Published in final edited form as:

Clin Cancer Res. 2013 October 15; 19(20): . doi:10.1158/1078-0432.CCR-12-1554.

Molecular Pathways: Inflammation-associated nitric-oxide production as a cancer-supporting redox mechanism and a potential therapeutic target

Elizabeth A. Grimm^a, Andrew G. Sikora^b, and Suhendan Ekmekcioglu^a

^aDepartment of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas, USA

^bDepartments of Otolaryngology, Immunology, Oncological Science, and Dermatology Mount Sinai School of Medicine, One Gustave L. Levy Place Box 1189, New York, New York. USA

Abstract

It is widely accepted that many cancers express features of inflammation, driven by both microenvironmental cells and factors, and the intrinsic production of inflammation-associated mediators from malignant cells themselves. Inflammation results in intracellular oxidative stress, with the ultimate biochemical oxidants composed of reactive nitrogens and oxygens. Although the role of inflammation in carcinogenesis is well accepted, we now present data that inflammatory processes are also active in the maintenance phase of many aggressive forms of cancer. The oxidative stress of inflammation is proposed to drive a continuous process of DNA adducts and crosslinks, as well as posttranslational modifications to lipids and proteins that we argue support growth and survival. In this Perspective we introduce data on the emerging science of inflammation-driven posttranslational modifications on proteins responsible for driving growth, angiogenesis, immunosuppression, and inhibition of apoptosis. Examples include data from human melanoma, breast, head and neck, lung, and colon cancers. Fortunately, numerous anti-oxidant agents are clinically available, and we further propose that the pharmacological attenuation of these inflammatory processes, particularly the reactive nitrogen species, will restore the cancer cells to an apoptosis-permissive and growth inhibitory state. Our mouse model data using an arginine antagonist that prevents enzymatic production of nitric oxide, directly supports this view. We contend that selected antioxidants be considered as part of the cancer treatment approach, as they are likely to provide a novel and mechanistically justified addition for therapeutic benefit.

Background

The most commonly recognized features of cancer-associated inflammation are those also expressed by the innate immune system, normally activated in response to stress or infection and which function teleologically during the initiation of wound control ¹. The observed chronic inflammatory milieu in notable subsets of human cancers, and particularly in melanoma, is proposed to support tumor growth, plasticity, and resistance to therapy ^{2, 3, 4, 5, 6}. Unfortunately, dysregulated persistent inflammation contributes to the chronic phase of many diseases, including maintenance of many cancers. It is accepted that inflammation drives development of some cancers which adapt to thrive in the oxidant-rich

Correspondence to Elizabeth Grimm, PhD, Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, Tel.: 713-792-3367; fax: 713-792-2070, egrimm@mdanderson.org.

The authors have no conflicts of interest to declare for information included in this manuscript.

microenvironment as described initially in the review by Coussens and Werb⁵, by “co-opting” expression of inflammatory mediators⁷. From our view, this continues to provide a persistent and self-perpetuating oxidative stress composed of both Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS), and derived from pro-inflammatory interleukins, chemokines, NOSs (nitric oxide synthases) often via growth factor receptors⁴. The critical oxidant sources are now realized to be more than reactive oxygen molecules, as the chronic production of another oxidant, NO (nitric oxide), also plays a major role in oxidative stress in melanoma and other cancers^{4,8,9,10,11} and the aberrant constitutive RNS is argued here as possibly a more important source of oxidative stress in many cancers. In comparison to the oxygen radicals, NO is a more stable oxidant, easily crosses lipid bilayers, and generates several types of posttranslational modifications with known ability to alter protein function and stability; these modifications are dependent on both NO concentration and temporal availability, resulting in a dynamic and reversible situation¹². A mathematical model of cutaneous melanoma predicted sufficient concentrations of NO at the periphery of a tumor to stimulate cell proliferation, lymphangiogenesis, and inhibit apoptosis¹³.

Inflammation driven NO supports melanoma growth, and apoptosis resistance

In melanocytes, the precursor cell of melanoma, the pigment eumelanin provides a redox function supporting an antioxidant intracellular environment; however in melanoma, a pro-oxidant status develops⁸. The enzymatic production of NO is cell type specific with cytokine-driven inducible NOS (iNOS) noted initially for the burst of higher levels as part of the pathogen defense system. Neuronal cells use nNOS (to produce NO for signaling, and as melanocytes are of neuroectoderm origin, it is not surprising to find nNOS also expressed. The third NOS, endothelial NOS, regulates NO production in endothelia and is responsible for vascular relaxation, and has also been reported to be expressed in melanoma¹⁴.

