Hypoxia-driven immunosuppression: A new reason to use thermal therapy in the treatment of cancer?

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Abstract
Hypoxia within the tumour microenvironment is correlated with poor treatment outcome after radiation and chemotherapy, and with decreased overall survival in cancer patients. Several molecular mechanisms by which hypoxia supports tumour growth and interferes with effective radiation and chemotherapies are now well established. However, several new lines of investigation are pointing to yet another ominous outcome of hypoxia in the tumour microenvironment: suppression of anti-tumour immune effector cells and enhancement of tumour escape from immune surveillance. This review summarises this important information, and highlights mechanistic data by which hypoxia incapacitates several different types of immune effector cells, enhances the activity of immunosuppressive cells and provides new avenues which help ‘blind’ immune cells to detect the presence of tumour cells. Finally, we discuss data which indicates that mild thermal therapy, through its physiologically regulated ability to alter vascular perfusion and oxygen tensions within the tumour microenvironment, as well as its ability to enhance the function of some of the same immune effector activities that are inhibited by hypoxia, could be used to rapidly and safely release the tight grip of hypoxia in the tumour microenvironment thereby reducing barriers to more effective immune-based therapies.

Keywords: hypoxia, anti-tumour immunity, thermoregulation, hyperthermia, tumour microenvironment

Introduction
Until recently, the underlying rationale guiding most cancer therapies was that the sensitivity of tumour cells to treatment arises from intrinsic characteristics of the tumour cells themselves and that these are expressed independent of the microenvironment. In other words, cancer cells within tumours have been treated to exploit potential vulnerable characteristics that are constitutively present, for example, increased tumour cell mitotic index which can aid in the efficacy of many chemotherapeutic drugs. Similarly, in the case of immunotherapies, the majority of studies have focused on the inherent immunogenicity (or lack thereof) of tumour cells. As a result, much work has been expended on efforts to strengthen weak tumour recognition by vaccinating patients with tumour antigens. Even in the much less studied field of thermal medicine, an area of intense interest to most readers of this review, one of the earliest and persistent rationales underlying the delivery of excess heat at the site of the tumour is focused on the ability of heat shock temperatures to enhance radiation damage through thermal modulation of the DNA repair pathway within individual tumour cells. As a result of this rationale, current hyperthermia treatment protocols aim at getting sufficient heat within tumours to engage these intrinsic radiation enhancing mechanisms.

However, current research indicates that the response of solid tumours to chemo-radiation therapy, immunotherapy, and perhaps even thermal therapy is not just a function of the inherent molecular properties of tumour cells or their ability to be recognised and killed by immune cells.
Now there is an emerging appreciation of the role of the tumour microenvironment and of the normal cells that surround and infiltrate tumours in modulating the response of individual tumour cells to a variety of treatments and in altering the intrinsic phenotype of tumour cells. Important factors that influence the response of tumours to chemotherapy are the metabolic environment of the tumour cells and the basic vascular access of the drug to the tumour microenvironment. Defective vascular channels, combined with lack of homeostatic regulation seen in normal tissues, not only reduce drug uptake, but also contribute to the formation of hypoxic regions within tumours, a condition now known to promote growth of tumours and their ability to attract sufficient vascularisation. Similarly in the case of radiation therapy, a lack of microenvironmental oxygen is the critical factor in preventing maximal DNA damage from radiation, limiting damage to a fraction of what would be possible if sufficient oxygen were available. In the case of immunotherapy, there is now a much greater appreciation for the fact that the degree of inflammation, and concentration of certain cytokines and chemokines within the tumour can significantly alter immunogenicity of tumour cells as well as immune cell activation potential.

This review covers new evidence indicating that the efficacy of the anti-tumour immune system may also be highly dependent upon the degree of hypoxia in the tumour microenvironment; thus hypoxia may not only blunt the effectiveness of chemotherapy and radiation, but may also contribute to an environment which inhibits the efficacy of natural host antitumour immune cells and improves the ability of tumours to avoid immunosurveillance. Further, this review will highlight some reasons why the use of thermal therapy, (particularly mild thermal therapy) through its ability to activate naturally occurring thermoregulatory homeostatic processes (which in turn could modulate tumour vascular perfusion and reduce regions of hypoxia), as well as its ability to stimulate immune cell activity, could enhance the natural immune response against cancer. We speculate that reversal of hypoxia-induced tumour immunosuppression could, at least in part, help to explain the positive survival benefit and reduction in local tumour recurrence seen following the use of hyperthermia in combination with other cancer therapies in multiple Phase II and Phase III trials. Specifically, over the past 15 years there has been an exciting accumulation of data indicating a positive survival benefit when hyperthermia is added to radiotherapy and/or chemotherapy, validating early literature suggesting just such a benefit [1]. There have been positive phase III trials for cancers including: melanoma [2], oesophageal cancer [3, 4], locally advanced head and neck cancer [5], locally advanced cervix cancer [6] and gliomas [7]. Addition of hyperthermia contributes to superior local control and durable responses in chest wall recurrences of breast cancer, when combined with radiotherapy, as compared with radiotherapy alone [8, 9]. These successes are driving a resurgence of interest in understanding the potential mechanisms by which temperature affects the efficacy of cancer therapy.

These exciting clinical trial data support the value of renewed, vigorous research efforts to test this and other new hypotheses regarding mechanisms by which thermal therapy improves cancer patient survival, and to develop and test new heating strategies which may maximise immunological and physiological anti-tumour effects.

