence or presence GTN (1 μM). Hypoxia did not appear to have an effect on B7-H3 protein expression nor did treatment with GTN (Figure 3.1.1). The results shown are representative of six independent experiments.

3.1.2 Effect of hypoxia on HIF-1α and B7-H3 protein expression in DU-145 cells

Western blot analysis was used to evaluate the effect of hypoxia on the expression of HIF-1α and B7-H3 simultaneously in DU-145 cells. Compared with cells incubated in standard conditions (20% O₂), incubation in hypoxia (0.5% O₂) did not have a significant effect on either HIF-1α or B7-H3 protein levels. Furthermore, addition of 10nM GTN did not appear to have affected the levels of HIF-1α in DU-145 cells (Figure 3.1.2). The results are representative of three independent experiments.
Figure 3.1.1 Effect of hypoxia and GTN treatment on the levels of B7-H3 in DU-145 cells

The effect of hypoxia on the levels of B7-H3 was analyzed by Western blot. Bars indicate the relative levels of B7-H3 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure did not increase the levels of B7-H3 in DU-145 cells. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Statistical analysis was conducted using one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=6.
Figure 3.1.2  Effect of hypoxia and GTN on HIF-1α and B7-H3 protein expression in DU-145 cells
The effect of hypoxia on the levels of HIF-1α and B7-H3 was analyzed by Western blot. Bars indicate the relative levels of B7-H3 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure did not have a significant effect on the expression of B7-H3 or HIF-1α in DU-145. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Statistical analysis was conducted using a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=3.
3.1.3 Effect of Hypoxia and GTN on the surface levels of B7-H3 in DU-145 cells

Analysis of cell surface B7-H3 by flow cytometry was performed on cells exposed to 20% O₂ or hypoxic (0.5% O₂) conditions in the absence or presence of 1 μM GTN for 24 hours. The results indicated that exposure to hypoxia did not alter the surface expression level of B7-H3, nor did treatment with GTN (Figure 3.1.3). The results shown are representative of six independent experiments.

3.1.4 Effect of hypoxia and GTN on B7-H3 mRNA expression in DU-145 cells

Quantitative Real-Time PCR analysis was used to determine the levels of B7-H3 mRNA in DU-145 cells exposed for 24 hours to standard culture conditions of 20% O₂ or hypoxic conditions of 0.5% O₂ in the absence or presence GTN (1 μM). Hypoxia resulted in a significant increase in B7-H3 mRNA levels (P = 0.0101), though treatment with GTN did not (Figure 3.1.4). The results shown are representative of three independent experiments.
The effect of hypoxia on the cell surface protein expression of B7-H3 was analyzed by flow cytometry. Bars indicate the relative levels of B7-H3 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure did not have a significant effect on the expression of B7-H3 in DU-145 cells. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=6.
Figure 3.1.4  Effect of hypoxia and GTN on B7-H3 mRNA levels in DU-145 cells
The effect of hypoxia on the mRNA levels of B7-H3 was analyzed by qPCR. Bars indicate the relative levels of B7-H3 mRNA in cells cultured under various conditions. The results indicate that hypoxic exposure had a significant effect on the expression of B7-H3 in DU-145 cells (*, P = 0.0101). Values represent the mean relative densities normalized using actin levels to control for pipetting differences. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=3.
3.2 Effect of Hypoxia and GTN on B7-H1 expression in breast cancer cell lines

