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Themed Section: Nitric Oxide 20 Years from the 1998 Nobel Prize

REVIEW ARTICLE

Understanding the tumour micro-environment communication network from an NOS2/COX2 perspective

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Recent findings suggest that co-expression of NOS2 and COX2 is a strong prognostic indicator in triple-negative breast cancer patients. These two key inflammation-associated enzymes are responsible for the biosynthesis of NO and PGE_2, respectively, and can exert their effect in both an autocrine and paracrine manner. Impairment of their physiological regulation leads to critical changes in both intra-tumoural and intercellular communication with the immune system and their adaptation to the hypoxic tumour micro-environment. Recent studies have also established a key role of NOS2–COX2 in causing metabolic shift. This review provides an extensive overview of the role of NO and PGE_2 in shaping communication between the tumour micro-environment composed of tumour and immune cells that in turn favours tumour progression and metastasis.

LINKED ARTICLES

This article is part of a themed section on Nitric Oxide 20 Years from the 1998 Nobel Prize. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.2/issuetoc

Abbreviations

AHR, aryl hydrocarbon receptor; Arg, arginase; BC, breast cancer; DC, dendritic cells; DKK, Dickkopf; ECs, endothelial cells; EMT, epithelial–mesenchymal transition; ERS, endoplasmic reticulum stress; ER^-, ER-negative; GLUT1, glucose transporter 1; HER2, human EGF receptor 2; HIF-1α, hypoxia-inducible factor 1-α; ICAM-1, intercellular adhesion molecule 1; IDO, indoleamine 2,3-dioxygenase; MDSCs, myeloid-derived suppressor cells; MET, mesenchymal–epithelial transition; MHC, major histocompatibility complex; mTOR, mechanistic target of rapamycin; NFAT, nuclear factor of activated T-cells; NSAIDs, nonsteroidal anti-inflammatory drugs; OXPHOS, oxidative phosphorylation; RNS, reactive nitrogen species; TAMs, tumour-associated macrophages; TCA, tricarboxylic acid; TIDC, tumour-infiltrating DC; TILs, tumour-infiltrating lymphocytes; TLRs, toll-like receptors; TME, tumour micro-environment; TNBC, triple-negative breast cancer; TRAF2, TNF receptor-associated factor 2; TSP1, thrombospondin-1

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Introduction

Chronic inflammation is a hallmark of many tumour types that leads to tumour growth, migration and metastasis (Elinav *et al.*, 2013). Tumour growth is determined not only by the cancer cells but also by communication with cells in the tumour micro-environment (TME) such as endothelial cells (ECs), macrophages, tumour-infiltrating immune cells such as T-cell, B-cell, dendritic cells (DCs), neutrophils and natural killer (NK) cells (Junttila and de Sauvage, 2013). The functional status of the immune cells in the TME is a critical determinant of the ability of the tumour to escape immune surveillance, a concept introduced over 60 years ago (Burnet, 1957). These cell populations in the TME vary in their ability to produce key inflammatory enzymes **NOS2** and **COX2**, responsible for biosynthesis of **NO** and PGE_2. Together, they contribute to tumour initiation and progression, as well as well as stasis and tumouricidal effects (Ghosh *et al.*, 2010; Bogdan, 2015). To comprehensively understand the multifaceted and often dichotomous role of NO and PGE_2 in tumour biology, it is essential to consider that (i) both NOS and COX enzymes have constitutively expressed isoforms **NOS1**, **NOS3** and **COX1**, respectively, which have overlapping roles with the inducible isoforms NOS2 and COX2 in cancer, and (ii) they have the ability to affect the cell intrinsically as well as neighbouring cells extrinsically. The effects of NO do not require cell surface receptors, while those of PGE_2 are mediated by the PG receptors **EP_1**, **EP_2**, **EP_3** and **EP_4**, combinations of which are expressed in a variety of cells (Sugimoto and Narumiya, 2007). Thus, NO and PGE_2 are critical in initiating an inflammation-driven communication network in the TME. This review discusses their role in the tumour, highlighting the collaboration between the tumour cell and the immune system with a focus on immune regulation and cellular metabolism in human physiology.

NOS2–COX2 as a driver of breast cancer progression and metastasis leading to poor prognosis

Breast cancer (BC) is the most common type of cancer among women with an estimated 252 710 new cases and 40 610 deaths in 2017 in the USA alone (DeSantis *et al.*, 2017). It is now widely accepted that BC is highly heterogeneous with multiple subtypes. Triple-negative breast cancer (TNBC), which lacks the expression of **oestrogen receptors (ERs)**, **progesterone receptors** and **human EGF receptor 2** (HER2), is an aggressive subtype that accounts for 15% of all BC patients, is highly metastatic and has poor prognosis. The most common treatment for BC is surgery, often in conjunction with radiation therapy, chemotherapy or blocking receptors using antibodies or receptor antagonists. In the case of TNBC, there is a lack of targets for receptor-blocking therapies, and multi-drug resistance is a major problem. Over the last decade, many reports have demonstrated the association between NOS2 and poor outcome in a variety of cancers, including ER-negative (ER^-) BC (Glynn *et al.*, 2010a; Granados-Principal *et al.*, 2015). Molecular mechanisms of NO function include regulation of metalloproteinase activity (Ridnour *et al.*, 2007), a crucial factor in tissue remodelling, and induction of cancer stem cell (CSC)-like characteristics by up-regulating c-Myc and CD44 that are unfavourable prognostic markers associated with a basal-like signature in BC (Sorlie *et al.*, 2001; Nielsen *et al.*, 2004; Ben-Porath *et al.*, 2008; Glynn *et al.*, 2010a; Ambs and Glynn, 2011). Inhibition of NOS2 as a therapeutic approach has shown promising results in a TNBC xenograft model (Heinecke *et al.*, 2014; Granados-Principal *et al.*, 2015). Administration of a pan-NOS inhibitor and, to a lesser extent, a NOS2 inhibitor, extended radiation-induced tumour growth delay by suppression of the immunosuppressive cytokine **IL-10** and pro-inflammatory immune polarization in the non-metastatic SCC/C3H tumour model, thus demonstrating potential role of NOS2 in immunomodulation of the TME (Ridnour *et al.*, 2015).

The other inflammation-associated enzyme, COX2, enhances the metastatic phenotype of breast tumours and is associated with poor patient outcome (Ristimaki *et al.*, 2002). Increased COX2 expression occurs early in BC. It has been detected in ductal carcinoma *in situ* (Half *et al.*, 2002), as well as invasive breast carcinoma (Takeshita *et al.*, 2005) and metastatic lesions (Costa *et al.*, 2002). High expression of COX2 in ER^- breast tumours is associated with activation of the Akt pathway and poor patient outcome (Prueitt *et al.*, 2007; Glynn *et al.*, 2010b), suggesting that co-expression of NOS2–COX2 in ER^- BC may be a strong predictor of poor outcome. In our recent epidemiological study in ER^- disease, co-expression of NOS2–COX2 in tumours predicted 33% patient survival compared with 95% survival of ER^- patients with low NOS2–COX2 expressing tumours, indicating an important interaction between these two enzymes in determining the outcome of ER^- BC patients. Furthermore, the use of COX inhibitors [nonsteroidal anti-inflammatory drugs (NSAIDs)] along with NOS2 inhibition significantly reduced primary tumour load in a TNBC xenograft mouse model (Basudhar *et al.*, 2017).

Investigation of the molecular mechanisms driving TNBC showed that NO induced COX2 and PGE2 induced NOS2, thus creating a positive feedforward loop as a result of NOS2–COX2 crosstalk (Figure 1). TNF receptor-associated factor 2 (TRAF2) was identified as a key protein in NO-mediated induction of COX2. Interestingly, in basal-like MDA-MB-468 BC cells, TRAF2 was activated in a **TNF-α**-dependent manner, while in the more aggressive mesenchymal-like MDA-MB-231 cells, endoplasmic reticulum stress (ERS) was the main activator of this pathway even though TNF-α-mediated activation of COX2 was also accessible. This was supported by elevated levels of X-box binding protein 1, a marker of ERS in TNBC (Chen *et al.*, 2014). This highlights BC subtype-specific effects of NO in driving poor prognosis. Apart from TNF-α, NO also induced **IL-8**, **CCL2** and **GM-CSF**, while PGE_2 up-regulated **IL-6**, and all of these factors may contribute to TME remodelling. Thus, together, NOS2 and COX2 generate a highly immunosuppressive TME and drive poor disease prognosis.

Exosomes have generated much interest in cancer biology in the last decade (King *et al.*, 2012; Azmi *et al.*, 2013; Boelens *et al.*, 2014) with several reports showing its key role in cancer development, metastasis, immunosuppression and drug resistance by driving CSC phenotype, angiogenesis, and the