The importance of hypoxia in tumour biology and patient prognosis

For decades, cancer researchers have been aware of the fact that there are regions of mild to severe oxygen deprivation in solid tumours [10] that are most likely due to aberrant vascular function as well as metabolic abnormalities associated with rapid tumour growth [11]. Clinical investigations have reported that a large fraction of locally advanced solid tumours demonstrate a prevalence of hypoxic tissue [12]. Determining the extent of hypoxia within patient tumours has been achieved using techniques such as microelectrode probes, nitroimidazole-based compounds, PET imaging, and biomarker expression by immunohistochemistry [13–15]. Solid tumours with measured oxygen tension levels less than ~2.5 to 10 mm Hg are considered hypoxic and are positively correlated to enhanced tumour progression and increased therapeutic resistance [16]. In a cervical cancer study published in 1993, it was found that the extent of hypoxia positively correlated with a more negative prognosis [17, 18]. In recent years, hypoxia inducible factor (HIF)-1α expression in both pancreatic cancer and surrounding stromal cells has been correlated to poor patient survival [19]. Tumour hypoxia and patient outcome have been further studied in other malignancies such as head and neck [20], prostate [21], and breast [22, 23] and in each case, hypoxia was seen to predict a more negative clinical outcome. In a study on patients with soft tissue sarcoma, Brizel et al. showed that tumour oxygenation status predicts for the likelihood of distant metastases [24].

While it may seem intuitive that hypoxic conditions could slow or even block the growth of a tumour, unfortunately tumour tissues are able to respond to this stress in a manner which protects and supports their growth. Indeed, it has been
reported that certain clones within the tumour can react favourably to hypoxic conditions, leading to tumour progression [25, 26]. Moreover, a state of hypoxia promotes the development of new blood vessels for supply of nutrients and oxygen [27]. Hypoxic conditions have been reported to affect gene expression associated with increased angiogenesis, resulting in an increase in VEGF and receptor levels [28]. These angiogenic signals induced by hypoxic conditions permit the continued growth and survival of the tumour cells leading to a potentially progressive and invasive phenotype.

How does malignant tissue become hypoxic? There have been numerous investigations into this important question and several outstanding reviews of the literature have been published. While the amount of oxygen carried by the blood to normal organs and tissues is more than sufficient to meet their metabolic requirements, the consumption rate of oxygen in neoplastic and stromal cells in locally advanced tumours appears to be greater than the supply, resulting in areas of low O2 levels [22]. Possible pathogenic mechanisms involved in development of tumour hypoxia according to one analysis of the literature by Vaupel [29] are (1) perfusion-limited O2 delivery, (2) diffusion-limited O2 delivery, and (3) anaemic hypoxia. Perfusion-limited O2 delivery is the result of aberrations and functional changes in the tumour microvessels, resulting in limited O2 into the tissue [30]. Diffusion-limited delivery is caused by cells that are located too far away from nutrients and oxygen supplied from blood vessels, leading to enhanced hypoxia in that tissue area [31].

In a more recent review of the literature, Dewhirst et al. [11] identified some similar and a few additional causes of tumour hypoxia. These include (1) the relatively sparse arteriole supply in many tumours, (2) an inefficient orientation of tumour blood vessels leading to an overabundance of vasculature in some regions and insufficient vasculature in others, (3) large variations in flow velocity and in the number of red blood cells that traverse a microvessel per unit time, (4) effects of hypoxia on red blood cells, which have been reported to shrink and become more stiff than normoxic cells, and increase blood viscosity thus slowing the flow, (5) the existence of large diameter shunts between arteriolar and draining veins diverting blood and oxygen away from the tumour mass, (6) increased metabolic demand for oxygen in tumours, in addition to the observation that the binding of oxygen or heightened metabolism of tumour cells nearest the microvessels may limit penetration to deeper cell layers. These authors also highlight the importance of intra-tumoural pressures in helping to compress tumour blood vessels, interfering with effective delivery of blood [33]. All of these mechanisms contribute to the occurrence of hypoxic areas in tissues which can lead to enhanced tumour progression and increased malignancy. Subpopulations of tumour cells that survive and progress under the nutrient deprived conditions are hypothesised to produce more aggressive, therapeutically resistant disease [34]. For example, in one recent study, evidence was provided that prostate cancer cells exposed to chronic hypoxia caused these tumour cells to have a more aggressive phenotype and display a higher invasion activity in Matrigel assays than cells cultured under normal conditions [25].

Following acute oxygen deprivation in normal cells, a key regulatory protein HIF-1α functions to maintain homeostasis. HIF-1α becomes stabilised, heterodimerises, and translocates to the nucleus and binds to hypoxia-responsive elements (HRE) of several genes responsible for increasing oxygen and nutrients within the hypoxic tissue. However, under conditions of chronic hypoxia within a tumour microenvironment, constitutive HIF-1α expression can occur and aid in tumour progression, invasion and metastasis. HIF-1α expression in tumours has been reported to induce epithelial to mesenchymal transition (EMT) in colon cancer, increase expression of angiogenic factors such as VEGF, and increased genetic instability [35, 36]. These factors can lead to hypoxia-mediated resistance to apoptosis, decreased DNA repair and increased mutagenesis rates.

**Tumour hypoxia results in enhanced resistance to radiation and chemotherapy**

Tumour blood flow and related microenvironmental parameters (tumour tissue oxygenation, pH, nutrient supply, and interstitial fluid pressure) are believed to significantly impair cancer therapies [37-43]. It has been previously shown that radiosensitivity significantly decreases when pO2 pressure is less than 30 mmHg in the tumour [22]. A two- or three-fold higher radiation dose is needed to kill hypoxic tumour cells compared to well-oxygenated cells [14, 44, 45]. Many clinical studies have reported pO2 of less than 15 mmHg in different types of tumours and therefore it is clear that an improved ability to predict the extent of hypoxia (and modify it) before or during radiation therapy could provide invaluable clues for designing alternative treatment schemes.

