


Improving the synergistic combination of programmed death-1/programmed death ligand-1 blockade and radiotherapy by targeting the hypoxic tumour microenvironment

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Background

Immunotherapy is a validated cancer therapy that functions to activate the body's natural immune response against cancer cells.¹ Over the past decade, there have been significant clinical advances in cancer immunotherapy, especially in the field of immune checkpoint inhibition (ICI).² Immune checkpoints are receptor-ligand pairs that regulate T-cell activation at various stages of an immune response and help prevent autoimmunity.³ The two most studied immune checkpoint receptors that are expressed on the T cells include cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1). CTLA-4 competes with a costimulatory molecule CD28,

Summary

Immune checkpoint inhibition with PD-1/PD-L1 blockade is a promising area in the field of anti-cancer therapy. Although clinical data have revealed success of PD-1/PD-L1 blockade as monotherapy or in combination with CTLA-4 or chemotherapy, the combination with radiotherapy could further boost anti-tumour immunity and enhance clinical outcomes due to the immunostimulatory effects of radiation. However, the synergistic combination of PD-1/PD-L1 blockade and radiotherapy can be challenged by the complex nature of the tumour microenvironment (TME), including the presence of tumour hypoxia. Hypoxia is a major barrier to the effectiveness of both radiotherapy and PD-1/PD-L1 blockade immunotherapy. Thus, targeting the hypoxic TME is an attractive strategy to enhance the efficacy of the combination. Addition of compounds that directly or indirectly reduce hypoxia, to the combination of PD-1/PD-L1 inhibitors and radiotherapy may optimize the success of the combination and improve therapeutic outcomes. In this review, we will discuss the synergistic combination of PD-1/PD-L1 blockade and radiotherapy and highlight the role of hypoxic TME in impeding the success of both therapies. In addition, we will address the potential approaches for targeting tumour hypoxia and how exploiting these strategies could benefit the combination of PD-1/PD-L1 blockade and radiotherapy.

Key words: hypoxia; immune suppression; PD-1/PD-L1 blockade; radiotherapy; tumour microenvironment.

also expressed on the T cells, and both these molecules bind to CD80 and CD86 on antigen-presenting cells (APCs). In contrast, PD-1 binds to its ligands, programmed death ligand-1 (PD-L1) and programmed death-ligand 2 (PD-L2), which are also present on APCs. The binding of these checkpoint receptors to their respective ligands inhibits T-cell activation and proliferation, thus dampening the immune response.^{4,5}

In recent years, PD-1/PD-L1 blockade has shown promise in clinical trials and is at the forefront of cancer immunotherapy.⁶ It has been trialled in various solid malignancies, resulting in significant anti-tumour effects including in melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), colorectal cancer, urothelial

carcinoma, head and neck carcinomas, and hepatocellular carcinoma (HCC).⁷⁻¹⁴ However, despite long-term remission and potentially cure-like benefits, inherent resistance to PD-1/PD-L1 blockade monotherapy remains a clinical challenge. Additionally, most responders may experience acquired resistance and eventually develop clinical relapse after a few years.^{15,16}

The tumour microenvironment (TME) has a major role in determining tumour immunogenicity and resistance to PD-1/PD-L1 blockade.¹⁷ Tumours are normally divided into immunogenic (or hot) and poorly immunogenic (or cold) tumour types. A hot TME is infiltrated with more immune cells, for example effector T cells, natural killer (NK) cells and dendritic cells (DCs), compared with a cold TME with low-to-no immune cell infiltration. Tumours with an immunogenic phenotype have improved prognosis and can respond well to ICI.¹⁸⁻²⁰ Moreover, the presence of immunosuppressive cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and anti-inflammatory macrophages in the TME also plays an important role in resistance to anti-PD-1/PD-L1 therapy.¹⁷ This suggests that modifying the TME to create an immunogenic phenotype and limit the recruitment and function of immunosuppressive cells could enhance the efficacy of PD-1/PD-L1 blockade therapy. Therefore, combination of anti-PD-1/PD-L1 antibodies with various other immunostimulatory therapies that target the TME, including radiotherapy, is actively being investigated.⁴ The combination of PD-1/PD-L1 blockade with radiotherapy can boost the anti-tumour responses and result in better clinical outcomes.

It is also important to understand that the success of the combination approach can be compromised by additional TME-associated factors such as tumour hypoxia. Hypoxia, a common feature of most solid tumours, generates an immunosuppressive TME, thereby suppressing the anti-tumour immune responses.^{21,22} Hence, overcoming the immunosuppressive mechanisms driven by the hypoxic TME may be an effective approach to elicit durable anti-tumour immunity and improve the responses of combined PD-1/PD-L1 blockade and radiotherapy. In this review, we will provide a brief overview of the combination of PD-1/PD-L1 blockade with radiotherapy. Furthermore, we will discuss how tumour hypoxia can act as a barrier to blunt the efficacy of both therapies. Finally, we will address how targeting the hypoxic TME has the potential to improve the synergistic interaction of PD-1/PD-L1 blockade and radiotherapy.

