The Role of Reactive Oxygen Species on Cellular Fate and Function of Tumor-Infiltrating Lymphocytes

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Abstract

Reactive oxygen species (ROS) produced as a result of cellular metabolism play key role in signaling for cell fate decisions. Imbalance in cellular redox conditions results in damage of cellular components such as lipids, proteins, and DNA. Chronically increased ROS causes irreversible cellular damage and leads to tumorigenesis. One of the critical effects of dysregulated ROS in tumors is its immunosuppressive effects. This promotes inflammation and facilitates the tumor to modulate and evade host immune responses. ROS-mediated modulation of tumor-infiltrating lymphocytes (TILs), the primary cell-mediated response to tumors facilitates tumor invasion, metastasis, and resistance. There are several contributors to ROS in the tumor microenvironment. The increased ROS level in the tumor microenvironment (TME) causes the recruitment, activation, and promotion of the function of immunosuppressive cells and prevents the recognition of cancer cells by TILs. In addition, the ROS generated inhibits the activation and function of TILs and can cause apoptosis of TILs in the TME. Immune-based therapies, particularly engineered TILs, for the effective eradication of tumors is an emerging area of cancer therapeutics. Given the tight control of redox state that is required for normal immune cell function, combining the targeting of ROS signaling in the TME along with other immune-based therapies may yield potential personalized therapeutics against cancer. This chapter explores the role of ROS in the TME on immune suppression and how ROS drive the inhibition of TILs and contribute to cancer immunomodulation, which results in metastasis.

Keywords

Reactive oxygen species · Oxidative stress · Immune system · Tumor-infiltrating lymphocytes · Redox

Abbreviations

AICD	Activation-induced T cell death
DAMPs	Damage-associated molecular patterns
ERR	Estrogen-related receptor α FOXP3: forkhead box protein P3
GPx	Glutathione peroxidase
HIF	Hypoxia-inducible factor
Il-2	Interleukin-2
Il-4	Interleukin-4
JNK	C-Jun N-terminal kinase
MDSC	Myeloid-derived suppressor cells
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOX1	Nicotinamide adenine dinucleotide phosphate oxidase-1

NOX2	Nicotinamide adenine dinucleotide phosphate oxidase-2
PAMP	Pathogen-associated molecular patterns
PGC-1β	PARγ -coactivator-1βROS: reactive oxygen species
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
TAMs	Tumor-associated macrophages
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor
Tregs	Regulatory T lymphocytes

Introduction

Reactive oxygen species (ROS) are defined as reactive radicals as well as nonradical derivatives of oxygen. The different types of ROS have varied reactivity and function. In physiology, they are by-products of metabolism. It is increasingly apparent that ROS function as signaling intermediates to regulate physiological processes, particularly in immune function against diseases. In contrast, elevated levels of ROS have been shown to reflect a state of oxidative stress affecting lipids, proteins, and DNA, and are associated with many pathologies (Alfadda and Sallam 2012). Physiological mechanisms and checkpoints have evolved to regulate ROS levels in all these processes. Dysregulation in these checkpoints results in alterations to signaling pathways and induction of carcinogenesis. Elevated ROS levels are reported across several cancer types. ROS-dependent oxidative stress resulting in DNA damage has been shown to promote tumor initiation and progression (Aggarwal et al. 2019). The altered metabolic activity of the cancer cells, the hypoxic core, as well as the several components of the tumor microenvironment (TME) contribute to the increased ROS levels within a tumor. The ROS in the TME have been shown to activate several types of cells such as fibroblasts and endothelial cells to facilitate tumor growth and invasion (Weinberg et al. 2019). In contrast, several studies have shown that ROS can also induce oxidative stress–dependent cancer cell death responses (Reczek and Chandel 2016). To overcome this double-edged ROS response, tumor cells show a concomitant increase in antioxidants signaling (Peiris-Pagès et al. 2015).

The immune system has several functions such as defense against foreign organisms, homeostasis, the destruction of damaged cells, and surveillance. Cancer cells evolve different mechanism[s] to mimic the peripheral immune tolerance. This results in one of the key hallmarks of tumor progression – immune evasion – the inability of the immune system to recognize and respond to tumor antigens in the body. Tumor-infiltrating cytotoxic T cells are the key players in the host antitumor immune response. However, as tumor growth progresses, the TME becomes immunosuppressive and the homing and/or function of these cytotoxic T cells are

inhibited. The signaling from the tumor cells can result in formation of tumor-associated macrophages which are key contributors the immunosuppressive TME. This is supported further by increased recruitment of regulatory T cells and myeloid-derived suppressor cells (MDSCs) to the TME. These cells, along with cancer-associated fibroblasts (CAFs), augment the immune evasion of the tumor. They modulate certain immune checkpoint pathways, resulting in evasion of host immune surveillance and attack. The relative balance between pro- and antitumor immune cells will determine the fate of tumor including dormancy, progression, or regression (Spranger and Gajewski 2018; Lei et al. 2020).