In melanoma tumors, we^{9,10,4,11} and others^{6,8} document expression of NOS, particularly *iNOS* and *nNOS*. The iNOS protein was found *in vivo* in the melanoma tumor cytoplasm of ~60% of advanced patients, and provided independent prognostic value by predicting decreased survival, so that the hazard ratio of iNOS positive patients was 4.6 by multivariate analysis⁹. This was an unexpected finding as the anti-iNOS antibody was employed to identify activated macrophages, which were also often positive, but for which the positivity did not prove prognostic. Further evidence supporting intracellular NO production was by use of DAF-2DA staining⁴ as well as identification of irreversible protein nitration and the reversible thiol modifications known as “S-NO” (S-nitrosylation)^{4,9,10,11}. Using a human cell line model, *in vitro* experiments were performed to scavenge endogenous NO which resulted in melanoma cell growth inhibition; the growth was restored with an RNS donor, providing data to support a pivotal functional role of NO in cell growth and proliferation¹⁵. Therefore, molecular analysis supports the hypothesis that NO can drive proliferation as well as resistance to apoptosis, and the chemical quenching of NO resulted in G2 growth arrest followed by a gain of cisplatin-induced apoptosis, now confirmed in several reports^{15,11,16}.

As iNOS is downstream of Toll-like receptors, recently described in an earlier CCR Pathways¹⁷, and results in NO production leading to a feed-forward loop and tumor progression,¹⁷ we asked whether we could identify a signature of the downstream common inflammatory markers. Although many markers have been noted in an extensive literature, in melanoma we have identified inflammatory cytokines IL-1 α and β ¹⁸, IL-6, and IL-8; and in a more recent study we have observed that an MIF-CD74 autocrine interaction upregulated by IFN- γ , promoting the phosphorylation of AKT Ser473, and driving the expression of IL-6, IL-8, BCL-2 and BCL-xl (unpublished data). IFN γ also regulates iNOS gene expression via Interferon Regulatory Factors (IRFs). IRFs are nuclear transcription

factors that respond to IFN- γ via the JAK-STAT signaling pathway¹⁹. The importance of IRF-1 and STAT-1 for the induction of iNOS gene expression in response to IFN- γ has been well documented^{20, 21}. Our earlier study showed that IL-24 (*aka* Melanoma Differentiation Antigen (MDA)-7) signaling modulates the IRF transcriptional system, to the extent that IL-24-treated melanoma cells exhibit a decline in IRF-1 and an increase in IRF-2 which blocks the IRF1 pathway. This alteration in the IRF balance predicts the result in inhibition of iNOS expression²². We have also employed gene array studies, followed by validation of protein in patient tumor samples, and identified iNOS, arginase, VEGF α , CXCL-10, IL-8, IL1 α / β , and TNFSF9 as produced constitutively^{23, 24}. Using melanoma patient's tumors, recent results from our lab continue to support iNOS protein associating with NT, COX2, pSTAT3, and arginase, consistent with the report of Johansson et al⁶, and with other recent studies^{25, 26}. Figure 1 includes these cytokines and their hypothesized connections to either drive NOS, or result from iNOS, as well as growth and apoptosis signaling molecules modified by RNS.

Biochemical and molecular mechanisms of NO-mediated dysregulation of cancer signaling

NO in the presence of equimolar O₂⁻ forms ONOO⁻ (peroxynitrite), which under physiological conditions rapidly reacts with available tyrosine or thiol- containing proteins to form nitrotyrosine or reversible nitrosylation of thiols (S-nitrosylation *aka* S-NO). Nitration of tyrosines is considered a surrogate marker for higher but generally nontoxic μ M concentrations of NO. Global analysis of melanoma patient samples for tyrosine nitration has been used by several groups as a biomarker of NO exposure^{27, 9, 4, 11} in melanoma tumors as well as micro-environmental cells. This nitrotyrosine modification is considered irreversible in eukaryotes, and once modified nitrated proteins will continue to express altered function; also such modified proteins are also proposed in some cases to alter antigenicity²⁸. Due to the lower nM levels of NO functional in generating S-NO, this modification is considered more physiologic relevant. Studies on cardiovascular and neuronal signaling regulation by NO have identified numerous protein S-NO modifications, and their reversibility suggests a novel paradigm for attempting to restore dysregulated apoptotic pathways in the inflammation-driven tumors.

Therefore, we now propose S-NO as not only a marker of nitrosative stress, but based on the specific molecule modified also serves functionally to activate oncogenes, inhibit apoptosis, drive growth and angiogenesis, and inhibit tumor suppressor functions. In a recent report by Switzer et al., oncogene activation include S-NO of Ras in in ER-negative breast cancer²⁹. Using a model ER-negative breast cancer cell line, it was shown that NO not only induces S-nitrosylation of wild-type Ras, but led to phosphorylation and activation of Ets-1 through the Ras/MEK/ERK pathway. Ets-1 is a key transcriptional mediator of oncogenic NO signaling, promoting the development of an aggressive disease phenotype in ER- breast cancer²⁹. S-NO-modified proteins involved in the apoptotic machinery include bcl-2 in lung carcinoma³⁰, the death receptor FAS in colon and breast cancer cells³¹ and the associated FLICE inhibitory protein (FLIP;³²), and caspase 9 in cholangiocarcinoma cells³³. Other early notable evidence supporting a role for NO in apoptosis resistance is the classic *in vitro* work of Mitchell and Marletta³⁴ with caspase 3 inactivation by reversible S-NO modification. Earlier *in vivo* data also indicates the NO-driven inhibition of caspase3³⁵. Nitrosylation has also been shown to stabilize mitogen-activated protein kinase phosphatase-1 (MKP-1) in head and neck cancer, thus decreasing radiosensitivity by inhibiting apoptosis³⁶.