The PD-1 pathway

As a member of the B7/CD28 family of costimulatory T-cell receptors, PD-1 is upregulated upon T-cell activation and during persistent antigen encounter, such as in the cases of chronic infections and cancers. Along with activated T cells, PD-1 is also widely expressed by Tregs, B cells, myeloid cells, DCs, NK cells and tumour-infiltrating

lymphocytes (TILs) from different tumour types.³ Its ligand PD-L1 is present on T cells, B cells, DCs, macrophages and other non-immune cells.²³ PD-L1 is also found to be upregulated on several solid tumours and is linked to increased TILs and poor patient prognosis.²⁴⁻²⁶ In contrast, the expression of PD-L2 is limited, and it is mainly expressed on DCs and activated macrophages.²⁷

Under normal conditions, the PD-1/PD-L1 pathway plays an essential role in maintaining immune tolerance and preventing autoimmune diseases.⁴ PD-1/PD-L1 inhibit T-cell responses and suppress overstimulation of the immune response, essentially acting as a break or an off-switch for the immune system. Tumours can exploit this pathway by overexpressing PD-L1 which impairs anti-tumour immunity and inhibits the activation and function of T cells by decreasing the production of inflammatory cytokines while increasing the secretion of inhibitory cytokines, decreasing lymphocyte proliferation and cytotoxicity, and stalling the T-cell cycle.^{4,28,29} The increased expression of PD-1 is also a hallmark of T-cell exhaustion, resulting in T-cell dysfunction and lack of immune surveillance in infections and tumours.^{30,31} Thus, blocking the PD-1/PD-L1 pathway can preserve the anti-tumour capacity of T cells and prevent immune evasion of the tumour cells.

Radiotherapy

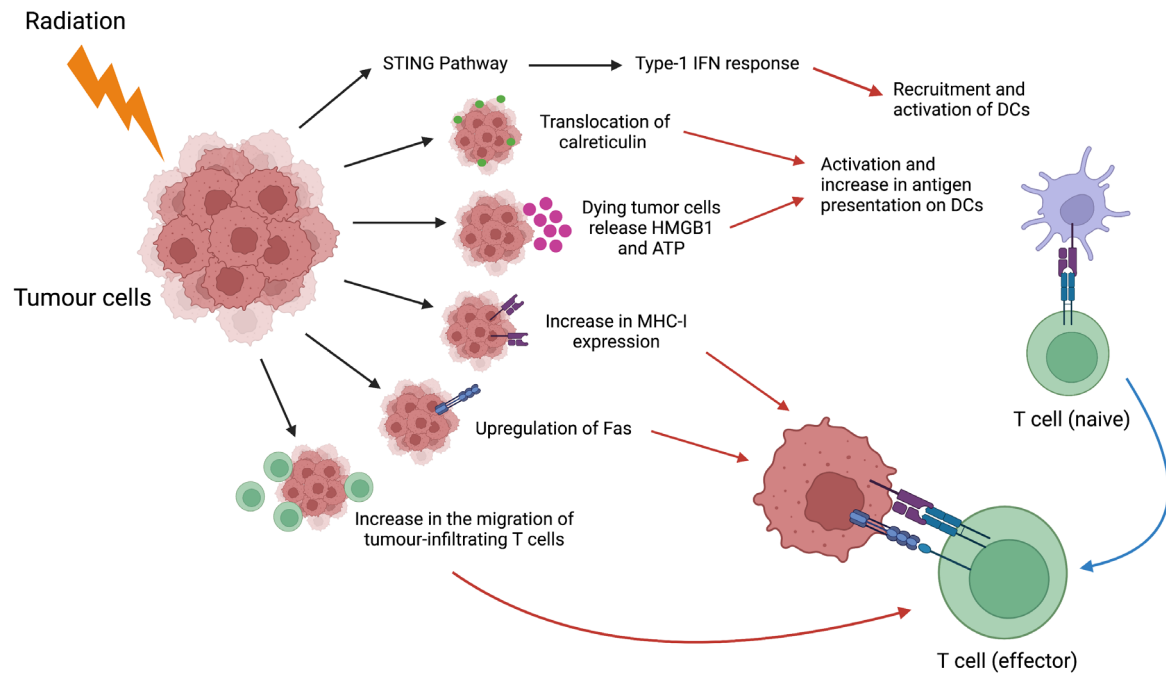
Radiotherapy remains a key component of cancer treatment, with curative or palliative roles in different cancer types. It uses ionizing radiation to directly damage the DNA by inducing DNA breaks. Indirect effects of radiation are mediated via reactive oxygen species (ROS) that oxidize lipids and proteins, and these also induce damage in the DNA. The consequence of radiation-induced DNA damage is programmed cancer cell death and mitotic failure.³² Unfortunately, many cancers are either inherently radiation resistant or they can develop acquired radioresistance following radiotherapy. Thus, the effectiveness of radiotherapy is frequently improved by combination with surgery, chemotherapy and immunotherapy.

Combination of PD-1/PD-L1 blockade and radiotherapy

Influence of radiation on the immune system

Radiation acts as an immune system modulator as it can influence the immune state of the tumour in various ways (Fig. 1). Radiotherapy has been shown to promote tumour-specific immune responses both in animal models and humans.^{33,34} Radiation causes activation of the stimulator of interferon genes (STING) pathway that induces interferon-I production, which is essential for recruiting and activating DCs in the TME.³⁵ Radiation can induce various damage-associated molecular pattern (DAMP) molecules that also activate the DCs and promote the uptake and presentation of tumour cell antigens by DCs.