ROS-regulated signaling is important for cancer progression as well as tumor-mediated immune evasion. The high ROS production within the TME contributes immensely to immunosuppression and immunomodulation within the tumor. In this chapter, we discuss the role of ROS signaling on the function of tumor-infiltrating lymphocytes (TILs). In addition, we also explore the data available on the effect of disrupting ROS levels on antitumor immunomodulation.

Role of ROS as Signaling Molecules in Cancer

ROS have been shown to act as "second messengers" (Costa et al. 2014). ROS accumulate abnormally in tumor epithelial cells, and stimulate a series of signaling events that mediate the oncogenic phenotype. In addition to affecting tumor epithelial cells, ROS also impact other cellular components of the TME. In fact, ROS production results in increased tumor angiogenesis and initiates inflammation. ROS are also known to induce the conversion of fibroblasts into myofibroblasts, which have been shown to be associated with aggressive adenocarcinomas. Chronic oxidative stress further supports tumor growth and metastasis via these modified myofibroblasts. Most interestingly, ROS also help cancer cells to adapt to oxidative stress, which as can be expected as this helps tumorigenesis. Thus, there is strong evidence to support important roles for ROS in the initiation and progression of tumors, as well as their metastasis (Costa et al. 2014).

ROS appear to promote the initiation of cancers and malignant transformation of cells by causing DNA damage. Immunosuppression in the TME supports tumor invasion and metastasis. The production of ROS has been shown to promote the function of immunosuppressive cells, which in turn result in increased tumor invasion (Kotsafti et al. 2020). Oxidative stress causes the production of the cytokine tumor necrosis factor (TNF)-α, which in turn promotes tumor invasion. In malignant melanoma, oxidative stress boosted the secretion of TNF-α from TAMs (Lin et al. 2013). Thus, ROS aids in immune editing to promote tumor progression and metastasis. It should be remembered that at low levels ROS play useful roles in antitumor immunity, but are antagonistic to immune cell function at higher levels, thus facilitating tumor survival and progression.

The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is extensively documented as being of critical importance in the inflammatory reactions and immune defense reactions. Additionally, dysregulation

of NF-κB is known to be associated with cancer progression (Taniguchi and Karin 2018). There is accumulating evidence to point to a relationship between ROS and NF-κB (Morgan and Liu 2011). ROS block the activation of the NF-κB pathway mainly by inhibiting phosphorylation. The classical activation pathway for NF-κB is triggered in response to microbial products, stress, and inflammatory cytokines; the phosphorylation of IκB-kinase (IKK) β and IκBa is an important step in this pathway. H_2O_2 has been shown to block the phosphorylation of IκBα and thus the activation of the NF-κB pathway. It is of interest to note that IKK is also the primary target for ROS in influencing NF-κB. MEKK1, a redox-sensitive kinase upstream of IKK, also seems to be inactivated by ROS. ROS also disrupt the ubiquitination and degradation of IκB and thus the activation of NF-κB.

ROS and the Immune System

Redox states play key functions in host immune responses. ROS are essential across different steps in both innate and adaptive immune surveillance and response. Innate immunity distinguishes self from nonself through pattern recognition receptors (PRRs) as well as germline-encoded receptors. Thus, innate immunity serves as the front line to monitor and detect signs of infection or tissue injury. This can further initiate the adaptive cell–based immune responses.

ROS and Innate Immunity

The cellular components of innate immunity include phagocytes (neutrophils, monocyte, and macrophages) and cells that release cytokines and mediators of inflammation (macrophages, mast cells, and natural killer cells). ROS derived from nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase and mitochondria are the primary weapon of destruction used by neutrophils and macrophages against pathogens. The ROS signaling is also used to organize other antipathogenic strategies. Several studies have shown that ROS are involved in sensing the presence of pathogens, as well as tissue damage by the immune system (West et al. 2011).

When a phagocyte encounters a pathogen, cell eating or phagocytosis is initiated. During phagocytosis, ROS production occurs on the membranes of the phagocytic cells. This synthesis is triggered by the activation of NADPH oxidase, a physiological response to the trigger of several events including apoptosis and cell proliferation. The cytosolic and membrane components of NADPH oxidase are otherwise separated in the inactive state. Blocking of NADPH oxidase and ROS production causes limited pathogen clearance. ROS produced by the NADPH oxidase (NOX2) complex expressed mainly in phagocytes. The ROS generated in phagocytes are used to kill pathogens through the direct oxidation of biomolecules as well as through activation of host proteases. Similar to the phagocytes, in neutrophils recognition of pathogens results in generation of excess

amount of extracellular ROS by the plasma membrane NOX. This results in an NF-κB-dependent increase in interleukin1β expression and neutrophil recruitment. Mitochondria in macrophages contribute to ROS production in response to *E. coli* or Toll-like receptor (TLR)1, 4, and 6 agonists. Reduction in mitochondrial ROS (mtROS) resulted in defective bacterial killing by macrophages. In addition, NOX2-dependent mtROS have been shown to be critical for formation of neutrophil extracellular traps (NETs), a specialized microbial defense mechanism. ROS also induce dendritic cell differentiation and their antigen representing functions. ROS function as regulators of the production of macrophage cytokines via mechanisms involving NF-κB. This is crucial for the induction of neutrophil and macrophage flux in inflammation (Mittal et al. 2014).