Tumor growth was also noted to be regulated by NO in a variety of tumor models via S-NO of Ras³⁷ and S-NO of the epidermal growth factor receptor³⁸. Cellular migration in

prostate cancer is enhanced by S-NO on the integrin alpha chain³⁹, and breast cancer invasiveness enhanced by nitrosylation of c-Src⁴⁰. In addition to the functional effects of nitrosylation of caspase 3 and 9, Ras, p53, PTEN, iNOS, COX2, AKT referenced above, other molecules that may have altered function as a result of S-NO as reported and include Bcl-2^{41,42}, HIF1 α ⁴³, PI3K⁴⁴, NF κ B and AP-1^{45, 46}.

S-nitrosylation of a number of proteins can be linked to tumor proliferation. Many GTPases within the Ras superfamily contain redox-sensitive Cys residues that are susceptible to S-nitrosylation. Ras S-nitrosylation is associated with the initiation of tumorigenesis and maintenance of established tumors. In tumor cells, oncogenic K-Ras activates eNOS by PI3K–Akt-dependent phosphorylation of eNOS at S1117, which results in S-NO of wild-type N- and H-Ras; S1117-phosphorylated eNOS is elevated in tumors isolated from patients with pancreatic cancer, compared to matched and unmatched normal tissue controls from these patients.³⁷

Our additional data is consistent with the model that p53 in melanoma is inactivated by NO¹⁵ and the quenching of NO in melanoma led to cisplatin-mediated apoptosis, which was blocked with biochemical donation of NO indicating that this was not due to toxicity of the quencher. Tang further reported that the apoptosis was dependent on p53 via use of siRNA. In melanoma, p53 does not express the usual driver mutations and is considered as rarely mutated yet functionally inactivated, and the NO is somehow responsible for p53 inactivation¹⁵. We have preliminary data of S-NO modification of wt p53 (Qin, manuscript in preparation), which likely adds another major protein to the list above.

From the molecular view, we hypothesize that endogenous NO in the presence of stoichiometric availability of O₂- chemically nitrosylates accessible thiols to activate oncogenes, inhibit multiple apoptosis and growth pathway proteins, and support enhanced growth. Local production of NO as a plausible mechanism for tumor resistance to therapy was elegantly presented by Dedon and Tannenbaum⁴⁷ almost a decade ago, and while still accepted to be provided by microenvironmental macrophages as in that first report, we now extend this understanding that NO is produced by tumor cells including melanoma. Although studies of S-NO modifications were performed initially by redox chemists, more accessible methods are becoming available to cancer biologists, such as the biotin-switch method for direct S-NO protein characterization⁴⁸, and even more recently the mass-spec/peptide sequencing methods for biochemical validation of the specific site and protein verification such as the SNO-Flo^{49, 50, 51}. The S-NO modifications are dynamic, can be identified biochemically, and can be reversed with various antioxidant approaches, some of which may prove useful in melanoma for inhibition of growth and restoration of apoptosis pathway function.

A second mechanism resulting in S-NO on signaling proteins in cancers is called trans-nitrosation, as reported with NO transferred from PTEN to AKT⁵², or from iNOS to COX2⁵³, as iNOS and PTEN are both themselves reported to have S-NO as well in those reports; the actual transfer of NO is based on differential nucleophilic profiles of the cysteine donor and receptor sites on each molecule. Therefore, after NO is generated and proteins are S-NO modified, the transfer of NO radicals can also regulate the dynamic and reversible post-translational modifications.

Currently there is no evidence that inflammation is uniquely linked to a specific driver mutation in melanoma; however in some recent data testing melanoma tumors containing mutated BRAF, it was reported that the mutation may actually support expression of IL-1 β expression⁵⁴, and in another report antisense *BRAF* inhibited iNOS expression⁵⁵. More analysis is needed to understand the driving forces for NO production and associated effects.

Inflammation-associated molecules drive formation of the pro-tumor cancer microenvironment and tumor-mediated immunosuppression

By upregulating key inflammatory molecules, including iNOS, COX2, and proinflammatory cytokines and chemokines, tumor cells invoke a chronic inflammatory state that also induces tumor-supporting myeloid cells such as tumor-associated macrophages and myeloid-derived suppressor cells (MDSC), and drives their infiltration of the tumor microenvironment^{56,57}. While many host cell types including T cells are involved in creating an inflammatory pro-tumor microenvironment, in this Pathway, we focus on inflammation-directed recruitment of MDSC and macrophage polarization. As tumors progress, the macrophage population is thought to switch from a tumoricidal M1 to a pro-tumor M2 phenotype, under the influence of IL-4, IL-10, IL-13 and TGF β . M2 macrophages are associated with immunosuppression via arginase expression, the release of TGF β and IL-10⁵⁸ and Treg recruitment⁵⁹. M2 macrophages also contribute to formation of the pro-tumor microenvironment and tumor progression by promoting angiogenesis⁶⁰.