Immunostimulatory effects of radiation



Immunosuppressive effects of radiation

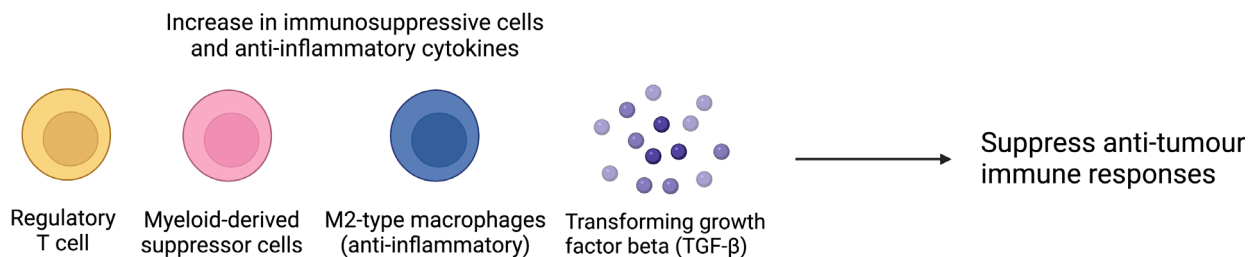


Fig. 1. Radiation can stimulate the immune response and induce immunogenic cell death in several ways. It activates the stimulator of interferon genes (STING) pathway that induce type-1 interferon (IFN) response which aids in the recruitment and activation of dendritic cells (DCs). Radiation can induce various damage-associated molecular pattern (DAMP) molecules including calreticulin, high-mobility group box 1 (HMGB1) and adenosine triphosphate (ATP). All these DAMPs promote activation of DCs and increase antigen presentation to the naïve T cells, which then convert to effector T cells. Radiation also increases the expression of major histocompatibility complex class I (MHC-I) that present antigens to T cells, and Fas death receptor that can induce apoptotic cell death of the tumour cells. Radiation increases the migration of tumour-infiltrating T cells to the tumour region that is required for tumour recognition and anti-tumour effect. The immunosuppressive effects of radiation are mediated by radiation-induced increase in the recruitment of immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells and M2-type macrophages, and increase in the generation of anti-inflammatory cytokines including transforming growth factor-beta (TGF- β). The immunosuppressive cells and cytokines can impair anti-tumour immune responses, thus sustaining immunosuppression in the tumour microenvironment. (●) Calreticulin, (●) HMGB1 and ATP, (M) MHC-I, (F) Fas death receptor, (T) T cell, (D) dendritic cell (DC), (T) tumour cell, (T) T cell receptor. Figure created using Biorender.com.

Calreticulin, an endoplasmic reticulum protein, is a DAMP molecule that serves as a signal for DCs to phagocytose the dying tumour cells.³⁶ Other DAMPs including high-mobility group box 1 (HMGB1) promote antigen cross-priming on the T cells, and adenosine triphosphate (ATP) which activates inflammasome signalling in DCs, inducing IL-1 β that primes the T cells.^{37,38} Chronic exposure of

these DAMPs in the TME stimulates long-lasting anti-tumour immunity and results in immunogenic cell death. Additionally, irradiated tumour cells can upregulate the expression of major histocompatibility complex class I (MHC-I) and cell surface death receptor Fas. Increased MHC-I enhances recognition of irradiated tumour cell antigens by effector T cells, and upregulated Fas promotes

apoptotic cell death.³⁹⁻⁴¹ Moreover, radiation also enhances the trafficking and homing of tumour-specific T cells to the tumour region, which is necessary for the anti-tumour effect.⁴² Collectively, these immunostimulatory properties of radiation suggest that it can be exploited to improve the efficacy and overcome resistance to PD-1/PD-L1 blockade immunotherapy.

Along with the immunostimulatory effects, several immunosuppressive effects are also reported following exposure to radiotherapy (Fig. 1). Radiation increases the recruitment of MDSCs, Tregs and anti-inflammatory macrophages, all of which help sustain immune suppression in the TME.⁴³⁻⁴⁵ Furthermore, radiation-induced ROS increases the anti-inflammatory cytokine *transforming growth factor-beta* (TGF- β), which inhibits the activation of DCs and antigen presentation to T cells.^{46,47} The immunosuppressive effects mediated by radiotherapy need to be controlled as these may oppose the pro-inflammatory states induced by radiation.

Although radiation could enhance the immunogenicity of tumours, it is still unable to elicit a coordinated and effective immune response as a monotherapy. This is because single-site radiotherapy rarely results in tumour regression in unirradiated sites (known as abscopal effect).⁴⁸ Increasing evidence indicates that abscopal effects are immune-mediated responses and their infrequency in clinical settings following radiotherapy alone is likely due to the counterbalance of immunostimulatory and immunosuppressive effects of radiotherapy.⁴⁹⁻⁵¹ Given that PD-1/PD-L1 blockade immunotherapy can activate anti-tumour immune responses, it is possible that radiotherapy combined with PD-1/PD-L1 blockade immunotherapy can amplify immune responses against tumours and therefore increase the occurrence of abscopal effects.

Synergistic interaction of PD-1/PD-L1 blockade and radiotherapy

Preclinical studies have demonstrated improved anti-tumour responses from the combination of PD-1 or PD-L1 blockade and radiotherapy. As an example, the combination of anti-PD-1 antibody and radiation showed synergistic improvement in survival and significantly decreased tumour volume in preclinical mice models of intracranial glioma, melanoma and breast cancer.^{52,53} Other studies in mice have also reported better tumour control with the combination of anti-PD-L1 antibody with radiation compared to either treatment alone.^{54,55} More importantly, abscopal effects were observed in the combined therapy group only.⁵⁴ Furthermore, in a PD-1 resistant lung cancer model, radiation increased MHC-1 production and interferon-beta (IFN- β) levels and sensitized this model to PD-1 blockade therapy, suggesting that radiation has the potential to overcome PD-1 resistance.⁵⁶

Based on these promising findings, many clinical trials have also confirmed the synergistic interaction of PD-1/

PD-L1 blockade and radiotherapy.^{57,58} In particular, the KEYNOTE-001 (phase 1), PEMBRO-RT (phase 2) and PACIFIC (phase 3) trials have all supported the feasibility and effectiveness of combined PD-1/PD-L1 inhibition and radiotherapy.⁵⁸⁻⁶⁰ More clinical trials assessing the synergy between the combination are currently ongoing (as indicated by searching these terms on the ClinicalTrials.gov website).