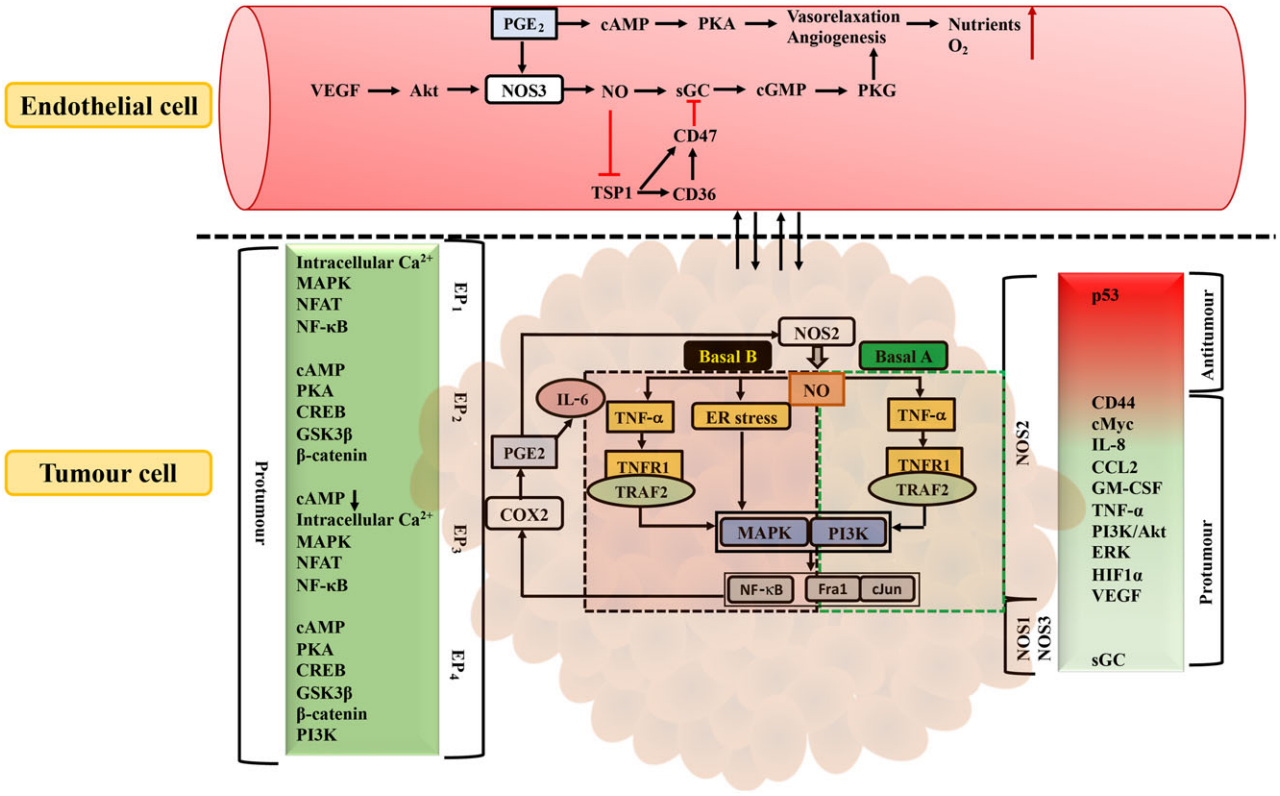


Figure 1

Increased levels of NO and PGE_2 drives tumour growth and its vascularization. PGE_2 effects are mediated by EP_1–EP_4 receptors, while NOS1 and NOS3 produce physiological levels of NO, and NOS2 is important in pathophysiological function. In tumour biology, co-expression of NOS2–COX2 is associated with poor patient survival. In basal A-like cells, TRAF2 is activated in a TNF-α dependent manner, while in the more aggressive mesenchymal-like cells, ERS was the main activator of COX2 even though TNF-α-mediated pathway is also accessible. NO and PGE_2 also lead to tumour vascularization and angiogenesis.

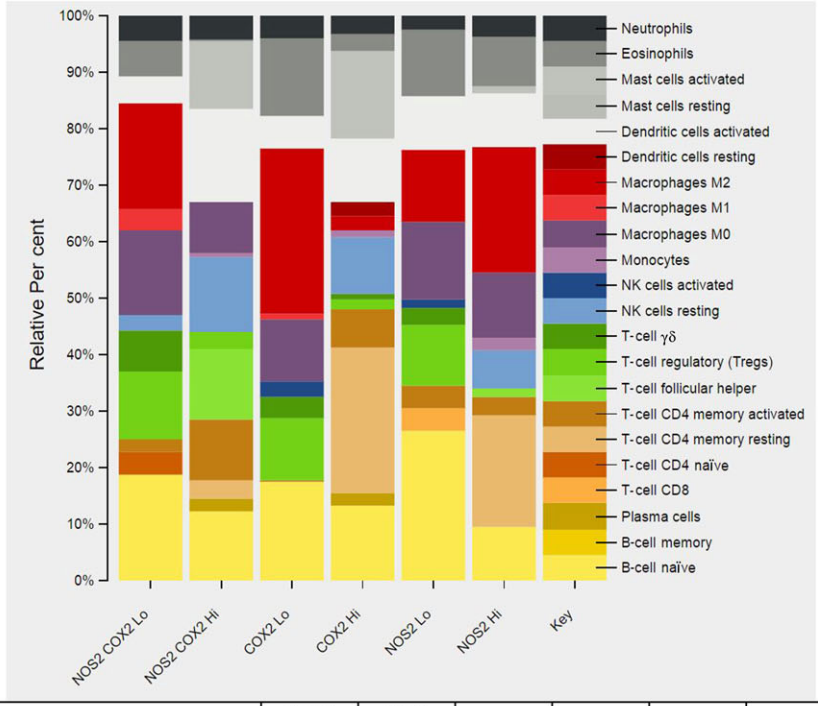
hypoxia-driven epithelial–mesenchymal transition (EMT). Both NOS2 and COX2 are involved in exosome-mediated immune regulation. In a lung cancer model, exosomes were involved in transferring COX2 to neighbouring immune cells (Kim *et al.*, 2018). Furthermore, breast tumour-derived exosomes are critical in macrophage polarization and metastasis (Piao *et al.*, 2018). PGE_2 and TGF-β from tumour-derived exosome are important mediators of myeloid-derived suppressor cell (MDSC)-mediated tumour progression (Xiang *et al.*, 2009).

A key difference between murine models and humans is the differential induction of NOS2. In humans, NOS2 expression is highly regulated and modest compared with mouse physiology. In this regard, small non-coding regulatory RNAs are becoming increasingly important in NOS2 regulation. MicroRNA (miR)-939 has emerged an important regulator of NOS2 translation as it is believed to bind to 3′-UTR to block cytokine-mediated NOS2 expression in human hepatocytes (Guo *et al.*, 2012). Interestingly, Di Modica *et al.* (2017) showed that higher levels of miR-939 are expressed in TNBC compared with other BC subtypes. Apart from miR-939, miR-146a and miR-26a also can down-regulate NOS2 expression in humans by modulating levels of inflammatory cytokines in glial cells and binding to 3′-UTR in T-cell lymphoma respectively (Li *et al.*, 2011; Zhu *et al.*, 2013). Similar to NOS2 regulation, miRs also play a role in regulation of COX2. Some of the miRs that have been invoked in COX2 regulation are miR101, miR-146a, miR-26b, miR-16, miR-199a and miR-122 (Ochs *et al.*, 2011). In BC, these miRs have been reported to play a critical role. Down-regulation of both miR-146a and miR-146b expression in breast tissues is correlated with development and increased tumourigenic potential of BC and associated with high levels of IL-6, a cytokine associated with COX2 overexpression in TNBC (Li *et al.*, 2015; Basudhar *et al.*, 2017). A similar profile was established for lower miR-26b expression in inflammatory BC compared with normal breast tissue along with association of miR-26a to tumour cell proliferation again emphasized the role of NOS2–COX2 in BC (Liu *et al.*, 2011; Zhao *et al.*, 2015; Ding *et al.*, 2018). Recently miR-122, a regulator of COX2, has been associated with reprograming oncometabolism in the TME by modulating glucose metabolism in the premetastatic niche to promote metastasis (Fong *et al.*, 2015). While its association with NOS2 is not reported, miR-122 mimics have been shown to down-regulate NOS2 (Liu da *et al.*, 2016). Thus, the collaboration of NOS2 and COX2 to drive poor disease prognosis led us to investigate the infiltration of immune cells in the TME.

Leukocyte profiling of patient samples based on RNAseq analysis of the TCGA PanCancer Atlas ER^- samples showed that the immune signature of patients with high

NOS2–COX2 levels is vastly different from high NOS2 or high COX2 alone (Figure 2). To comprehensively understand the role of NO and PGE_2 in tumour biology, it is important to appreciate that their cellular signalling is tightly controlled based on their rates of production from different cell types and cellular proximity in TME, which determines the intracellular flux of NO and PGE_2 and hence their pro-tumorigenic and anti-tumorigenic properties.

A



B

	NOS2 COX2 Lo	NOS2 COX2 Hi	COX2 Lo	COX2 Hi	NOS2 Lo	NOS2 Hi
B-cell naïve	18.7%	12.2%	17.4%	13.3%	26.4%	9.6%
B-cell memory						
Plasma cells		2.2%		2.2%		
T cells CD8					4.1%	
T-cell CD4 naïve	4.1%					
T-cell CD4 memory resting		3.4%		25.7%		19.6%
T-cell CD4 memory activated	2.2%	10.7%	0.3%	6.8%	3.9%	3.3%
T-cell follicular helper	0.1%	12.5%				1.6%
T-cell regulatory (Tregs)	12.0%	3.0%	11.1%	1.7%	10.8%	
T-cell γδ	7.1%		3.7%	0.9%	3.0%	
NK cells resting	2.7%	13.4%		10.2%		6.6%
NK cells activated			2.8%		1.6%	
Monocytes		0.5%		1.2%		2.5%
Macrophages M0	15.0%	9.1%	11.0%		13.8%	11.3%
Macrophages M1	3.7%		0.9%			
Macrophages M2	18.8%	0.1%	29.2%	2.5%	12.6%	22.4%
Dendritic cells resting				2.4%		
Dendritic cells activated	4.8%	16.4%	5.8%	11.3%	9.5%	9.4%
Mast cells resting						
Mast cells activated		12.0%		15.5%		1.1%
Eosinophils	6.1%	0.4%	13.7%	2.9%	11.7%	9.0%
Neutrophils	4.6%	4.1%	4.0%	3.3%	2.6%	3.6%

Figure 2

Leukocyte profiling in ER^- patient samples. The RNA-Seq leukocyte subset data for the TCGA PanCancer Atlas ER^- samples were downloaded from the cBioPortal.org, and the predefined leukocyte signature markers' information was downloaded from the cibersort.stanford.edu. Data on differentially expressed leukocyte markers, generated from RNA-Seq, were uploaded to the CIBERSORT and processed against the LM22, a predefined leukocyte marker subset. The relative percentage for each leukocyte subtype were presented as (A) stacked bar chart, and (B) the table represents cell types from the signature genes files and columns that represent deconvolution results of each mixture sample. All results are reported as relative fractions normalized to 100% across all cell subsets.