Increased HIF-1α levels both experimentally and clinically have been shown to be correlated
with a decrease in tumour radiosensitivity. For example, in a recent study it was shown that lung cancer cells express high levels of HIF-1α and are resistant to radiation-induced cell death [46, 47]. Thus, HIF-1α is a promising target for increasing radiosensitivity of tumours that display a hypoxic phenotype. Using inhibitors to HIF-1α or proteins involved in its regulation could provide a way to increase the sensitivity of tumours to radiation therapy [48, 49].

Many cytotoxic drugs are also dependent on oxygen, and tumour hypoxia could confer resistance to chemotherapeutic drugs. Constitutive activation of HIF-1α and subsequent activation of many HRE genes such as STAT3 leads to chemoresistance. Constitutive activation of STAT3 in ovarian cancer cells cultured under hypoxic conditions renders them resistant to chemotherapeutic drugs such as Cisplatin and Taxol [50]. A recent study by Huang et al. observed that hypoxia-induced HIF-1α expression in ovarian cancer cells decreased the cells susceptibility to Paclitaxel [51]. Another study reported that hypoxia can also reduce p53 protein levels in tumour cells and results in resistance to Etoposide therapy [52]. It is clearly evident that hypoxic conditions can confer resistance of tumour cells to chemotherapies and hypoxic-induced proteins such as HIF-1α need to be targeted to increase efficacy of these chemotherapies.

The above data summarise some of the literature which indicates the importance of hypoxia in blunting more effective radiation and chemotherapy. The immune system is also a potentially powerful force that can prevent or slow tumour growth and major efforts are underway to develop new therapies that exploit anti-tumour immune responses. Unfortunately, there is now growing evidence that hypoxia can also negatively impact immune cell function and limit effective immunosurveillance. In the following section, we summarise some of the information on this important field of research.

Immunosuppressive effects of the hypoxic tumour microenvironment

Accumulating evidence suggests that a hypoxic microenvironment may protect tumours from natural anti-tumour immune responses (and from immunotherapies) by inhibiting anti-tumour immune effector cells and facilitating immune escape. Examples that will be described more completely below include (1) tumour hypoxia-induced tumour cell shedding of immune recognition molecules, reducing sensitivity to Natural Killer (NK) or Cytotoxic T Lymphocyte (CTL)-mediated killing, (2) hypoxia-induced inhibition of dendritic cells and T cells, and (3) hypoxia-induced promotion of suppressive cells (T regulatory cells and tumour-associated macrophages) which in turn, block immune effector cells (see Table I for a summary of key points from this literature). However, as will also be indicated below, several of these same immune effector cells may be positively affected by mild thermal stress, thus providing a potential rationale for using thermal therapy in cancer treatment.

Hypoxia-induced shedding of immune recognition cell surface markers

NKG2D is an activating receptor expressed by natural killer (NK) cells, CD8+ T cells and γδ T cells. The binding of NKG2D to its ligands activates NK and T cells and promotes cytotoxic lysis of the cells expressing these molecules. The MHC class I chain-related (MIC) molecules (MICA, MICB, and UL16-binding proteins), one family of the NKG2D ligands, are not expressed by the majority of benign cells, but are up-regulated on numerous tumour cells [53, 54]. The MIC molecules expressed on tumour cells are important for tumour immune surveillance through their interaction with NKG2D receptors on NK and cytotoxic T cells and subsequent tumour lysis [55-57]. Several lines of evidence show that shedding of MIC molecules is one of the mechanisms used by tumour cells to escape from immune surveillance. Tumour-derived soluble MIC ligands (sMIC) down-regulate and degrade NKG2D on T cells and impair these tumour-antigen-specific effector T cells [58]. Serum levels of sMIC from colorectal cancer patients are elevated and mediate down-regulation of activating NKG2D receptors on NK cells [59].

Studies on prostate cancer cells have shown that hypoxia increases tumour cell shedding of MIC molecules through impaired nitric oxide (NO) signalling. This hypoxia-induced MIC shedding decreases the sensitivity of tumour cells to peripheral blood lymphocyte-mediated killing. This finding is important because previous studies have shown that hypoxia-induced tumour invasiveness and chemo resistance are linked to reduced nitric oxide (NO) signalling [60, 61]. NO mimetic treatment attenuates hypoxia-induced shedding of MIC molecules and decreases prostate tumour growth in a murine xenograft model [62]. These results suggest that reactivation of NO signalling through administration of NO mimetic agents to increase cancer cell MIC expression can be a potential immunotherapy and help to overcome hypoxia-driven tumour escape.
Table I. Immunosuppressive effects of the hypoxic tumour microenvironment.