Inflammation is a complex and coordinated series of events that involves several types of immune cells, primarily neutrophils and macrophages. The immune surveillance carried out by these cells screens for injury or infiltration of pathogens by recognizing both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Inflammasomes are key cellular organelles, existing as cytoplasmic multiprotein complexes in order to activate caspase-1 and secrete pro-inflammatory cytokines. The Nod-like receptor (NLR) family inflammasome has been extensively studied due to its nonspecific response to stimuli. NLRP3 is activated in response to microbial stimuli, pore-forming toxins, extracellular ATP, uric acid, and environmental particulate irritants. NLRP3 activation has been proven to induce ROS production as an upstream event; however, the mechanism for this still remains elusive. There is evidence that both mtROS and ROS generated by the NOX2 complex can activate NLRP3 (Harijith et al. 2014). Increased mitochondria-derived ROS have been observed to cause caspase-1 activation and IL-1β secretion, which can be blocked by mitochondria-targeted antioxidant. Mitophagy of dysfunctional mitochondria causes its accumulation and thereby triggers the NLRP3 activation. Chronic inflammatory diseases have been proven to be linked to ROS, due to NLRP3 activation triggering cytokine production. Further, activation of TLR1, 2, and 4 causes increased mtROS production and subsequent antimicrobial pro-inflammatory reactions. This increase in mtROS is mediated by tumor necrosis factor receptor-associated factor 6 (TRAF6), a TLR signaling adaptor. In macrophages, the mtROS is produced by IFN-γ -estrogen-related receptor α (ERRα) and PARγ -coactivator-1β (PGC-1β) signaling. In certain cases, ROS accumulation has been observed at the site of inflammation, which worsens the condition further (Mittal et al. 2014). Tight regulation of ROS is crucial to ensure inflammation subsides after removal of the foreign object. Natural killer T (NKT) cells also play critical roles in killing infected and altered cells. ROS regulates the function of NKT cells (Kim et al. 2017).

ROS in Adaptive Immunity

The adaptive immune response occurs after exposure to an antigen either from a pathogen or any abnormal cells. The T cells and the B cells are the major components

of adaptive immunity. Similar to its function in innate immunity, ROS signaling is also instrumental for the activation of B and T cells. It is well recognized that ROS levels increase after T cell activation by T cell receptor (TCR) signaling. ROS signaling controls T cell proliferation and clonal expansion in response to antigens (Franchina et al. 2018). NOX2-derived ROS was responsible for CD3/CD28 stimulation-mediated CD8+ T cell activation. TCR activation resulted in mtROS-dependent expression of cytokines IL2 and IL4. Further, ROS regulate the differentiation and effector functions of various T cell subsets. Higher microenvironmental levels of ROS result in T helper (Th)2-skewed immune phenotype. In contrast, low levels of ROS promote differentiation of CD4+ T cells into Th1 and Th17 cells. NOX-derived ROS were also important for Treg differentiation and function. Mutations in components of the NOX complex resulted in downregulated Treg induction and T cell suppression in mice. The levels of ROS as well as spatial localization of ROS within the cells is strictly regulated for an effective T cell–mediated immunity. Although ROS are essential for T cell activation, prolonged ROS signaling can inhibit NF-κB phosphorylation, resulting in T cell hyporesponsiveness. Additionally, ROS generated during T cell activation have to be compartmentalized to prevent deregulation of the mitochondrial pore permeability during TCR stimulation. ROS are also involved in maintenance of T cell balance under homeostatic and disease conditions by modulating T cell apoptosis through both intrinsic and extrinsic mechanisms (Hildeman 2004).

In primary resting murine B cells, BCR stimulation induced rapid ROS production. While NOX2-dependent ROS are essential in the early stage of B cell activation, the mtROS are required at a later stage of B cell activation (Wheeler and DeFranco 2012). H_2O_2 also regulates B cell activation and subsequent signaling. Subsequently, these activated B cells may generate H_2O_2 kill cells recognized by specific antibodies. Collectively, ROS signaling is a key intermediate and regulator of the adaptive immune response.

ROS Production in the Tumor Microenvironment

In the tumor microenvironment, ROS signaling is known to suppress immune responses. The TME includes tumor cells, MDSCs, TAMs, and regulatory Tregs. Several of these cellular components of TME contribute to regulate the ROS levels in TME.