MDSC are a myeloid cell type shown to play a critical role in establishing a cancer-supporting microenvironment⁶¹ and implicated in suppression of anti-tumor immune responses⁶². The role of MDSC has been the focus of a recent Molecular Pathways by Lu and Gabriolovich²⁷ describing MDSC producing NO and ONOO- leading to protein modifications *in vivo* and *vitro*. A recent paper by us also identified a pivotal role for iNOS and ROS as mediators of MDSC recruitment and cancer-mediated immunosuppression, as *in vivo* melanoma tumor-expressed iNOS regulated MDSC induction by modulating VEGF release, and that pharmacologic iNOS inhibition depletes intratumoral MDSC and unmasks anti-tumor immunity⁶³. We have further investigated the role of iNOS in orchestrating MDSC migration in response to iNOS or NO inhibition, leading to upregulation of CXCL-10²³, as well as that iNOS inhibition blocks release of a number of inflammatory mediators by melanoma cells, including VEGF, which we subsequently showed to be required for accumulation and functional activation of MDSC⁶³. Another interesting paper shows in several preclinical cancer models that nitration of the chemokine CCL2 abolished intratumoral infiltration of anti-tumor CD8+ T cells, and in clinical specimens that tumor-infiltrating T cells are depleted in NO-expressing tumors^{64, 65}. COX2 can also play a role in MDSC induction, via the direct MDSC-stimulating effects of its enzymatic product PGE2^{66; 67; 68}. Nagaraj et al also showed that pharmacologic modulation of ROS with an antioxidant CDDO-Me triterpenoid compound resulted in dramatic reduction of ONOO- and suppressed MDSC function and boosted immunity in tumor-bearing mice and in cancer patients receiving the drug in a clinical trial⁶⁹. Each of these papers suggests potential therapeutic strategies for reversing tumor-mediated immunosuppression by blocking inflammation-induced MDSC, particularly involving NO regulation.

Clinical-Translational Advances

Based on the contention that a cancer's "inflammatory niche" drives alterations in critical protein function via identifiable S-NO post translational modifications as proposed above, two clinical-translational advances are in progress. The first is development of a marker set or predictive signature of inflammation that would prove useful for targeting. This work is active currently with a clinically useful melanoma protein signature around iNOS, IL-1s, IL-6, MIF, pSTAT3 and at a global level of total nitrated tyrosines and total S-NO at both the cellular and microenvironmental levels still being resolved. As our detection methods become more sensitive and specific, it is likely the modified proteins such as SNO-PTEN or AKT will also be useful markers. Another important complimentary marker in the tumor microenvironment was MIF itself also from both melanoma cells and infiltrating immune cells, for which its presence negatively associated with CD74 expression. Our work in progress as well as that of others continues to support the growing evidence that "an

inflammatory protein signature” is likely to continue as a consideration of an independent prediction model for clinical outcome, still to be validated. A large set of markers is undergoing analysis on both blinded patient tumor samples, and prospective testing on tumor samples from ongoing clinical trials. Ideally, a blood test and specific anti S-NO-protein epitope is envisioned, and underway using a new method named the SNO-Flo system⁴⁹. Direct measurements of SNOed proteins and their loss of this adduct after use of an anti-inflammatory agent is currently a view considered as a future consideration as a set of predictive markers.

A second advance we view is the development of clinically useful pharmacologic approaches with carefully selected anti-oxidants and/or small molecule inhibitors of RNS, possibly combined with inhibitors of other pro-oxidant enzymes to permit the restoration of susceptibility to immune or targeted therapy in melanoma patients. The known reversibility of SNO post-translational modifications suggests a novel paradigm for attempting to restore the apoptotic pathway in the inflammatory-driven tumors of patients for therapeutic goals. We now propose pharmacologic approaches with carefully selected anti-oxidants which will inhibit the reactive nitrogen species of oxidants to permit the restoration of susceptibility to immune or targeted therapy in melanoma patients, possibly predicted by reversal of SNO on selected markers, for which much research is currently underway.

