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Biopterin metabolism and nitric oxide recoupling in cancer

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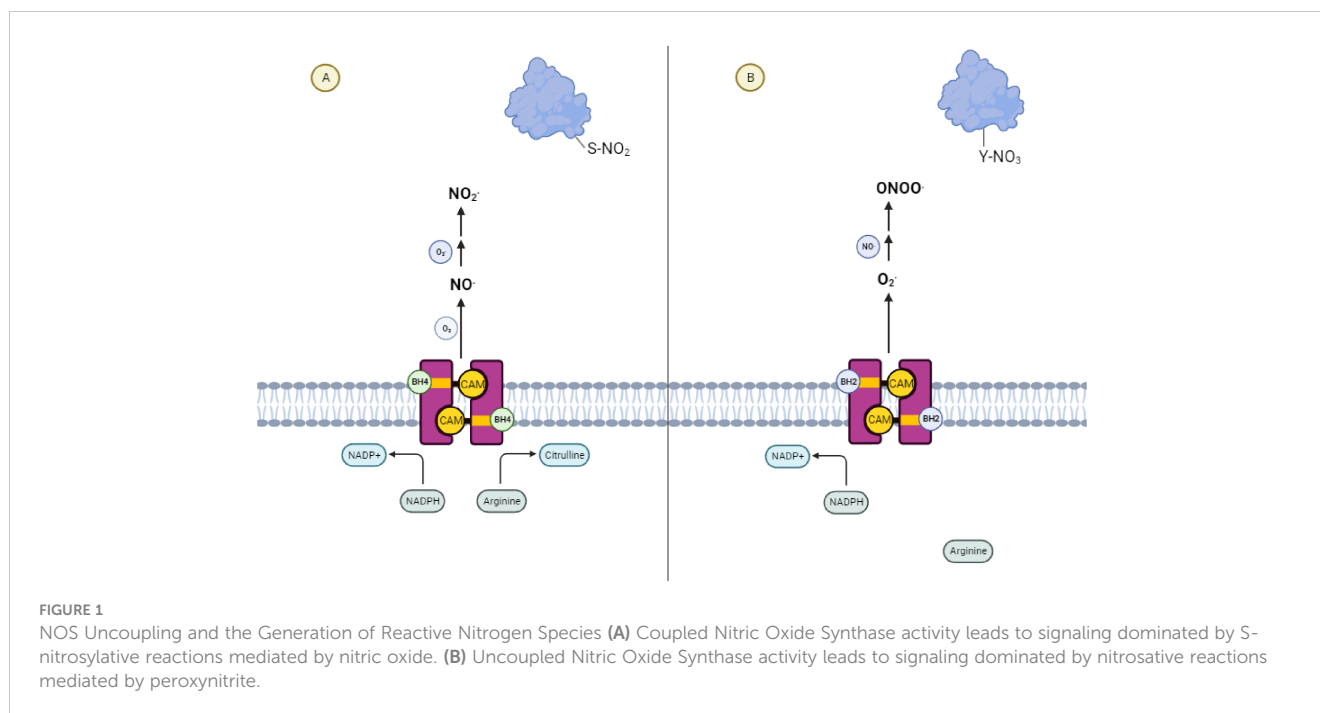
Tetrahydrobiopterin is a cofactor necessary for the activity of several enzymes, the most studied of which is nitric oxide synthase. The role of this cofactor-enzyme relationship in vascular biology is well established. Recently, tetrahydrobiopterin metabolism has received increasing attention in the field of cancer immunology and immunotherapy due to its involvement in the cytotoxic T cell response. Past research has demonstrated that when the availability of BH4 is low, as it is in chronic inflammatory conditions and tumors, electron transfer in the active site of nitric oxide synthase becomes uncoupled from the oxidation of arginine. This results in the production of radical species that are capable of a direct attack on tetrahydrobiopterin, further depleting its local availability. This feedforward loop may act like a molecular switch, reinforcing low tetrahydrobiopterin levels leading to altered NO signaling, restrained immune effector activity, and perpetual vascular inflammation within the tumor microenvironment. In this review, we discuss the evidence for this underappreciated mechanism in different aspects of tumor progression and therapeutic responses. Furthermore, we discuss the preclinical evidence supporting a clinical role for tetrahydrobiopterin supplementation to enhance immunotherapy and radiotherapy for solid tumors and the potential safety concerns.

KEYWORDS

tetrahydrobiopterin, radiotherapy, nitric oxide synthase, immunotherapy, immunometabolism, vascular normalization, cancer, S-nitrosylation

Introduction

Nitric oxide synthase (NOS) exhibits two alternative activities depending on the relative availability of its cofactor tetrahydrobiopterin (BH4) (1). (Figure 1) In the presence of sufficient BH4, NOS catalyzes the production of NO and citrulline from the amino acid arginine. A necessary component of this process is the transfer of NADPH-derived



electrons by BH₄ to the ferrous dioxygen complex within the NOS active site (2, 3). When the availability of BH₄ is low, as it is in chronic inflammatory conditions (4) and tumors (5), electron transfer in the active site becomes uncoupled from the oxidation of arginine, resulting in the production of a superoxide radical (6, 7). In the presence of trace amounts of NO, these two species react to form the short-lived, tyrosine-nitrating radical, peroxynitrite. This further reduces BH₄ levels by direct oxidation (1, 8–10), producing a destructive feed-forward loop (11, 12). Due to this evolutionarily conserved (13) phenomenon of “NOS uncoupling,” the restriction of this cofactor is reinforced upon depletion of local BH₄ until NOS activity ceases.

Due to its capacity for antigen-specific cell killing and immune memory, the T lymphocyte is a major focus in studies on therapeutic anti-tumor immunity. T cells recognize peptides derived from intracellularly degraded proteins and loaded onto Major Histocompatibility Complex (MHC) proteins on the cell surface in a process commonly referred to as antigen presentation (14). Two broad classes of T cells exist and are necessary for anti-tumor immunity (15, 16). T cells that express the co-receptor CD4 recognize antigens loaded onto MHC-II proteins on the surfaces of APCs. These “helper” T cells organize the adaptive immune response to tumors by secreting pro-inflammatory cytokines and chemokines (16). T cells that carry the co-receptor CD8 on the other hand recognize peptides loaded onto MHC-I molecules on the surfaces of cancer cells and carry out cell killing by secreting cell toxins or presenting cancer cells with FAS Ligand (15). CD8⁺ T cells can also be activated by MHC-I⁺ dendritic cells in a process called cross-presentation, which is crucial for generation of an effective anti-tumor immune response (17). CD4 and CD8 T cells are necessary for both the responses of tumors to immunotherapy and for the immunological memory required for durable responses to anti-cancer therapy (18, 19). Recently, it has been reported that

ambient BH₄ availability is a rate limiting factor required for the growth and proliferation of T cells after T cell receptor (TCR) engagement (20). Furthermore, pharmacological augmentation of BH₄ levels has been demonstrated to enhance T cell function in multiple models including anti-tumor immunity (20). This suggests that mechanisms that alter BH₄ metabolism within the tumor microenvironment (TME), such as NOS uncoupling, may play have a significant impact on immune evasion and the resistance of tumors to immunotherapy (14).