Discrete levels of NO and PGE_2 in cancer determine pro-tumorigenic and anti-tumorigenic properties

The role of NO in tumour biology is complex and tightly controlled by its cellular concentration, duration of release and location due to its short half-life and high diffusion constant in contrast to signalling by ligand–receptor binding. This leads to differential effects on tumourigenic versus tumouricidal signalling pathways (Ridnour *et al*., 2008). The balance between ROS/reactive nitrogen species (RNS) and antioxidants is dysregulated in tumours, thus initiating tumour progression. The perceived role of NO in signal transduction has grown over the years (Basudhar *et al*., 2016). Most of its biological signalling is due to either its direct reaction with metal centres or nitrosation of biomolecules. Nitrosylation of metal centres occur at a low concentration of NO and can be broadly classified as physiological response, while protein nitrosation is an indicator of nitrosative stress, which is critical for the understanding of various pathophysiological conditions. Thiol nitrosation is involved in inhibition as well as activation of key signalling pathways.

The effect of different fluxes of NO in tumour cells was studied using donor compounds and has been reviewed recently (Somasundaram *et al*., 2018). In brief, low flux (<100 nM) NO regulates normal physiological functions such as BP control. Intermediate levels (200–700 nM) lead to tumour proliferation and metastasis (or wound healing and tissue restoration in normal tissue) by activating the **PI3K/PKB (Akt)** pathway, ERK and stabilization of hypoxia-inducible factor-1 (HIF-1α). At high levels (500–1000 nM), NO is associated with an anti-tumour effect through activation of p53 and other anti-proliferative proteins. This dichotomous role of NO provides two distinct therapeutic windows: (i) utilization of NOS inhibitors or (ii) increasing NO concentration using NO donor compounds.

The effect of COX2-derived PGE_2 occurs in autocrine or paracrine manner through four different receptors in humans, EP_1–EP_4, of which EP_3 exists in at least eight different isoforms due to differential mRNA splicing, providing an additional level of functional control (Sugimoto and Narumiya, 2007). While EP_1 (K_D = 25 nM) and EP_2 (K_D = 5 nM) receptors have low binding affinity to PGE_2, the EP_3 (K_D = 0.33 nM) and EP_4 receptors (K_D = 0.59 nM) can bind to PGE_2 at a very low concentration (Abramovitz *et al*., 2000). This provides a concentration-dependent signalling mechanism in the TME. EP_2 and EP_4 receptors are key mediators of anti-inflammatory and immune-suppressive effects through activation of the **cAMP**/PKA/cAMP response element-binding protein pathway (Subbaramaiah *et al*., 2008). In addition, EP_2/EP_4 receptor-mediated induction of the **GSK-3β/β-catenin** pathway leads to the production of several pro-tumourigenic transcription factors, for example, cyclinD1, c-Myc and **VEGF**. EP_4 receptors can also activate the PI3K pathway, leading to tumour growth and metastasis (Fujino *et al*., 2002). PGE_2 levels are further controlled by the rate of desensitization upon ligand–receptor interaction. EP_4 receptors are rapidly desensitized, leading to a rapid burst of PGE_2 -mediated signalling, while EP_2 receptors havea long-lasting effect (Kalinski, 2012). EP_1 and EP_3 receptors can activate PLC-mediated stimulation of calcium, leading to activation of the MAPK, nuclear factor of activated T-cells and NF-κB signalling pathways. Activation of EP_3 receptors can also lead to inhibition or induction of cAMP, based on the isoform present (Woodward *et al*., 2011). The involvement of EP_1 and EP_4 receptors has been demonstrated in breast tumour growth and metastasis (Kawamori *et al*., 2001; Ma *et al*., 2006). Targeted inhibition of these receptors is emerging as new therapeutic area in cancer.

The tumour biology of PGE_2 is based on a large body of literature that shows tumour regression in BC and other cancers with NSAIDs, which are COX inhibitors (Cha and DuBois, 2007). Recently, a crosstalk between osteoprotegerin, fatty acid synthase and COX2 has also been implicated in highly invasive BC (Goswami and Sharma-Walia, 2016). PGE_2 may also mediate **CXCR2** signalling, playing a role in BC metastasis and chemoresistance (Xu *et al*., 2018). Like IL-8 and TNF-α, high levels of IL-6 in BC patient serum are also a poor prognostic marker. In TNBC cell lines, PGE_2 induces IL-6 that controls growth, metastasis and CSC renewal (Basudhar *et al*., 2017). Although the role of PGE_2 in tumour biology and immune system regulation is well established, there are not many reports demonstrating the underlying mechanism. Recently, Zelenay *et al*. (2015) showed for the first time that PGE_2 -mediated pro-tumourigenic effects are due to immune evasion in a murine model of BrafV600E-mutated melanoma, where a synergistic effect of COX inhibition with anti-PD-1 blockade showed promise as adjuvant to immunotherapy. Another study demonstrated NOS2 and COX2 are important regulators of Dickkopf (DKK)1 expression, a predictor of lung versus bone marrow metastasis in BC. DKK1 expression suppressed COX2-mediated recruitment of neutrophils and macrophages in lung metastasis through the non-canonical WNT pathway but at the same time DKK1 promoted bone metastasis by regulating canonical WNT signalling of osteoblasts (Zhuang *et al*., 2017). On the other hand, NOS2 induces the WNT pathway by inhibition of DKK1 in BC (Du *et al*., 2013). Together, NOS2 and COX2 are important modulators of tumour progression and metastasis.

Role of NOS2–COX2 in tumour vascularization

Vascular endothelium is the barrier between blood and the tissues, which controls tumour initiation, development and metastasis. A rapidly proliferating tumour requires extensive tumour vascularization and elevated angiogenesis. The immature neovasculature can inhibit immune cell extravasation, limiting tumour surveillance. Endothelial NOS (eNOS or NOS3), a mediator of VEGF activity, is the primary endogenous source of NO in ECs. NOS3 activity is regulated by post-translational modification, binding to regulatory proteins such as heat shock protein 90 and CaM and intracellular localization. In addition, PGE_2 can also induce VEGF via EP_2 and EP_4 receptors. Recently, the crosstalk between NO and PGE_2 was demonstrated by PGE_2/EP_4-mediated dephosphorylation of eNOS at Thr^{495}, stimulating NO production and vasodilation (Hristovska *et al*., 2007). Low levels of NO (<1 nM) inhibit thrombospondin-1 (TSP1), a secreted protein involved in EC proliferation that blocks NO-mediated

angiogenesis through the ERK pathway (Ridnour *et al.*, 2005). During inflammation, as NO flux increases, TSP1 levels start to increase, partly due to a protein tyrosine phosphatase MKP-1-mediated dephosphorylation of ERK. This leads to TSP1-mediated inhibition of the NO–cGMP pathway, causing inhibition of angiogenesis through its binding to CD47 receptor, which has emerged as an immune checkpoint (Matlung *et al.*, 2017). At high TSP1 levels, it also binds to CD36, a free fatty acid transporter that is involved in eNOS activation by myristate, thereby inhibiting NO signalling (Isenberg *et al.*, 2007, 2009). In endothelial biology, both NO and PGE_2 play important roles in the tumour vasculature.

In the BC TME, TNBC cells can produce GM-CSF and CCL2 in the presence of NO, while PGE_2 -mediated IL-6 release leads to recruitment of monocytes through tumour vasculature, which are then activated to become tumour-associated macrophage (TAM) (Roca *et al.*, 2009; Qian and Pollard, 2010; Basudhar *et al.*, 2017). GM-CSF also induced EC angiogenesis emphasizing the interplay between BC and EC in the TME (Bussolino *et al.*, 1991). NO also has a concentration-dependent role in expression of intracellular adhesion molecules that are involved in recruitment of T lymphocytes and monocytes. At low concentration, NO is critical for activation of **vascular cell adhesion protein 1**, intercellular adhesion molecule 1 (**ICAM-1**) and E-selectin, while high flux of NO down-regulates their expression inhibiting recruitment of immune cells (Sektioglu *et al.*, 2016).

A crosstalk between tumour cells and ECs can be mediated by PGE_2 as well. For example, high concentration of PGE_2 can act through EP_1 receptor signalling to enhance expression of ICAM-1 in oral cancer cells leading to increased cell motility (Yang *et al.*, 2010). In brain ECs, PGE_2 induced ICAM-1 expression through EP_4 receptors (Park *et al.*, 2013). Moreover, cellular communication between metastatic BC and ECs is also controlled by α3β1 integrin, which is highly expressed in BC and regulates invasion and metastasis through **MMP-9**. Integrin α3β1 is an upstream regulator of COX2 in BC and a potential therapeutic target for immunomodulation (Mitchell *et al.*, 2010). The role of NO in MMP-mediated signalling is well documented, revealing a role of both NO and PGE_2 in regulating BC and EC interactions (Ridnour *et al.*, 2007; O'Sullivan *et al.*, 2014). Thus, low NO flux and short-term PGE_2/EP_4 receptor signalling can induce T lymphocyte and monocyte recruitment at the tumour site.

Dysregulated metabolism in the TME characterized by low nutrient and oxygen supply and low pH due to increased lactate secretion makes it challenging for ECs to survive and proliferate in the TME. Low oxygen and glucose deprivation-mediated stabilization of HIF-1α induces VEGF and VEGF-R2 leading to tumour vascularization (Tang *et al.*, 2004; Yun *et al.*, 2005). Moreover, IL-8 from tumours can also drive EC proliferation and angiogenesis (Li *et al.*, 2003). Like tumour cells, ECs overexpress the glucose transporter 1 (**GLUT1**) and utilize the glycolytic pathway for energy requirements even in the presence of oxygen (Yeh *et al.*, 2008; Parra-Bonilla *et al.*, 2010). This allows maximum oxygen transport to the tumour and is beneficial for their survival and increased tumour vascularization. In tumour-associated EC, induction of VEGF by COX2 can increase the expression of phosphofructokinase 2, a key enzyme involved in synthesis of fructose-2,6-bisphosphate, which is an allosteric activator of phosphofructokinase 1 in the glycolysis pathway (Zhang *et al.*, 2018). A hallmark of increased glycolysis in TME is high lactate concentration, which can be taken up by ECs through the monocarboxylate transporter 1 and converted to pyruvate for use in the tricarboxylic acid (TCA) cycle in the presence of oxygen (Sonveaux *et al.*, 2012). This implies a role of NOS2–COX2 in regulation of BC–EC interaction in the TME as well as regulation of EC cellular metabolism, thereby promoting tumour progression and metastasis.