<table>
<thead>
<tr>
<th>References</th>
<th>Target cell type</th>
<th>Effect of hypoxia on the target cells</th>
<th>Outcome of hypoxia-induced modification</th>
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<tbody>
<tr>
<td>[62]</td>
<td>Tumour cells</td>
<td>Increased tumour cell shedding of MIC molecules through impaired nitric oxide signalling and decreased sensitivity to MK can CTL-mediated killing.</td>
<td>Tumour immune escape</td>
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<tr>
<td>[67]</td>
<td>Dendritic cells</td>
<td>Decreased sensitivity to NK and CTL-mediated killing</td>
<td>Impaired DC maturation and cytokine production</td>
</tr>
<tr>
<td>[69]</td>
<td>Dendritic cells</td>
<td>Accumulation of extracellular adenosine leads to decreased antigen presentation and enhanced IL-10 secretion</td>
<td>Diminishes the capacity of DCs to initiate and amplify Th1 immune responses</td>
</tr>
<tr>
<td>[74]</td>
<td>T cells</td>
<td>Diminished IL-2 production</td>
<td>Impaired T cell growth and survival</td>
</tr>
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<td>[77]</td>
<td>T cells</td>
<td>Downregulation the expression and activity of Kv1.3 channels</td>
<td>Inhibition of TCR-mediated T cell proliferation</td>
</tr>
<tr>
<td>[78]</td>
<td>T cells</td>
<td>Inhibition of the accumulation of IL-2 and IFN-γ during TCR-driven differentiation of CTL</td>
<td>Less CTL development</td>
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<td>[83]</td>
<td>T regulatory cells</td>
<td>Upregulation of cAMP-elevating A2A and HIF-1α to enhance the transcription of immunosuppressive molecules and extracellular adenosine accumulation</td>
<td>Beneficial for Treg cell development and increased anti-tumour immune suppression</td>
</tr>
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<td>[85]</td>
<td>T regulatory cells</td>
<td>Induction of Foxp3 expression through A2A-cAMP pathway and may enhance the expression of HIF-1α</td>
<td>Increased intensity of cAMP-elevating A2A and HIF-1α pathway on Treg cells</td>
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<tr>
<td>[84]</td>
<td>T regulatory cells</td>
<td>Upregulation of Foxp3 expression in a HIF-1α dependent manner</td>
<td>Increased number and suppressive properties of naturally occurring CD4⁺CD25⁺ Treg cells</td>
</tr>
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<td>[92]</td>
<td>Tumour-associated macrophages</td>
<td>Tumour cells secrete higher amounts of chemoattractants</td>
<td>Enhance monocyte attachment to and migration through the tumour vasculature and differentiate to TAM</td>
</tr>
<tr>
<td>[93]</td>
<td>Tumour-associated macrophages</td>
<td>Decreased mobility and inhibition of the chemoattractant signalling cascade</td>
<td>Entrap TAMs in the tumour hypoxic environment</td>
</tr>
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<td>[94]</td>
<td>Tumour-associated macrophages</td>
<td>Inhibition of the CCR5 chemokine receptor expression on macrophages</td>
<td>Immobilisation of TAMs</td>
</tr>
<tr>
<td>[91]</td>
<td>Tumour-associated macrophages</td>
<td>Activation of a protumour phenotype in macrophages to promote tumour growth</td>
<td>Increased TAMs differentiation</td>
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Impaired dendritic cell maturation and cytokine production within hypoxic tumour microenvironments

Dendritic cells (DC) and tumour antigen-specific T cells are essential for optimal anti-tumour immunity. Immature dendritic cells have acute ability to capture antigens, including tumour antigens, but low capacity to stimulate T cell activation. Upon antigen uptake, DC can undergo maturation and express high levels of MHC, CD40, CD80 and CD86. DCs will then migrate to lymph nodes during their maturation process and present antigen to T cells for activation and initiate adaptive immune responses [63–65]. Factors which could inhibit DC maturation or function in the tumour microenvironment might be a very important mechanism for tumour immune escape. Triozzi et al. generated autologous DCs in vitro from peripheral blood of melanoma and breast carcinoma patients. Intratumoural injection of these in vitro-generated DCs induced tumour regression in some patients along with lymphocyte infiltration and tumour necrosis. However, these DCs failed to induce a systemic response because they apparently could not migrate normally out of the tumour tissue to draining lymph nodes [66]. Recently, Yang et al. used human monocyte-derived DC and cultured these cells under normoxic or hypoxic (1% O2) condition for five days in the presence of GM-CSF, IL-4. Maturation was further induced by LPS treatment. They found that hypoxia could inhibit DC maturation and cytokine production by inhibiting CD40 and MHC expression, as well as Th1-type cytokine production (eg, IFN-γ, TNF-α and IL-12). These hypoxia-modified DCs displayed poor T cell stimulatory activity and were skewed to a Th2-stimulating phenotype by polarising naïve T cells to secrete IL-4 instead of IFN-γ [67]. Th1 cells can enhance the function of tumour-specific CTL through co-stimulatory molecules present on their surface and also indirectly by secretion of IL-2 [68]. Therefore, hypoxia-skewed Th2 cell development will compromise anti-tumour immunity [69]. Overcoming hypoxia within tumours would therefore seem to be necessary for achieving effective dendritic cell function leading to a Th1 response.

Hypoxia reduces T cell proliferation and survival and inhibits CTL development

Activation of tumour antigen-specific T cells is a critical event needed for anti-tumour immunosurveillance. T cells are exposed to different oxygen tensions, including hypoxic levels, during their development and during migration between blood and tissue [70]. A hypoxic environment can exist normally in lymphoid organs, inflamed tissues and tumours. T cells have been shown to increase expression of certain genes which are regulated by HIF (VEGF, glycolytic enzymes) after exposure to hypoxia [71–73]. Many studies report that hypoxia has inhibitory effects on T cell growth. For example, T cell growth and survival are impaired at low oxygen levels because hypoxia will down-regulate T cell IL-2 mRNA expression [74]. Lymphocytes express both voltage-dependent potassium (Kv) and Ca2+-activated potassium (Kca) channels, and their activities are essential for T cell activation [75]. K+ channels modulate the resting potential of the T cell membrane and indirectly regulate Ca2+ signalling which is important for cell proliferation and cytokine production. It is well established that blocking Kvä channels inhibits T cell proliferation by inducing membrane depolarisation and decreasing calcium influx [76]. Conforti et al. have shown that hypoxia selectively inhibits T cell receptor (TCR)-mediated T cell proliferation by down-regulating the expression, as well as activity, of Kvä channels. On the other hand, T cell proliferation induced by agents bypassing the membrane (such as ionomycin with or without phorbol-myristate-acetate (PMA)) is not affected by hypoxia. Therefore, the Kvä channels expressed in T cells are sensitive to hypoxia and are responsible for hypoxia-mediated inhibition of T cell proliferation [77].