MDSCs

MDSCs have been shown to produce ROS, and therefore play a vital role in the suppression of TME (Umansky et al. 2016). ROS reduce T cell immune responses by blocking the interaction between the T cell receptor (TCR) and MHC peptide complex. In in vivo models, ROS and peroxynitrite generated by MDSCs modify TCR and CD8 molecules (Nagaraj et al. 2007). This causes $CD8^+$ T cells to lose

their ability to bind specific peptide–major histocompatibility complex (pMHC) dimers by nitration of tyrosine residues in a (TCR)-CD8 complex, and thus displaying a mechanism of prompting T cell tolerance in cancer. In silico analysis has suggested that on nitration, TCR domains become more rigid and less flexible and thereby influence the specific binding to epitopes between TCR and pMHC. Furthermore, due to the highly reactive and short-living characteristics of ROS and peroxynitrite, they remain active only in a localized, narrow vicinity in the environment within which MDSCs and CD8+ T cells interact during antigen-TCR recognition. The peroxynitrite molecules from MDSCs are sufficient to nitrate the tyrosine present in these cells that are in contact in the TME (Nagaraj et al. 2007).

Another notable feature of MDSCs is that they suppress T cell stimulation by depleting cystine and cysteine, which are critical for the synthesis of glutathione, which in turn eliminates ROS production. Cysteine is required by cells for protein synthesis and proliferation, and can be produced via the plasma membrane cystine transporter x_c^-, which permits the uptake of disulfide-bonded cystine from the oxidizing extracellular environment, and when in the reducing intracellular environment, the cystine gets reduced to cysteine (Arnér and Holmgren 2000). T cells require cysteine for antigen presentation and T cell activation, but they lack both cystathionase and x_c^- transporter. Therefore, they depend on other cells (such as macrophages and/or dendritic cells) as their source of cysteine, which they take up via the plasma membrane. ASC neutral amino acid transporter MDSCs express cystine transporter x_c-, meaning the import of cysteine works, but they do not express the ASC neutral amino acid transporter, so they cannot export cysteine (Srivastava et al. 2010). MDSCs were also seen to not express cystathionase, thus relying on their uptake of cysteine from their environment. Due to this, MDSCs limit the amount of cysteine in their extracellular environment as they take up cystine but do not release cysteine. Hence, due to the presence of MDSCs in the microenvironment, macrophages and dendritic cells do not aid the cysteine uptake by T cells for their proliferation, and therefore, T cells are not activated. In other words, this is a cause for the suppression of the antitumor immune mechanism (Srivastava et al. 2010). Further, the scavenging of H_2O_2 by catalase, a ROS inhibitor, resulted in differentiation of immature myeloid cells into macrophages (Gabrilovich and Nagaraj 2009).

MDSCs exist as two major subgroups in cancer patients, namely polymorphonuclear MDSCs (PMN-MDSC) and monocytic MDSCs (M-MDSCs) (Dolcetti et al. 2010). PMN-MDSCs, which are pathologically activated immature neutrophils, differentiate into neutrophils (PMN). PMN-MDSCs are seen to have elevated levels of ROS and myeloperoxidase but lower amounts of lysosomal enzymes than PMN. On the other hand, M-MDSCs are pathologically activated macrophages and differentiate into TAMs. It should be emphasized that ROS and reactive nitrogen species are produced by all myeloid cells and are the major effectors of myeloid cell–induced immune suppression (Lu and Gabrilovich 2012).

Tumor-Associated Macrophages

Tumor-associated macrophages are another group of tumor-infiltrating leukocytes that are largely present in the microenvironment of solid tumors. Mature monocytes in the blood and tissue macrophages are recruited by signals and cytokines from the tumor. These are the first host cells to enter the TME in order to kill the cancer cells (Munn 2017). Their role is to produce cytokines and chemokines to recruit other immune cells, and to produce factors such as growth factors and angiogenic factors for tissue repair. TAMs have been shown to have impacts on tumor initiation, promotion, angiogenesis, and metastasis. TAMs are believed to be critical links between inflammation and cancer development [as reviewed by (Chen et al. 2016)]. They play a major role as an inflammatory component in tumors, and are known to promote tumor progression and metastases. TAMs are recruited to solid tumor sites by chemokines and CSF-1. TAMs exert immunosuppressive effects by the production of contact-dependent H_2O_2. The level of ROS production in TAMs was higher than in MDSCs (Hamilton et al. 2014). ROS signaling promotes TAM immunosuppressive function by upregulating the expression of immune checkpoint ligand, PD-L1 (Roux et al. 2019).

ROS generated by TAMs play a vital role in inducing apoptosis in lymphocytes and may also stimulate Tregs (Kraaij et al. 2010). Tregs are known to regulate the immune system, maintain tolerance to self-antigens, and bring about suppression of effector T cells and inhibit T cell proliferation. Furthermore, they produce immunosuppressive cytokines, specifically TGF-β, prostaglandin E2, and adenosine (Mandapathil et al. 2010).