Regulation of NO and PGE_2 in the immune system

The immune system consists of a wide variety of cell types located in specific niches throughout the body. An appropriate balance of immune cells is required to mount an effective response to disease. Infection has been established as a precursor of approximately 20% of malignant tumours (de Martel *et al.*, 2012). The levels of NOS2 and COX2 produced by the immune system and their effect on tumour cells and the TME determine tumourigenic versus tumouricidal response. A complete understanding of these effects is required to determine the mechanisms underlying chemoresistance and effectiveness of immunotherapy.

NOS2–COX2 in the innate immune system

Persistent chronic inflammation, marked by increased release of pro-inflammatory and oncogenic mediators, makes the micro-environment susceptible to tumourigenesis. The innate and adaptive immune system can inhibit or enhance tumour initiation. Mediators released by these cells can influence each other's activity. Many innate immune cells such as monocytes/macrophages, NK cells, DC, eosinophils and mast cells biosynthesize NO as discussed below. PGE_2 and NO levels in the macrophages can determine patient prognosis in several disease conditions especially infections, diabetes and cancer (MacMicking *et al.*, 1997; Tessaro *et al.*, 2015; Liu da *et al.*, 2016; Brune *et al.*, 2017; Oleson *et al.*, 2018). Therefore, response to inflammation and hence inflammation-associated cancers (like BC) is dependent on NOS2–COX2 signalling in macrophages, which is important for macrophage polarization and the regulation of tumour progression. The COX2/ PGE_2 signalling pathway contributes to immune evasion and resistance to cancer therapy by suppressing the activity of innate immune cells such as DC and NK cells (Liu *et al.*, 2015). In the past decade, several studies have shown how bioenergetic status controls the fate and function of immune cells. In the following sections, we will address NOS2/NO and COX2/PGE_2 signalling and how it modulates innate immune cell function, along with a discussion of their metabolic pathways.

Macrophages

The presence of TAMs in BC has been associated with improved tumour vasculature, increased invasion and metastasis and immune escape (Figure 3) (Ruffell *et al.*, 2012). Studies have shown that local low-dose irradiation and cytosine–phosphorothioate–guanine oligodeoxynucleotides, a toll-like receptor (TLR) agonist, can differentiate TAMs into $NOS2^+$ macrophages, which in turn can facilitate infiltration of T-cells into the TME (Klug *et al.*, 2013; Sektioglu *et al.*, 2016). However, more recent studies have shown that these infiltrated T-cells are often exhausted in TNBCs as they become more invasive (Gil Del Alcazar *et al.*, 2017). This points towards the possibility of several alternative modes of immune escape that may be employed by the more aggressive cancers. The polarization of TAMs and downstream signalling events are dependent on the interaction between cancer cells and macrophages. COX2 inhibitors have been found to have chemopreventive effects in many cancers by reducing **arginase** (*Arg*)1 expression, increasing the chemokine CXCL1 and potentially reprogramming macrophages into NOS2-expressing, anti-tumour cells (Ruffell *et al.*, 2012). This also throws light on the involvement of PGE_2 in tumour progression. Chemokines such as colony-stimulating factor 1 and CCL2 produced by the tumour cells recruit macrophages into the TME, while tumour-derived PGE_2 and cytokines such as IL-10 signal through $EP_2/_4$ receptors and IL-10R, respectively, to activate a loop that regulates NOS2 and/or Arg1, hence determining the immune status of the tumour (Ruffell *et al.*, 2012). An additional determinant of the functional effects of these pathways within the TME is HIF-1α (Ruffell *et al.*, 2012). The relationship between NOS2, hypoxia and $COX2/PGE_2$ in macrophages within the TME has been succinctly described by a few reviews (Obermajer *et al.*, 2012; Ruffell *et al.*, 2012; Ridnour *et al.*, 2013; Brune *et al.*, 2017). Behaviour of inflammatory cancers such as breast, ovarian, pancreatic, gastric (mainly colorectal and oesophageal) and bladder carcinomas within the TME during tumourigenesis and response to therapy is regulated by the metabolic status of the micro-environment contributed largely by macrophage metabolism (Na *et al.*, 2018).

NO produced by stimulated macrophages regulates the micro-environment, and this paracrine effect modulates response to infections and anti-cancer therapies. NO can affect cellular respiration and reduce O_2 consumption, thus converting the TME into an environment that utilizes 'aerobic glycolysis' for energy production (Beltran *et al.*, 2000). This oncometabolism helps tumours escape the surveillance of the immune system. A recent paper reveals that NO production within macrophages is differentially regulated at the level of transcription between various species, and this could contribute to pathogen host restriction (Young *et al.*, 2018). This species-specific regulation of NOS2 and associated NO production/arginine metabolism could also lead to differences between interactions of tumours with the immune system as well as response to immunotherapy. Human macrophages have been found to produce low amounts of NO in response to inflammatory stimuli, and the detection of NO in this model has been difficult (Weinberg *et al.*, 1995). Hence, the effects of macrophage NO in the TME especially with regard to immunometabolism have largely been studied in murine macrophages.

In murine macrophages, IFN-γ + LPS induces NOS2 and COX2, while in human macrophages, it induces COX2 and **indoleamine 2,3-dioxygenase** (IDO), an enzyme that metabolizes tryptophan to kynurenine. IDO has been linked to immunosuppression through attenuation of T-cell activity. In macrophages, IDO1 up-regulated M2-associated effector molecules (IL-10, **CXCR4**) and reduced M1 [C–C chemokine receptor type 7 (CCR7) and IL-12p35] phenotypic markers (Wang *et al.*, 2014). Conversely, IDO knockdown in THP-1 cells showed preference for M1 compared with M2 markers suggesting a role of IDO in macrophage differentiation towards an M2 phenotype. Though IFN-γ is generally associated with M1 polarization, it can also facilitate immunosuppression, perhaps via the induction of IDO.

Espey *et al.* (2000) showed that IFN-γ with different combinations of cytokines and TLR activators could produce different levels of NO, which in turn determines their nitrosative capacity and downstream intracellular and intercellular effects within the local environment. The level of NO has been shown to directly regulate the metabolic state of macrophages by inhibiting oxidative phosphorylation (OXPHOS) and making them glycolytically committed. Furthermore, NO inhibition of OXPHOS resulted in IL-10 production antagonizing NOS expression. Thus, NO regulation of OXPHOS and the negative feedback of IL-10 form a metabolic rheostat (Baseler *et al.*, 2016). The same study by Baseler

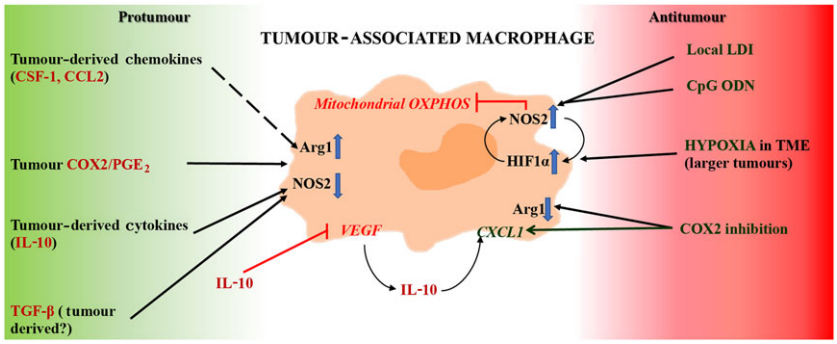


Figure 3

NOS2–COX2-mediated signalling in TAMs. LDI, low-dose irradiation; CpG ODN, CpG oligodeoxynucleotide.

et al. (2016) delineating the effect of IL-10 on macrophage metabolism suggests that IL-10, anti-inflammatory cytokine produced by stimulated macrophages can control their propensity for glycolysis by regulating the levels of NO. More recently, it was found that IL-10 had a multi-pronged effect on metabolically reprogramming macrophages in inflammatory bowel disease (Ip *et al*., 2017).

Apart from regulating glycolysis, IL-10 inhibited caspase 1-mediated inflammasome activation and also helped maintain healthy mitochondria as well as prevent accumulation of dysfunctional mitochondria by turning on an autophagic mechanism (Ip *et al*., 2017). Increased IL-10 can inhibit VEGF production and subsequently neovascularization, from 'M1'-polarized and not 'M2'-polarized macrophages (Wu *et al*., 2010). Hypoxia-induced VEGF was also not affected by IL-10 but IFN-γ-induced VEGF was. This study showed that high NOS2 expression was required for IL-10 to inhibit VEGF, but if the cells expressed high NOS2 and Arg1, IL-10 again had no anti-inflammatory effect (Wu *et al*., 2010). NO-mediated inhibition of mitochondrial OXPHOS has also been implicated to be the reason for the inability of IL-4 to convert M1 macrophages back to M2 (Van den Bossche *et al*., 2016).

Infantino *et al*. (2013) found that the enzyme ATP citrate lyase plays a role in human macrophage response to inflammation by directly regulating NO, ROS and PGE_2 levels, thus hinting at a possible link (direct or indirect) between NO flux and metabolism-driven response in human macrophages as well. These studies point towards an unequivocal role for NO in macrophage metabolism. However, there is a need to understand that *in vivo* macrophages are exposed to a plethora of stimuli, further complicated by fuel restrictions and do not always fully conform to either the M1 or the M2 definition (Van den Bossche *et al*., 2017).

Neutrophils

Neutrophils are one of the first responders in the host immune system and produce high ROS and RNS (in rodents). The flux of ROS in neutrophils is a major determinant of tumour outcome. NO/cGMP signalling and direct NO-mediated modifications lead to tuning of these ROS levels. Unlike infectious disease, in the tumour-associated neutrophils, there is a reduction in ROS levels, thus switching their function from anti-tumour to pro-tumourigenic (Sagiv *et al*., 2015). In BC, high neutrophil-to-lymphocyte ratios are associated with poor patient survival (Ethier *et al*., 2017). Tumour cells play an important role in recruiting neutrophils by secreting chemoattractants such as IL-8 and **LTB_4**, an eicosanoid signalling pathway parallel to COX2 pathway (Lammermann *et al*., 2013). Furthermore, IL-8 is produced by tumour cells in the presence of NO and associated with poor prognosis in TNBC (Glynn *et al*., 2010a; Hartman *et al*., 2013; Basudhar *et al*., 2017). NO also induces GM-CSF in TNBC, which can in turn amplify IL-3 receptor expression, thus inducing responsiveness of neutrophils to IL-3 that is commonly produced by T-cells or mast cells (Smith *et al*., 1995).