Considering the inducible nature of NOS expression within the tissues of the body (21), NOS-mediated BH₄ consumption may also, ostensibly, limit the availability of this cofactor for other BH₄-dependent enzymes such as alkylglycerol monooxygenase (AGMO) and the three aromatic amino acid hydroxylases: phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase. As reviewed elsewhere (22–24), these enzymes also make contributions to the immunosuppressive TME, making this an attractive area for further study (22–24). NOS uncoupling also has significant tissue level effects beyond the consumption of BH₄. NO plays an essential role in the genesis and maturation of nascent vessels as well as the quiescence of mature vasculature (25, 26). As such, its absence in the TME contributes to the irregular structure and dysfunction that is common to tumor vasculature (27–30). This altered vasculature is both a hallmark of tumorigenesis in its own right and leads to therapeutic resistance through multiple mechanisms (31). Thus, exogenous BH₄ supplementation may alter the characteristics of TME to improve traditional anti-cancer therapy (32), and also potentially make them more susceptible to immune attack (20, 33–35).

NOS can be effectively “recoupled” by supplementation with BH₄ or its precursor sepiapterin. The therapeutic effect of NOS recoupling on endothelial dysfunction in hypertension, diabetes, and myocardial infarction is well recognized (36–40). However, the

therapeutic potential of NOS recoupling in the context of cancer has only just begun to be explored. It has been demonstrated that the BH4/BH2 ratio is low in multiple *in vivo* tumor models and patient biopsies (5, 27, 32). Reconstitution of coupled NOS signaling with sepiapterin resulted in vasculature normalization, alleviating tumor hypoxia and enhancing the efficacy of chemotherapy (5, 27, 32). Lastly, sepiapterin supplementation has the added benefit of enhancing cytotoxic T cell proliferation and activity *in vivo* (20). Considering the potential of BH4 supplementation to synergize with existing cancer therapies as well as the excellent and well-established safety of both sepiapterin and BH4 in humans (41–45), BH4 supplementation may become an important tool in the anti-cancer arsenal of the future. In this review, we set out to describe the relevance of local BH4 depletion to existing anti-cancer therapies such as radiotherapy and immune checkpoint blockade with a focus on molecular mechanisms.

NOS uncoupling and BH4 depletion

Biopterins are reductive, enzymatic cofactors required by organisms in all kingdoms of life (13). BH4 is the fully reduced form of biopterin in mammals and its availability is tightly regulated in normal tissues by elegant, cell-type specific control of its synthetic enzymes. Multiple mechanisms of NOS uncoupling and BH4 depletion within tumors have been reported. First, persistent generation of high levels of reactive oxygen and nitrogen species (ROS/RNS) is a hallmark of tumorigenesis (46). BH4 is highly reductive and therefore easily oxidized to its non-productive metabolite 7,8-dihydrobiopterin (BH2) by free radicals such as superoxide or peroxynitrite. Because these two biopterin species bind to the active site of NOS with equal affinities (47), the abundance of BH4 relative to BH2 is a crucial determinant of NOS activity. Additionally, exogenous producers of ROS/RNS, such as radiation, have been demonstrated to initiate NOS uncoupling, leading to reduced BH4 metabolism (48). Second, BH4 levels are regulated by transcriptional control of its synthetic enzymes. In chronic inflammation, BH4 production is limited due to the downregulation of GTP cyclohydrolase-I (49, 50) (GCH-I), the rate limiting enzyme in BH4 synthesis as well as by multiple signaling factors such as the immunosuppressive cytokines IL-10 and TGF- β (13). On the other hand, proinflammatory stimuli such as TNF α or TCR ligation stimulate the expression of GCH-I in their respective cell types (13). This dichotomous regulation of BH4 production by anti-inflammatory and proinflammatory signaling supports a central role of this cofactor in the immune response. Because once NOS uncoupling is initiated by low BH4 levels, its activity directly results in further ROS/RNS-mediated depletion of BH4, the effects of any one of these initial stimuli may be perpetuated until NOS activity ceases or is re-coupled to NO production. This feed-forward loop may represent a molecular switch, flipped off and on by anti-inflammatory and proinflammatory signals, that governs the local availability of BH4 and its impact on the TME and other pathological states (51).

NOS uncoupling is also accompanied by a consequential shift in NO-mediated signaling, which is relevant both to tumor

progression as well as the anti-tumor immune response. The first reported physiological role of NO was discovered when it was found to act as a secondary messenger that binds and activates soluble guanylate cyclase (sGC) in endothelial cells. However, in addition to the activation of sGC and its downstream effectors such as protein kinase G, NO also mediates signaling by oxidative S-nitrosylation of cysteine residues to form S-nitrosothiol groups within target proteins (52). This bona fide post-translational modification, functioning analogously to phosphorylation to activate or inactivate proteins by various physical mechanisms (52). Comparably to phosphorylation, this mechanism modulates proteins of all functional classes and is governed by enzymatic machinery that includes S-nitrosylases and denitrosylases, reflecting its long presence over the course of evolution (53–56). More on the functional role of this post-translational modification on the activation and inactivation of proteins relevant to the immune cascade is provided below.

As NOS becomes uncoupled, an accompanying shift from S-nitrosylation to tyrosine nitration occurs and is the direct product of the changing ratio between ambient NO and superoxide produced by the enzyme. Despite its unpaired electron, NO itself is relatively non-reactive, reacting only with transition metals and heme iron. However, NO can also be “activated” via interactions with low levels of superoxide to form more reactive intermediates such as peroxynitrite (ONOO⁻), nitrogen dioxide (NO₂), and dinitrogen trioxide (N₂O₃), all of which are capable of interacting with nucleophiles like cysteine (57). Similar to NO, NO₂ is lipophilic, migrating across cell membranes and S-nitrosylating proteins at a relative distance. ONOO⁻, on the other hand, is not lipophilic and reacts rapidly with CO₂ to form a CO₂-ONOO⁻ intermediate that quickly degrades into NO₂ and N₂O₃ in the presence of high levels of NO. For this reason, when NO levels are higher than superoxide levels, as they are when NOS is coupled, cyclic-GMP cyclase, protein kinase G signaling, and nitrosative reactions predominate over the oxidative reactions caused by peroxynitrite (58). In contrast, when superoxide levels are higher than NO levels, as they are when NOS is uncoupled, oxidative reactions mediated by peroxynitrite predominate while NO signaling is inhibited due to its impaired production and rapid local consumption. Due to their charge and short half-life, the oxidative reactions mediated by peroxynitrite, such as tyrosine nitration, are relegated to the immediate area of superoxide production.

