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Emerging novel mechanisms of action for nitric oxide in cancer progression

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Aberrant expression of the nitric oxide synthases (NOS) is frequently found in cancer. Nitric oxide (NO) can have both anti-tumor and pro-tumor effects, depending on its cellular source, and concentration. Predominantly tumor epithelial derived NO promotes proliferation, angiogenesis, and metastasis. Recent advances focus on identifying signal transduction networks involved in NO signaling, impact on epigenetic regulation, regulation of cancer stem cells and inflammation mediated carcinogenesis. The impact of NO on non-epithelial cell within tumors is more varied. NO signaling regulates the pro-tumorigenic immunosuppressive effects of myeloid derived suppressor cells and mesenchymal stromal cells, while NO has anti-tumorigenic effects when derived from M1-polarized macrophages and specific T cell subsets. These findings highlight the complexity of NO/NOS in tumor carcinogenesis and progression.

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Introduction

Nitric oxide (NO) is generated by three different nitric oxide synthase (NOS) isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS) inducible NOS (iNOS), and plays an important role in the development and progression of cancer [1]. NO has both pro-tumor and anti-tumor effects depending on its concentration and duration of exposure [2]. In this review, we focus on recent developments investigating the impact of NO on cancer progression, in the context of carcinogenesis, metastatic progression, cancer stem cell regulation and the interactions between different cell types within the tumor microenvironment (Figure 1).

NO regulation of carcinogenesis and tumor progression

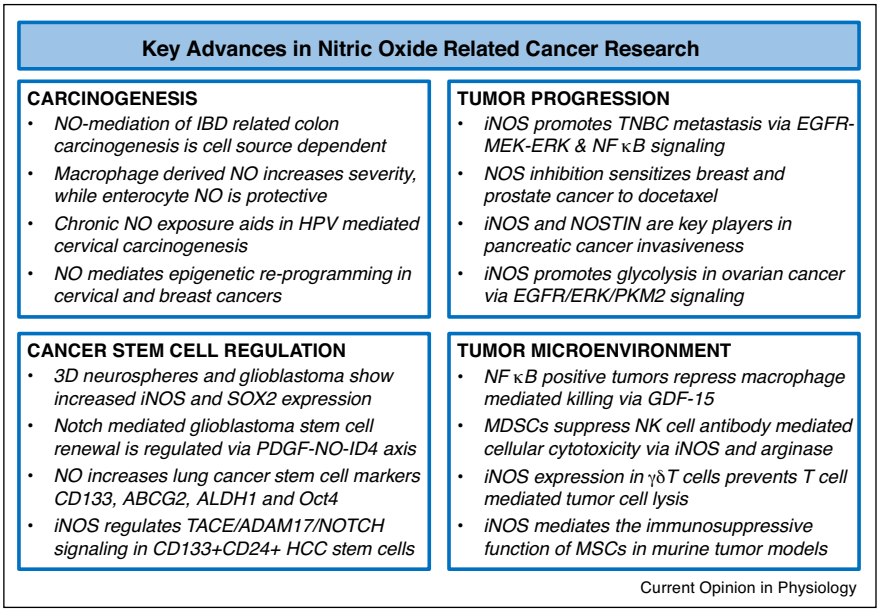
Role of NO in inflammation mediated carcinogenesis

Increased NO signaling has been implicated in the pathogenesis of human inflammatory bowel diseases including Crohn's disease and ulcerative colitis (UC) which are strongly associated with increased risk of colon cancer. It is unclear whether the cellular source of NO has implications in regulating intestinal inflammation. Wang *et al.* investigated the role of iNOS in a RAG2 deficient murine model of *Helicobacter hepaticus* (Hh) induced colitis leading to lower bowel cancer [3]. Hh induced inflammation resulted in increased colonic epithelial iNOS expression, increased DNA damage, crypt cell proliferation and dysplasia, in an IL-22-STAT3-dependent manner, coupled with expansion of inflammatory macrophages ($MHCII^+$ $Ly6C^+$) and dysbiosis (increased *Proteobacteria* and *Enterobacteriaceae*, decreased *Bacteriodetes*) [3]. Rafa *et al.* demonstrated elevation of plasma NO and serum TNF-α levels and increased colonic mucosal TNF-α, TNFR, and iNOS mRNA in both UC and UC-associated colon cancer patients via the TLR4-NFκB signaling pathway, accompanied by increased inflammatory infiltrates and crypt destruction, reversible with SN-40 NFκB inhibitor or all-trans retinoic acid [4].