While the role of PGE_2 in neutrophil function in BC is still being elucidated, activated neutrophils can also be a source of PGE_2. Early literature suggested a role of PGE_2 in neutrophil migration in general (Van Epps *et al*., 1978). In colonic epithelial cells, PGE_2 stimulates IL-8 leading to neutrophil recruitment to TME, thus contributing to tumour progression and metastasis (Yu and Chadee, 1998; Hartman *et al*., 2013; Wang *et al*., 2015). Furthermore, IL-8 is also responsible for a neutrophil-mediated increase of Arg1 in TME in a non-small cell lung cancer model, which can then reduce influx of L-arginine to the NOS pathway and suppress cytotoxic T-cell functions through arginine deprivation, thus leading to an immunosuppressive TME (Rotondo *et al*., 2009). T-cells also secrete cytokines that lead to increased neutrophil infiltration. Recent studies showed that γδ T-cells enhanced **IL-17**-mediated recruitment of neutrophils in turn leading to metastasis in the TME of BC (Benevides *et al*., 2015; Coffelt *et al*., 2015). Tumour-derived **IL-1β** is a key mediator of γδ T-cell activation as well as tumour NOS2 expression. ROS-producing neutrophils mediate suppression of macrophage NOS2 resulting from the anti-tumour activity of cytotoxic $CD8^+$ T-cells (Bingisser *et al*., 1998; Mazzoni *et al*., 2002; Governa *et al*., 2017). Taken together, these results suggest that neutrophils can have pro-tumourigenic properties.

Another important property of neutrophils is the reorganization of the extracellular matrix by MMPs, a key component of tumour progression and metastasis. MMP-9 has been associated with cancer progression and is mainly produced by neutrophils. MMP-9 activation is tightly controlled by NO levels. As mentioned earlier, there is a reduction in RNS levels produced by neutrophils in the TME. At low levels, the NO–cGMP pathway inhibits tissue inhibitor of metalloproteinase-1 (TIMP-1), thus in turn increasing MMP-9. Moderate levels of NO can directly activate MMP-9 presumably by attacking the zinc thiolate site (Ridnour *et al*., 2007). MMP-9 also induces angiogenesis by releasing VEGF. Thus, MMP-9 supports neutrophil-mediated metastasis in the TME.

Neutrophils are almost entirely dependent on glycolysis for their energy requirements, as they have extremely few mitochondria (Fossati *et al*., 2003; Chacko *et al*., 2013). In a TME with depleted nutrients and oxygen, neutrophils show distinct properties. Tumour-associated neutrophils demonstrate increased expression of NOS2, IL-6 and IL-10 compared with splenic MDSCs (Elpek *et al*., 2014). In neutrophils, NOS can also increase cGMP and decrease TSP-1 as discussed above. This can lead to decreased ROS due to antioxidant properties of NO. Thus, it is reasonable to conclude that ROS-mediated killing would be attenuated by an increase in NOS2 in the TME. Furthermore, IL-8 generation is maintained, and elastase is also up-regulated in neutrophils under hypoxic conditions and taken up by tumour cells leading to proliferation (McGovern *et al*., 2011; Kerros *et al*., 2017). TGF-β is also induced under hypoxia, leading to pro-tumourigenic effects of neutrophils (Fridlender *et al*., 2009).

NK cells

NK cells, a major component of innate immunity and one of the three major lymphoid cell populations in blood, play an important role in host resistance against viral infections and tumours without prior sensitization (Figure 4). NK cells produce several cytokines and are notable for their production

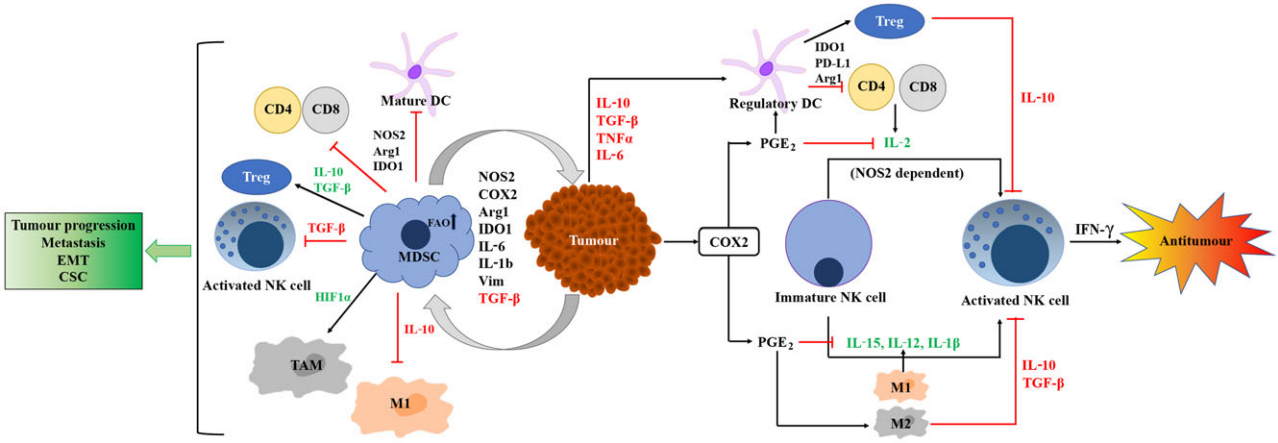


Figure 4

Tumour cells interact with MDSCs to mediate an immunosuppressive TME with NO and PGE_2 as the key players.

of high levels of IFN-γ. The maturation and differentiation of NK cells are regulated by various stimuli, including **IL-15** that is essential for normal NK cell development (Becknell and Caligiuri, 2005). Cytokines such as IL-15, **IL-12** and **IL-2** augment the cytolytic activity of NK cells against tumours (Wu and Lanier, 2003). Studies have previously shown that increased NO production through NOS2 contributes to IL-2-mediated enhanced cytotoxicity and IFN-γ production (Hibbs Jr. *et al.*, 1992; Diefenbach *et al.*, 1998; Cifone *et al.*, 1999; Furuke *et al.*, 1999; Cifone *et al.*, 2001). Depletion of L-arginine, the substrate of NOS and Arg, has a profound impact on NK cells functions that was reflected in lower cytotoxicity and decreased NK cell viability (Lamas *et al.*, 2012). Increased production of NO has been linked to enhanced cytotoxic activity of NK cells in s.c. tumour-transplanted animals when compared with i.p. tumour-bearing animals. This discrepancy was attributed to substantially reduced NOS activity in the latter case (Jyothi and Khar, 1999).

Association of PGs (PGE_2 and PGD_2) with suppressed cytolytic activity of NK cells was established early in the 1980s with cAMP identified as a key meditator for PGE_2-induced suppression (Bankhurst, 1982; Goto *et al.*, 1983). While IL-2-stimulated NO production and enhances NK cytotoxicity, PGE_2 suppressed IL-2 activated NK cell cytotoxicity (Baxevanis *et al.*, 1993). Also, in resident splenic NK cells, PGE_2 suppressed NK cell activity primarily via EP_4 receptors (Holt *et al.*, 2011). PGE_2 is also known to down-regulate IL-15-mediated human NK cell function such as IFN-γ production at the protein and transcriptional levels. The down-regulation of surface expression of the common γc-chain that is used by the **IL-2**, **IL-4**, **IL-7**, **IL-9**, **IL-15** and **IL-21** receptors was suggested as the potential mechanism (Joshi *et al.*, 2001). In another study, PGE_2 antagonized the potent synergistic induction of IFN-γ production from NK cells by IL-12 and IL-18 (Walker and Rotondo, 2004). These studies showed that PGE_2 is capable of suppressing NK cell activity in various scenarios, thereby limiting innate inflammatory processes. As NK cells are crucial for killing tumour cells, inhibition of COX should be considered as an adjuvant in cancer therapy. In immune competent Balb/cByJ mice, tumour growth of s.c. implanted mammary tumour cells was suppressed in indomethacin (COX1/COX2 inhibitor) and celecoxib (COX2 inhibitor) treatment groups (Kundu *et al.*, 2005). Both inhibitors limited tumour metastasis, an effect that was found to be dependent on NK but not T-cell function.

Several mechanisms exist by which NK cells can kill cancer cells, such as antibody-dependent cell-mediated cytotoxicity even in tumours that are resistant to T-cell killing. In spite of this, NK cell-based immunotherapy has been unsatisfactory in clinical settings in part due to evasion mechanisms used by cancer cells to avoid NK cell-mediated killing. As an example, tumour-derived mesenchymal stem cells exert their immunosuppressive activity by secretion of soluble factors including PGE_2, that suppressed NK cell function. Inhibition of PGE_2 synthesis and IL-6 activity restored NK cell activity (Galland *et al.*, 2017). Tumour-derived PGE_2 was also critical in NK cell dysfunction in several cancers such as gastric cancer, melanoma and colorectal carcinoma (Pietra *et al.*, 2012; Li *et al.*, 2013, 2016; Mao *et al.*, 2014; Liu *et al.*, 2015).