Although NSAIDs have been popularized and continue to be assessed for prevention of cancers, their application to inhibiting inflammation supporting metastatic disease remains to be considered. The *in vivo* use of other classes of antioxidants in treatment of established cancers has been explored. As one of the most investigated antioxidant, *Resveratrol*, shown to be protective against DNA damage by inhibiting hydro peroxidases and scavenging free-radicals⁷⁰. In melanoma, *Resveratrol* has been shown to inhibit growth and induce apoptosis in cell lines, potentially by regulating p53 expression, CDKs, and COX-2^{71, 72}. Another much studied agent, *Curcumin* (*Curcuma longa*), has been shown by many groups, including us, as an apoptosis inducer in human melanoma cells, most known pathways is through the inhibition of NF- κ B which drives iNOS^{73, 74}. Other approaches include clinically applicable arginine antagonists, such as L-NIL which was developed for treating asthma and used in our recent mouse model¹¹ and for which an entire class of agents is currently available. Other drugs with anti-cancer potential known to downregulate iNOS include WP-760⁷⁵ which was found to inhibit iNOS production, and also localize to the mitochondria and activate caspase 3. As aberrant expression of inflammatory molecules is common to many cancers, it stands to reason that therapeutic approaches targeting these molecules have the potential for anti-tumor efficacy across a broad range of tumor types. In fact, much of the preclinical literature describing these approaches uses targeted inhibition of inflammatory molecules to simultaneously prove their relevance to cancer, and invoke therapeutic efficacy. As discussed above, we have shown that pharmacologic inhibition of iNOS has anti-tumor effects in various melanoma models which may be directly related to tumor cell-intrinsic growth and survival pathways^{11; 15} or indirect and mediated by effects on the tumor-promoting microenvironment including vascular¹¹ or immunoregulatory cells⁶³. The role of MDSCs is particularly relevant here as Nagaraj et al also showed that an antioxidant triterpenoid compound can suppress MDSC function and boost immunity in tumor-bearing mice and in cancer patients receiving the drug in a clinical trial⁶⁹.

In conclusion, many human cancers express inflammatory molecules that lead to an intrinsic pro-oxidant environment in the cancer cell, as well as potentiate a microenvironment that drives immune escape and resistance to apoptosis. We contend that by attenuating the NO production resulting from this inflammation, possibly by repurposing currently available agents, that the prospects for cancer control will be greatly increased. Our data and that of others provides an emerging molecular model by which the RNS drive growth,

immunosuppression, angiogenesis, and apoptosis resistance by specifically altering the function of signaling molecules by nitration and/or nitrosylation. We propose that the NO-contributed oxidants and the pathways that drive them may be useful targets as part of a therapeutic consideration which may be useful when combined as part of targeted and immune therapy approaches.

Acknowledgments

Grant Support: EA Grimm and S Ekmekcioglu: this work was partially supported by the following grants: MD Anderson Cancer Center SPORE in Melanoma P50 CA093459; Cancer Center Support Grant CCSG P30 CA016672; Miriam and James Mulva Foundation; and Dr. Miriam and Sheldon G. Adelson Medical Research Foundation

AG Sikora: this work was partially supported by grants from the National Institutes of Health (NIH) National Institute of Dental and Craniofacial Research (5R03DE021741-02) and the NIH National Cancer Institute (1K08CA154963-01A1)

References

1. Tabas I, Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science*. 2013; 339:166–72. [PubMed: 23307734]
2. Landsberg J, Kohlmeyer J, Renn M, Bald T, Rogava M, Cron M, et al. Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation. *Nature*. 2012; 490:412–6. [PubMed: 23051752]
3. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-kappaB as the matchmaker. *Nature immunology*. 2011; 12:715–23. [PubMed: 21772280]
4. Grimm EA, Ellerhorst J, Tang CH, Ekmekcioglu S. Constitutive intracellular production of iNOS and NO in human melanoma: possible role in regulation of growth and resistance to apoptosis. *Nitric Oxide*. 2008; 19:133–7. [PubMed: 18472017]
5. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002; 420:860–7. [PubMed: 12490959]
6. Johansson CC, Egyhazi S, Masucci G, Harlin H, Mougiakakos D, Poschke I, et al. Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma. *Cancer ImmunolImmunother*. 2009; 58:1085–94.
7. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer*. 2006; 6:24–37. [PubMed: 16397525]
8. Meyskens FL Jr, McNulty SE, Buckmeier JA, Tohidian NB, Spillane TJ, Kahlon RS, et al. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. *Free RadicBiol Med*. 2001; 31:799–808.
9. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, et al. Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. *Clin Cancer Res*. 2000; 6:4768–75. [PubMed: 11156233]
10. Ekmekcioglu S, Ellerhorst JA, Prieto VG, Johnson MM, Broemeling LD, Grimm EA. Tumor iNOS predicts poor survival for stage III melanoma patients. *IntJCancer*. 2006; 119:861–6.
11. Sikora AG, Gelbard A, Davies MA, Sano D, Ekmekcioglu S, Kwon J, et al. Targeted inhibition of inducible nitric oxide synthase inhibits growth of human melanoma in vivo and synergizes with chemotherapy. *Clin Cancer Res*. 2010; 16:1834–44. [PubMed: 20215556]
12. Ridnour LA, Thomas DD, Donzelli S, Espey MG, Roberts DD, Wink DA, et al. The biphasic nature of nitric oxide responses in tumor biology. *Antioxid Redox Signal*. 2006; 8:1329–37. [PubMed: 16910780]
13. Chin MP, Deen WM. Prediction of nitric oxide concentrations in melanomas. *Nitric Oxide*. 2010; 23:319–26. [PubMed: 20854923]
14. Tu YT, Tao J, Liu YQ, Li Y, Huang CZ, Zhang XB, et al. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in human malignant melanoma and their relation to angiogenesis. *Clinical and experimental dermatology*. 2006; 31:413–8. [PubMed: 16681591]