Considerations for the combination approach

Several factors need to be considered to maximize the therapeutic effect and minimize the risk of toxicity from the combination of PD-1/PD-L1 blockade and radiotherapy. These include the sequence of application (concurrent or sequential), radiation dose and fractions, and safety of the combination treatment.^{61,62} While preclinical investigations and some clinical trials have favoured the concurrent application sequence of PD-1/PD-L1 blockade and radiotherapy,^{55,63,64} other trials have supported the effectiveness of sequential radiotherapy combined with PD-1/PD-L1 inhibition.^{59,60} Moreover, preclinical studies found that radiation delivered as single high dose of 20 Gy could impair tumour immunogenicity, whereas fractionated radiotherapy could stimulate anti-tumour immunity and demonstrated effective abscopal responses when combined with anti-CTLA-4 antibody.^{65,66} However, clinical data to clearly support a difference between single and multi-fraction radiotherapy schedules in combination with immune checkpoint blockade is still lacking. Currently, there is no consensus on the most effective treatment schedule for the combination approach. More clinical studies are needed to justify the dosages and mode of the combination therapy for optimal therapeutic benefit.

Along with the above-mentioned considerations, the immunosuppressive mechanisms of hypoxia in the TME can also negatively affect the outcomes of the combined therapy approach. Tumour hypoxia reduces the efficacy of both radiotherapy and immunotherapy when used as monotherapies; therefore, it is highly likely that the hypoxic TME could impede the success of the combination approach by suppressing the anti-tumour responses. Thus, to fully realize the benefits of this combination, another strategy could involve the incorporation of drugs that target hypoxia and/or the downstream effects of hypoxia to amplify the synergistic interaction of PD-1/PD-L1 blockade and radiotherapy.

Hypoxia as a barrier to radiotherapy and PD-1/PD-L1 blockade immunotherapy

Establishment of the hypoxic tumour microenvironment

Hypoxia occurs when the oxygen pressure in the TME drops below normal for the surrounding tissue. It arises

due to the high oxygen consumption and rapid proliferation of tumour cells, leading to a mismatch between oxygen supply and demand, and resulting in the development of hypoxic niches.^{67,68} The hypoxic TME induces angiogenesis, promoting the growth of neovascular networks which are often disorganized and aberrant, which further enhances tumour hypoxia.^{69,70} Hypoxia influences tumour biology in several ways including promoting tumour invasiveness and metastasis, altering tumour metabolism, suppressing apoptotic responses and generating an immunosuppressive TME.^{22,71–74}

Hypoxia also stabilizes the transcription factors, hypoxia-inducible factors (HIFs), which maintain tumour cell adaptation to the hypoxic TME. There are three isoforms of the oxygen regulated component of HIF, that is HIF-1 α , HIF-2 α and HIF-3 α , and the hypoxic responses in the TME are primarily regulated by HIF-1 α . HIF pathways are upregulated in many tumour types and increased expression of HIFs is correlated with poor patient prognosis.^{75–78} HIF activity also promotes several other biological pathways that contribute to resistance to radiotherapy and immunotherapy, which will be discussed in the following sections.^{79,80}

Hypoxia impairs radiation response

Hypoxia is a major barrier to the effectiveness of radiotherapy. It limits the efficacy of radiotherapy by attenuating the radiation-induced DNA damage as oxygenation is essential for permanent DNA damage (Fig. 2).^{81,82} Hypoxia confers a radioresistant phenotype as hypoxic tumours require a 2- to 3-fold higher radiation dose than normoxic tumours to achieve the same biological effect.⁸³ As hypoxia drives radioresistance of most cancers, alleviating tumour hypoxia is an attractive strategy to improve the radiosensitivity of tumours and enhance radiation outcomes.

Hypoxia suppresses the anti-tumour immune responses

The hypoxic TME strongly influences the immune cells and emerging evidence suggests that hypoxia is a key component in immunotherapy resistance (Fig. 2).^{22,84,85} Links between the hypoxic TME and the mechanisms of immunotherapy resistance are further supported by several preclinical and clinical studies.⁸⁶ Hypoxia-driven mechanisms, including those regulated by HIF, can result in immune tolerance and immune evasion by damaging the key regulatory anti-tumour immune responses.⁸⁰ Hypoxia also creates an immunosuppressive TME by recruiting immunosuppressive cells, upregulating inhibitory checkpoint proteins and altering the tumour metabolic landscape.⁸⁷ Moreover, hypoxic regions of the tumours have poor infiltration of anti-tumour immune cells and the limited immune cells that do reach the

hypoxic areas are still unable to fully exhibit their anti-tumour functions.⁸⁰ In this section, we will provide a detailed overview of how hypoxia affects the different immune cell types and immune responses, with a particular focus on HIF-1 regulated processes.

Effector T cells

Effector T cells, consisting of CD8⁺ cytotoxic and CD4⁺ helper T cells, are vital components of adaptive immune response against tumour antigens. Hypoxia alters the effector T-cell population in several ways, including through inducing apoptosis, delaying the differentiation of T cells and reducing the release of proinflammatory cytokines such as interleukin-2 and interferon-gamma (IFN- γ).^{22,88} Hypoxia can impair the activation of T cells by decreasing the activation markers, CD40L and CD69.⁸⁹ Furthermore, hypoxia via HIF-1 α pathway converts CD4⁺ T cells into the Tregs, which suppress the effector function of CD8⁺ T cells.^{90,91} Hypoxia-induced HIF-1 α enhances the accumulation of metabolic by-product lactate, creating an acidic TME, which further suppresses T-cell proliferation, cytokine production and inhibits their cytolytic activity.^{92,93} Moreover, under hypoxic conditions, there is an increase in adenosine, an immunosuppressive metabolite that also hampers anti-tumour immunity.^{94,95} The binding of adenosine with its receptors (A2AR and A2BR) on the T cells, leads to the production of intracellular cyclic adenosine monophosphate (cAMP) which impair effector T-cell functions such as T-cell trafficking and the release of proinflammatory cytokines.^{95,96}