Dendritic cells

Dendritic cells (DCs) are key regulators of innate immunity and play a crucial role in forming the bridge between the innate and adaptive arms of the immune system (Figure 4). As a part of the innate response, immature DCs constantly scan for pathogens through pattern recognition receptors such as the TLR. Upon binding of TLR ligands, the DC undergoes maturation that involves various transformations, such as increased expression of major histocompatibility complex (MHC) molecules and migration to lymph nodes to present antigens to T-cells, and expression of wide range of proteins, including cytokines and chemokines. DCs play a crucial role in maintaining the balance of adaptive immunity and immune tolerance. Tumour-infiltrating DC (TIDC) are present in the immune-suppressive TME and have been well documented in different cancer types. They can have good or poor prognostic properties depending upon antigen-presenting capability and expression of co-stimulatory molecules (Karthaus *et al.*, 2012; Janco *et al.*, 2015). In colorectal

carcinoma, higher TIDC numbers were associated with shorter disease-free and overall survival (Sandel *et al.*, 2005), whereas in melanoma, TIDC correlated with regression of tumour (Ladanyi *et al.*, 2007). NO can sensitize tumour towards DC-mediated apoptosis. Pretreatment of lymphomas cells with NO donor sensitized them towards DC-mediated cytotoxicity, and this activity enhancement involved Fas engagement and loss of survivin protein expression (Huang *et al.*, 2005). In BC, 42% of breast adenocarcinomas contain TIDC, and their population decreased in fibrous tumours (Lespagnard *et al.*, 1999).

DC-derived NO controls effector and regulatory functions of DCs by inhibiting effector DC development (Si *et al.*, 2016). NO not only influences effector DC development but also modulates cytokine expression and release from LPS-matured DC. NO does so by sustaining IL-1β and **IL-23** expression that is inhibited in the presence of NO scavenger carboxy-PTIO. These cytokines in turn are crucial for DCs to induce **IL-17**-producing T-cells, thereby effectively maintaining inflammation during infection (Obregon *et al.*, 2015). It has also been reported that NO donor treatment or overexpression of either NOS2 or NOS3 alone can induce expression of MHC II and essential co-stimulatory molecules CD80 and CD86 in immature DCs. Enhancement of surface localization of MHC II was attributed to interaction of NOS2 with CD74 that prevents CD74 degradation by caspase (Huang *et al.*, 2008).

To meet the increased metabolic demands upon activation, DCs undergo rapid glycolytic reprogramming. Binding of TLR agonist leads to metabolic transition from OXPHOS to aerobic glycolysis and is promoted by PI3K/Akt signalling (Krawczyk *et al.*, 2010). Mechanistic target of rapamycin (mTOR), a kinase involved in regulating different cellular process, is a downstream target of many growth factor receptors and is also activated by the PI3K/Akt signalling pathway. It plays a critical role in regulating DC life span upon activation after TLR stimulation, and its inhibition by rapamycin prolongs life span of DC (Amiel *et al.*, 2012). Hypoxia, a hallmark of the cancer micro-environment, has also been studied extensively for DC activation (Jantsch *et al.*, 2008; Kohler *et al.*, 2012; Naldini *et al.*, 2012). It has been previously shown that hypoxia alone did not activate murine DCs, but hypoxia combined with LPS resulted in an increased DC activation signature compared with LPS alone (Jantsch *et al.*, 2008). It was accompanied by increased accumulation of HIF-1α and enhanced glycolytic activity that indicates hypoxia stabilized HIF-1α plays a crucial role in DC activation in inflammatory states under low oxygen tension. Moreover, HIF-1α also plays a crucial role in the differentiation and migration of DCs generated under hypoxia (Kohler *et al.*, 2012). Enhanced glycolysis is a trademark of several immune cells that enables them to generate sufficient ATP and other required biosynthetic intermediates quickly that can in turn help them to carry out their specific immune functions. As an example, NADPH has multiple functions in immune cells: to generate ROS by the enzyme NADPH oxidase, or lipid synthesis to support endoplasmic reticulum synthesis (Everts *et al.*, 2014).

Serbina *et al.* (2003) identified a TNF-α/NOS2-producing (Tip)-DC subset in spleens of *Listeria monocytogenes*-infected mice. (TIP)-DC subset was shown to exert a direct role in killing microbes, thereby mediating the innate immune response. A recent study showed that interaction between anti-tumour $CD8^+$ T-cells and NO producing Tip-DCs regulates tumour growth in mouse model (Marigo *et al.*, 2016). In contrast, NOS2 in human DCs is yet to be identified. It has been previously shown that conventional GM-CSF and IL-4 differentiated monocyte-derived human DC do not produce NO (Nishioka *et al.*, 2003). However, a recent report suggests maturation of human DCs, upon inflammatory cytokine exposure that led to pronounced expression of neuronal NOS (NOS1), suggesting a regulatory role for NO (Adler *et al.*, 2010).

There are several reports that show NO plays a complex role in regulating DC immune responses as well as their cellular metabolism. Similar to macrophages, down-regulation of OXPHOS in activated DCs has been attributed to NO (Everts *et al.*, 2012). Using real-time metabolic flux analysis, researchers have previously shown that in inflammatory blood monocyte-derived DCs that express NOS2, mitochondrial activity is lost gradually after activation by TLR agonists (Everts *et al.*, 2012). During the early stage of activation, there is a transient increase in OXPHOS followed by collapse in mitochondrial function, which coincides with increased NOS2 expression and NO production. Inhibition of this early glycolytic reprogramming severely decreases the DC capability to migrate and stimulate T-cells (Everts *et al.*, 2014). Early glycolytic reprogramming occurs through the kinases TBK1-IKKε and is responsible for supporting the *de novo* synthesis of fatty acids required for DC activation. While TBK1-IKKε are responsible for the early increase of glycolysis, long-term glycolytic commitment happens through NOS2 and HIF-1α, whose expression are increased via PI3K signalling (Everts *et al.*, 2014). More recently, it has been shown that glucose represses DC inflammatory outputs via a signal transduction mechanism that involves mTOR complex 1 (mTORC1), HIF-1α and NOS2 (Lawless *et al.*, 2017). In the AMP-activated protein kinase/mTORC1 glucose-sensing signalling axis, decreasing glucose concentrations leads to loss of HIF-1α expression and decreased NOS2 expression and NO production. Alternatively, HIF-1α expression can be attenuated by glucose levels independently of mTORC1 signalling, presumably through GlcNAcylation. Glucose-based repression could shed light on how T-cells regulate the DC micro-environment, thereby controlling DC-induced T-cell responses.

In the absence of functional OXPHOS, DC depend heavily on glycolysis for ATP production, a phenomenon that has been observed under *in vitro* as well as *in vivo* conditions (Everts *et al.*, 2012). This NO-mediated commitment to glycolytic metabolism occurs only in DC subsets that biosynthesize NO. In conventional DC (cDC) that do not express NOS2, the switch to glycolytic metabolism following TLR stimulation was not observed. However, a recent *in vivo* study in cDC showed long-term diminished mitochondrial activity and enhanced glycolysis, albeit in a NO-dependent manner (Pantel *et al.*, 2014). These immunometabolism studies in DC are still in their infancy, and much needs to be discovered before a detailed picture can be drawn (Dong and Bullock, 2014; Everts and Pearce, 2014; Thwe and Amiel, 2018).

Expression of COX2 and/or production of PGE_2 in DC cells in response to inflammatory stimuli have also been well documented (Harizi *et al*., 2001, 2010; Fogel-Petrovic *et al*., 2004; Long *et al*., 2004; Cho *et al*., 2011). Mouse DC, produced *in vitro* from bone marrow cells, showed significant PGE_2 production that was inhibited in the presence of the COX-inhibitor indomethacin (Harizi *et al*., 2001). Upon LPS simulation, expression of both COX1 and COX2 was evident, and addition of exogenous PGE_2 led to diminished MHC II expression. Similarly, upon activation with agonist anti-CD40 monoclonal antibody, dose-dependent induction of PGE_2 synthesis via COX2 was observed (Harizi *et al*., 2010). CD-40 stimulated PGE_2 production was proposed to represent a negative feedback mechanism whereby it limits the propagation of Th1 responses and involves EP_2 receptors. Human DCs express little COX2 constitutively. However, upon LPS stimulation, increased COX2 mRNA and PGE_2 synthesis were observed, while pro-inflammatory PGD_2 was not detected (Fogel-Petrovic *et al*., 2004).

The role played by PGE_2 in DC biology is varied and often contrasting. (Harris *et al*., 2002; Scandella *et al*., 2002). The effect of several PGs on DC maturation has been examined, and PGE_2 was found to be the most potent (Steinbrink *et al*., 2000). PGE_2 also inhibited TNF-α release from activated bone marrow-derived DCs as well as **IL-27** in murine DCs (Vassiliou *et al*., 2003; Hooper *et al*., 2017). PGE_2 elicits differential migratory patterns in monocyte-derived DC (MDC) as compared with peripheral blood DC (PDC) (Luft *et al*., 2002). MDC acquired a migratory phenotype when exposed to pro-inflammatory cytokines or CD40L or intact bacteria only in the presence of PGE_2. Pro-inflammatory-type MDCs were generated when stimulated with CD40L or intact *Escherichia coli* in the absence of PGE_2. In contrast, CD1b/c(+) PDC acquired migratory potential irrespective of the activator. Functional CCR7 (a key factor in DC migration into draining lymph node) was shown to be enhanced on co-stimulation with PGE_2 in MDCs. CCL2, the key DC-produced Treg-attracting chemokine, is up-regulated when DCs are matured in the presence of PGE_2. Elevated production of CCL2 persists even when PGE_2 is removed. The study showed how DC targeting of the regulatory versus pro-inflammatory T-cells is imprinted at the stage of DC maturation (Muthuswamy *et al*., 2008). Previously, it has been shown that a positive feedback loop between PGE_2 and COX2 redirected the development of $CD1a^+$ DCs to $CD14^+CD33^+CD34^+$ monocytic MDSCs and up-regulated production of several MDSC-associated suppressive factors such as IDO1, IL-4Rα and IL-10 (Obermajer *et al*., 2011). Tumour COX2 has been shown to suppress DC function by reducing cell surface expression of CD11c, MHC class I, MHC class II, CD80, and CD86 (Sharma *et al*., 2003). Thus, NO-mediated and PGE_2-mediated alteration in DC function and differentiation is likely a key player in ability of tumour cells to escape immunoediting.