Stettner *et al.* established an immune competent cell-specific conditional argininosuccinate lyase (ASL) knockout mouse model in combination with genetic and chemical colitis models to assess cell-specific functions of NO in intestinal inflammation [5••]. ASL is the only enzyme able to produce arginine, the substrate for NO generation by NOS isoforms [5••]. The findings convincingly demonstrate complex cell-specific roles for NO. NO derived from enterocytes alleviates colitis by decreasing macrophage infiltration and tissue damage, whereas immune cell-derived NO is associated with macrophage activation, resulting in increased severity of inflammation. These findings are in contrast to that shown by Wang *et al.* [3], but further serve to emphasize limitations in interpretation of data in RAG-deficient mice that lack functional B and T cells. Rafa's [4] findings on the other hand, could be further interpreted by the Stettner study, that the cellular source of the NO detectable in plasma levels may be an important predictor of disease severity. Macrophage produced NO may serve to increase the severity of inflammation in the intestine.

Su *et al.* explored the effects of chronic NO exposure on precancerous HPV-16 and HPV-18 infected Z172 and

Figure 1



Summary of key advances in the field of nitric oxide and cancer.

Z183A cervical cell lines [6]. Chronic NO exposure led to increased cell proliferation, migration, invasion, and anchorage-independent growth. Chronic NO exposure led to protein tyrosine phosphatase receptor-type R *hypermethylation*, which inhibits ERK 1/2 phosphorylation, and AP1 and HPV oncogenes E6/E7 expression. Approximately a further 700 genes were hypermethylated and 600 genes hypomethylated after chronic NO exposure [6], indicating NO may play a role in epigenetic reprogramming of cervical cancer cells. This is further supported by Vasudevan *et al.* who showed that NO can also modulate epigenetic regulatory histone demethylases [7]. NO treatment of the triple negative breast cell line MDA-MB-231 altered histone methylation patterns at key lysine residues of the H3 and H4 histones. Additionally NO altered the distribution of H3K9me2 and H3K9ac across genomic loci, with H3K9ac being enriched at sites of genes commonly upregulated in breast cancer [7].

NO regulation of tumor progression

Further evidence has emerged supporting iNOS as a mediator of poor outcome in numerous tumor types. Garrido *et al.* showed that high levels of iNOS was associated with increased risk of distant metastasis and poor survival in triple negative breast cancer (TNBC) [8]. NO increased the activation of the EGFR-MEK-ERK and NFκB signaling pathways, resulting in increased cytokine secretion and invasive potential [8]. Basudhar *et al.* showed co-expression of COX2 with iNOS in estrogen negative breast cancer further enhances the effects of iNOS on poor outcome, augmenting Akt and TNFα receptor-2 (TRAF2) activation [9]. Co-inhibition of iNOS and COX2 reduced tumor proliferation in a murine MDA-MB-231 TNBC xenograft model [9]. Davila-Gonzalez showed L-NMMA NOS inhibition in combination with docetaxel significantly increased cellular apoptosis via ATF4 endoplasmic reticulum stress response, and decreased tumor proliferation in TNBC patient derived xenograft models [10•]. Collectively, these studies support a role for iNOS in aggressive hormone independent breast cancer. In a study of 4T1/BALBc murine model of breast cancer lung metastasis, increased pulmonary expression of MMP2, MMP9, and MMP14, and reduced elastin was seen with the progressive development of pulmonary metastasis. This was accompanied by reduced pulmonary NO, reduced eNOS activation and reduced endothelial markers VE-CAD, CD31, vWF, or VEGFR2, resulting in a weakened endothelial barrier against metastasis [11].