3.2.1 Effect of hypoxia on the levels of B7-H1 and HIF-1α protein in MDA-MB-231 cells

Immunofluorescence analysis was used to visualize the expression and localization of HIF-1α and B7-H1 in MDA-MB-231 cells exposed to standard culture conditions of 20% O₂ or hypoxic conditions of 0.5% O₂ for 24 hours. Hypoxia resulted in increased levels of HIF-1α (Figure 3.2.1). Immunofluorescence associated with HIF-1α appeared to be restricted to the nucleus. B7-H1 immunofluorescence also appeared to increase in cells exposed to hypoxia, though to a lesser extent than HIF-1α. The B7-H1 expression was predominantly cytoplasmic, with some increased perinuclear staining in some slides.
Figure 3.2.1 Effect of hypoxia on HIF-1 and B7-H1 levels in MDA-MB-231 cells
The effect of hypoxia on the levels of HIF-1α and B7-H1 was analyzed by immunofluorescence. The results indicated that hypoxic exposure resulted in increased staining of HIF-1α (A) and B7-H1 (B) in MDA-MB-231 cells. HIF-1α and B7-H1 were stained with alexa-fluor 594 and nuclei were visualized with DAPI.
3.2.2 Effect of hypoxia and cobalt chloride on HIF-1α and B7-H1 levels in MDA-MB-231 cells

Western blot analysis was used to determine the levels of B7-H1 and HIF-1α exposed for 24 hours to either standard culture conditions of 20% O₂, hypoxic conditions of 0.5% O₂, or treated with the HIF-1α stabilizing compound CoCl₂ (100 μM). Hypoxia did not appear to have an effect on B7-H1 protein expression nor did treatment with CoCl₂. Exposure to hypoxia and CoCl₂ resulted in a significant increase in HIF-1α levels (P < 0.05; Figure 3.2.2). The results shown are representative of nine independent experiments.

3.2.3 B7-H1 and HIF-1 correlation

Upon further analysis of the Western blots, it was apparent that there was an inconsistent upregulation of HIF-1α at 20% O₂. The expression of HIF-1α correlated to B7-H1 expression (r² = 0.48) and this was significant (P< 0.0001), as determined by linear regression and Pearson correlation analysis (Figure 3.2.3). The results shown are representative of nine independent experiments.
Figure 3.2.2 Effect of hypoxia and cobalt chloride on HIF-1α and B7-H1 levels in MDA-MB-231 cells

The effect of hypoxia on the whole cell protein expression of HIF-1α and B7-H1 was analyzed by Western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure and CoCl₂ treatment did not have significant effects on the expression of B7-H1, though hypoxia and CoCl₂ did result in a significant increase of HIF-1α protein (P < 0.05), (*) denotes significance between 20% O₂ and 0.5% O₂/CoCl₂ treatments. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc multiple comparisons test. Error bars represent standard deviation. N=9.
The effect of hypoxia on the whole cell protein expression of HIF-1α and B7-H1 was analyzed by Western blot. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Linear regression of B7-H1 versus HIF-1α indicated a significant positive correlation ($r^2 = 0.48$, $P < 0.0001$). $N=9$. 

**Figure 3.2.3 B7-H1 and HIF-1 correlation**
3.2.4 Effect of HIF-1α knockdown on B7-H1 levels in MDA-MB-231 cells

Western blot analysis was used to determine the levels of B7-H1 and HIF-1α exposed for 24 hours to either hypoxia alone (0.5% O₂), or hypoxia in combination with the HIF-1α siRNA or control scrambled RNA (SC). The knockdown efficiency was approximately 50%, and resulted in a significant decrease in HIF-1α protein levels from untransfected (N/A) to HIF-1α siRNA samples (P = 0.0292; Figure 3.2.4). HIF-1α siRNA did not have a significant effect on B7-H1 protein expression. The results shown are representative of three independent experiments.

3.2.5 Effect of HIF-1α knockdown on B7-H1 levels in MCF-7 cells

Western blot analysis was used to determine the levels of B7-H1 and HIF-1α in cells exposed for 24 hours to either hypoxia alone (0.5% O₂), or hypoxia in combination with the HIF-1α siRNA or control scrambled RNA (SC). The knockdown efficiency was approximately 50%, and the overall resulting decrease in HIF-1α protein expression was significant between the untransfected (N/A) and scrambled RNA (SC) with the HIF-1α siRNA (P=0.0032; Figure 3.2.5). HIF-1α siRNA did not have a significant effect on B7-H1 protein expression. The results shown are representative of four independent experiments.
Figure 3.2.4 Effect of HIF-1α knockdown on B7-H1 levels in MDA-MB-231 cells