Dendritic cells

Dendritic cells are the dominant APCs that present tumour antigens to T cells and initiate immune responses. Hypoxia affects DCs by impairing their circulation and downregulating the expression of both differentiation and maturation markers (CD80, CD83 and MHC-II), and costimulatory molecules (CD40, CD80 and CD86) on the DCs.^{97,98} Hypoxia downregulates the chemokine receptor CCR7, which is required for mature DCs migration to the lymph nodes.⁹⁸ Hypoxia also upregulates vascular endothelial growth factor (VEGF) and the interleukin-10, which inhibit the maturation and differentiation of DCs.^{99–101} Furthermore, the secretion of large amounts of osteopontin by the hypoxic DCs promotes tumour cell migration.¹⁰²

Natural killer cells

Natural killer cells are components of innate immune system and are also affected by the hypoxic TME. Hypoxia can impair the function of NK cells by downregulating the expression of several NK-cell receptors such as NK p44, NK p30 and NK p46.¹⁰³ Moreover, hypoxia-

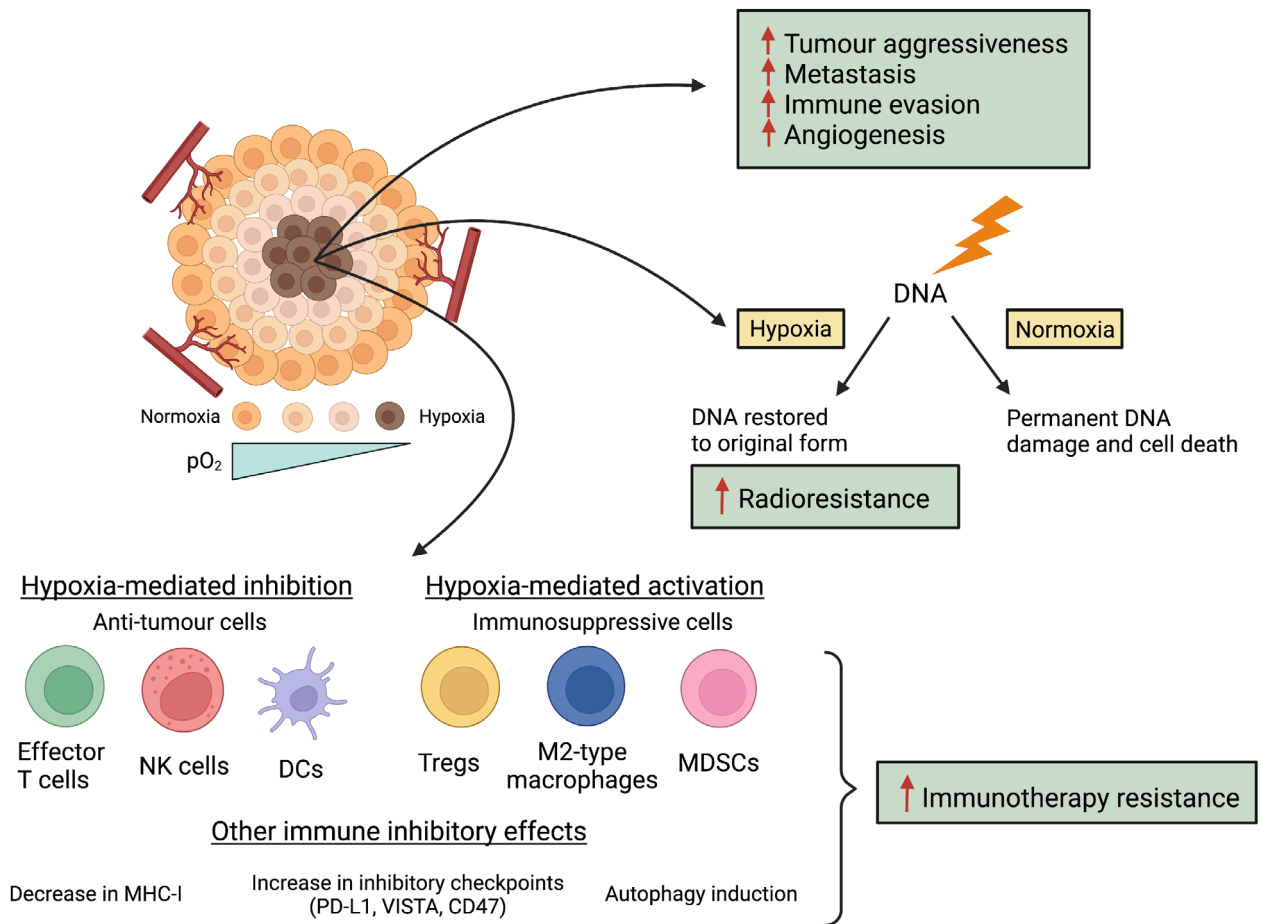


Fig. 2. Hypoxia promotes tumour progression and leads to radioresistance and immunotherapy resistance. Hypoxia is a key player in the tumour aggressiveness and promotes metastasis, angiogenesis and tumour immune evasion. Hypoxia contributes to radioresistance by preventing permanent DNA damage from ionizing radiation and instead restoring the DNA back to its original form. However, under normoxic conditions, damaged DNA undergoes permanent fixation that eventually leads to cell death. Hypoxia also leads to immunotherapy resistance by modulating the tumour microenvironment (TME) in several ways. It inhibits the anti-tumour immune cells such as effector T cells, natural killer (NK) cells and dendritic cells (DCs), while activating and increasing the recruitment of immunosuppressive cells which include regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and anti-inflammatory M2-type macrophages. Hypoxia can impair tumour-antigen presentation to T cells by downregulating the expression of major histocompatibility complex class I (MHC-I). It also increases various inhibitory checkpoint molecules such as programmed death-ligand 1 (PD-L1), V-Domain Ig Suppressor of T-cell Activation (VISTA) and cluster of differentiation 47 (CD47), all of which contribute to immune evasion. Moreover, hypoxia enhances autophagy, which can impair tumours susceptibility to immune cell attack such as inhibiting NK-cell-mediated killing of tumour cells. Figure created using Biorender.com.