Contrasting results emerged for prostate cancer. While Erlandsson *et al.* showed high levels of iNOS protein were associated with lethal prostate cancer [12], a study by Nesbitt *et al.* found that low levels of NO donor NOC-18 increased RUNX2 in LnCAP prostate cancer cells, while high levels of NO reduced the expression of RUNX2 [13]. L-NAME treatment inhibited the expression of eNOS, RUNX2, and BCL-2, and sensitized cells to docetaxel, in a ERK-dependent and PI3K-dependent manner, while RUNX2 expressing LnCAP xenografts showed increased tumor growth and metastasis formation [13]. Arora *et al.*

showed that NO donor *S*-nitrosoglutathione (GSNO) inhibited tumor progression in a SCID xenograft model of androgen independent 22Rv1 prostate cancer cells. GSNO treatment suppressed androgen receptor signaling and the macrophage M2 polarization cytokines G-CSF and GM-CSF [14]. GSNO treated tumors showed an increase in M1 markers (iNOS) and a decrease in M2 markers (F4/80, CD206, Arginase). These results suggest that studies using low levels of NO donors exert pro-tumorigenic effects, while high levels of NO donors may have anti-tumorigenic effects on prostate cancer.

iNOS deletion in the *LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx-1-Cre* (KPC) murine model of pancreatic cancer resulted in lower grade disease and improved survival. iNOS knockout KPC cells had reduced invasive capacity. iNOS deficiency reduced tumor macrophage infiltration, mir-21 expression and CCL-2 mRNA [15]. In a second study, reduced expression of endothelial nitric oxide synthase traffic inducer (NOSTRIN) was a predictor of worse survival in pancreatic cancer. NOSTRIN binds eNOS and translocates eNOS to a vesicular compartment reducing its activity. Forced expression of NOSTRIN in pancreatic tumor cells, suppressed invasive capacity, reduced eNOS phosphorylation and increased apoptosis [16•]. Research by Chakraborty and Rupasri showed that NOSTRIN interacts with TNF receptor-associated factor 6 (TRAF6), suppressing NFκB activity and AKT activation. Other genes of interest downregulated by NOSTRIN included ADAM17, CCL2, CCL5, IL-6, MMP2, and MMP9, all of which have previously been reported to be increased in iNOS related tumors [17].

Papaevengelou examined the effects of iNOS inhibition on C6 glioma tumor dynamics [18]. iNOS inhibition reduced tumor growth and angiogenesis including VEGF secretion and blood vessel maturation (as indicated by perivascular cell content and fraction of mature vessels (α-SMA: CD31 stained areas)). Li *et al.* demonstrated the importance of iNOS and NO in the promotion of glycolysis in ovarian cancer with low levels of NO equivalent to that derived from tumor cells, activating EGFR/ERK/PKM2 signaling resulting in PKM2 translocation to the nucleus and glycolytic gene expression. iNOS was also associated with poor outcome in ovarian cancer [19].

NO modulation of cancer stem cells

There has been a renewed focus on NO signaling in the modulation of cancer stem cell regulation (Figure 2). Palumbo *et al.* investigated the relationship between stem cell markers and iNOS in glioblastoma cell line neurospheres. iNOS mRNA and nitrite production was increased in all glioblastoma cell lines tested when grown as 3D neurospheres versus 2D monolayers. This was accompanied by an increase in SOX2 mRNA. iNOS and SOX2 levels were also increased surgical glioblastoma specimens when grown in 3D versus 2D [20]. Eun *et al.* [21••] expanded their previous work which had shown that platelet derived growth factor (PDGF) activates sustained Notch-mediated glioblastoma stem cell renewal through NO activation of inhibitor of differentiation 4 (ID4) in glioblastoma [22]. ID4 activation in glioblastoma cells (A172 and A1207) upregulated PDGFB and increased iNOS in a PDGF pathway dependent manner, which could be reversed with the PDGFR inhibitor sunitinib. This indicates a positive-regulatory circuit in the PDGF-NO-ID4-signalling axis which aides in glioblastoma self-renewal and sphere formation [21••]. Similarly, in H460, H23, and H292 lung cancer cell lines, NO treatment increased spheroid formation and increased CD133, ABCG2, ALDH1A1, and Oct4 expression. NO inhibited Oct4 ubiquitination. Additionally NO increased Akt activation promoting Oct4 expression by dissociating caveolin 1 (Cav1) from the Oct4-Cav1 complex, preventing Cav1 repression of Oct4 activity [23]. Wang *et al.* showed that CD133^{+}CD24^{+} hepatocellular carcinoma stem cells (HCC) display increased SOX2, Nanog expression, and iNOS expression, and have increased hepatosphere and tumor formation capacity, compared to CD133^{-}CD24^{-} HCC non-stem cells [24••]. Increased CD133^{+}CD24^{+} was associated with poor HCC patient outcomes and elevated iNOS expression. iNOS shRNA reduced CD133^{+}CD24^{+} hepatosphere formation and tumor initiation, accompanied by reduced expression of TACE/ADAM17 which is required for Notch activation. Similarly iNOS overexpression in CD133^{+}CD24^{+} HCC cells promoted hepatosphere formation, and increased ALDH expression and Notch signaling, in a TACE/ADAM17-dependent manner [24••]. Furthermore in a Drosophila eye tumor model, iNOS induction due to PTEN depletion contributed to Notch-PI3K/Akt-induced tumourigenesis [25]. The pro-tumorigenic iNOS effects were found to be sGC/cGMP-dependent [25].