The effect of HIF-1α knockdown on the levels of HIF-1α and B7-H1 in cells incubated in hypoxia was analyzed by Western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that HIF-1α knockdown did not have a significant effect on the expression of B7-H1. The knockdown efficiency was approximately 50%, and resulted in a significant decrease in HIF-1α levels (*, P = 0.0292). Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc multiple comparisons test. Error bars represent SEM. N=3.
Figure 3.2.5  Effect of HIF-1α knockdown on B7-H1 levels in MCF-7 cells

The effect of HIF-1α knockdown on the levels of HIF-1α and B7-H1 in cells exposed to hypoxia was analyzed by Western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that HIF-1α knockdown did not have a significant effect on the expression of B7-H1. The knockdown efficiency was approximately 50%, and resulted in a significant decrease in HIF-1α levels (P = 0.0032). (*) and (#) denote significance between N/A and SC samples to HIF-1α siRNA respectively. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Data was analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=4.
3.2.6 HIF-1α binds a hypoxia response element in the B7-H1 promoter in MDA-MB-231 cells

A chromatin immunoprecipitation (ChIP) assay was used to determine if HIF-1α bound to the HRE in the B7-H1 DNA. The MDA-MB-231 cells were exposed to 20% O$_2$ or 0.5% O$_2$. The HIF-1α antibody bound to the B7-H1 DNA and as such B7-H1 DNA was amplified by PCR (Figure 3.2.6). Vascular endothelial growth factor (VEGF - a known target of HIF-1α) was used as a positive control and was also amplified by PCR. These results indicate that when HIF-1α was present (under hypoxic conditions), the B7-H1 DNA was bound.
The ability of HIF-1α to bind B7-H1 DNA in cells exposed to hypoxia was analyzed by ChIP assay. The results indicate that HIF-1α did bind to the B7-H1 DNA (top panel) and the positive control VEGF (bottom panel). Lane 1: 0.5% O₂ whole cell lysate – positive control, immunoprecipitation. Lane 2: 0.5% O₂ + HIF-1α antibody immunoprecipitation. Lane 3: 20% O₂ + HIF-1α antibody immunoprecipitation. Lane 4: 0.5% O₂ + control IgG antibody immunoprecipitation.

The experiment that generated the result shown above was conducted in conjunction with Dr. Ivraym Barsoum and is shown here with his permission.

**Figure 3.2.6  HIF-1α binds HRE on B7-H1**

The ability of HIF-1α to bind B7-H1 DNA in cells exposed to hypoxia was analyzed by ChIP assay. The results indicate that HIF-1α did bind to the B7-H1 DNA (top panel) and the positive control VEGF (bottom panel). Lane 1: 0.5% O₂ whole cell lysate – positive control, immunoprecipitation. Lane 2: 0.5% O₂ + HIF-1α antibody immunoprecipitation. Lane 3: 20% O₂ + HIF-1α antibody immunoprecipitation. Lane 4: 0.5% O₂ + control IgG antibody immunoprecipitation.

The experiment that generated the result shown above was conducted in conjunction with Dr. Ivraym Barsoum and is shown here with his permission.
3.2.7 Effect of hypoxia and GTN treatment on HIF-1α and B7-H1 levels in MDA-MB-231 cells

Western blot analysis was used to determine the levels of HIF-1α and B7-H1 in cells exposed for 24 hours to standard culture conditions of 20% O₂ or hypoxic conditions of 0.5% O₂, in the absence or presence of GTN (10 nM). Hypoxia did not appear to have a significant effect on HIF-1α or B7-H1 protein levels nor did treatment with GTN (Figure 3.2.7). The results shown are representative of three independent experiments.