induced recruitment of Tregs in the TME activates TGF- β , an immunosuppressive cytokine that also inhibits the function of NK cells.¹⁰⁴

Regulatory T cells

Tregs, a subset of CD4⁺ T cells, are responsible for immune suppression, thereby maintaining self-tolerance and preventing autoimmunity. Hypoxia/HIF-1 α pathway is shown to upregulate the forkhead box P3 (FOXP3), a transcription factor and specific marker for Tregs, indicating that hypoxia enhances the formation of Tregs from CD4⁺ T cells.⁹¹ Hypoxia also promotes the recruitment of Tregs by inducing the chemoattractant CCL28. A positive

correlation between CCL28 and HIF-1 α expression was observed in ovarian cancer, and this was linked to poor patient prognosis.⁹⁰ Similarly, hypoxia-induced recruitment of Tregs is found to be a negative prognostic factor in HCC and basal-like breast cancer.^{105,106}

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells are immature myeloid cells that undergo differentiation to mature myeloid cells in the presence of specialized cytokines. MDSCs consist of DCs, immature macrophages and granulocytes. These regulate self-tolerance, impair T-cell functions and are also responsible for tumour metastasis and

angiogenesis.¹⁰⁷ Hypoxia-induced HIF drives the accumulation of MDSCs and blocking HIF was found to decrease the recruitment of MDSCs, tumour growth and angiogenesis.¹⁰⁸

Tumour-associated macrophages

Tumour-associated macrophages (TAMs), derived from myeloid progenitors, can be classified into M1-type (anti-tumour and proinflammatory) and M2-type (pro-tumour and anti-inflammatory) macrophages.¹⁰⁹ Hypoxia via HIF-1 α mediates the differentiation of MDSCs into TAMs that prevent immune destruction by decreasing T-cell infiltration.¹¹⁰ High number of M2-type TAMs in the tumour region contributes to immunosuppression, tumour progression and angiogenesis, and is mostly associated with poor clinical prognosis in cancer patients.¹¹¹ Tumour hypoxia increases the recruitment of M2-type macrophages in the TME by inducing the expression of migratory stimulatory factors such as VEGF and endothelins.²¹ Similarly, enhanced production of lactate and the secretion of VEGF, TGF- β , interleukin-4 and interleukin-6 under hypoxic conditions, create a suitable environment for the differentiation of macrophages into suppressive M2 TAMs.¹¹² Moreover, hypoxia upregulates the secretion of matrix metalloproteinase (MMP7) by TAMs, which cleaves the Fas ligand from neighbouring cells, thus decreasing tumour cell lysis by T cells and NK cells.^{113,114}

Other regulatory pathways

Hypoxia attenuates anti-tumour immunity by upregulating the expression of PD-L1 on tumour cells, MDSCs, TAMs and DCs via HIF-1 α regulated pathways.¹⁰⁴ The positive correlation and links between PD-L1 and HIF-1 α has been reported in various studies.^{115,116} HIF-2 α is also reported to increase PD-L1 expression in clear cell renal cell carcinoma (ccRCC).¹¹⁷ Inhibition of PD-L1 was found to promote MDSCs-mediated T-cell activation, thus decreasing the immunosuppressive function of MDSCs.¹¹⁸ Another checkpoint molecule overexpressed in hypoxic conditions is V-Domain Ig Suppressor of T cell Activation (VISTA). VISTA is expressed on MDSCs, TAMs and DCs and hypoxia-induced VISTA is shown to suppress T-cell proliferation and function.¹¹⁹ Moreover, hypoxia upregulates the expression of cluster of differentiation 47 (CD47), a macrophage immune checkpoint protein, thus, enabling tumour cells to escape phagocytic cell death.¹²⁰ Overexpression of CD47 has been linked to poor clinical prognosis.¹²¹ Furthermore, hypoxia-induced HIF-1 α can increase the expression of metalloproteinase ADAM10, which regulates the shedding of the MHC-I-chain related molecule A (MICA) from the tumour cell surface. MICA is a ligand for activating the natural killer group 2, member D (NKG2D) receptor on the NK cells, and the shedding of MICA downregulates the NKG2D

receptor, leading to tumour cell escape from T cells and NK cells.^{122,123} Additionally, evidence suggests that hypoxic tumour cells can induce autophagy to degrade the NK-cell derived proteases granzymes B, thus decreasing tumour cell susceptibility to NK-cell-mediated killing.^{124,125}

Overall, all the above-mentioned mechanisms indicate that the hypoxic milieu dampens the anti-tumour immune responses and can suppress immunotherapy approaches. As hypoxia promotes radioresistance and immunosuppression,^{80,83} a sustained reduction in tumour hypoxia appears to be an attractive strategy to lower immunosuppression and improve the efficacy of combined PD-1/PD-L1 blockade therapy and radiotherapy. Thus, the prospect of targeting hypoxia in combination with PD-1/PD-L1 blockade and radiotherapy appears to be encouraging (Fig. 3).

Targeting tumour hypoxia to overcome resistance to radiotherapy and PD-1/PD-L1 blockade immunotherapy

Increasing oxygen supply to the tumour

Since hypoxia develops due to lack of oxygen supply, the most relevant approach to resolve hypoxia and improve radiotherapy and immunotherapy outcomes is to increase the oxygen supply to the tumour region. Initial attempts involved the use of blood transfusions to increase haemoglobin levels and therefore improve responses to radiotherapy. However, clinical studies reported that these transfusions did not improve treatment outcomes significantly.^{126,127} Other approaches that attempted to increase oxygen supply included hyperbaric oxygen breathing or the use of carbogen (95% oxygen and 5% carbon dioxide). Despite promising preclinical and early-phase data, both approaches failed to show significant radiosensitization outcomes in patients.^{82,128,129} Interestingly, the combination of carbogen with nicotinamide targets hypoxia and is reported to improve radiation responses in clinical trials.^{130,131} However, the temporary increase in oxygenation from radiotherapy combined with carbogen and nicotinamide maybe too short to initiate effective and durable immune responses.¹³² This suggests that other approaches are needed to result in sustained oxygenation and improve the responses to combined PD-1/PD-L1 blockade immunotherapy and radiotherapy.