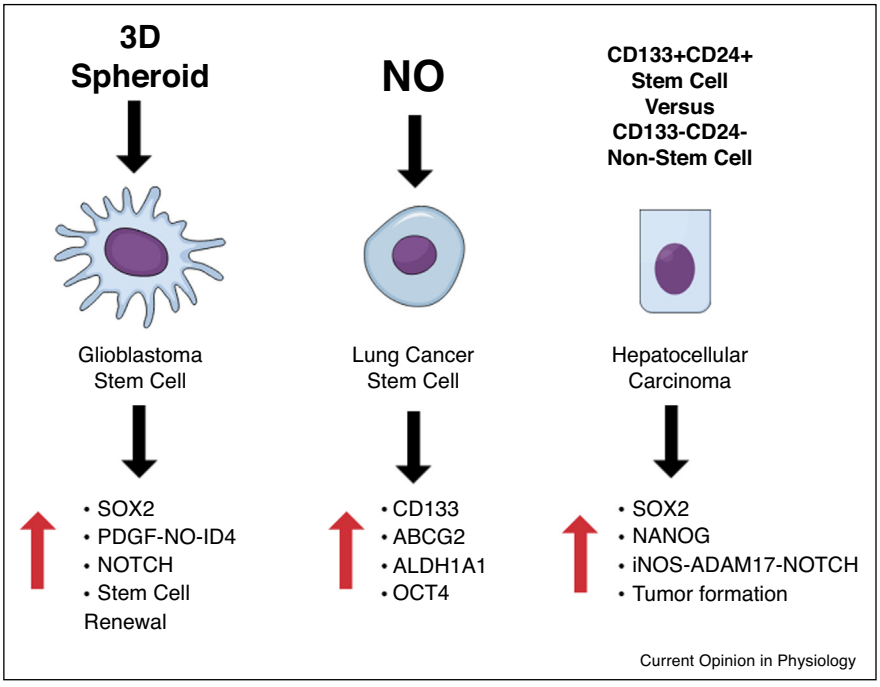
Role of NO in tumor microenvironment

The effects of NO on the tumor microenvironment are far reaching and cell type dependent (Figure 3). Recent focus has been on the role of NO in a range of immune cells including myeloid-derived suppressor cells (MDSCs), T cells, macrophages, and dendritic cells.

Role of NO in innate immunity tumor responses

Tumor associated macrophages are commonly defined as M1 or M2 polarized, being anti-tumorigenic or pro-tumorigenic. Monteiro *et al.* investigated the predominance of M1 versus M2 macrophages in canine mammary tumors finding M2 macrophages were associated with malignant tumors while iNOS expressing M1 macrophages were associated with benign tumors [26]. Sektioglu *et al.* explored the role of macrophages in the efficacy of CD8^{+} T cell transfer/CpG treatment in a pancreatic neuroendocrine tumor model [27•]. CpG (to polarize macrophages to iNOS expressing M1) and CD8^{+} T cell