15. Tang CH, Grimm EA. Depletion of endogenous nitric oxide enhances cisplatin-induced apoptosis in a p53-dependent manner in melanoma cell lines. *J BiolChem*. 2004; 279:288–98.
16. Godoy LC, Anderson CT, Chowdhury R, Trudel LJ, Wogan GN. Endogenously produced nitric oxide mitigates sensitivity of melanoma cells to cisplatin. *Proc Natl Acad Sci U S A*. 2012; 109:20373–8. [PubMed: 23185001]
17. Ridnour LA, Cheng RY, Switzer CH, Heinecke JL, Ambs S, Glynn S, et al. Molecular Pathways: Toll-like Receptors in the Tumor Microenvironment--Poor Prognosis or New Therapeutic Opportunity. *Clin Cancer Res*. 2013; 19:1340–6. [PubMed: 23271799]
18. Qin Y, Ekmekcioglu S, Liu P, Duncan LM, Lizée G, Poindexter N, et al. Constitutive aberrant endogenous interleukin-1 facilitates inflammation and growth in human melanoma. *Mol Cancer Res*. 2011;1537–50. [PubMed: 21954434]
19. Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, et al. Interferon regulatory factors: the next generation. *Gene*. 1999; 237:1–14. [PubMed: 10524230]
20. Saura M, Zaragoza C, Bao C, McMillan A, Lowenstein CJ. Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription. *Journal of molecular biology*. 1999; 289:459–71. [PubMed: 10356322]
21. Dell'Albani P, Santangelo R, Torrisi L, Nicoletti VG, de Vellis J, Giuffrida Stella AM. JAK/STAT signaling pathway mediates cytokine-induced iNOS expression in primary astroglial cell cultures. *Journal of neuroscience research*. 2001; 65:417–24. [PubMed: 11536325]
22. Ekmekcioglu S, Ellerhorst JA, Mumm JB, Zheng M, Broemeling L, Prieto VG, et al. Negative association of melanoma differentiation-associated gene (mda-7) and inducible nitric oxide synthase (iNOS) in human melanoma: MDA-7 regulates iNOS expression in melanoma cells. *Mol Cancer Ther*. 2003; 2:9–17. [PubMed: 12533668]
23. Tanese K, Grimm EA, Ekmekcioglu S. The role of melanoma tumor-derived nitric oxide in the tumor inflammatory microenvironment: Its impact on the chemokine expression profile, including suppression of CXCL10. *Int J Cancer*. 2011
24. Qin Y, Deng W, Ekmekcioglu S, Grimm EA. Identification of unique sensitizing targets for anti-inflammatory CDDO-Me in metastatic melanoma by a large-scale synthetic lethal RNAi screening. *Pigment Cell Melanoma Res*. 2013; 26:97–112. [PubMed: 23020131]
25. Messina JL, Fenstermacher DA, Eschrich S, Qu X, Berglund AE, Lloyd MC, et al. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? *Scientific reports*. 2012; 2:765. [PubMed: 23097687]
26. Meyer S, Fuchs TJ, Bosserhoff AK, Hofstadter F, Pauer A, Roth V, et al. A seven-marker signature and clinical outcome in malignant melanoma: a large-scale tissue-microarray study with two independent patient cohorts. *PLoS One*. 2012; 7:e38222. [PubMed: 22685558]
27. Lu T, Gabrilovich DI. Molecular pathways: tumor-infiltrating myeloid cells and reactive oxygen species in regulation of tumor microenvironment. *Clin Cancer Res*. 2012; 18:4877–82. [PubMed: 22718858]
28. Ohmori H, Kanayama N. Immunogenicity of an inflammation-associated product, tyrosine nitrated self-proteins. *Autoimmunity reviews*. 2005; 4:224–9. [PubMed: 15893716]
29. Switzer CH, Cheng RY, Ridnour LA, Glynn SA, Ambs S, Wink DA. Ets-1 is a transcriptional mediator of oncogenic nitric oxide signaling in estrogen receptor-negative breast cancer. *Breast Cancer Res*. 2012; 14:R125. [PubMed: 22971289]
30. Chanvorachote P, Nimmannit U, Stehlik C, Wang L, Jiang BH, Ongpipatanakul B, et al. Nitric oxide regulates cell sensitivity to cisplatin-induced apoptosis through S-nitrosylation and inhibition of Bcl-2 ubiquitination. *Cancer Res*. 2006; 66:6353–60. [PubMed: 16778213]
31. Leon-Bollotte L, Subramaniam S, Cauvard O, Plenchette-Colas S, Paul C, Godard C, et al. S-nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. *Gastroenterology*. 2011; 140:2009–18. 18 e1–4. [PubMed: 21354149]
32. Chanvorachote P, Nimmannit U, Wang L, Stehlik C, Lu B, Azad N, et al. Nitric oxide negatively regulates Fas CD95-induced apoptosis through inhibition of ubiquitin-proteasome-mediated degradation of FLICE inhibitory protein. *J Biol Chem*. 2005; 280:42044–50. [PubMed: 16246840]