This dichotomy of RNS directed signaling has a profound impact on the character and biology of the TME. The baseline NO produced by coupled endothelial NOS plays an important role in maintaining vascular integrity via activation of endothelial sGC as well as the S-nitrosylation and silencing of inflammatory mediators such as NF- κ (59–61). The consequence of these effects is a quiescent and functional vasculature. When NOS is uncoupled, as it often is in the TME, peroxynitrite becomes the predominant RNS species produced. This metabolite exhibits a much different chemistry, preferring to react with immediately available tyrosine groups on nearby proteins, resulting in a relatively long-lived Tyr-nitration. The effect of Tyr-nitration is analogous to Tyr-phosphorylation and its effects are different for different residues and proteins. Although this mechanism is less

well studied, peroxynitrite mediated Tyr-nitration and degradation of I κ B is an example, and coupled with the loss of NF- κ B silencing S-nitrosylation, has been suggested to lead to self-perpetuating and potentially unresolved vascular inflammation characteristic of tumors tissues (62, 63). In the setting of the aforementioned T cell dysfunction that comes with NOS mediated BH4 depletion, these effects of NOS uncoupling may greatly contribute to the pro-tumorigenic character of the TME by facilitating immune escape, the development of dysfunctional tumor vasculature, and the propagation of tumor-promoting inflammation.

BH4 metabolism in tumorigenesis

BH4 and immune escape

Although BH4 is necessary for multiple cellular processes, the availability of this essential cofactor is a key determinant of immune cell activity (20, 34, 35). Of the cells of the immune system, the activity of the T lymphocyte is especially susceptible to restriction of BH4, making its control a crucial factor in the adaptive immune response. Since NOS activity is often associated with local BH4 availability, NOS uncoupling within tumor vasculature may represent a novel pathway involved in tumor immune escape.

BH4 availability has been shown to act as a rate-limiting factor for T cell proliferation and activity in diverse models of immunity, including within solid tumors (20, 64). This acceleration in T cell proliferation may occur due to increased iron-redox recycling of T cell mitochondrial cytochrome c which otherwise results in defective oxidative phosphorylation and superoxide production upon TCR stimulation (20, 35). It has been reported that expression of GCH1, the rate-limiting enzyme in BH4 production, is enhanced upon TCR stimulation and its inhibition antagonizes T cell proliferation (13). However, exogenous supplementation of BH4 has been demonstrated to enhance T cell activity in models of autoimmunity, suggesting that endogenous BH4 production by T cells does not support maximum activity and environmental BH4 is rate limiting (20). This supports the idea that the control of BH4 availability by NOS-expressing cells such as endothelial and myeloid cells may be a direct mechanism of impaired T cell activity both within and outside of the TME.

In this way, NOS uncoupling may have an indirect, albeit potent, effect on anti-tumor T cell activity by metabolizing BH4 to non-reducing metabolites. Therefore, supplementation of BH4 may counteract this effect both by supplying BH4 directly to proliferating T cells as well as recoupling NOS, alleviating further NOS-mediated BH4 consumption. Whether BH4 available for use by T cells is represented by free-floating, ambient BH4 or BH4 that is stored in the surrounding stroma has yet to be determined. However, it has been demonstrated that both BH4 production and sequestration occur in various cell types in response to pro-inflammatory cytokine stimulation (13).

In addition, much evidence exists that the restoration of NO signaling via recoupled NOS may support anti-tumor immunity as well. For instance, human T cells express NOS (65–69) and produce NO upon TCR activation, leading to enhanced activity of the TCR

CD3-zeta chain, the adaptor kinase ZAP-70, as well as N-Ras/ERK pathways mediated by N-nitrosylation (70–72). Furthermore, endogenous levels of NO, such as those produced by eNOS, promote the differentiation of Th1 T cells via a cGMP dependent pathway (73), while NOS uncoupling and oxidative stress support Th2 differentiation (74, 75). Additionally, FOXP3⁺ regulatory T cells have also been reported to be negatively regulated in a cGMP dependent manner although it is unclear if this is a direct result of NOS activity (76, 77).

Interestingly, two other classic immunosuppressive mechanisms in the TME, Arginase 1 (Arg1) and Indoleamine 2,3-Dioxygenase 1 (IDO1), can also cause BH4 restriction indirectly via NOS uncoupling (11, 78). Arg1 produces arginase, which breaks down ambient arginine that is required for T cell activity and proliferation. The metabolism of arginine by arginase expressing cells such as M2 macrophages and MDSCs, is a well-recognized mechanism causing T functional impairment within the TME (79). Arginine is also the ultimate electron acceptor in the coupled NOS reaction and required for the production of NO. In the absence of arginine, NOS activity will proceed by donating electrons directly to molecular oxygen, generating free radicals (13). This initiates the uncoupled NOS cascade, leading to ambient BH4 depletion and further restriction of T cell activity. IDO1, which causes T cell restraint by restricting ambient tryptophan (79), may also induce NOS-mediated BH4 depletion via a secondary metabolite of tryptophan metabolism, xanthuric acid. This metabolite has an inhibitory effect on sepiapterin reductase (SR), a necessary enzyme in the BH4 salvage pathway that catalyzes the production of BH4 from BH2 (80). The enhanced concentration of BH2 competes with BH4 for binding to NOS, leading to uncoupling of NOS activity and BH4 depletion (48). Considering the evolutionarily conserved phenomenon of NOS uncoupling as well as the sophisticated mechanisms governing its activation, NOS uncoupling may be a deliberate and physiological mechanism of T cell restriction that is co-opted by solid tumors to achieve immune escape (Figure 2).

In addition to depleting available BH4, peroxynitrite produced by uncoupled NOS has been demonstrated to antagonize T cell activity via more direct effects (81–83). This leads to both the direct nitration and inactivation of the TCR as well as nitration and inhibition of the chemokines CCL2 and CCL5, leading to decreased T cell accumulation within tumors (84–86). Taken together, these reports suggest that endogenous NO expression may contribute to immune surveillance in normal tissues and that NOS uncoupling is a necessary step in the development of immune escape. Highlighting the direct antagonistic effect of NOS uncoupling on T cell activity, de Sanctis et al. found that inhibiting ARG1 and NOS2 expression inhibited accumulation of nitro-Tyrosine moieties in T cells and sensitized pancreatic tumor models to attack by TERT specific CAR-T (33). In addition to modulating the negative effects of NOS uncoupling, restoring native NO production and downstream signaling by activated T cells has also been proposed as an attractive therapeutic strategy (87).