Figure 2

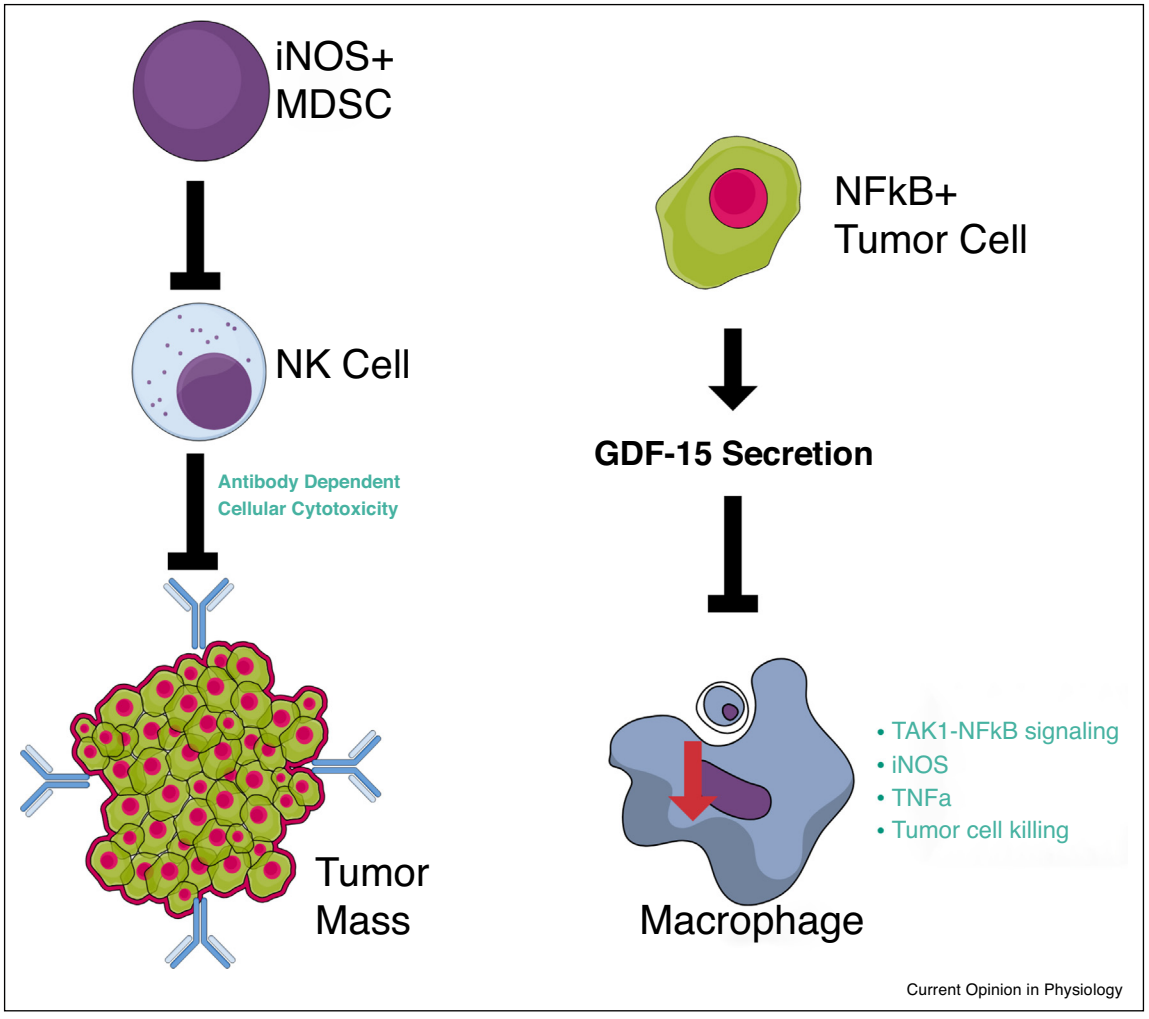


NO/iNOS regulation of cancer stem cells (Figure generated using MindTheGraph).