Differentiation of T cells is both TCR-driven and cytokine-dependent. Therefore, it is very important to evaluate the effect of hypoxia on T cell cytokine production. Caldwell et al. have shown decreased CD8+ T cells development under hypoxic conditions (2.5% O2) compared to normal oxygen concentration (20% O2). Hypoxia also alters CTL cytokine secretion patterns. Hypoxia exposure enhances the transcription of HRE-containing genes, such as VEGF but inhibits the accumulation of non-HRE-containing genes, such as IL-2 and IFN-γ during TCR-driven activation. This suggests that T cell activation under hypoxic conditions in vivo may lead to different patterns of cytokine secretion [78].

Since reduction of IL-2 production is the major consequence of hypoxia-induced T cell immunosuppression, Kim et al. engineered human tumour-specific cytotoxic T cells which express hypoxia-inducible human IL-2 genes (HRE-IL-2) to rescue CTL function within a hypoxic tumour microenvironment. They found that these modified CTLs sustained their proliferation and survival by increasing IL-2 production under hypoxic conditions. HRE-IL-2 transduction also increased CTL cytotoxic activity even under hypoxia. After adoptive transfer into tumour-bearing mice, these HRE-IL-2-modified CTLs migrated into the tumour and promoted more
rapid and complete tumour regression than parental CTls. Overall survival was also increased after the HRE-IL-2-modified CTL transfer [79]. Therefore, increase of T cell growth and survival within tumour hypoxic microenvironments, by providing IL-2, can restore their anti-tumour functions and may overcome hypoxia-induced immunosuppression.

Hypoxia- and adenosine receptor-mediated T regulatory cell development and suppressive function

Cells which are now known to help suppress effective anti-tumour immunity include T regulatory cells (Tregs) and tumour-associated macrophages (TAMs). Tregs cells play an important role in suppressing immune responses for central or peripheral tolerance but they also protect cancer cells from anti-tumour immunity. Treg cells exert their suppressive effects by direct contact with T effector cells, inhibition of DC-induced T cell priming and secretion of suppressive molecules (TGF-β, IL-10, galectin-1, and CTLA-4) [80]. A more complete understanding of the development and activities of Treg cells may help generate new therapies to inhibit their tumour growth promoting functions.

A very intriguing hypothesis regarding the effects of hypoxia-induced immunosuppression is now emerging: the tumour hypoxic microenvironment not only actively inhibits anti-tumour immune cells, but also promotes development of immune suppressor cells and this effect involves a role for adenosine in the tumour microenvironment. It has been shown that one consequence of local hypoxia is the accumulation of extracellular adenosine. Specifically, hypoxia up-regulates adenine nucleotide-metabolising ectoenzymes, ATPase/ADPase, CD39 and 5'-nucleotidase, CD73 to increase extracellular adenosine production [81]. The extracellular adenosine signals through high-affinity A2A adenosine receptors on activated immune cells and increases immunosuppressive intracellular cAMP. Signals triggered from A2A adenosine receptors result in an ‘off’ signal to inhibit immune cell activation, such as inhibiting the release of tissue-damaging oxygen radicals in polymorphonuclear neutrophils, down-regulating APC cytokine production and antigen presentation, and inhibiting peripheral T cell proliferation and activation. This mechanism may help to down-regulate immune responses to protect normal tissues from collateral inflammation-induced tissue damage [82]. However, the potential for hypoxia-adenosinergic signalling may be underestimated because the majority of current in vitro studies are at non-physiologically high (21%) oxygen concentration [78].

Natural Treg cells express high levels of CD39 and CD73, which are responsible for extracellular adenosine production. Treg cells also have high intracellular cAMP and produce extracellular adenosine themselves. Therefore, based on the immunosuppressive cytokine up-regulation in other cell types under hypoxic conditions, Sitkovsky et al. [83] have proposed a model for Treg cell development and suppressive function via hypoxia-driven and adenosine receptor-mediated (hypoxia-adenosinergic signalling) within the hypoxic tumour microenvironment. They propose that TCR-activated Treg cells which express cAMP-elevating A2A and HIF-1α might lead to enhanced transcription of immunosuppressive molecules such as TGF-β, IL-10 and galectin-1 which have hypoxia response element (HRE) and cAMP response element. In addition, A2A and HIF-1α up-regulate CD39 and CD73 on Treg cells to mediate extracellular adenosine accumulation to directly inhibit DC and T effector cell functions. Hypoxia also increases FoxP3 expression in a HIF-1α-dependent manner [84, 85]. Therefore, the A2A and HIF-1α pathways might be required for Treg cell development [83]. These new data suggest that tumour hypoxia is beneficial for Treg cell development and function, thus leading to significant anti-tumour immune suppression.

Tumour-associated macrophages: A pro-tumour phenotype activated by tumour hypoxia

Phagocytic cells such as neutrophils and macrophages are important cells of the innate immune system and they function in response to tissue injuries or invading pathogens during inflammation. One feature of inflamed tissue is hypoxia, and therefore the function of phagocytic cells could be modulated by altered concentrations of oxygen or other aspects of abnormal metabolic activity. Phagocytes may need to adapt to the hypoxic environment to generate energy and function efficiently [86]. Compared to other immune cells, phagocytes switch their metabolic activity to use anaerobic glycolysis to generate ATP [87–90]. Deletion of HIF-1α in phagocytes revealed that HIF controls major defence functions in phagocytes including phagocytosis, production of bactericidal molecules and pro-inflammatory cytokine production. Under hypoxic conditions within inflamed tissues, HIF can synergise with the NF-κB pathway to increase phagocytosis of bacteria, release of bactericidal molecules and pro-inflammatory cytokines and inhibit apoptosis to increase phagocyte lifespan [91].
Cancer cells can secrete many chemoattractants to recruit monocytes into tumours [92]. Recruited monocytes rapidly differentiate into immunosuppressive tumour-associated macrophages (TAMs) and accumulate in the hypoxic area. In comparison to conventional macrophages, TAMs have a relatively immature macrophage phenotype and have poor antigen-presenting ability and produce factors that suppress T cell proliferation and activity. TAMs also up-regulate several genes (e.g. growth factors, VEGF, MMP-7) to promote tumour growth, invasion and metastasis. New data suggests that hypoxia appears to entrap TAMs by decreasing their mobility [91, 93, 94]. These results suggest that hypoxia can recruit blood monocytes and activate a pro-tumour phenotype in macrophages to promote tumour growth.