Conversely, it should be noted that some studies suggest that very high levels of NO, such as those produced by NO donors or potentially iNOS, may actually negatively regulate Th1 differentiation required for anti-tumor immunity by leading to

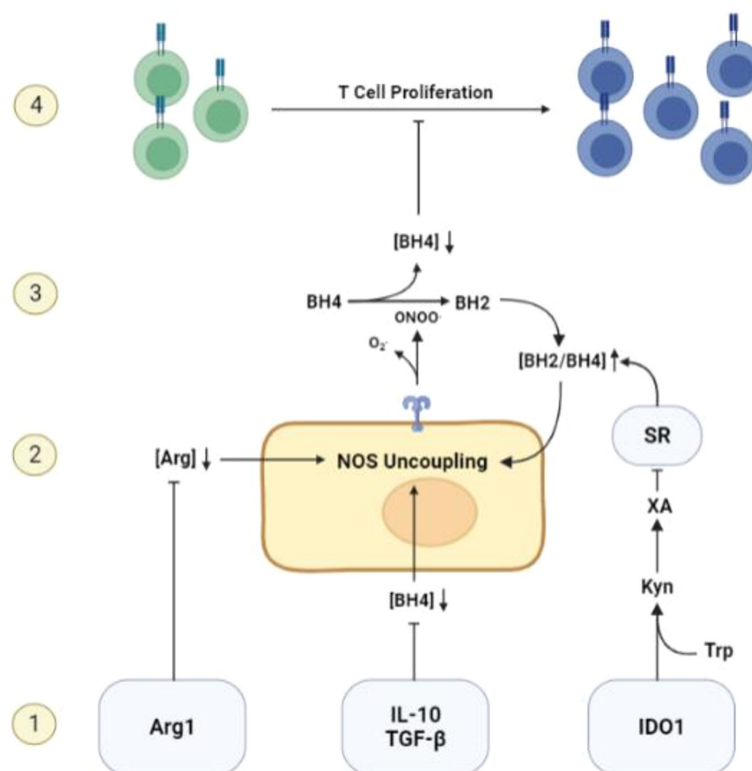


FIGURE 2

Mechanisms of BH4 depletion in the tumor microenvironment. 1) BH4 levels are suppressed by multiple common features of the TME including, but not limited to, the activity of Arg1 and IDO1, the expression of immunosuppressive cytokines such as IL-10 and TGF- β , and high levels of reactive oxygen species. 2) Low levels of ambient BH4 leads to uncoupling of NOS activity, leading to the production of ROS/RNS such as peroxynitrite, a potent oxidizer of BH4. 3) NOS-mediated BH4 depletion initiates a destructive feedforward loop, leading to local BH4 depletion and reinforcing uncoupled NOS activity. 4) local BH4 availability is limited and T cell proliferation is subsequently restricted.

the S-nitrosylation of key post-transcriptional regulators of Th1 cytokines and transcription factors (88–90). One report suggests that a FOXP3⁺ population of regulatory T cells may exist whose differentiation may be NO dependent (91). The discrepancy of these effects of NO on T cell phenotype may be explained by the time and dose dependence reported to characterize this pathway. Given the potential of BH4 supplementation to create high levels of NO via forced coupling of iNOS by immune cells within TME, their effects on immune functions need to further investigated.

BH4 and tumor vasculature

One hallmark of tumorigenesis is the acquired ability of tumors to recruit and maintain their own blood supply. Many excellent reviews of our understanding of the mechanisms of tumor vascular recruitment exist (31, 92, 93) and these mechanisms will not be extensively reviewed here. Compared to vasculature elsewhere in the body, tumor vasculature is typically erratic and highly dysfunctional, characterized by blunt ends and inconsistent blood flow, resulting in insufficient tumor perfusion and high interstitial pressure (94–97). These characteristics lead to the poor penetration of chemotherapy (98–100) and can cause intra-tumoral hypoxia and an accumulation of acid (95, 101–104) which in turn

antagonizes anti-tumor immunity by stimulating immune checkpoint expression (105, 106) and immunosuppressive myeloid cells. Activity of cytotoxic lymphocytes in the tumor may also be antagonized due to the abnormal nature of tumor vasculature (107–109). As a result, the potential of vasculature normalizing agents to alter the immunosuppressive TME is the subject of considerable ongoing research.

Coupled eNOS activity is a key mediator of vascular function throughout the body. eNOS mediated NO gradients are necessary for the recruitment of pericytes and smooth muscle cells required for vascular integrity (29). NO is also necessary for the migration of endothelial cells, the formation of new capillary structures (26, 110) such as tertiary lymphoid tissues, which are important for the proliferation of anti-tumor lymphocytes, and for the maturation of normal vasculature through the expression of Angiotensin 1 (25, 111–113). Finally, constitutive, physiologic levels of NO provided by eNOS is an important part of maintaining vascular quiescence. Underscoring the importance of NO signaling to vascular function, NOS recoupling with BH4 or sepiapterin has been demonstrated to stimulate neoangiogenesis and normalize the reactivity of dysfunctional endothelium in cardiovascular disease (38, 39, 114, 115).

The positive role of NO in regulating angiogenesis raises the question of whether recoupling NOS and restoring NO signaling

has a beneficial effect on tumor biology. Within the TME, where NOS is uncoupled, NO signaling is absent and vascular inflammation is exacerbated by the effects of peroxynitrite. This leads to endothelial dysfunction, which is typical of tumor vasculature, and may partly explain the irregular structure and leakiness of the TME. Recoupling of endothelial NOS using oral sepiapterin leads to the normalization of tumor vasculature as demonstrated by the enhanced oxygenation of tumors, decreased hypoxia, and increase in pericyte coverage of tumor blood vessels (32). This is likely due to the inhibition of ROS/RNS (29) as well as the vasculature normalizing effect of physiological levels of NO (29, 116–118) enhancing tumor oxygenation and blood flow, thereby contributing to an enhanced efficacy of both radiotherapy and treatment with doxorubicin. Considering their potential synergy with immunotherapy, it is important to note that strategies that aim to restore physiological NO signaling by recoupling NOS appear to lead to vascular normalization as opposed to vascular disruption (27). This is contrary to the NOS inhibitor L-N^G-nitroarginine which disrupts tumor vasculature causing hypoxia and tumor cell death (119–121). For interventions that rely on enhanced immunogenicity of the TME via vasculature modulation, normalization is more likely than vascular disruption to alleviate hypoxic immunosuppression. This is consistent with multiple studies demonstrating that the efficacy of immunotherapy could be enhanced by treatment with combination with anti-angiogenic agents at low doses, but that the use of high dose anti-angiogenic therapy antagonized immunotherapy due to the hypoxia induced by excessive vessel pruning (94, 96, 97).