transfer induced strong immune infiltration ($CD8^+$ T cells, $CD4^+$ T cells, B cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages) into RT5 mouse tumors, resulting in increased survival. The effects were reversed upon macrophage depletion or iNOS inhibition. iNOS expressing macrophages were required for CD31/VCAM-1 co-expression and T-cell diapedesis. Macrophage depletion or iNOS inhibition decreased $CD8^+$ T cell/CpG induction of IFN-γ, CCL-5, CXCL9 and CXCL10 tumor expression. Perrotta *et al.* explored the role of unique iNOS expressing M2 polarized macrophages in glioma [28]. Their results show the presence of both M1 and M2 polarized iNOS expressing macrophages; however, the M2 iNOS expressing macrophages produced low levels of NO, and were found to induce resistance to cisplatin treatment, reversible with iNOS inhibitor L-NAME. Solis-Martinez *et al.* examined the effect of PC3 metastatic prostate cancer cell line on human peripheral derived macrophages [29]. PC3 secretome induced macrophage STAT3 phosphorylation (reversible with IL-10 blockade) and polarization to a M2 phenotype, with increased expression of CD206 and CD163 and loss of M1 associated CD14, TNF-R1, CD86, and CD11a [29]. Ratnam *et al.* showed that tumor NFκB inhibition of macrophage anti-tumor responses is dependent on GDF-15 [30••]. NFκB expressing $p65^{+/+}Ras$ MEFs, have significantly elevated GDF-15 and are resistant to macrophage mediated killing. GDF-15 knockdown resulted in decreased tumor proliferation in a $p65^{+/+}Ras$ MEF subcutaneous SCID model, and increased macrophage killing of $p65^{+/+}Ras$ MEFs. Similar results were also seen in murine Panc02 and KPC pancreatic models. Tumor derived GDF-15 inhibits macrophage anti-tumor responses by inhibition of TAK1 signaling to NFκB, thus blocking TNF-α and NO production.

MDSCs play a key role in tumor immunosuppression via secretion of reactive oxygen species and reactive nitrogen species [31]. MDSCs have the ability to inhibit the FcR mediated NK cell functions required for monoclonal antibody (mAb) mediated therapies. NK cells express low-affinity, activating FcR (FcγRIIIa) allowing them to identify mAb coated neoplastic cells, and initiate cell killing. MDSCs ($CD33^+CD11b^+HLA\text{-}DR^{low}$) isolated from melanoma patients, suppressed NK antibody-dependent cellular cytotoxicity (ADCC), IFNγ secretion, and FcR mediated ERK phosphorylation [32••]. MDSC iNOS and arginase inhibition rescued NK ADCC function, highlighting the importance of iNOS/arginase in MDSC regulation of NK function. Inhibition of MDSC iNOS also enhanced mAB trastuzumab therapy *in vivo* [32••]. Cao *et al.* showed L-arginine supplementation in 4T1 murine models of breast cancer [33], inhibited tumor proliferation, and reduced the number of tumor and spleen associated MDSCs. L-arginine supplementation promoted $Gr\text{-}1^+CD11b^-F4/80^+$ macrophages and suppressed $Gr\text{-}1^+CD11b^+F4/80^+$ macrophages, while

Figure 3



NO/iNOS mediates of MDSC and macrophage effects in the tumor microenvironment (Figure generated using MindTheGraph).

iNOS/NO levels were significantly elevated in tumors and spleens of these mice; however, the cellular source of NO is unclear. L-arginine also promoted the differentiation and activation of DCs and Th1 immune responses [33]. Redd *et al.* demonstrated that iNOS in tumor derived MDSCs is regulated by SETD1B rather than NFκB Rel or STAT1 [34]. SETD1B increased H3K4me3 (marker of active chromatin and gene activation) localization to the iNOS promoter region in MDSCs [34]. In ovarian cancer, Wong *et al.* reported the importance of IFNγ and TNFα in MDSC-suppressive activity [35]. IFNγ and TNFα were key for the induction of IDO1, iNOS and COX2 in MDSCs, and required intact COX2 signaling for the suppression of type I $CD8^+$ T-cell proliferation and granzyme B anti-tumor immune responses. Markowitz *et al.* reported that patients with advanced melanoma have an increased number of MDSCs with high levels of iNOS, which decreased DC antigen presentation to $CD4^+$ T cells in an NO-dependent manner, via nitration of STAT1 [36].