3.2.8 Effect of hypoxia and GTN on HIF-1α and B7-H1 levels in MCF-7 cells

Western blot analysis was used to determine the levels of HIF-1α and B7-H1 in cells exposed for 24 hours to standard culture conditions of 20% O₂ or hypoxic conditions of 0.5% O₂, in the absence or presence of GTN (10 nM). Hypoxia did not appear to have a significant effect on HIF-1α or B7-H1 protein levels nor did treatment with GTN, though an effect was occasionally observed (Figure 3.2.8). The results shown are representative of three independent experiments.
Figure 3.2.7  Effect of hypoxia and GTN on the levels HIF-1α and B7-H1 protein in MDA-MB-231 cells

The effect of hypoxia on the levels of HIF-1α and B7-H1 was analyzed by Western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure did not have a significant effect on the expression of B7-H1 or HIF-1α in MDA-MB-231 cells, nor did GTN treatment. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. Error bars represent SEM. N=3.
Figure 3.2.8 Effect of hypoxia and GTN on the levels of HIF-1α and B7-H1 protein in MCF-7 cells

The effect of hypoxia on the levels of HIF-1α and B7-H1 was analyzed by Western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure did not have a significant effect on the expression of HIF-1α in MCF-7 cells (# represents significance between 20% O₂ and 0.5% O₂ +/- GTN, * represents significance between 20% O₂ + GTN and 0.5% O₂ +/- GTN). Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Statistical analysis included a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons (P < 0.0001). There was no significant effect of treatments on B7-H1 expression. Error bars represent SEM. N=7.
3.2.9 Effect of hypoxia and GTN treatment on the levels of HIF-1α and B7-H1 protein in murine 4T1 mammary carcinoma cells

Western blot analysis was used to determine the levels of HIF-1α and B7-H1 in cells exposed for 24 hours to standard culture conditions of 20% O$_2$ or hypoxic conditions of 0.5% O$_2$, in the absence or presence of GTN (10 nM). Hypoxia resulted in an increase of HIF-1α protein though this was not statistically significant (P = 0.0679). Hypoxia did not have a significant effect on B7-H1 protein expression, nor did treatment with GTN (Figure 3.2.9). The results shown are representative of five independent experiments.
Figure 3.2.9  Effect of hypoxia and GTN on the levels HIF-1α and B7-H1 in 4T1 cells
The effect of hypoxia on the levels of HIF-1α and B7-H1 in 4T1 cells was analyzed by western blot. Bars indicate the relative levels of HIF-1α and B7-H1 protein in cells cultured under various conditions as determined by densitometric analysis of bands. The results indicate that hypoxic exposure had a significant effect on the expression of HIF-1α in 4T1 cells as compared to 20% O₂ (P = 0.0179), (*) indicates significance between 20% O₂ and 0.5% O₂ +/- GTN. Values represent the mean relative densities normalized using tubulin levels to control for loading differences. Statistical analysis included a one-way ANOVA followed by Bonferroni’s post-hoc test for multiple comparisons. There was no significant difference of treatments on B7-H1 expression. Error bars represent SEM. N=5.
3.3 Effect of GTN on tumour growth *in vivo*

3.3.1 4T1 tumour growth in BALB/c Mice treated with GTN

A study was conducted using 41 female BALB/c mice injected with $3.5 \times 10^3$ 4T1 mammary carcinoma cells to determine the effect of continuous transdermal administration of GTN (0.4mg/h) on tumour growth. Results indicated that GTN treatment slowed tumour growth *in vivo* ($P < 0.0001$; Figure 3.3.1). Data was analyzed using a two-way ANOVA followed by Bonferroni’s multiple comparison test.