To conclude this discussion, the immune system normally up-regulates many genes to adapt to hypoxic conditions in order to maintain their functions in various regions of the body that may differ in terms of oxygen tensions. However, as outlined above, cancer cells take advantage of hypoxia to alter activities of several immune effector mechanisms and increase tumour cell escape from immune surveillance. A better understanding of the mechanisms of hypoxia-induced immunosuppression is needed to develop more efficient therapies to boost anti-tumour immune responses. Moreover, these data contribute to the need for testing new ideas for reducing hypoxia within tumours.

Mild, fever-range hyperthermia positively affects several of the same immune mechanisms negatively affected by hypoxia

Previous studies from our lab [95–99] have shown that fever-range thermal stress can modulate some of the same immune targets that are affected by hypoxia, and fortunately these data suggest the effects of mild thermal stress are opposite to that of hypoxia. For example, we observed that the cytotoxic activity of human NK cells isolated from peripheral blood is enhanced after exposure to fever-range thermal stress and this correlates with an increase in NKG2D clustering but not total level of NKG2D surface expression. Unlike hypoxia, mild temperature elevation (i.e. 39.5°C) also results in the up-regulation of MICA on tumour target cells that is associated with increased sensitivity to cytolysis in our in vitro studies. These results suggest that fever-range thermal stress not only enhances tumour cell recognition through up-regulation of molecules on tumours needed for NK cell recognition, but also increases NK cell cytotoxicity [95, 99].

Dendritic cell mobility is also sensitive to temperature changes. Unlike hypoxia, fever-range thermal stress enhances DC activation by increasing MHC II and CD86 expression. Our in vivo studies show that DCs exposed to mild fever temperatures have increased mobility which is likely associated with increased DC trafficking to the draining lymph nodes. Besides DC phenotype and migration patterns, hyperthermia allows for greater T cell activation in a mixed lymphocyte assay [96–98, 100]. Therefore, opposite to the effects of hypoxia, mild thermal stress enhances DC activation and antigen presentation which may lead to an increased anti-tumour T cell response. In our new in vitro studies, we have observed that fever-range thermal stress directly increases CD4 T cell IL-2 production in response to a suboptimal activation stimulus. IL-2 is a critical cytokine needed to increase and mobilise immune effector cells, and further work indicates that its enhanced production may be related to thermally induced changes in T cell membrane lipid raft organisation (M.Yuan and M.Grimm et al., unpublished MSS). Overall, these results suggest that thermal therapy could be used to help reverse at least some of the negative effects of hypoxia on the anti-tumour immune response. However, to date, there are few studies that evaluate the effects of mild thermal stress on some of the other immune suppressive mechanisms (e.g. recruitment of Tregs or TAMs) discussed above and therefore this should be a top research priority.

Thus mild thermal therapy may have a dual benefit: direct enhancement of immune cell activity through thermally sensitive molecular pathways associated with immune cell function/activation, and indirect enhancement of immunosurveillance through a reduction in hypoxia-induced immune suppression via improved tumour vascular perfusion. In the next section we discuss the possibility that mild thermal therapy can also be used to relieve, at least temporarily, the grip of hypoxia in the tumour microenvironment through its effects on vascular function.

Hyperthermia-induced effects on vascular perfusion of tumours: Can thermal therapy help to overcome hypoxia-driven immunosuppression in the tumour microenvironment?

Several lines of research suggest that tumour vasculature may be an important target of hyperthermia. Indeed, a well studied aspect of fundamental vertebrate physiology is the exquisite neuromuscular-mediated vascular homeostatic mechanisms in normal tissue that are launched rapidly and reversibly to change vascular flow patterns whenever
there is even a very small change in tissue temperature. Extensive literature has revealed the fact that heat-induced changes in blood flow (from exercise, fever, or changes in ambient temperature) is due to controlled amounts of both vasoconstriction and vasodilation of blood vessels which are well supplied by sympathetic nerves, which in turn are regulated through the action of the temperature-regulating centres of the hypothalamus, as well as exquisitely sensitive warm and cold receptors in the skin and in deep tissues [101–105]. But, does this same high degree of neuronal-vascular thermoregulation occur in the tumour microenvironment? Currently, little evidence is available to answer this question. For example, it is not known whether tumour blood vessels differ from normal vessels in terms of the density of warm/cold receptor nerve endings. Cancer researchers believe that the blood vessels which supply tumours come from nearby normal host vessels which become incorporated into growing tumours and/or from newly formed vessels. Whatever their source, tumour vasculature often exhibits severe functional and morphological abnormalities; sympathetic nerve endings are missing on newly formed blood vessels, and these vessels are often seen to be highly irregular in their course, with dilated and compressed regions, irregular branching, and poor perfusion [106]. Incomplete or missing endothelial cells and smooth muscle cells, interrupted basement membranes may result in increased vascular permeability, contributing to an increased interstitial fluid pressure (IFP) [32]. The lack of normal innervation and the existence of numerous structural defects, including deficient smooth muscle coating cells would strongly predict that the tumour microenvironment may actually escape the active neuro-regulatory control which exists in the rest of the body following heating. But, curiously, within the tremendous volume of literature on neurovascular thermoregulatory mechanisms, there is no comparison of normal and tumour blood vessels, nor an examination of whether tumours differ from normal tissue in terms of the density of thermal nerve endings or in terms of their ability to actively constrict or dilate in response to temperature shifts. Nevertheless, early studies in the field of hyperthermic oncology have looked at blood flow in tumours following local heating, and this information is briefly summarised here. Although the data are complex (with some differing conclusions that may depend upon the local heating protocol/duration, temperature achieved or tumour model used) a series of studies have shown that hyperthermia can change tumour oxygen concentration, blood flow and vascular permeability, and these factors could contribute significantly to overcome hypoxia-induced immnosupression. Below is a brief summary of some of this pioneering literature on tumour oxygenation and blood flow in response to local hyperthermia, followed by some new information from our own studies using systemic heating at mild fever-range temperatures.