BH4 and tumor-promoting inflammation

Chronic inflammation is a hallmark of the TME, promoting tumor growth and invasion while paradoxically providing the means for escape from the adaptive immune response (122). While appropriate, physiological inflammation is an essential

component of an anti-tumor immune response, the inappropriately prolonged, disordered inflammation associated with the TME actually serves as a barrier to T cell function and immune surveillance (123). It is partially for this reason that initiation of as many as a fifth of human cancers is linked to inflammation caused by chronic infection or exposure to irritants (124) as illustrated by the chemo-preventative effects of daily anti-inflammatory drug use on certain types of cancer.

As discussed above, coupled NOS activity plays a major role in maintaining vascular quiescence. This is due to the pleiotropic anti-inflammatory effects of NO-mediated signaling on the cells of the vasculature itself. Possibly the most important pathway regulated by NO is that of the master inflammatory transcription factor NF- κ B, (Figure 3) which is susceptible to silencing by S-nitrosylation. NO-dependent S-nitrosylation of the p50 subunit inhibits the transcriptional activity of NF- κ B in multiple cell types (59–61). The p50 subunit is also nitrosylated at a cysteine located in its DNA binding domain, resulting in an inhibition of inflammation (61). IKK β has also been demonstrated to be negatively regulated by S-nitrosylation mediated by NO, resulting in the inhibition of NF- κ B activity (125). In addition to the loss of these silencing mechanisms, uncoupled NOS may directly activate this pathway via the Tyr-nitration and proteasome independent inhibition of I κ B α (126). Whether this additional positive regulation of the NF- κ B pathway occurs with NOS uncoupling *in vivo* remains to be seen.

Other mediators of inflammation are directly inhibited by NO mediated S-nitrosylation as well. CD40 is a member of the TNF receptor family, responsible for activating NF- κ B in response to stimulation with CD40L in what has been called the non-canonical NF- κ B pathway. S-nitrosylation of CD40 on the surface of resting monocytes and macrophages has been observed to inhibit signal transduction in response to CD40 activation, suggesting that denitrosylation may be a prerequisite for activation of this pathway (127). S-nitrosylation of S100A8 has also been suggested to be an important step in the resolution of inflammation by

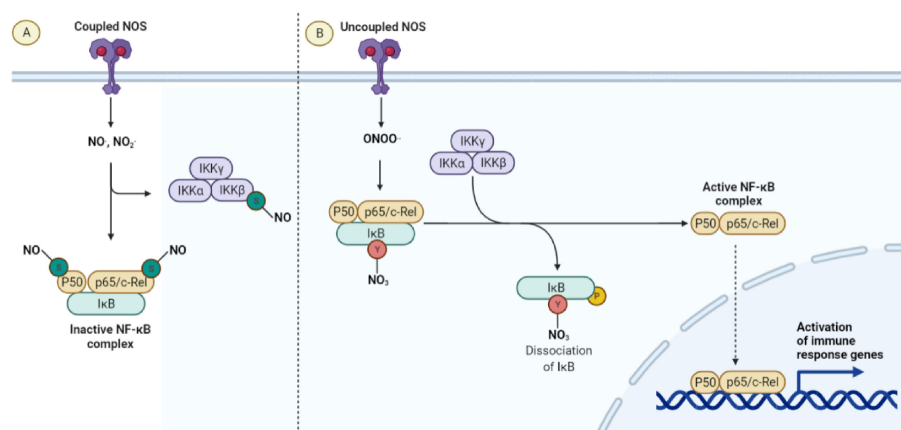


FIGURE 3

The anti-inflammatory effect of coupled NOS. (A) When NO is the predominant nitrogen species generated by NOS, anti-inflammatory S-nitrosylations of the NF- κ B pathway lead to quiescence and normal endothelial function. (B) When NOS is uncoupled, peroxynitrite and ROS are the predominant species produced, leading to a loss of anti-inflammatory S-nitrosylation as well as the Y-nitration and inactivation of I κ B.

limiting the endothelial cell-lymphocyte interactions in circulation (128). AP-1 is a transcription factor responsible for cytokine production and proliferation in response to multiple stimuli. It is activated by a MAPK cascade resulting in the activation of the kinase c-Jun N-terminal Kinase (JNK) which phosphorylates and activates c-Jun, one of its components. JNK1 is susceptible to NO mediated S-nitrosylation at Cys-119, further contributing to the anti-inflammatory effect of NO (129, 130). Finally, iNOS itself is inhibited by S-nitrosylation (131, 132), suggesting that NOS uncoupling may be further perpetuated by an increase in iNOS expression under conditions that support superoxide production and BH4 depletion supporting the idea that NOS uncoupling may function as a molecular switch.

NO has also been recognized for its inflammation suppressing effects in quiescent tissues by inhibiting leukocyte adhesion to the vascular wall, preventing the extravasation of immune cells into the underlying tissue (133–135). More recently, it has been suggested that this may be due in part to the suppression of P-selectin expression in the vascular endothelium mediated by constitutive, eNOS dependent sGC activation (136). Previous reports demonstrated that inactivation of sGC is a critical step in the recruitment of myeloid cells to the colon after DSS treatment. It was found by our group that the recruitment of inflammatory macrophages and neutrophils to the colon could be inhibited by treatment with sepiapterin, an effect that was partially inhibited by a cGC inhibitor (4). The consequence of therapeutically augmenting BH4 levels within tumors was the resolution of NF- κ B driven inflammation. This resulted in the inhibition of tumor formation in a spontaneous colon cancer model (4, 5). This finding is relevant to this tumor type for which inflammation plays such a well-recognized role in progression as well as due to previous findings that suggest BH4 levels are low in human colorectal tumor biopsies when compared to normal tissue (32). All of these are important co-stimulators of T-cell mediated anti-tumor immunity and extravasation. However, the inappropriate, persistent activity of these inflammatory mediators leads to T cell dysfunction due to