Tumor Cells

Interestingly, tumor cells are also a source of ROS in the TME (Weinberg et al. 2019). This is possibly due to mutations that would alter genes involved in the electron transport chain and mitochondrial DNA, specifically a deficiency in respiratory complex I activity. Such mutations in mitochondrial enzymes can boost ROS production from several sites on the mitochondria. For example, a loss of p53 causes altered homeostasis of mitochondrial ROS wherein p53-depleted cells showed a decrease in mitochondrial and cellular superoxide, and an increase in cellular hydrogen peroxide (Lebedeva et al. 2009). Mitochondrial ROS can furthermore contribute to the promotion of neoplastic transformation by initiating nuclear or mitochondrial DNA mutation (Sabharwal and Schumacker 2014).

ROS in cancer cells have two seemingly opposing effects in the progression of cancer (Chen et al. 2016). Depending on the concentration of ROS, it appears that carcinogenesis and cancer progression can either increase or can be inhibited. At mild to moderately increased levels of ROS, cancer progression is facilitated;

however, excessive ROS can damage cancer cells and may even kill them. This contributes to the complexity of the roles of ROS in the activity of TILs and indicates that the potential use of ROS in cancer therapy needs to be carefully strategized, such as targeted disruption of mitochondria-to-cell redox communication represents (Sabharwal and Schumacker 2014).

ROS and Tumor-Infiltrating Lymphocytes

Host immune cells can both antagonize and stimulate cancer growth. Tumors express tumor antigens that distinguish the tumor cells from healthy cells, and thus provide an immunological stimulus. In the early stages of cancer, the host immune system recruits TILs to recognize, infiltrate, and attack the tumor. Tumor-infiltrating cytotoxic T cells (i.e., $CD8^+$ T cells) play an essential role in host antitumor immune response to cancer (Weinberg et al. 2019). TILs are components of the adaptive immune response. They comprise cytotoxic lymphocytes, natural killer cells, and T helper 1 lymphocytes, which are pivotal for tumor cell recognition and elimination. As the tumor progresses, immune selection will produce tumor cell variants that produce decreasing amounts of tumor antigens. The tumor also expresses factors that mimic peripheral tolerance, facilitating the escape from immune attack and modulating the function of TILs.

The two major subsets of CD4+ T helper (Th) cells are Th1 and Th2 cells. Th1 cells have a crucial role in activating cytotoxic T lymphocytes, and CD8+ cytotoxic T lymphocytes are directly capable of killing tumor cells. Th2 lymphocytes stimulate humoral immunity, and are less effective than Th1 activation in terms of antitumor immunity. Besides the Th1 and Th2 subsets, CD4+ regulatory T lymphocytes (Treg) have immunosuppresive effects of effector T lymphocytes (Curiel et al. 2004). In cancer, Tregs preferentially migrate to tumors, attracted by chemokines produced by tumor cells and microenvironmental macrophages (Curiel et al. 2004).

TILs have generated great interest both in terms of basic understanding of their roles in antitumor immunity and also in terms of clinical application by way of exploring their therapeutic potential. TILs and adoptive T cell therapy have shown promise in several types of cancer.

Despite this potential anti-tumor property of TILs, their cytotoxicity is inhibited by the immunosuppressive nature of the TME during cancer progression. One of the many mechanisms by which tumor progression occurs is by ROS-mediated suppression of immune cells (Weinberg et al. 2019). ROS produced by tumor cells have been shown to inhibit the proliferation and anti-tumor functions of T cells. Additionally, ROS produced by other cells within the TME have been reported to cause T cell hyporesponsiveness in cancer patients (Cemerski et al. 2002). Such an immunosuppressive microenvironment encourages tumor invasion, metastasis, and resistance to treatments. The schematic on ROS-mediated immunomodulation in the TME is depicted in Fig. 1.