**Hyperthermia-induced changes in tumour oxygenation and vascular perfusion**

Hyperthermia has been suggested to increase the responses of tumour cells to radiation by several mechanisms, including improving tumour oxygenation [107, 108]. See Table II for a brief summary of some of these studies on tumour oxygenation measurements.

This idea has been supported by data as far back as the 1980s. Bicher et al. [109], Tanaka et al. [110] and Vaupel et al. [111] had reported that increased tumour oxygenation occurred after heating at mild temperature in rodent tumours. Several more recent studies in rodent tumours, human xenografts, patient and canine tumours support that local heating (40–43°C) results in an overall improvement of tumour oxygenation. The changes in tumour oxygenation after heating correlated with changes of tumour blood flow [109, 112-115]. The increase of oxygen delivery into the tumour through increased blood flow after heating could reduce hypoxic regions within the tumour microenvironment. A higher thermal dose (>43°C) leads to decrease of tumour oxygenation possibly because of blood vessel damage [40, 108, 114, 116–118].

In these studies, heat-induced tumour oxygenation is transient, persisting for several hours and then begins to decrease depending on the heat dose and tumour type [119]. Other studies also show that heating tumours grown at different sites (in the hind limb, leg or flank) may result in different changes in tumour pO2 [110]. Collectively, these important studies show that the effects of hyperthermia on tumour oxygenation depend not only on the heat dose but also on the tumour site. Moreover, it has been suggested that the late increase in tumour pO2 (24 h after heating) is not as proportional as that found immediately after heating. This suggests that the late increase in tumour pO2 might also be controlled by other mechanisms such as a decrease in oxygen consumption [120, 121].

In addition to studies on tumour oxygen levels there are also many studies on tumour blood flow after local hyperthermia which reveals complex results. The changes in blood flow caused by hyperthermia seem to be dependent on heating temperatures, lengths and tumour types. Heating at lower temperatures (41–43.5°C) significantly increases the blood flow in SCK tumours in A/J
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Table II. Heat-induced changes in tumour oxygenation.

<table>
<thead>
<tr>
<th>References</th>
<th>Tumour type</th>
<th>Site</th>
<th>Tumour oxygenation</th>
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<tbody>
<tr>
<td>[109]</td>
<td>C3H mammary tumour</td>
<td>Mouse leg (i.m.)</td>
<td>$pO_2$ increased at $&lt;41^\circ C$ and decreased at $&gt;42^\circ C$</td>
</tr>
<tr>
<td>[110]</td>
<td>S-180 tumour</td>
<td>Mouse limb (s.c.)</td>
<td>$pO_2$ increased during heating at $41^\circ C$ for 30 min</td>
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<td></td>
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<td>$pO_2$ increased at $42^\circ C$ for 30 min, decreased after heating, but increased again 14–18 h after heating</td>
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<td></td>
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<td>$pO_2$ decreased at $45^\circ C$ for 30 min and failed to recover after heating</td>
</tr>
<tr>
<td>[111]</td>
<td>DS carcinoma</td>
<td>Rat foot</td>
<td>Oxygenation (Hb $O_2$ in blood vessels) peaked at $39.5^\circ C$ and decreased at $42^\circ C$</td>
</tr>
<tr>
<td>[74]</td>
<td>R3230 AC tumour</td>
<td>Rat leg (s.c.)</td>
<td>$pO_2$ increased during and 12–15 min after heating at 40.5–43.5 $^\circ C$ for 30 min</td>
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<tr>
<td></td>
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<td>$pO_2$ increased during and 12–15 min after heating at 40.5–41.5 $^\circ C$ for 60 min but decreased during heating at $43^\circ C$ for 60 min</td>
</tr>
<tr>
<td>[77]</td>
<td>R3230 AC tumour</td>
<td>Rat leg (s.c.)</td>
<td>$pO_2$ decreased during heating at $42.5^\circ C$ for 60 min but increased 24 h after heating</td>
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<td>$pO_2$ decreased during and 24 h after heating at $43.5^\circ C$ for 60 min</td>
</tr>
<tr>
<td>[85]</td>
<td>C3H mammary tumour</td>
<td>Mouse flank or leg (s.c.)</td>
<td>$pO_2$ increased 24 h after heating at $43.5^\circ C$ for 60 min</td>
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<td>$pO_2$ decreased 4 h after heating at $43.5^\circ C$ for 120 min</td>
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<td>[84]</td>
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<td>Heat-induced changes in $pO_2$ depend on tumour site because $pO_2$ in the legs did not increase by heating</td>
</tr>
<tr>
<td>[119]</td>
<td>SCK mammary carcinoma</td>
<td>Mouse leg (s.c.)</td>
<td>$pO_2$ increased during and 12–15 min after heating at $41.5^\circ C$ for 60 min</td>
</tr>
<tr>
<td>[115]</td>
<td>Human soft tissue sarcoma</td>
<td>Human extremity</td>
<td>$pO_2$ increased 1 day after first hyperthermia</td>
</tr>
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</table>

mice [122], RIF-1 tumours in C3H mice [123], and spontaneous canine tumours [124]. Similar results have also been found in human xenograft tumours [125]. However, heating at higher temperatures ($44.5^\circ C$) will decrease the blood flow [64, 122–124] and this may be due to direct damage to blood vessels. The responses of multiple heating on tumour vessels are quite different from that of single heating. In R3230 AC tumours, the tumour blood flow after heating at $42.5^\circ C$ for 1 h is less than in the control tumours due to vascular damage. However, when tumour are preheated at $42.5^\circ C$ for 1 h and reheated 16–24 h later at $42.5^\circ C$, the tumour blood flow increases two to three times after the second heating. The increase in tumour blood flow by the second heating following the conditioning of blood vessels by the first heating has been suggested to be due to development of vascular tolerance [126].