3.3.2 Characteristics of tumours in BALB/c mice treated with GTN

At necropsy, important tumour characteristics were recorded as the tumour was dissected. These characteristics include if the tumour was adherent, invasive, necrotic, and whether the mouse died prematurely. No observable differences were found between the GTN treated group and the placebo group (Figure 3.3.2).
Figure 3.3.1  4T1 tumour growth in mice treated with GTN
Curves represent mean tumour volume relative to first measurement until sacrifice. The placebo group at Day 0 consisted of 17 mice, and GTN treated group at Day 0 consisted of 24 mice. The points represent mean relative tumour volume to Day 0 of surviving mice. Statistical difference between the treatment groups was determined by a two-way ANOVA, non-repeated measures, followed by Bonferroni’s multiple comparisons test. Both time and treatment were significant (P < 0.0001) as well as the interaction (P = 0.0004). Error bars represent standard deviation, (*, P < 0.05), (**, P < 0.01).
Figure 3.3.2  Characteristics of tumours from mice treated with GTN
The relative frequencies of tumour characteristics (adherence, invasiveness, necrosis, and premature death) are displayed above. These characteristics are important and were noted to determine if treatment with GTN had an effect. There was no observable difference between placebo and GTN groups.
Chapter 4

Discussion

4.1 General discussion

The work of this thesis sought to determine whether hypoxia, through HIF-1, plays a role in a potential mechanism of escape from acquired immunity. Specifically, the aim of the research presented in this thesis was to determine whether HIF-1 is mechanistically linked to increased expression of the coinhibitory ligands B7-H3 and B7-H1 in tumour cells. The most important and novel finding that the levels of the T cell coinhibitory ligand B7-H1 correlate with HIF-1α levels in MDA-MB-231 breast cancer cells indicates that a HIF-1-dependent mechanism of acquired immune escape is possible. The second aspect involved exploring whether administration of an NO mimetic interferes with this mechanism of immune escape. And finally, the effect of GTN in a syngeneic breast cancer mouse model was explored. A novel finding was that GTN administration in vivo had a significant effect on the mammary carcinoma tumour volume, though did not have a significant effect in vitro on the levels of HIF-1α or B7-H1. While hypoxia and GTN treatment did not appear to have an effect on B7-H3 protein expression in DU-145 cells, there was a correlation between HIF-1α and B7-H1 protein in MDA-MB-231 cells that did not extend to MCF-7 cells. The data indicate that the mechanism of escape from adaptive immunity is complex, and point to a role of HIF-1 in the regulation of B7-H1.

The role of B7-H1 in immune function has been well established. Its receptor PD-1, upon engagement, leads to T cell apoptosis. Still, there remains much to be determined
in terms of regulation of B7-H1 in both immunology and cancer progression. B7-H1 expression levels were visualized using fluorescence in MDA-MB-231 cells in conjunction with HIF-1α expression. The expression of B7-H1 protein in response to hypoxia was variable. However, analysis of the blots revealed that there was a significant correlation ($r^2=0.48$, $P<0.0001$) between HIF-1α and B7-H1 that was originally masked due to the upregulation of HIF-1α observed at 20% O$_2$. To further confirm this observation, a hypoxia response element (HRE) was found in the B7-H1 promoter. Though a perfect correlation was not detected, this is to be expected considering that there are alternative mechanisms of B7-H1 regulation (especially post-transcriptional). For example, it is known that IFN-γ can increase the expression of B7-H1 in various cell lines and in vivo (41, 44). Another mechanism that has been revealed is that PI3K activation of Akt through phosphatase and tensin homolog (PTEN) loss can result in a subsequent increase in B7-H1 expression (46). Indeed, a combination of PTEN loss and stimulation with IFN-γ resulted in superinduction of B7-H1 leading to highly immunoresistant glioblastomas (101).