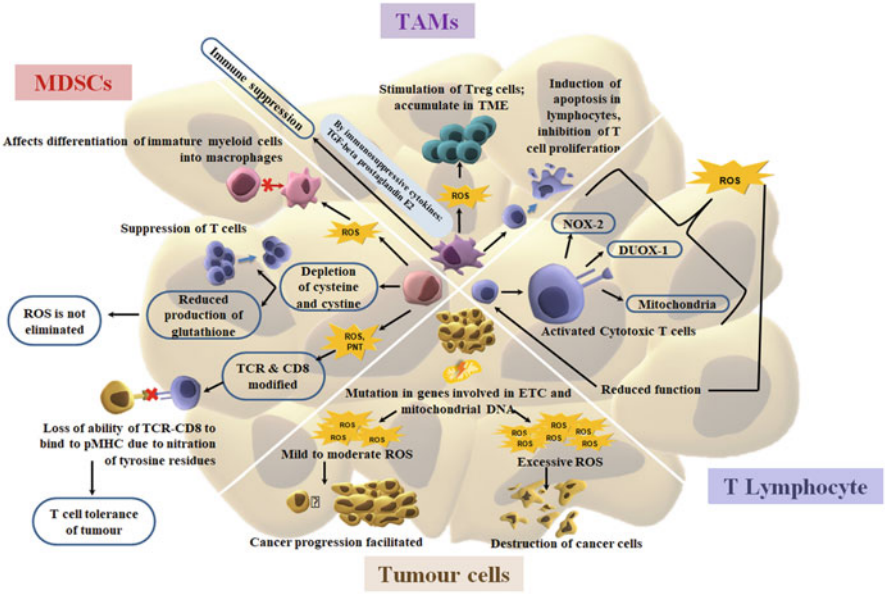

Fig. 1 ROS and immune cells in TME

Mechanisms of Action of ROS on Tumor-Infiltrating T Lymphocytes

ROS produced in the tumor microenvironment discussed previously are still critical for, and govern, activities of antitumor T cells, such as their activation, proliferation, differentiation, and apoptosis. Thus, at low concentrations, ROS are actually essential for T cell activation, expansion, and effector function (Jackson et al. 2004). However, an excessive level of ROS in the TME causes a decrease in T cells' antitumor function and proliferation, and an increase in apoptosis (Cemerski et al. 2002). Moreover, a downregulation of T cell activity on exposure to high levels of ROS is also observed. ROS signaling also affects the antigen presentation ability of dendritic cells in the TME.

In response to signals from the TME, T cells become activated and differentiate into cytotoxic T lymphocytes. These cells destroy the tumor cells by recognition of specific tumor peptide–MHC complexes expressed on the tumor cells. Studies have reported enhanced production of ROS by peripheral blood T lymphocytes from patients with cancer as compared to those from healthy individuals (Toyokuni et al. 1995). ROS that have accumulated in the TME may cause TILs to have reduced function.

Various immune processes can induce the production of ROS. T cell activation provokes the production of ROS via NOX-2 (where T cells from mice lacking NOX2 showed a decrease in ROS production on TCR stimulation), via dual-substrate

oxidase 1 (DUOX-1), and via the mitochondria (Kwon et al. 2010). Tregs are critical immunosuppressive cells in cancer patients. Macrophage-derived ROS have been shown to induce the accumulation of Tregs in the TME. In addition to T cells and macrophages, other immune cells such as eosinophils, neutrophils, and monocytes may secrete ROS in the TME; this could lead to increased growth of the tumor and enhanced antitumor immune reactivity.

T Cell Hyporesponsiveness

T lymphocytes were rendered hyporesponsive to activation stimulus when exposed to an oxidative environment produced by synovial fluid-derived (activated) neutrophils, and were made hyporesponsive (Cemerski et al. 2002). Furthermore, when catalase, a ROS inhibitor, was added to the coculture, T cell proliferation was restored, which indicates that hydrogen peroxide influences T cell hyporesponsiveness. The same effect was seen again in a coculture of buffy coat-derived T cells and fMLP (N-formylmethionylleucylphenylalanine)-activated neutrophils. Here, the induced T cell hyporesponsiveness directly correlated with the amount of O^2 produced, and on treatment with antioxidants, proliferation was recovered. There was a decrease in the mobilization of $[Ca^{2+}]_i$ in hyporesponsive T cells due to exposure to ROS.

Altered T Cell Activation

Excessive ROS in the TME results in reduction of function and proliferation of T cells. ROS signaling causes oxidative stress and induces T cell hyporesponsivenes in cancer patients. MnSOD is a mitochondrial antioxidative enzyme that neutralizes $O_2^{\bullet-}$ generated from the electron transport chain. Inactivation of the extracellular SOD causes an accumulation of ROS in the TME. Consistently, silencing MnSOD resulted in increased intracellular oxidative stress in TME. In contrast, by increasing MnSOD, enhanced anti-tumor effect of T cells was observed (Kamiński et al. 2012). This enzyme plays a major role as a control switch in activation-induced oxidative signal generation in T cells. Evaluation of TCR-triggered ROS production showed an increase in activity of MnSOD and a shutdown phase of oxidative signal generation (Kamiński et al. 2012). Loss of regulation of MnSOD resulted in aberrant T cell development and function. High levels of ROS also inhibited the mTOR pathway, which is crucial in T cell activation and metabolism. Further, ROS inhibit the function of cytotoxic T lymphocytes by regulating the phosphorylation of proline-rich tyrosine kinase 2 (Pyk2) by Erk signaling.