All of the above studies have utilised local or local/regional heating models in rodents and used relatively high, non-physiological temperatures ($40^\circ C$ and above) to mimic clinical protocols in which heat shock temperatures are used. Because thermoregulation is optimally regulated at physiologically relevant temperatures (below $40^\circ C$), we have concentrated our studies on temperatures from $38^\circ C$ to $39.5^\circ C$ and have developed a systemic heating model rather than trying to use a local heating model. Using this model, earlier studies from our lab showed that systemic mild (fever-range) hyperthermia results in an obvious expansion in the diameters of many tumour blood vessels and an increase in the percentage of perfused blood vessels with discernible erythrocytes [99, 100]. This thermally increased perfusion can persist for 24–48 h after heating [127]. As mentioned earlier, high tumour interstitial fluid pressure may contribute to insufficient blood perfusion and reduced oxygenation and is recognised as the barriers for tumour therapy. New data from our lab shows that systemic or whole body hyperthermia treatment can significantly decrease tumour interstitial fluid pressure (IFP) to a comparable level achieved in Taxol treatment, a common chemotherapy drug that has been shown to decrease tumour IFP (Arindam Sen et al., unpublished MSS).

Systemic heating may indeed trigger powerful thermoregulatory responses which could, in turn, be responsible for increased vascular perfusion within tumours and reduced tumour IFP.

While there are still many unanswered questions, collectively the data in this field suggest that hyperthermia (both local applications at higher target temperatures and systemic application at physiologically relevant temperatures) increases tumour oxygenation and vascular perfusion. The expected resultant decrease in hypoxia could provide a tumour-specific window of opportunity for decreasing the immunosuppressive environment within the hypoxic tumour microenvironment. Moreover, direct immune activation by thermal signalling can further enhance anti-tumour immune effector activity. See the article by Multhoff in this special issue for a more in depth study on immune cell activation/heat shock protein function and...
hyperthermia). More research is needed to compare various temperatures and protocols for achieving tumour hyperthermia and to more completely test these exciting assumptions.

Summary and identification of additional important research questions

While hypoxia is well known to contribute to radioor chemoresistance of tumours, this article summarises a growing body of evidence showing that the hypoxia within the tumour microenvironment can also be highly detrimental to the activity of the anti-tumour effector mechanisms. Specifically, we summarise data which shows that hypoxia not only helps tumour cells to escape recognition of cytolitic cells (e.g. by inducing shedding of targets for NK cells) but also prevents the proper maturation and function of dendritic cells and T lymphocytes and increases the potential for recruitment of immunosuppressive T lymphocytes and tumour-associated macrophages. Hypoxia-induced immunosuppression can be added to other known strategies by which tumours escape effective immune control either by natural immunity or following immunotherapy.

Although the mechanism is far from clear, local hyperthermia can increase oxygenation of tumours and increase vascular perfusion in experimental animal tumour models. Whether the effect of local heating is on the tumour vasculature itself, or from secondary thermoregulatory responses in normal vessels (which are equipped by adequate neuro-muscular regulation to recognise thermal signals) draining the heated tumour is not yet clear. Our data shows that mild, fever-range systemic hyperthermia can also significantly increase the percentage of perfused blood vessels within tumours and that this effect can last for hours. Moreover, systemic heating is associated with a significant depression in interstitial fluid pressure. Other recent research has also revealed that exposure of immune cells and tumour cells to mild hyperthermia have positive effects on the same immune mechanisms that are negatively impacted by hypoxia. Thus treatment with heat could have a two-pronged ability to improve anti-tumour immunity: reduced hypoxia-mediated immune suppression via heat-induced vascular changes in the tumour microenvironment and direct stimulation of anti-tumour immune mechanisms.

Many questions remain. If thermal regulation of blood flow is the key target for hyperthermia’s ability to improve immunotherapy (or radiation or chemotherapy), are we using the optimal target temperature? The literature summarised here suggests that blood flow is highly sensitive to temperature, and that heat shock temperatures often aimed for in clinical protocols can actually damage blood vessels and/or inhibit blood flow. Moreover, would it be better to also heat normal vasculature surrounding a tumour in order to maximise thermoregulatory signals that increase blood flow to the tumour? Clearly, new detailed analyses on the differences between tumour and normal vasculature in terms of thermal sensitivity, thermoregulation and neural regulation are needed. Another question is raised when considering the types of tumour models which have been used by investigators who have studied hypoxia and the ability of hyperthermia to improve blood flow and oxygen tensions. Essentially, all of the published literature utilises transplantable tumours derived from long-term cell lines. Will these same effects be observed in spontaneous tumours in which both the tumour and the vasculature derived from the host? Moreover, how does hyperthermia affect oxygen tension and the blood vessel function in metastatic tumours? As was stated above, the clear benefit seen when hyperthermia is added to chemotherapy and/or radiation in actual patient tumours strongly suggests that there will be a positive effect on tumour vascular function and hypoxia status. But many more studies are needed to truly assess the full potential of thermal therapy. A deeper understanding of the relationships among hypoxia, anti-tumour immunity and tumour blood flow regulation is necessary before we can achieve the most optimal thermal therapy strategies designed not only to improve radiation and chemotherapy, but also long-term immunological control of tumour growth or metastasis.

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