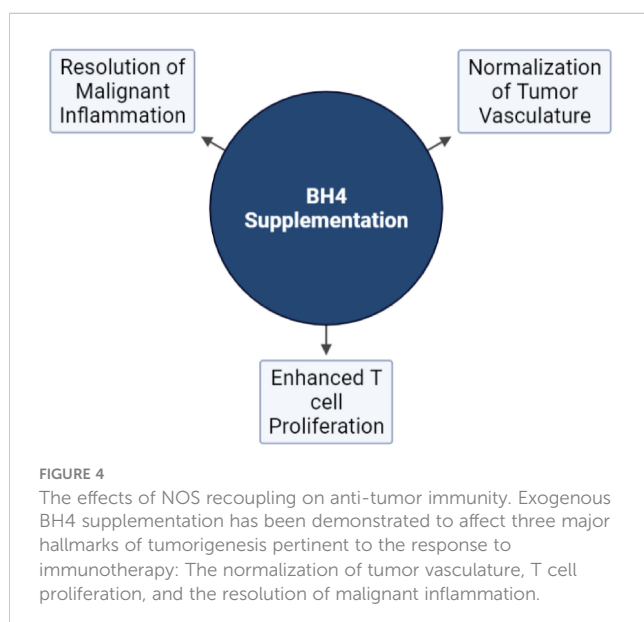
the effects of long-term inflammation characteristic of the TME. The various mechanisms of this effects are expertly reviewed elsewhere (123, 124, 137, 138).

The Effects of BH4 supplementation on Cancer Therapies

Immunotherapy

Immune checkpoint blockade (ICB) has become the standard of care for multiple cancer types and members of this family of drugs have been approved for use as monotherapies for more than a dozen malignancies (101, 139, 140). However, compared to hematological malignancies (102), only a small fraction of patients with solid tumors respond to ICB monotherapy while as many as 87% of patients derive no clinical benefit (102, 141). It is believed that the TME stands as a major impediment to the effector function of CTLs, limiting the efficacy of therapies that rely on their activity. This has led to development of combination strategies designed to alter the TME, making tumors more susceptible to immune attack (123, 142–144). BH4 supplementation represent a novel approach to enhancing immunotherapy both by its direct proliferative effects on T cells outlined above and by its impact on multiple aspects of the TME including its effects on vascular normalization and the resolution of malignant inflammation (Figure 4) (92). As monotherapies, anti-angiogenic agents have also failed to yield clinical benefits, due in part to both the toxic effects that these agents have on normal tissues as well as the rapidity with which tumors develop resistance (102, 145). On the other hand, multiple anti-angiogenesis and immunotherapy combinations have achieved significant clinical results (146–151), demonstrating the potential of this treatment approach. Combination therapy has been shown to sensitize tumors to ICB by enhancing T cell penetration, supporting the formation of high endothelial venules, reducing T cell exhaustion, suppressing immunosuppressive cells types, and increasing the recruitment of activated antigen presenting cells (APCs) to the tumor (144). This illustrates the role that the dysfunctional tumor vasculature has on immune escape. However, combination with existing anti-angiogenic faces significant hurdles. For instance, the efficacy of combination relies on using strategies that normalize tumor vasculature and avoids detrimental vascular pruning as described above (27). That makes combination therapy a delicate balance, which may be difficult to achieve reliably in the clinic. In addition (144), most anti-angiogenic strategies are complicated by toxic sequelae due to their effects on normal tissues. This includes hypertension, proteinuria, impaired wound healing, and dose limiting cardiotoxicity (152–156). Finally, most anti-angiogenesis and vasculature normalizing strategies are only effective for a brief window of time (157). This is due to the rapid development of resistance to agents that target angiogenic pathways and represents a major obstacle to their clinical effectiveness.

For these reasons BH4 supplementation maybe be a more robust and reliable agent for combination therapy. Firstly (32, 158) the vascular normalizing function of sepiapterin does not



rely on antagonizing angiogenic pathways but instead causes signaling within tumor vasculature to better resemble that of vasculature elsewhere in the body, as detailed above. Therefore, the risk of inducing vascular pruning as opposed to normalization is minimal and it likewise may not be as susceptible to the same mechanisms of therapeutic resistance as other vasculature normalizing strategies. Additionally, we have been able to demonstrate that enhanced tumor oxygenation caused by oral treatment with sepiapterin lasts for a significant amount of time after treatment has ceased (32), possibly reflecting its different mechanism of action. In addition, it demonstrates none of the toxic effects seen with other agents and has minimal detectable impact on normal tissues where BH4/BH2 levels are tightly regulated (158). This significantly widens the therapeutic window of BH4 supplementation compared to other agents.

Somewhat paradoxically, the quality of the unresolving, malignant inflammation typical of TME has long been recognized as a hallmark of tumorigenesis that aids in immune escape. As such, many strategies have been attempted to make tumors more susceptible to immunotherapy by limiting this inflammation (123, 159). Despite compelling pre-clinical reports, clinical trials aimed at inhibiting chronic inflammation to enhance therapeutic outcomes of immunotherapy have yet to yield significant results (123). Among other things, this may reflect the necessity of physiological inflammation for the function of immune cells in the TME. Similar to other anti-inflammatory/immunotherapy combination strategies, the resolution of tumor-supporting, malignant inflammation caused by uncoupled NOS may leave tumors vulnerable to more appropriate, T cell mediated responses. However, as with all such strategies timing and dosage need careful consideration, as immunotherapy relies on inflammation driven penetration and activation of both myeloid cells and lymphocytes into tumors. Interventions that limit myeloid cell activation in the TME or immune cell trafficking to the tumor tissue may limit immunotherapy when not given concomitantly with drivers of appropriate inflammation such as ICB or PRR agonists.

Radiotherapy and the optimization of radio-immunotherapy

The majority of cancer patients with solid tumors will receive some form of radiotherapy (RT) during their course of treatment. However, treatment is limited by the toxic effects of off-target radiation on normal tissues. Much research in the field of radiation oncology is focused on limiting these off-target effects or enhancing the efficacy of RT in order to widen its therapeutic window. Numerous studies have demonstrated that NOS can be activated or upregulated by cancer cells in response to exposure to ionizing radiation (160, 161). However, it has also been demonstrated that oxidative stress caused by ionizing radiation may reduce BH4 availability resulting in a shift in BH4/BH2 ratios at both systemic and local levels (48, 162), resulting in uncoupled NOS activity (48). Suggested mechanisms for this phenomenon

include the direct oxidation of BH4 by radiation-stimulated ROS production and the upregulation of the negative regulator of GCH1, GTP cyclohydrolase I feedback regulator (GFRP) (163, 164), although other pathways have been implicated (48). It is still unknown which pathway is dominant within the irradiated TME. As a result, multiple investigations are currently studying the role of NOS uncoupling in the response of tumors and normal tissues to RT as well as the therapeutic potential of BH4 supplementation.