Antigen-specific T cell activation is also governed by ROS signaling, and it has been shown that CD3 activation stimulates an influx of calcium, which in turn regulates ROS production as calcium is an essential component for generation of ROS (Sena et al. 2013). mtROS oxidation results in higher release of high mobility group box1 (HMGB1), a key player in regulating T cell immune responses. Loss of

mitochondrial function was observed in TILs consistent. Restoring this loss of mitochondrial function in TILs by expression of a key player in mitochondrial biogenesis (PGC1α) restores the TILs' antitumor activity.

T Cell Death

T cell activation–induced cell death is highly influenced by mitochondrial ROS, as well as from other sources by the induction of expression of FasL, an important mediator in activation-induced cell death (Kamiński et al. 2012). This expression of FasL leads to the activation of NOX-2, an essential constituent of the apoptotic program via ROS-mediated AKT activation and MEK inhibition (Li-Weber et al. 2002). Programmed death-1 (PD-1) is a checkpoint that controls T cell function and proliferation. PD-1 expression is increased in T lymphocytes in cancer patients compared to normal individuals. The expression of PD-1 is correlated with production of cellular ROS and oxidative metabolism in T cells.

Human T cell subsets vary in their extent of susceptibility to H_2O_2-induced apoptosis, i.e., T cells' resistance to exogenous H_2O_2 decreases from effector T cells > regulatory T cells > naive T cells > memory T cells. CD8+ effector memory T cells are the most sensitive to ROS among T cell subsets (Mougiakakos et al. 2009). ROS also manipulate factors that participate in T cell proliferation and survival. For instance, the nuclear factor of activated T cell 5 (NFAT5), which plays a role in T cell proliferation and survival, is inhibited in its binding to IL-6 promoter by ROS (Trama et al. 2000).

Therapeutic Strategies that Affect ROS and Influence Anti-tumor Immunity

ROS signaling is critical for cancer initiation, progression, and metastasis. Some of the drugs that regulate ROS levels in the TME exhibit antitumor immune modulation (Table 1). Bortezomib, an inhibitor of the ubiquitin-proteasome proteolytic pathway, enhanced cytotoxic T lymphocyte responses against immune-resistant melanoma (Shanker et al. 2015). Doxorubicin induces ROS generation and shows selective MDSC cytotoxicity (Alizadeh et al. 2014). Treatment with this drug results in increased effector T cells and NK cells. 5-flurouracil and cisplatin have been individually shown in murine models of breast and colorectal cancers to induce ROS production and subsequent MDSC apoptosis. This treatment also generates enhanced CD8+ T cell and NKT cell responses. Photodynamic therapy (PDT) or photochemotherapy results in death of tumor cells by generating excess ROS. Through a localized oxidative stress–induced strong inflammatory reaction, PDT has been shown to modulate the host immune response to the tumor (Castano et al. 2006).

In contrast, drugs that cause a reduction of ROS have resulted in increased antitumor immunity. Celecoxib, a COX-2 inhibitor used for the treatment of

Table 1 Immune cell responses to ROS modulators in cancer treatment

SI. No	Drug	Immune cell type	Model systems	Phenotype	Cancer treated	References
1.	Metformin	Tumor-infiltrating CD4+ CD25+ regulatory T cells (Ti-Treg)	BALB/c and C57BL/6 (B6) mice	Differentiation of naïve CD4+ T cells into inducible Tregs (iTregs) is inhibited by reducing forkhead box P3 (Foxp3) protein expression	Pancreatic and gastric cancers	Kunisada et al. (2017)
2.	Doxorubicin	Tumor-induced MDSC	Six- to eight-week-old Balb/c and C57BL/6 mice. Six- to eight-week-old gp91*phox*−/− (C57Bl6-Cybbtm1Din)	Selective cytotoxic effects on MDSC and increase in effector lymphocytes and NK cells	Hematological malignancies, soft tissue sarcomas, and breast cancer	Alizadeh et al. (2014)
4.	5-fluorouracil (5FU)	MDSC	Nude mice and TLR4−/− C57BL/6 mice	Selectively induces MDSC apoptotic cell death and enhances antitumor CD8+ T cells, NK cells, and B lymphocyte cell functions	Colorectal and breast cancers	Vincent et al. (2010)
5.	Cisplatin	Tregs and MDSC	Female C57BL/6 mice (5–8 week old)	Cisplatin and CRT/E7 DNA vaccine generated E7-specific CD8+ T cell immune responses, which reduced MDSC and Tregs	Breast, ovarian, lung, and gastric cancers	Reviewed in De Biasi et al. (2014)
6.	Bortezomib	CD4+	Peripheral blood samples from 53 patients with multiple myeloma treated with bortezomib	The selective and reversible proteasome inhibitor targets the catalytic 20S core of the proteasome and induces apoptosis in myeloma and lymphoma cells	Head and neck cancers and multiple myelomas	Shanker et al. (2015)
7.	Celecoxib	MDSC	BALB/c mice	Impaired function of all MDSC subtypes due to the reduction in ROS and NO levels	Prostate cancer	Veltman et al. (2010)

colorectal cancer, resulted in impaired MDSC function with reduced ROS levels (Veltman et al. 2010). Similarly, metformin inhibits ROS production and prevents formation of inducible Tregs (Kunisada et al. 2017). The addition of ROS scavengers resulted in enhanced activation of CD8+ TILs in kidney tumors by activation of SOD2 (Siska et al. 2017). Collectively, these studies suggest that modulating the redox homeostasis in TME can induce antitumor immunity.