Hypoxia-activated prodrugs

Hypoxia-activated prodrugs (HAPs) have also been explored to selectively target hypoxic tumour cells. HAPs are inactive compounds that convert to active drugs in hypoxic regions.¹³³ Despite preclinical data showing cytotoxicity from various HAPs, the clinical studies of HAPs as single agent or combined with chemoradiotherapy were generally disappointing.¹³³ Recently, a second-

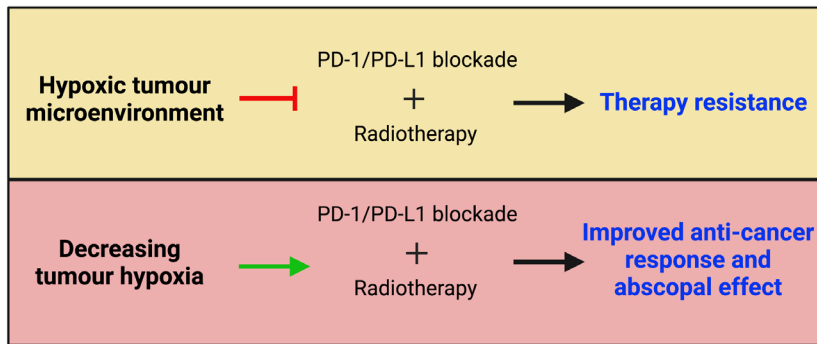


Fig. 3. Hypoxic tumour microenvironment can act as a barrier to the effectiveness of PD-1/PD-L1 blockade therapy and radiotherapy. As hypoxia acts as a barrier to both radiotherapy and immunotherapy, the hypoxic tumour microenvironment (TME) may negatively influence the synergistic combination of PD-1/PD-L1 blockade and radiotherapy and contribute to therapy resistance. Decreasing the hypoxic TME can overcome the immunosuppressive effects mediated by hypoxia and may benefit combined PD-1/PD-L1 blockade therapy and radiotherapy. Incorporation of compounds that directly or indirectly target hypoxia may boost the synergistic combination of these therapies and lead to improved anti-cancer responses and abscopal effects. Figure created using Biorender.com.

generation HAP evofosfamide (TH-302) has demonstrated favourable outcomes in combination with CTLA-4 and PD-1 blockade by curing more than 80% of tumours in a mouse prostate-derived model. This was most likely achieved by a significant decrease in tumour hypoxia, promoting the influx of T cells and decreasing in MDSCs in the TME.¹³⁴ Currently, a clinical trial (NCT03098160) is investigating the efficacy of TH-302 in combination with ipilimumab (anti-CTLA-4) against a range of tumour types. Moreover, TH-302 has also been assessed in combination with radiation, showing increased tumour growth delay in various preclinical tumour models.^{135–137} Research on TH-302 is still ongoing and because previous clinical studies suggest that TH-302 may only provide benefit to patients with high tumour hypoxic fractions, determining the hypoxia status of the patient's tumour will be beneficial before the clinical application of TH-302.¹³⁸ Future preclinical studies could use tumour models with high hypoxic burden to assess whether TH-302 could improve tumour control and enhance the efficacy of combined radiotherapy and anti-PD-1/PD-L1 antibodies.

Decreasing oxygen consumption of the tumour

In contrast to the aforementioned strategies, another alternative to overcome hypoxia is to reduce tumour cells demand for oxygen by inhibiting the mitochondrial oxidative phosphorylation (OXPHOS).¹³⁹ Increasing evidence suggests that along with active glycolysis, cancer cells also have increased mitochondrial OXPHOS, which results in increased oxygen consumption and ATP production in the tumour.^{140–144} OXPHOS is a metabolic pathway comprising of an electron transport chain with complexes I, II, III and IV where a series of redox reactions occur, eventually resulting in the generation of ATP at complex V. Oxygen is critical for OXPHOS as it is the

terminal electron acceptor in this process.¹⁴⁰ The hypothesis is that OXPHOS inhibition will decrease the oxygen consumption rate (OCR) of tumour cells, allowing for the diffusion of unmetabolized oxygen into hypoxic regions, reserving more available oxygen, and thus reducing tumour hypoxia. Mathematical modelling also indicates that decreasing cellular oxygen consumption appears to be a more efficient method to alleviate tumour hypoxia than increasing oxygen supply.¹³⁹

The assumptions underlying the use of OXPHOS inhibitors are that elevated oxidative metabolism in cancers is associated with increased tumour hypoxia, which impedes the success of radiotherapy and immunotherapy. Indeed, several studies have now demonstrated that OXPHOS inhibitors could decrease OCR and tumour hypoxia. Metformin, an antidiabetic drug and a mitochondrial complex I inhibitor, is the first compound tested in this context. It decreased tumour hypoxia and improved tumour radiation response in colorectal and brain tumour preclinical models.^{145,146} As a result, metformin has been evaluated in clinical trials as a potential radiosensitizer.¹⁴⁷ In another study, metformin-induced reduction in tumour hypoxia enhanced the effect of PD-1 blockade immunotherapy by improving the function of cytotoxic T cells.¹⁴⁸ Another compound, atovaquone, an anti-malarial drug and a mitochondrial complex III inhibitor, was found to reduce the OCR in numerous cancer cell lines.¹⁴⁹ It alleviated tumour hypoxia in colorectal and hypopharyngeal carcinoma xenograft models and caused a significant tumour growth delay in combination with radiation.¹⁴⁹ In a recent study, atovaquone/albumin nanoparticles combined with anti-PD-1 antibody significantly improved tumour control in mice xenograft models. The observed effect was due to atovaquone-mediated hypoxia reduction, leading to increased recruitment of CD8⁺ T cells, thereby enhancing the efficacy of anti-PD-1 immunotherapy.¹⁵⁰ The findings using