To further examine the relationship of HIF-1α and B7-H1, knockdown studies were performed using HIF-1 siRNA. The MDA-MB-231 cells demonstrated that the overall HIF-1α knockdown efficiency in MDA-MB-231 cells was 46%. HIF-1α was detectable in MDA-MB-231 cells exposed to 20% O$_2$ (discussed later), and as such there would be stabilized HIF-1α protein in the cells that would not be susceptible to knockdown with siRNA. The HIF-1α knockdown was significant ($P = 0.0292$), and though B7-H1 was not significantly different in the knockdown cells, its expression appeared to follow the same general pattern. Since the correlation was not perfect, it
follows that B7-H1 may not always respond to HIF-1α knockdown. To test whether the lack of knockdown was due to stabilized HIF-1α in 20% O₂, the same experiment was performed using MCF-7 cells. These cells did not express HIF-1α at 20% O₂, perhaps because unlike MDA-MB-231 cells, which are more mesenchymal in phenotype, MCF-7 cells display a less invasive epithelial phenotype (96, 97). Upon HIF-1α knockdown, the levels of HIF-1α did decrease significantly (P=0.0032). The overall HIF-1α knockdown efficiency was 64% in MCF-7 cells. Compared to the 46% knockdown efficiency observed in MDA-MB-231, the HIF-1α knockdown was overall more efficient in MCF-7 cells. Interestingly, B7-H1 expression had no apparent correlation to HIF-1α expression in MCF-7 cells, as such it may be possible that B7-H1 is differentially regulated depending on cell type, or perhaps the hypoxia-mediated increase in B7-H1 expression could be linked with more malignant phenotypes. Indeed, high levels of tumour cell B7-H1 have been linked with poor prognosis in patients with renal cell carcinoma, breast cancer, and esophageal cancer (102-104).

B7-H1 has been shown to be post-transcriptionally regulated by IFN-γ as well as PTEN loss through PI3K which indicates that though HIF-1α may regulate B7-H1 at a transcriptional level, the mRNA remains subject to post-transcriptional regulation. Furthermore, re-distribution of B7-H1 from the cell surface to the nucleus where it has an anti-apoptotic role upon doxorubicin treatment (105), in addition to the presence of an active soluble B7-H1 (106), indicates that the regulatory and downstream pathways of B7-H1 are multifactorial and remain to be fully elucidated. Thus, while an increase in HIF-1α may lead to an increase in B7-H1 transcription, due to potential post-transcriptional regulation, this does not necessarily correlate with increased protein
expression. So, while not always observed, the HIF-1α – B7-H1 interaction is still potentially biologically relevant.

B7-H3 is a ligand that has been suggested to have a role in cancer immune escape (25) and its susceptibility to hypoxia and the NO mimetic GTN was evaluated. The protein levels of B7-H3 in DU-145 cells were analyzed using Western blotting, with no observed effect of hypoxia on its expression. As HIF-1α was detected in the 20% O₂ samples, the potential for correlation between HIF-1α and B7-H3 cannot be excluded. Studies involving the knockdown of HIF-1α to determine the effect on B7-H3 expression levels would be useful in establishing a firm conclusion. Results of qPCR analysis indicate an upregulation of B7-H3 mRNA under hypoxic conditions (P = 0.0101), which indicate the potential for HIF-1 transcriptional regulation. Analysis of cell surface expression of B7-H3 by flow cytometry demonstrated no change in B7-H3 surface expression.

In addition to its immunological roles, B7-H3 has been shown to regulate carcinogenesis via migration and invasion (107) as well as increased metastatic capacity (108). Recent findings by Ingebrigsten et al. have illustrated that nuclear localization of B7-H3, rather than cytoplasmic localization, was correlated with poor prognosis in colorectal cancer patients (109). Furthermore, it was found that silencing B7-H3 in MDA-MB-231 breast cancer cells leads to an enhanced apoptotic response to paclitaxel, mediated by the Jak2/Stat3 pathway (110). This indicates that it may not be overall B7-H3 expression that is important for cancer progression but rather localization. Furthermore, these results support a non-immunological role for B7-H3. Thus, while
HIF-1 may still have a regulatory role over B7-H3 transcription, it may not be as relevant as the functional properties of B7-H3 and its cellular localization.

While B7-H3 has a T cell coinhibitory role and appears to have a function in drug resistance in cancer, its dual role should not be forgotten. Only one putative receptor for B7-H3 has been identified: the TLT-2 receptor that delivers stimulatory signals to T cells (31). The finding has been disputed, though this group maintains that not only is TLT-2 a receptor for B7-H3, but that it mediates anti-tumour effects through increased cytotoxicity of CD8+ T cells (111). It is possible that variants of B7-H3 exist that cannot be distinguished by the antibodies available, which could result in heterogeneous responses to T cells (a similar phenomenon was reported with CTLA-4 signaling) (25). Interestingly, Ling et al. have suggested that for coinhibitory function, there may be a requirement for B7-H3 to be in close proximity with a T cell receptor (26). Thus, until other receptors are identified and the signaling pathway of B7-H3 illuminated, the role of B7-H3 in anti-tumour immunity may be difficult to elucidate and as such, the significance of HIF-1 regulation.