Tumor immunotherapy including T cell adoptive immunotherapy (ACT) and checkpoint inhibitors functions by augmenting or reinforcing the host's immune system. Recent studies have demonstrated that combining immunotherapy with ROS modulation increases antitumor activity. Combining the use of chemo-photodynamic therapy with ROS-sensitive nanoparticle augmented the antitumor activity of the anti-PD-L1 (checkpoint) antibody (Hu et al. 2019). Inhibition of NOX4 resulted in an increased recruitment of CD8þ cells and susceptibility to different immune-based therapies in mouse models (Ford et al. 2020). Treatment of tumor-specific CD4-positive T cells with Cytoxan (ROS inducer) results production of inflammatory cytokines (such as TNF-α, interferon γ). Adoptive transfer of these cells promoted the decay of vessel-intensive tumor. In addition, the Cytoxan-treated CD4+ T cells altered the tumor metabolism resulting in deficiency of antioxidant GSH and enhanced antitumor activity. Cytotoxic T lymphocytes engineered with T cell receptors that coexpressed catalase showed protection from oxidative stress. Subsequently, they also exhibited increased antitumor activity (Ligtenberg et al. 2016).

A summary of the effect of modulating ROS in TME on immune cell function is summarized in Fig. 2.

Conclusions

Redox homeostasis plays an essential role in maintaining diverse cellular processes. The immune response to the cancer is tightly regulated by the redox state in the TME. Increased ROS levels are both NOX-2 dependent and mtROS, and result in an immunosuppressive environment in the TME. In addition to the tumor cells, several immune cells including MDSCs, Tregs, and TAMs contribute to this immunosuppression. Elevated ROS in the TME helps in recruiting, activating, and promoting the function of these suppressive cells. This results in attenuation of the cell-mediated immune response to the tumor. In particular, the activation and function of effector T cells are compromised.

Given its dichotomous nature, the maintenance of ROS levels is essential for potent cell–mediated immunity. Within the TME, the ROS levels are maintained to promote immunosuppression. Several lines of evidence have shown that disruption of this steady-state levels within the TME result in mounting successful antitumor host immune response. Emerging research suggests that combining ROS modulation of TIL during adoptive immunotherapy would augment their antitumor activity. Thus, further studies should focus on mapping the relationship of ROS to their

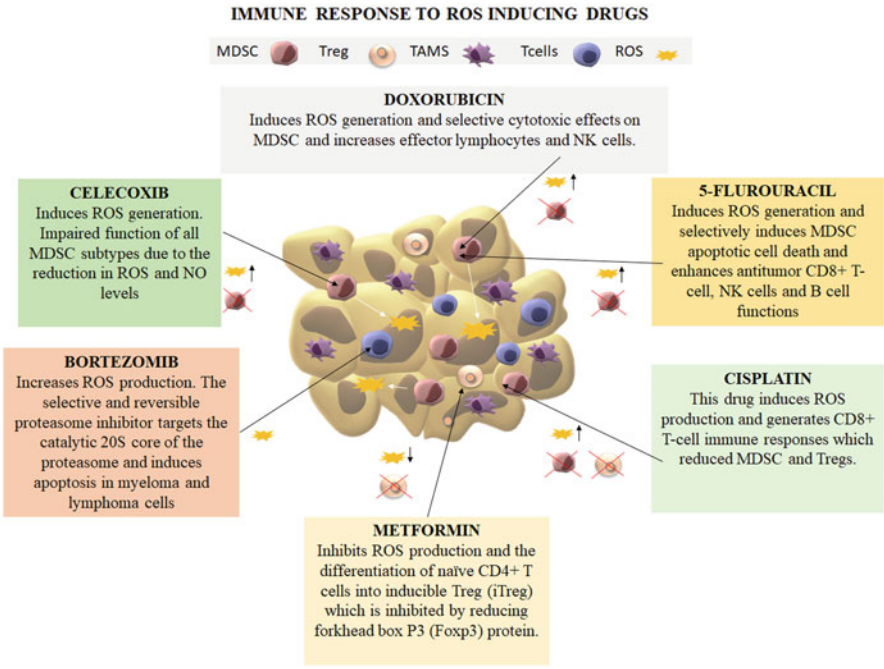

Fig. 2 Immune response to ROS modulating drugs

immunosuppressive effects in T cells across cancer types. This approach would combine the targeting of ROS with immune-based treatment of cancer patients to provide a more promising clinical outcome.

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