metformin and atovaquone combined with an anti-PD-1 antibody provide convincing evidence that modification of the hypoxic TME using OXPHOS inhibitors could improve the efficacy of immunotherapy. Additionally, the hypoxia modification efficacy of atovaquone was corroborated in a recent clinical trial (NCT02628080) showing increased tumour oxygenation and inhibition of hypoxic gene expression in NSCLC patients.¹⁵¹ These promising findings have initiated another ongoing trial testing the efficacy of atovaquone in combination with chemoradiotherapy (NCT04648033). Furthermore, papaverine, an antispasmodic drug and a mitochondrial complex I inhibitor, has provided further support towards hypoxia inhibition by OXPHOS inhibitors. This drug also reduced OCR *in vitro*, decreased tumour hypoxia and enhanced radiation response in *in vivo* breast and lung cancer models.¹⁵² Collectively, these findings provide a strong rationale to investigate OXPHOS inhibitors to eliminate the hypoxic TME and improve the efficacies of PD-1/PD-L1 blockade and radiotherapy.

Various studies have supported the effectiveness of OXPHOS inhibition in targeting cancers with upregulated OXPHOS. It also appears that cancers with increased oxidative metabolism might be resistant to PD-1/PD-L1 blockade immunotherapy. As an example, a study on melanoma patients reported that increased oxidative metabolism could act as a barrier to successful PD-1 blockade therapy.¹⁵³ Interestingly, transcriptional profiling data revealed enrichment of hypoxia gene sets in non-responders to anti-PD-1 therapy compared with responders, further supporting the notion that tumour hypoxia promotes resistance to anti-PD-1 immunotherapy.^{154,155} Similarly, links between elevated OXPHOS and immune checkpoint therapy resistance are reported by another study showing that PD-1 resistant NSCLC model appeared to have higher oxidative metabolism than PD-1 sensitive model.¹⁵⁶ This preclinical study highlighted the significance of combining IACS-010759 (mitochondrial complex I inhibitor) with radiotherapy to overcome PD-1 resistance. The addition of IACS-010759 to the combination of radiotherapy and anti-PD-1 antibody significantly prolonged survival and increased abscopal responses in the PD-1 resistant model. Furthermore, they demonstrated that IACS-010759 increases anti-tumour immunity by decreasing Tregs and increasing granzyme B⁺ CD8⁺ T cells.¹⁵⁶ These results suggest that IACS-010759 alleviates tumour hypoxia by blocking OXPHOS and improves anti-tumour response by overcoming radiation resistance and the immunosuppressive TME. The triple combination of OXPHOS inhibition, radiotherapy and anti-PD-1 checkpoint immunotherapy is assessed for the first time by this study. It provides valuable insight into targeting the hypoxic TME to improve the synergistic combination of PD-1/PD-L1 blockade and radiotherapy and increase the abscopal effects (Fig. 3). Future investigations on combined PD-1/PD-L1 blockade and radiotherapy could consider assessing other OXPHOS

inhibitors or compounds that are found to decrease the OCR, influence tumour hypoxia and modulate radiosensitivity. Some of these include the mitochondrial complex inhibitors (metformin, atovaquone and papaverine), non-steroidal anti-inflammatory drugs, glucocorticoids, antiangiogenic agents and MAPK inhibitors (reviewed in Ref. 157).

Inhibiting HIF activity

Hypoxia-inducible factor inhibitors combined with radiotherapy and PD-1/PD-L1 blockade may also prove to be an effective anti-cancer strategy. HIF increases the expression of PD-L1 and has numerous effects on the immune system, summarized above. HIF also has important roles in the tumour in driving metastasis, angiogenesis and tumour metabolism. Of the two main targets, HIF-1 and HIF-2, only a direct HIF-2 inhibitor, belzutifan, has been approved for clinical use.¹⁵⁸ Belzutifan is currently undergoing clinical assessment in combination with the anti-PD-1 antibody pembrolizumab and other compounds on ccRCC (NCT04736706). There are several HIF-1 inhibitors and clinical trials that have been reviewed elsewhere.^{159,160} While space constraints limit our discussion on the topic, preclinical studies indicate that combining HIF inhibition with immune checkpoint inhibitors can promote anti-tumour immunity in various cancers.^{161,162} Moreover, drugs including PX-478, bortezomib and chetomin that could indirectly inhibit HIF-1 protein expression have demonstrated radiosensitization in several cancers at preclinical level.¹⁶³⁻¹⁶⁷ Combinations of HIF inhibitors, radiotherapy and PD-1/PD-L1 blockade is a growing area of interest, and further studies will determine whether this is a viable strategy.

Conclusion

The combination of PD-1/PD-L1 blockade and radiotherapy is a promising development in cancer management; however, the complexity of inherent and acquired resistance to both these therapies can limit their effectiveness in the clinical settings. Evidence from literature suggests that hypoxic TME could impede the success of both radiotherapy and immunotherapy, and several studies have proved that inhibition of hypoxia can sensitize tumour cells to radiotherapy and improve immunotherapy outcomes. This suggests that hypoxia could also be exploited to improve the synergistic combination of PD-1/PD-L1 blockade and radiotherapy. Compounds that have shown promising reduction in tumour hypoxia could be investigated along with the combination of PD-1/PD-L1 blockade and radiotherapy. There is still need for more preclinical and clinical studies to address which of the hypoxia modifying compounds could boost the anti-tumour immunity without suppressing it to achieve optimal therapeutic results when combined with PD-1/PD-L1 blockade and radiotherapy.

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