Immature myeloid cells or myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs, $CD11b^+CD33^+HLA\text{-}DR^-$) are a population of immature myeloid cells that are a hallmark of an immunosuppressive TME due to their ability to inhibit NK, DC, CD4 and CD8 T-cells, thus blocking the innate and adaptive immune systems (Figure 4) (Gabrilovich *et al*., 2012). A recent study in BC patients showed high levels of tumour infiltration of immature myeloid cells that were not fully differentiated into monocytes or granulocytes [MDSC subtypes referred as monocytic (mMDSC) and granular (gMDSC)] and expressed high Arg1 (Toor *et al*., 2017). This is in line with reduced activity and proliferation of cytotoxic T-cells in BC (Gil Del Alcazar *et al*., 2017). In a mouse model, tumour-infiltrating mMDSCs induce EMT-mediated metastasis and CSC-like phenotype, while gMDSCs support metastatic niche by mesenchymal–epithelial transition (Ouzounova *et al*., 2017). NOS2 expression along with IL-6, **IL-1a**, Arg1, vimentin and TGFB1 gene induction by 4T1-mMDSC coculture was identified as an inducer of EMT and cancer stem cellness as well as a suppressor of the anti-tumour immune response. MDSCs regulate signalling and metabolism in the TME in multiple ways. L-Arginine is a key amino acid that serves as a substrate for NOS2 and Arg1. L-Arginine is rapidly depleted in the TME by Arg1, thus reducing proliferation and effector function of cytotoxic T-cells (Rodriguez *et al*., 2004). While NOS2-mediated NO production by immune cells is associated with anti-tumour activity, it is also involved in MDSC-mediated immunosuppression in advanced-stage non-small cell lung cancer patients (Liu *et al*., 2010). MDSCs can also inhibit T-cell signalling through RNS-mediated nitration of the T-cell receptor and inhibition of JAK/STAT, MAPK and PI3K-mediated IL-2 signalling, while MDSCs from the *NOS2* knockout mouse were not immunosuppressive (Mazzoni *et al*., 2002; Nagaraj *et al*., 2007). IDO-mediated tryptophan depletion linked to T-cell exhaustion also augments MDSC activity in BC and correlate to lymph node metastasis (Yu *et al*., 2013). Similar to NOS2, COX2 also supports MDSC-mediated suppressive activity. The 4Tl tumour model in EP_2 receptor knockout syngeneic mice had delayed tumour growth and reduced MDSC suggesting involvement of the PGE_2/EP_2 receptor axis (Sinha *et al*., 2007). MDSCs can also up-regulate PGE_2 in the TME, which also is an immunosuppressive molecule (Eruslanov *et al*., 2010). Furthermore, HIF-1α-mediated expression of programmed death-ligand 1 under hypoxia in MDSCs, TAMs and DCs also plays a critical role in T-cell exhaustion (Noman *et al*., 2014). Under hypoxic conditions, MDSCs can also differentiate into TAM, thus further supporting an immunosuppressive TME (Corzo *et al*., 2010). Human MDSCs are also involved in immune polarization by converting Th17 T-cells to Tregs via production of TGF-β and retinoic acid (Hoechst *et al*., 2011). Similar to Tregs, tumour-infiltrating MDSCs preferentially use fatty acid oxidation over glycolysis, and inhibition of this pathway showed a synergistic decrease in tumour size with both chemotherapy and adoptive cellular therapy (Hossain *et al*., 2015). Thus, MDSCs play a pivotal role in maintenance of an immunosuppressive TME.

NOS2–COX2 in the adaptive immune system

There are two main components in the adaptive immune system: B lymphocytes or B-cells and T lymphocytes or T-cells

that originate in the bone marrow then migrate to thymus, lymph nodes and spleen. Adaptive immune system is antigen specific and activated by the presence of danger signals. Tumour-specific antigens can lead to an adaptive immune response. The immune cells that migrate towards the malignant tumour are referred as tumour-infiltrating lymphocytes (TILs). This population consists mainly of T-cells and B-cells and also contains NK cells. High TIL levels are strongly associated with a positive patient prognosis. In this section, the effect of endogenous and exogenous NO and COX2 on the adaptive immune system and their crosstalk with the tumour is discussed.

T-cell

Niedbala *et al*. published a series of papers (Niedbala *et al*., 2007, 2013, 2014) that examined the effect of different levels of NO on different murine T-cell populations (Figure 5). Low concentrations of NO donor DETA/NO (5–10 μM) significantly enhanced the differentiation of Tc1 and Th1 subtypes through cGMP in both human and murine models without a significant effect on Tc2 or Th2 cells, while >40 μM DETA/NO inhibited Tc1 and Th1 differentiation. Furthermore, they found that the increased IFN-γ but not **IL-5** (characteristic of Tc2 and Th2) was dependent on a NO-mediated increase in the expression of IL-12Rβ2. Obermajer *et al*. (2013) showed NO–cGMP-dependent differentiation of human CD4+ cells to Th17 (producers of IL-17 and IFN-γ) below 50 μM DETA/NO.

NO exerts a key role in the modulation of Tregs characterized by expression of the Foxp3 transcription factor and CD25 on the cell surface. NO had no effect on proliferation of $CD4^+/CD25^+$ Tregs. However, NO was capable of converting $CD4^+CD25^-$ to $CD4^+CD25^+FOXP3^-$ Tregs through an NO–cGMP-independent pathway (Niedbala *et al*., 2007). This process was driven by (i) p53, a tumour suppressor protein; (ii) IL-2, a cytokine that stimulates the growth of T-cells; and (iii) CD134, a TNF receptor superfamily member. $CD4^+CD25^+FOXP3^-$ Tregs induced immunosuppressive cytokines IL-4 and IL-10 but not IL-2, IFN-γ or TGF-β unlike $CD4^+CD25^+FOXP3^+$ Tregs that up-regulate TGF-β and induce Th17 development (Xu *et al*., 2007). Another study demonstrated that the IL-12p40 homodimer induces NO production via IL-12Rβ1, which then subsequently down-regulates the FOXP3 marker of Tregs in naïve mouse splenocytes (Brahmachari and Pahan, 2009). These results are in line with the generation of NO-induced $CD4^+CD25^+FOXP3^-$ Treg subtype. While $CD4^+CD25^+FOXP3^+$ Tregs suppressed Th1 without affecting Th17, NO induced Tregs down-regulated Th17 response through the aryl hydrocarbon receptor (AHR) and increased IL-10 without affecting Th1 cell differentiation (Niedbala *et al*., 2013). AHR is known to have a dual function of up-regulation of phase I and II xenobiotic metabolizing enzymes such as P450s and binding to HIF-1α to activate the hypoxia response element (HRE) of target gene promoters. NO induces stabilization of HIF-1α, thus favouring HRE activation in response to metabolic changes in the TME. This leads to up-regulation of target genes in tumour as well as immune cells and supports tumour progression. These results suggest that the location and duration of NO production will control generation of Th1 versus Th17 cells. In BC, IL-17A is produced by TILs and has been implicated in proliferation and chemoresistance through the ERK pathway (Cochaud *et al*., 2013). In TNBC, $IL\text{-}17^+$ T-cells were associated with reduced patient survival, while there was no prognostic correlation in other BC subtypes (Allaoui *et al*., 2017). NO also induced Th9 subtype (IL-9 and IL-21 producers) at ~100 μM DETA/NO in a p53-dependent manner (Niedbala *et al*., 2014). This subtype is associated with anti-tumour activity in the TME (Vegran *et al*., 2015).

Similar to NO/NOS2, PGE_2/COX2 also plays an important role in T-cell function. This idea was first presented by Goodwin and Ceuppens (1983) and led to investigation of its role in different subtypes of T-cells. While COX2 is expressed in human T-cells and up-regulated upon activation, there was no corresponding PGE_2 synthesis (Pablos *et al*., 1999). However, inhibition of the COX pathway increased IL-2 production and T-cell proliferation, which was further verified by PGE_2-mediated inhibition of naïve T-cell expansion and activation through down-regulation of the JAK3 signalling pathway and increased cAMP, which reduced CD25 expression (Rincon *et al*., 1988; Kolenko *et al*., 1999; Pablos *et al*., 1999). PGE_2 can also shift the balance from pro-inflammatory Th1 towards the immunosuppressive Th2

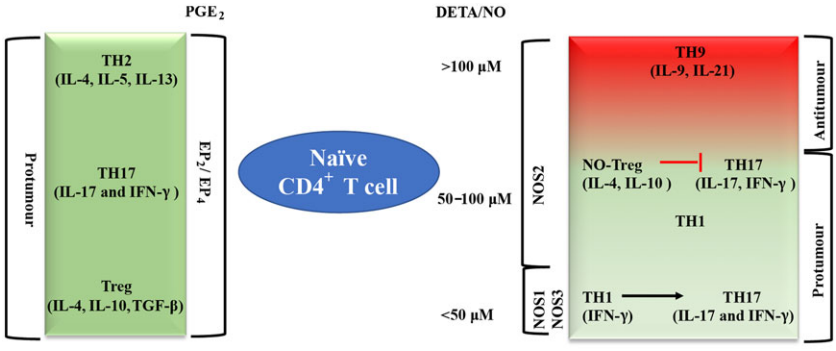


Figure 5

Differentiation of naïve $CD4^+$ T-cells to different subtypes in the presence of NO and PGE_2 in the TME. NO shows a concentration-dependent role in T-cell-mediated tumour response, while PGE_2 plays a mainly immunosuppressive role, in the TME.

subtype in the TME by selective inhibition of Th1 generation (Betz and Fox, 1991). However, this phenomenon is highly dependent on CD28. Yao *et al*. (2009) reported that strong CD28 stimulation induced the Th1 subtype, while weaker activation suppressed it. They also found that PGE_2 suppressed differentiation of naïve $CD4^+$ cells to Th17 subtype in the presence of TGF-β and IL-6, while facilitating Th17 expansion in the presence of **IL-23**. In humans, IL-1β and IL-23 are mainly responsible for Th17 generation. PGE_2 increased IL-17 and reduced IFN-γ production from activated CD4 cells (Boniface *et al*., 2009; Napolitani *et al*., 2009). It also increased EP_2 and EP_4 receptors leading to increase in ROR-γt and down-regulation of T-bet, thus shifting the balance towards the Th17 subtype. PGE_2 also enhanced the activity of $CD4^+CD25^+$ Tregs and induced FOXP3 in $CD4^+CD25^-$ cells, making them Treg-like. Thus PGE_2 plays an important role in modulating Treg mediated immunosupressive activity.