33. Torok NJ, Higuchi H, Bronk S, Gores GJ. Nitric oxide inhibits apoptosis downstream of cytochrome C release by nitrosylating caspase 9. *Cancer Res.* 2002; 62:1648–53. [PubMed: 11912135]
34. Mitchell DA, Marletta MA. Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. *Nature chemical biology.* 2005; 1:154–8.
35. Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, et al. Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *J Biol Chem.* 1999; 274:6823–6. [PubMed: 10066732]
36. Guan W, Sha J, Chen X, Xing Y, Yan J, Wang Z. S-Nitrosylation of mitogen activated protein kinase phosphatase-1 suppresses radiation-induced apoptosis. *Cancer Lett.* 2012; 314:137–46. [PubMed: 22014408]
37. Lim KH, Ancrile BB, Kashatus DF, Counter CM. Tumour maintenance is mediated by eNOS. *Nature.* 2008; 452:646–9. [PubMed: 18344980]
38. Murillo-Carretero M, Torroglosa A, Castro C, Villalobo A, Estrada C. S-Nitrosylation of the epidermal growth factor receptor: a regulatory mechanism of receptor tyrosine kinase activity. *Free Radic Biol Med.* 2009; 46:471–9. [PubMed: 19056486]
39. Isaac J, Tarapore P, Zhang X, Lam YW, Ho SM. Site-specific S-nitrosylation of integrin $\alpha 6$ increases the extent of prostate cancer cell migration by enhancing integrin $\beta 1$ association and weakening adherence to laminin-1. *Biochemistry.* 2012; 51:9689–97. [PubMed: 23106339]
40. Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. *J Biol Chem.* 2010; 285:3806–14. [PubMed: 19948721]
41. Azad N, Iyer AK, Wang L, Lu Y, Medan D, Castranova V, et al. Nitric oxide-mediated bcl-2 stabilization potentiates malignant transformation of human lung epithelial cells. *American journal of respiratory cell and molecular biology.* 2010; 42:578–85. [PubMed: 19556603]
42. Azad N, Vallyathan V, Wang L, Tantishaiyakul V, Stehlik C, Leonard SS, et al. S-nitrosylation of Bcl-2 inhibits its ubiquitin-proteasomal degradation. A novel antiapoptotic mechanism that suppresses apoptosis. *JBiolChem.* 2006; 281:34124–34.
43. Yasinska IM, Sumbayev VV. S-nitrosation of Cys-800 of HIF-1 α protein activates its interaction with p300 and stimulates its transcriptional activity. *FEBS letters.* 2003; 549:105–9. [PubMed: 12914934]
44. Leslie NR. The redox regulation of PI 3-kinase-dependent signaling. *Antioxid Redox Signal.* 2006; 8:1765–74. [PubMed: 16987030]
45. Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. *FASEB J.* 2000; 14:1889–900. [PubMed: 11023973]
46. Kroncke KD. Nitrosative stress and transcription. *Biological chemistry.* 2003; 384:1365–77. [PubMed: 14669980]
47. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. *Archives of biochemistry and biophysics.* 2004; 423:12–22. [PubMed: 14989259]
48. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol.* 2001; 3:193–7. [PubMed: 11175752]
49. Wiktorowicz JE, Stafford S, Rea H, Urvil P, Soman K, Kurosky A, et al. Quantification of cysteinyl S-nitrosylation by fluorescence in unbiased proteomic studies. *Biochemistry.* 2011; 50:5601–14. [PubMed: 21615140]
50. Li S, Wang H, Xian M, Whorton AR. Identification of protein nitrosothiols using phosphine-mediated selective reduction. *Nitric Oxide.* 2012; 26:20–6. [PubMed: 22100619]
51. Uehara T, Nishiya T. Screening systems for the identification of S-nitrosylated proteins. *Nitric Oxide.* 2011; 25:108–11. [PubMed: 21111056]
52. Numajiri N, Takasawa K, Nishiya T, Tanaka H, Ohno K, Hayakawa W, et al. On-off system for PI3-kinase-Akt signaling through S-nitrosylation of phosphatase with sequence homology to tensin (PTEN). *Proc Natl Acad Sci U S A.* 2011; 108:10349–54. [PubMed: 21646525]
53. Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science.* 2005; 310:1966–70. [PubMed: 16373578]