There were detectable levels of HIF-1α observed in DU-145 cells and MDA-MB-231 incubated in 20% O2. Though hypoxia exerts its effect predominantly through HIF-1α, HIF-1α is not regulated exclusively by hypoxia. This is evident in cancers especially due to genetic instability and ensuing mutations. In many cancers (including breast and prostate), the tumour suppressor PTEN may be dysfunctional or lost (112). This leads to activation of the PI3K pathway and subsequent HIF-1α stabilization. Zhong et al. found HIF-1α protein in their PC-3 prostate cancer cells under normoxic conditions (113). For example, murine double minute (Mdm2) binding to the tumour suppressor p53 leads to
HIF-1α degradation through VHL-independent ubiquitination. Thus, in cancers lacking p53, HIF-1α may be stabilized independently of oxygen status (114). Furthermore, breast cancer cell lines express variable levels of Mdm2 depending on ER status. The ER-positive MCF-7 cells express higher mRNA levels of Mdm2 than the ER negative MDA-MB-231 (115). Thus, if MDA-MB-231 express low levels of Mdm2, it follows that HIF-1α levels might be higher due to lack of degradation. Regardless of oxygenation status, the results of the present study reveal the importance of HIF-1α on the regulation of B7-H1 expression. This is an important and novel finding.

In conjunction with evaluating the effect of hypoxia through HIF-1α on the expression of B7-H3 and B7-H1 in vitro, the effect of administration of low dose GTN (1 μM – 10 nM) was explored. Previous studies in our lab have demonstrated that NO mimetics can interfere with the hypoxia-mediated acquisition of malignant phenotypes such as metastasis (116) and drug resistance (89, 90). We have also shown that NO signaling can inhibit the accumulation of HIF-1α in hypoxia in vitro (72). GTN did not affect HIF-1α or B7-H1 protein levels in MDA-MB-231 cells. It was postulated that if HIF-1α was upregulated at 20%, and if NO exerts its effects by interfering with the hypoxic upregulation of HIF-1α, then GTN may affect hypoxia-independent mechanisms of HIF-1α up-regulation. To test this, MCF-7 cells - which do not express HIF-1α - were exposed to 20% O₂ and 0.5% O₂ and the effect of GTN in these conditions was evaluated. The effect of GTN on HIF-1α expression by densitometry revealed that GTN had no effect on the hypoxic upregulation of HIF-1α in MCF-7 cells or the expression of B7-H1. The interaction of NO with HIF-1α remains largely to be resolved. For instance, NO may have stabilizing effects on HIF-1α under normoxic conditions due to S-nitrosylation,
though in hypoxia NO generally has a HIF-1α destabilizing effect (65). Moreover, the role of NO in cancer is multifaceted and a topic of debate. Since a complete mechanism of NO-mediated HIF-1α degradation in hypoxia has not been determined, it is difficult to draw conclusions from the present data. While some groups, including ours, have found that NO can interfere with the acquisition of malignant properties and resistance to chemotherapy, other groups have found that NO can promote cancer progression. The effect of NO depends on multiple factors such as the microenvironment, redox status of the tissue, pH, and concentration of NO, and the molecular structure of the NO donor, among others (85). Hence, in a heterogeneous tumour cell population, and between cancer types, one may expect the response to be heterogeneous as well. Furthermore, while GTN did not have an effect on the levels of HIF-1α or B7-H1 in vitro, the effect of GTN on these proteins in vivo has yet to be determined.