Alterations in glucose, amino acids and lipids together with oxygen content in the TME play an important role in T-cell differentiation and response. T-cells rely on glucose metabolism for their activation, differentiation and function. Glucose uptake is mainly mediated by Glut1 in T-cells, which is expressed at a significantly higher level in activated cells and regulated by PI3K–Akt pathway (Wieman *et al*., 2007; Macintyre *et al*., 2014). Glucose is then converted to pyruvate followed by two ATP molecules. The fate of pyruvate is dependent on the subtype of cell. Naïve and memory T-cells utilize TCA cycle and OXPHOS, while activated T-cells undergo aerobic glycolysis to form lactate along with OXPHOS to support the energy requirements of rapid proliferation and differentiation (Cao *et al*., 2014). A comparison of CD8 and CD4 showed that CD8 favoured glycolysis, while CD4 favoured OXPHOS due to higher mitochondrial content. A closer look at different subtypes showed that Th1, Th2 and Th17 rely on glycolysis and OXPHOS, while Tregs mainly depend on OXPHOS (Shi *et al*., 2011). While the effect of NO and PGE_2 on T-cell metabolism has not been established, there are indirect clues to their involvement. Hypoxia, a key modulator of the TME, leads to NO-mediated HIF-1α stabilization, which acts as a metabolic checkpoint in differentiation of Th17 through the mTOR pathway over Th1, Th2 or Treg subtypes (Shi *et al*., 2011). PGE_2 also induced HIF-1α stabilization in prostate cancer and thus can lead to immunomodulation of the TME (Liu *et al*., 2002). Hypoxia is also associated with overexpression of GLUT1 in activated T-cells, a phenomenon observed in TNBC, as well as increased migration and cancer stem cellness (Oh *et al*., 2017). Inhibition of glycolysis changes the balance between Th17 and Treg in favour of Tregs (Shi *et al*., 2011). NOS2 also promoted IL-2 production, proliferation and glycolysis in γδ T-cells, which along with neutrophils have been implicated as a driver of tumour progression and metastasis in BC (Coffelt *et al*., 2015; Douguet *et al*., 2016). In the TNBC TME, the T-cells have been reported to be exhausted (Gil Del Alcazar *et al*., 2017). This can be partly attributed to constant competition with the tumour for glucose requirement leading to hyporesponsiveness and low IFN-γ levels (Chang *et al*., 2015). Naïve T-cells, memory CD8 and Tregs also rely on fatty acid oxidation for their energy requirements (Michalek *et al*., 2011; van der Windt *et al*., 2012). These studies highlight the importance of glucose metabolism and fatty acid oxidation in T-cell proliferation and differentiation, which likely controls pro-tumour versus anti-tumour response.

Amino acids such as tryptophan, arginine and glutamine also play a critical role in T-cell proliferation and activation. In BC TME, Th1 cell activation led to IFN-γ-mediated induction of IDO that metabolizes tryptophan to kynurenine, while Th2 production of **IL-13** down-regulated IDO (Godin-Ethier *et al*., 2009). Depletion of L-tryptophan in the TME led to reduction of T-cell proliferation, as well as TGF-β-mediated Treg cell differentiation and inhibition of Th1 and Th17 subtypes, thus leading to an immunosuppressive TME (Munn *et al*., 2005; Yan *et al*., 2010). This shows the self-limiting ability of Th1 response, which is key to pathogen response without harming host system; however, it plays a debilitating role in tumour clearance and promotes CSC phenotypes. As seen with tryptophan, depletion of arginine also impairs T-cell activation and proliferation (Rodriguez *et al*., 2007). CD4 and CD8 cells utilize Arg2 for L-arginine metabolism for increased survival and anti-tumour activity (Geiger *et al*., 2016). High L-arginine levels favour OXPHOS over glycolysis, which in turn promotes memory cell formation. Thus, metabolism of L-tryptophan and L-arginine has contrasting effects on macrophage and T-cell function, demonstrating the complexity of the TME. Increased levels of glutamine in the TME is associated with favourable Th1 and Th17 differentiation *in vitro*, while Th2 cells do not depend on it (Nakaya *et al*., 2014). Glutamine deprivation is favourable for Treg differentiation (Klysz *et al*., 2015). Thus, crosstalk of glucose, amino acid and lipid metabolism controls the fate of T-cell.

B-cell

In BC, B-cell infiltration can be significant, and it is a strong prognostic indicator of metastasis-free survival (Schmidt *et al*., 2008; Erdag *et al*., 2012; Iglesia *et al*., 2014). Even though they are the second most abundant population of TILs, the role of B-cells is less well understood in cancer biology. In the TME, B-cells have the capability to recognize antigens through B-cell receptors as well as present antigen and modulates other innate and adaptive immune cells. Like T-cells, B-cells can differentiate to different subtypes and secrete cytokines that contribute to pro-tumour or anti-tumour effects. In the TME, regulatory B-cells are phenotypically similar to activated mature B2 cells with reduced proliferation and induce TGF-β-dependent conversion of resting $CD4^+$ T-cells to $FoxP3^+$ Tregs, thus promoting BC metastasis (Olkhanud *et al*., 2011).

The effects of NO and PGE_2 on B-cells have been examined in both normal B-cells and B-cell leukaemias (Bogdan, 2015). Splenic B-cells exposed to NO donors are rescued from programmed cell death. This was shown to be through a cGMP mechanism that maintained Bcl-2 expression. Conversely, NO inhibition *in vitro* decreased Bcl-2 while increasing Bax leading to more apoptosis (Genaro *et al*., 1995; Hortelano and Bosca, 1997). Further studies shown that in **IL-4** stimulated B-cells, both cAMP and cGMP contribute to increased IgE and sCD23 (Paul-Eugene *et al*., 1995). In Epstein–Barr virus-infected human B-cells, NOS2 expression inhibits apoptosis (Mannick *et al*., 1994). One

study suggests that nitrite at micromolar concentration was capable of enhancing DNA synthesis in LPS-stimulated splenic B-cells (Takagi *et al.*, 1992). Since nitrite can be reduced to NO and cGMP under hypoxia, this may be a pathway that stimulates B-cell-mediated conversion of Th1 to Th2 (Taylor-Robinson and Phillips, 1994). With respect to B-cell lymphoma, there was a correlation between apoptosis and NOS2 in human samples (Atik *et al.*, 2006), which is in contrast to other human cancers where NOS2 is associated with increased progression and poor outcome. Though there are less details, high levels of NOS2 may in fact be critical for the control of B-cell lymphoma. NO donor DETA/NO was shown to enhance killing of B-cell lymphoma with fludarabine. This effect of elevated levels of NO is consistent with a study by Stuehr and Nathan (1989) that suggested NO from macrophages kills leukaemic cells. However, another study suggests that IL-4 and LPS lead to increased NOS2 and anti-apoptotic effects in B-CLL (Levesque *et al.*, 2003). This suggests that a more detailed analysis of both normal B-cells and B-cell lymphomas, and the role of NO is needed.

While it has been shown that primary B-cells of human origin produce NOS2 and it plays an important role in immunoglobulin expression by B-cells in influenza virus A infection, the role in BC still needs to be investigated (Jayasekera *et al.*, 2006; Olkhanud *et al.*, 2011). Recently, Saini *et al.* (2014) showed that NOS2 through the NO–cGMP pathway plays an important role in the survival of plasma cells, which are terminally differentiated B-cells that produce large amounts of antibody. ERS and NOS2 crosstalk was also implicated in this signalling process. They also showed an association of NOS2 signalling through the IL-6 and APRIL pathway in B-cell maintenance and survival. As PGE_2 is a major inducer of IL-6, this hinted at a possible role of COX2 in the function of plasma cells. This is supported by reduction of $CD138^+$ plasma cells and Blimp-1, an essential plasma cell transcription factor, upon inhibition of COX2 activity and PGE_2-mediated IgE production (Carini *et al.*, 1981; Bernard and Phipps, 2010). However, COX2-mediated PGE_2-cAMP regulation leads to reduced proliferation and differentiation of B-cells (Carini *et al.*, 1981). B-cells can also produce COX2 upon activation with CD40L and anti-IgM antibody (Ryan *et al.*, 2005). This observation is in agreement with an association of high levels of CD40L in serum of BC with immunosuppression (Huang *et al.*, 2012). Furthermore, PGE2 in the TME can induce IL-6 production by TNBC that binds directly to $iCD5^+$ on B-cell and up-regulates its expression, thus forming a feedforward loop via STAT3, and these B-cells promoted tumour growth (Zhang *et al.*, 2016). Taken together, these studies show how ERS, NOS2 and COX2 may be necessary for maintaining homeostasis of B-cell-mediated immunity.

Conclusions

Over the past decade, it has become increasingly evident that the efficacy of cancer therapy will depend on (i) the ability to successfully target tumour cells and (ii) activation of the immune system to control tumour progression. NOS2/NO and COX2/PGE_2 have emerged as key players in shaping the TME through regulation of cytokine-mediated signalling as well as cellular metabolism. NO-mediated cellular effects are diffusion dependent, while the PGE_2 effect is mediated by EP_1–EP_4 receptors, which are expressed by specific cell types. TNBC is an inflammation-driven cancer, so it is not surprising that co-expression of high NOS2 and COX2 leads to dramatically reduced patient survival. This phenotype is supported by a feedforward mechanism of NOS2 activation by PGE_2 and COX2 activation by NO that also involves key cytokines such as IL-8, IL-6 and TNF-α. Moreover, BC is associated with a high frequency of PI3KCA mutations and loss of PTEN, a tumour suppressor. This leads to aberrant PI3K–Akt–mTOR signalling, and NO plays a critical role in this by promoting mutation and post-translational modification of key proteins. Growing evidence suggests that metabolic reprogramming of both the tumour cells and the immune cells in the TME due to depleted levels of glucose, amino acids and low oxygen leads to a competition for resources that is crucial in determining their crosstalk as well as dictating the phenotype and function of immune cells, for example, M1 versus M2 macrophages or NADPH utilization for producing ROS. Further insights into the interface between metabolism and immunity can lead to novel therapeutic approaches.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d,e).

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Conflict of interest

The authors declare no conflicts of interest.

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