54. Khalili JS, Liu S, Rodriguez-Cruz TG, Whittington M, Wardell S, Liu C, et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. *Clin Cancer Res.* 2012; 18:5329–40. [PubMed: 22850568]
55. Ellerhorst JA, Ekmekcioglu S, Johnson MK, Cooke CP, Johnson MM, Grimm EA. Regulation of iNOS by the p44/42 mitogen-activated protein kinase pathway in human melanoma. *Oncogene.* 2006; 25:3956–62. [PubMed: 16474847]
56. Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature.* 2009; 457:102–6. [PubMed: 19122641]
57. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol.* 2006; 176:284–90. [PubMed: 16365420]
58. Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J, et al. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J Immunol.* 2000; 164:762–7. [PubMed: 10623821]
59. Spence S, Fitzsimons A, Boyd CR, Kessler J, Fitzgerald D, Elliott J, et al. Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization. *Immunity.* 2013; 38:66–78. [PubMed: 23177319]
60. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002; 23:549–55. [PubMed: 12401408]
61. Meyer C, Sevko A, Ramacher M, Bazhin AV, Falk CS, Osen W, et al. Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. *Proc Natl Acad Sci U S A.* 2011; 108:17111–6. [PubMed: 21969559]
62. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1⁺/CD11b⁺ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res.* 2005; 11:6713–21. [PubMed: 16166452]
63. Jayaraman P, Parikh F, Lopez-Rivera E, Hailemichael Y, Clark A, Ma G, et al. Tumor-expressed inducible nitric oxide synthase controls induction of functional myeloid-derived suppressor cells through modulation of vascular endothelial growth factor release. *J Immunol.* 2012; 188:5365–76. [PubMed: 22529296]
64. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med.* 2011; 208:1949–62. [PubMed: 21930770]
65. Schaer DA, Lesokhin AM, Wolchok JD. Hiding the road signs that lead to tumor immunity. *J Exp Med.* 2011; 208:1937–40. [PubMed: 21948803]
66. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res.* 2007; 67:4507–13. [PubMed: 17483367]
67. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res.* 2011; 71:7463–70. [PubMed: 22025564]
68. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med.* 2005; 202:931–9. [PubMed: 16186186]
69. Nagaraj S, Youn JI, Weber H, Iclozan C, Lu L, Cotter MJ, et al. Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. *Clin Cancer Res.* 2010; 16:1812–23. [PubMed: 20215551]
70. Burkitt MJ, Duncan J. Effects of trans-resveratrol on copper-dependent hydroxyl-radical formation and DNA damage: evidence for hydroxyl-radical scavenging and a novel, glutathione-sparing mechanism of action. *Archives of biochemistry and biophysics.* 2000; 381:253–63. [PubMed: 11032413]

71. Hsieh TC, Wang Z, Hamby CV, Wu JM. Inhibition of melanoma cell proliferation by resveratrol is correlated with upregulation of quinone reductase 2 and p53. *Biochem Biophys Res Commun*. 2005; 334:223–30. [PubMed: 15993843]
72. Bishayee A. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. *Cancer Prev Res (Phila)*. 2009; 2:409–18. [PubMed: 19401532]
73. Uffort DG, Grimm EA, Ellerhorst JA. NF-kappaB mediates mitogen-activated protein kinase pathway-dependent iNOS expression in human melanoma. *J Invest Dermatol*. 2009; 129:148–54. [PubMed: 18668140]
74. Zheng M, Ekmekcioglu S, Walch ET, Tang CH, Grimm EA. Inhibition of nuclear factor-kappaB and nitric oxide by curcumin induces G2/M cell cycle arrest and apoptosis in human melanoma cells. *Melanoma Res*. 2004; 14:165–71. [PubMed: 15179184]
75. Zheng M, Priebe W, Walch ET, Roth KG, Han M, Tang CH, et al. WP760, a melanoma selective drug. *Cancer Chemother Pharmacol*. 2007; 60:625–33.

Figure.

Simplified examples of molecular pathways known to induce NO production in cancer cells, and examples of proteins directly modified by reactive nitrogens.

Numerous mediators of chronic inflammation are produced by melanoma and microenvironment cells including growth factors (GF), transcription factors, adhesion molecules, signaling molecules and Extracellular Matrix (ECM) proteins. Melanoma tumor cells also are known to produce NO from NOS and each of the four major NOS-inducing pathways are shown in different colors (green, purple, blue, and white). Once produced, NO combines chemically with oxygen radicals to form ONOO⁻ (peroxynitrite) resulting in direct nitrosylation/nitrosation or nitration on many important signaling proteins (in red with *) in the tumor cells. The concentration of NO also influences the anti-tumor immune response, as shown in the box on left, with increased NO associated with immune suppression. Immune system cells are functionally affected including the recruitment of activate myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), Tumor Associated Macrophages (TAM), and Th2 lymphocytes. Once activated, MDSCs also produce high amounts of NO in the tumor microenvironment, which further contributes to the inhibition of antitumor responses and nitrosative stress.

* Posttranslational confirmation of nitration or nitrosylation affecting function of each protein consistent with supporting growth, inhibiting apoptosis and/or driving angiogenesis and growth.