Based on previous work demonstrating that GTN could slow the growth of DU-145 prostate cancer cells in vivo (72), the role of GTN on breast cancer growth in vivo was evaluated. A syngeneic 4T1 mouse mammary carcinoma cell line was transplanted orthotopically into female BALB/c mice. Prior to injection the expression of B7-H1 in these cells was confirmed. The cells were positive for B7-H1, though correlation to HIF-1α could not be concluded. In addition, the effect of GTN on HIF-1α was not significant in the 4T1 cells. Upon injection, the 4T1 cells grew rapidly and many of the tumours became necrotic within the first 10 days of the tumour becoming palpable. Interestingly, GTN significantly inhibited tumour growth in vivo (P<0.0001). It remains to be determined whether the anti-tumour effect of GTN is a result of increased clearance of the tumour cells by cytotoxic T cells. The differing effects of GTN observed in cell
culture versus in the mouse model may be due to the multitude of factors present in vivo that are lacking in vitro.

4.2 Summary and concluding remarks

The data collected from the studies in this thesis indicate a novel correlation between HIF-1α and B7-H1 levels in a breast cancer cell line, and that GTN treatment slows tumour growth in a syngeneic mammary carcinoma mouse model. Thus, HIF-1α may regulate the expression of ligands involved in cancer immune escape, though the mechanism appears to be complex and must be further investigated. Furthermore, the use of GTN as an NO mimetic in cancer depends on the context of its use. The correlation of HIF-1α with B7-H1 levels in MDA-MB-231 cells as well as the in vivo use of GTN to slow tumour growth both provide encouraging preliminary evidence for the use of NO mimetics in breast cancer.
Chapter 5
Future Directions

The present studies have focused on the effect of hypoxia and GTN on the B7-H3 and B7-H1 coinhibitory ligands. Future studies should further test the findings that hypoxia did not have an effect on B7-H3 protein and further investigate the increase in mRNA expression – these should include HIF-1α knockdown studies. It might also be useful to determine the cellular localization of B7-H3 in DU-145 cells upon exposure to hypoxia by immunofluorescence studies.

The results indicating the correlation between HIF-1α and B7-H1 in conjunction with the ChIP assay that illuminates the HRE in B7-H1 should be further investigated to determine the effects on mRNA levels. The effect of HIF-1α on B7-H1 may be more pronounced when examining mRNA levels and this would indicate the degree to which B7-H1 may be post-transcriptionally regulated.

A further analysis of the dissected tumours from the mice would be useful in determining the effect of GTN on HIF-1α and B7-H1 expression in vivo. It should be noted that a tumour is temporally dynamic and spatially heterogeneous and as such, at the time of dissection, the results of histological and quantitative (via Western blot or qPCR) analyses may be difficult to interpret, and may not be representative of the evolving tumour phenotype.

The future in vivo studies will involve depletion of either the NK or T cell subsets, or both, and evaluating the effect of GTN on tumour growth (Figure 4.2.1). These studies will involve the use of anti-asialo GM-1 to deplete the NK subset, and an anti-CD8
antibody to deplete the cytotoxic T cells when the tumours become palpable. The 4T1 cells to be injected express luciferase and so, at the time of sacrifice, the mice will be imaged to look at metastases. Furthermore, anti-B7-H1 antibody will be administered to block B7-H1 activity and determine whether the effect of GTN in NK-deficient mice is due to signaling through B7-H1.

Finally, it would be interesting to evaluate the effect of hypoxia on PD-1 (on T cells). It is possible that in the tumour environment, up-regulation of PD-1 on infiltrating CTLs in addition to upregulation of B7-H1 on the tumour cells could have an additive effect resulting in tumour immune escape.
Future studies will involve depleting various immune subsets to determine whether the effect of GTN is mediated through the acquired immune system. This will involve injecting the mice with depleting antibodies, ensuring the removal of immune cells by flow cytometry, and harvesting tumours at endpoint.

**Figure 4.2.1** Future mouse model
The future studies will involve depleting various immune subsets to determine whether the effect of GTN is mediated through the acquired immune system. This will involve injecting the mice with depleting antibodies, ensuring the removal of immune cells by flow cytometry, and harvesting tumours at endpoint.
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