The bulk of irradiated cancer cells die due to mitotic crisis, an ill-defined form of necrotic cell death caused by continued cell cycling in the presence of unrepaired DNA double-strand breaks (DSBs). These breaks are caused by either direct attack by high-energy photons or, much more commonly, by reactive oxygen (ROS) released from mitochondria within irradiated cells (165). Because the availability of ambient oxygen is necessary for the majority of DNA damage produced by radiation in the X-ray range, tumor hypoxia reduces the effectiveness of the radiation (157). Multiple studies have demonstrated that the degree of hypoxia directly correlates with reduced radiosensitivity by limiting the oxygen available for the production of ROS (166–169). Due to its ability to normalize tumor vasculature via NOS recoupling, BH4 supplementation has been demonstrated to enhance RT induced cell killing by increasing perfusion and oxygen delivery to tumor cells (32, 170). It is important to note that BH4 itself has been shown to act as a free radical scavenger and inhibitor of apoptosis *in vitro*, which may directly limit RT cell killing (48). In addition, both positive and negative effects of NO on apoptosis have been reported to function through various mechanisms *in vitro* as well (171). However, these may not be salient features of BH4 supplementation *in vivo* as they appear to be overshadowed by the positive effect generated by alleviating tumor hypoxia.

The total dose of RT given to a singular disease site is often limited due to the need to avoid lethal fibrotic side effects in soft tissues such as the heart and lungs. Radiation-induced cardiomyopathy is a dose-dependent, progressive disease characterized by myocardial and pericardial fibrosis, loss of contractile reserve and LV systolic function, and ultimately, premature death (172–175). Radiotherapy-induced lung fibrosis is a fibrotic disease resembling idiopathic pulmonary fibrosis and is one of the most common side effects of thoracic RT. Unfortunately, options for limiting radiation off-target effects or treating exposure are limited (48). The mechanisms behind radiation-induced tissue injury have been reviewed by other authors and will not be deeply examined here (176). However, multiple studies demonstrate that radiation-induced injury to the heart and lungs is mediated in part by chronic inflammatory signaling maintained by persistent endothelial dysfunction (175–181). Radiation-induced oxidative stress has been demonstrated to induce endothelial dysfunction by reducing BH4 levels leading to the uncoupling of NOS in both the heart and lungs (162, 163, 182). Multiple groups have reported that BH4 supplementation has a radioprotective effect on normal tissues through reversing the effects of uncoupling, restoring NO levels, mitigating inflammation, and acting as a free radical scavenger (48, 183). Three clinical trials are currently underway to study the effects of BH4 supplementation on radiation-induced skin injury and enteritis (NCT05299203, NCT05114226,

NCT05138887). The results of these studies may open up a new avenue for the prevention of radiation-induced late effects and their life-threatening fibrotic sequelae, potentially allowing for more aggressive, sub-fractionated therapy without the fear of soft tissue fibrotic effects.

The response rates of solid tumors to immunotherapeutic strategies that have been effective for hematologic malignancies have been disappointing (142). It has been hypothesized that the cause of this discrepancy may lie in the ability of tumor-associated stroma to suppress the effector functions of anti-tumor lymphocytes. One proposed approach to overcoming this immunosuppressive barrier is combination with RT, which is thought to have multiple immunostimulatory effects (142, 165, 184–186). In brief, RT expands the repertoire of tumor-associated antigens (TAAs) available in the TME while directly stimulating type I IFN expression by activating cyclic-GAMP synthase (cGAS) and its downstream signaling partner stimulator of interferon genes (STING). This leads to the necessary cross-presentation of TAAs by newly recruited APCs and subsequent priming of tumor-specific CTLs. It is hoped that the addition of strategies that revitalize T cell function, such as ICB, may have synergistic effects to achieve tumor regression in patients with advanced disease. However, clinical trials exploring the efficacy of a radio-immunotherapy combination have so far failed to yield significant positive results.

Considering the negative effect that RT has on BH4 availability, RT-induced BH4 depletion may be an overlooked restraint to the immune response to irradiated tumors. BH4 supplementation may be exploited to potentiate the combination therapy for at meaningful clinical impact. First, systemic BH4 supplementation

may enhance the proliferation of lymphocytes stimulated by APCs in the lymph nodes in response to RT (Figure 5). By enhancing the expansion of tumor-reactive lymphocytes, BH4 may provide for a more robust response to subsequent immune augmentation with ICB. Second, it has been established by multiple groups that durable anti-tumor immune responses to immunotherapy require intra-tumoral proliferation of T cells (187), a process that may be directly inhibited by RT-induced NOS uncoupling and local BH4 depletion. BH4 supplementation is likely to alleviate this immune-inhibitory process, leading to a more robust immune effector response within the irradiated TME.

RT also induces other changes to the TME that are relevant to the discussion of NOS uncoupling and BH4 metabolism. For instance, within a certain dose range, RT has a transient, type I IFN-dependent, normalizing effect on the tumor vasculature. It is thought that this may aid immune cell infiltration into the tumor and synergize with other vasculature normalizing strategies such as BH4 supplementation and immune checkpoint blockade (188). However, with so many vascular modulators at play, this three-part interaction will have to be carefully studied for potential synergy as it may tip the scales from immunostimulatory vascular normalization to immunosuppressive vascular regression and hypoxia. The immunostimulatory effects of RT have also been attributed its ability to recruit lymphocytes and myeloid cells. Many of these cells are likely to carry active iNOS. However, further study is required to understand how forced coupling of this influx of NOS by BH4 saturation may influence the irradiated TME. Finally, the advent of stereotactic body RT has made the immune-mediated effects of RT more clinically relevant by