Role of NO in adaptive tumor immunity responses

Douguet *et al.* studied the role of iNOS in γδ T cells, in melanoma progression [37]. γδ T cells differ from $CD4^+$ and $CD8^+$ T cells by expressing γδ T cell receptors (TCRs), rather than αβ TCRs. iNOS/NOS2 KO in the Ret murine melanoma model, delayed melanoma formation and dissemination. VEGF, keratinocyte derived factor, G-CSF levels were reduced in RetNOS2KO mice. IL-17, IL-1β, and IL-6 levels were higher in RetNOS2 mice. While there were no major changes in DCs, macrophages and monocytic MDSCs (M-MDSCs) in the RetNOS2KO mice, there were significantly less polymorphonuclear MDSCs (PMN-MDSCs). RetNOS2KO tumors had decreased γδ T cells (in particular IL-17 expressing), known to recruit PMN-MDSCs.

RetNOS2KO γδ T cells displayed enhanced tumor lysis capacity compared to iNOS expressing γδ T cells [37]. Douguet *et al.* demonstrated that IL-1β and IL-6 drive iNOS expression in γδ T cells, and also regulate IL-17 [38]. Fauskanger investigated the mechanism of tumor killing initiated by $CD4^+$ T cells in a multiple myeloma model. They showed that $CD4^+$ T cell recognition of secreted tumor neoantigen, resulted in macrophage-induced killing of MOPC315 cells via the intrinsic apoptosis pathway. Macrophage-induced killing was iNOS driven and spatially limited [39].

Role of NO in stromal cell compartment regulation

NO may play an important role in stromal cell regulation in cancer. Fibroblasts and mesenchymal stromal cells (MSCs) promote wound healing, home to tumors and have immunosuppressive effects [40]. Lisanti *et al.* reported that a JNK1 mediated iNOS gene signature in fibroblasts of women with high breast density which is associated with increased breast cancer risk [41]. NO was shown to increase wound healing and actin microfilament remodeling in keratinocytes and fibroblasts in a cGMP/cGC-dependent manner [42]. NO/iNOS mediates the immunosuppressive effects of IFNγ primed murine MSCs. Treatment of MSCs with IFNα, inhibited iNOS expression and switched MSC effects from pro-tumorigenic to anti-tumorigenic in the B16 mouse melanoma model, via Stat1 dysfunction [43]. Langroudi *et al.* reported that tumor derived MSCs from a BALBc mammary murine model displayed higher immunosuppressive potential (iNOS, IDO and COX2 expression) compared to matching adipose derived MSCs [44]. Similar results were seen in murine lung cancer models, where MSCs suppressed the cytotoxicity of NK cells in an iNOS and IDO-dependent manner [45]. Interestingly Jia *et al.* showed that murine MSCs exposed to M1 macrophage conditioned media, displayed increased iNOS and promoted tumor proliferation [46], by inducing M1–M2 macrophage polarization, indicating that MSCs may play a role in tumor defense against M1 macrophages infiltration. A cautionary note is needed relating to murine model studies on the role of iNOS in MSC mediated tumor suppression. Human MSCs do not express iNOS, rather their immunosuppressive properties are driven by IDO and COX2 as reported by Lohan *et al.* [47]. While we have demonstrated that metastatic prostate cancer patient derived MSCs increase tumor cell migration and invasion to a greater extent than MSCs from healthy men, iNOS is not expressed in these cells [48].

Conclusions

Increased NO exposure has a role to play in carcinogenesis and tumor progression. This can be due to increased accumulation of pathological changes such as DNA damage and tissue destruction or more subtly via regulation cancer stem cell associated genes promoting tumor formation, maintenance and progression. Importantly NO is emerging as a major regulator of the tumor microenvironment with far reaching effects stromal cells including fibroblasts and MSCs, in addition to immune cells including macrophages, NK cells, MDSCs, and T cells. Murine models have particularly focused in on the role of iNOS in these settings. Future directions will need to translate this to the human setting particularly in the context of several immune and stromal functions in humans being more strongly driven by IDO and COX2 than iNOS. Whether human tumor epithelial derived NO compensates for the lack of iNOS in human MSCs, remains to be seen.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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