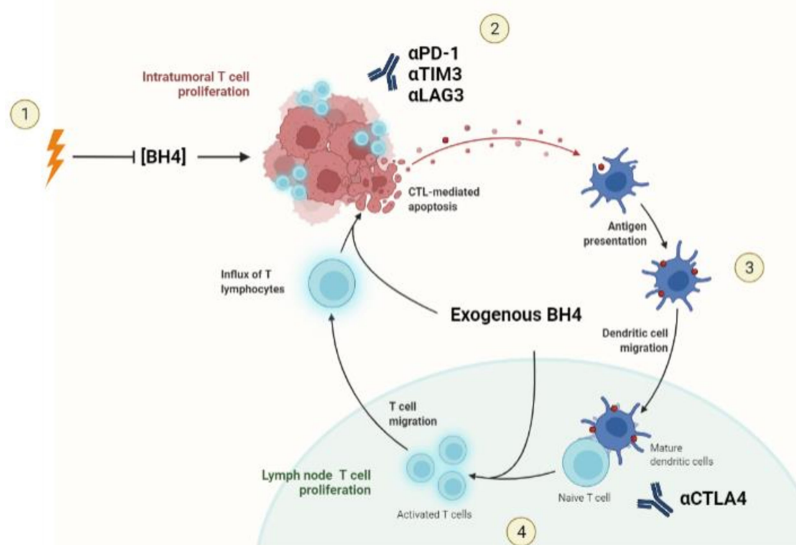


FIGURE 5

BH4 supplementation and Radiotherapy-Immunotherapy combination. 1) Radiotherapy stimulates an influx of lymphocytes and myeloid cells from the blood stream while simultaneously depleting BH4 in the TME. This depletion represents a potential barrier to ICB response in irradiated tumors. 2) Exogenous supplementation of BH4 leads to normalization of BH4 levels within the tumor, allowing for the intra-tumoral T cell proliferation necessary for the response to ICBs. 3) APCs recruited to the tumor acquire TAAs and migrate to the draining lymph nodes where they present antigen to naïve T cells. 4) Exogenous BH4 supplementation enhances tumor specific T cell expansion both enhancing the number of effector T cells available to attack the tumor as well as to be retained in the form of memory T cells.

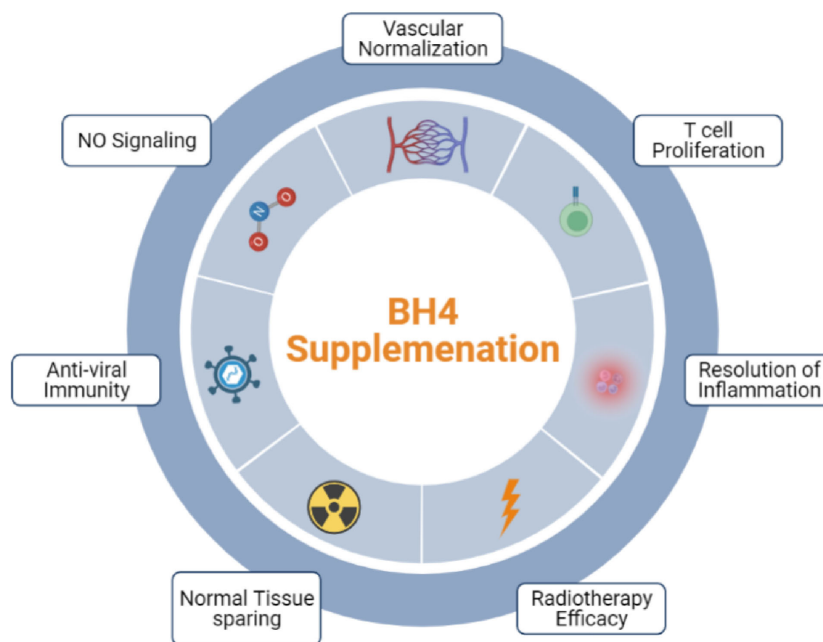


FIGURE 6

BH4 supplementation is poised to become a useful tool in the anti-cancer and anti-viral arsenal.

allowing the use of more immunogenic doses of radiation (8-15 Gy per fraction) (143). The radioprotective effect of BH4 supplementation on normal tissue may also aid in the use of these more immunogenic doses, leading to better synergy with immunotherapy. How these effects are incorporated into clinical practice will be exciting to see moving forward.

Safety of BH4 supplementation

BH4 supplementation, either with BH4 itself or with its precursor sepiapterin, has a well-established safety record in humans. Both have undergone numerous clinical trials and appear to cause few side effects, even at very high levels (41–45). BH4 is currently indicated in the long-term setting for certain types of phenylketouria (189). This is contrary to other immunomodulators, such as pattern recognition agonists, which cause global immune activation and may have a narrow therapeutic window (190). This superior safety profile is likely a result of BH4s water solubility, its ease of excretion, its highly regulated concentrations in normal tissues (158), and the restricted nature of its positive effects on proliferating T cells (35). T cell proliferation and cytokine production are usually coupled upon activation. Interventions such as pegylated IFN- α , PRR agonism, or ICBs typically enhance both the number and activation state of T cells. However, these therapies enhance T cell activity indiscriminately, putting patients at risk for off target effects and severe reactions caused by T cell cytokine production. These risks pose a major obstacle to the augmentation of cancer immunity in clinical trials. Due to its purely proliferative effect, BH4 supplementation enhances T activity only in those T cells that have

already been stimulated by their cognate antigen, without effecting global T cell activation and IFN- γ secretion (20). This makes it a much safer immunological option.

However, therapy with ICBs does carry the risk of life threatening immune-related adverse events (irADs) (102). In addition, it has previously been reported in preclinical models that forced overproduction of BH4 exacerbates certain T cell mediated autoimmune disease in mice (20). While it is true that immunological side effects have not been reported with long term BH4 treatment alone, combination therapy with ICB should be considered carefully for the risk of exacerbating the established risk for irADs. However, these events are clinically manageable, and we expect that existing interventions for irADs, namely corticosteroid treatment and ceasing therapy with the offending agent, would be effective in mitigating significant risk for irADs. Finally, due to its anti-inflammatory effect on myeloid cells, which are necessary for anti-tumor immune responses, prolonged BH4 supplementation as a single agent runs the theoretical risk of suppressing anti-tumor immunity, potentially allowing for tumor advancement, and making distant peripheral sites more hospitable to metastases. Although this currently remains a theoretical risk, care should be taken in trials not to expose patients with documented tumors to prolonged BH4 treatment alone.

Conclusions and future directions

In conclusion, the propensity of low intratumoral BH4 levels to affect chronic inflammation, tumor-associated vascular

dysfunction, and proliferation of CTLs contributes significantly to the tumor supportive characteristics of the TME. Here, we have provided pre-clinical evidence that supports exploring the clinical utility of BH4 supplementation to enhance existing anti-cancer therapies such as RT and immunotherapy. Whether BH4 restriction represents a bona fide, physiological mechanism of immune evasion or a pathological consequence of the disordered TME remains to be determined. Multiple strategies for enhancing BH4 production and recycling within cells exist beyond direct supplementation. One of these, supplementation with the BH4 precursor sepiapterin, is especially promising as it can be taken orally and can penetrate cells passively due to its relative hydrophobicity compared to BH4. This also has implications for tumors of the central nervous system as this form of biopterin can more easily pass through the blood-brain barrier (44). Therefore, BH4 supplementation may one day offer a new therapeutic avenue to oncologists and their patients (Figure 6).

Author contributions

GC: Conceptualization, Writing – original draft, Writing – review & editing. AL: Writing – original draft, Writing – review & editing. AA: Writing – original draft, Writing – review & editing. ZL: Supervision, Writing – original draft, Writing – review & editing. X-YW: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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