Survival of the fittest: How myeloid-derived suppressor cells survive in the inhospitable tumor microenvironment

By

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Précis: Myeloid-derived suppressor cells are ubiquitously present within solid tumors where they inhibit antitumor immunity. They survive in this hostile environment by activating the transcription factor Nrf2 and by entering an autophagic state.

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List of Abbreviations:

ARE	anti-oxidant response elements
CBI	checkpoint blockade inhibitors
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DAMP	damage associated molecular pattern
HMGB1	high mobility group box protein 1
Keap1	kelch-like ECH-associated protein
MDSC	myeloid-derived suppressor cells
M-MDSC	monocytic MDSC
mTOR	mammalian target of rapamycin
Nrf2	nuclear factor erythroid-2-related factor 2
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PI	propidium iodide
PMN-MDSC	polymorphonuclear or granulocytic MDSC

ROS	reactive oxygen species
TIMDSC	tumor-infiltrating MDSC
tBHQ	tert-butylhydroquinone
TME	tumor microenvironment

Abstract

Myeloid-derived suppressor cells (MDSC) are present in most cancer patients where they are significant contributors to the immune suppressive tumor microenvironment (TME). The TME is a hostile locale due to deficiencies in oxygen (hypoxia) and nutrients, and the presence of reactive oxygen species (ROS). The survival of tumor cells within the TME is partially governed by two mechanisms: (i) Activation of the transcription factor Nuclear Factor Erythroid-derived 2-like 2 (Nrf2) which turns on genes that attenuate oxidative stress; and (ii) The presence of High Mobility Group Box Protein-1 (HMGB1), a damage-associated molecular pattern molecule (DAMP) that induces autophagy and protects against apoptosis. Because Nrf2 and HMGB1 promote tumor cell survival, we speculated that Nrf2 and HMGB1 may facilitate MDSC survival. We tested this hypothesis using $Nrf2^{+/+}$ and $Nrf2^{-/-}$ BALB/c and C57BL/6 mice and pharmacological inhibitors of HMGB1. In vitro and in vivo studies demonstrated that Nrf2 increased the suppressive potency and quantity of tumor-infiltrating MDSC by up-regulating MDSC production of H_2O_2 and decreasing MDSC apoptosis. Decreased apoptosis was accompanied by a decrease in the production of MDSC, demonstrating that MDSC levels are homeostatically regulated. Pharmacological inhibition of autophagy increased MDSC apoptosis indicating that autophagy increases MDSC half-life. Inhibition of HMGB1 also increased MDSC apoptosis and reduced MDSC autophagy. These results combined with our previous findings that HMGB1 drives the accumulation of MDSC, demonstrate that HMGB1 maintains MDSC viability by inducing autophagy. Collectively, these findings identify Nrf2 and HMGB1 as important factors that enable MDSC to survive in the TME.

Introduction

T cell-mediated adaptive immunity is capable of controlling cancer cell progression and eliminating malignant cells, as shown by the successful treatment with the checkpoint blockade inhibitors (CBI) anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies. However, CBIs are only effective in a subset of patients with certain types of cancers [1]. This partial effectiveness is likely due to the presence of other immune suppressive mechanisms present in the TME (tumor microenvironment). Myeloid-derived suppressor cells (MDSC) are present at different levels in virtually all cancer patients. These immature myeloid cells are potent inhibitors of T cell-mediated antitumor immunity. They are derived from the common myeloid progenitor cell in the bone marrow, accumulate in response to a variety of pro-inflammatory mediators, and are chemoattracted to the TME by chemokines [2, 3]. They use a variety of mechanisms to suppress antitumor immunity and facilitate tumor progression including inhibition of T cell activation and function, polarization of macrophages towards an M2-like phenotype, induction of T regulatory cells, inhibition of T cell trafficking into lymph nodes, blocking NK cell-mediated cytotoxicity, promotion of neo-angiogenesis, and enhancement of cancer cell stemness (reviewed in [4, 5]). The chronic low-grade inflammation associated with obesity also drives the accumulation of MDSC and is at least partially responsible for the increased susceptibility of obese individuals to more rapid tumor progression [6, 7].

The local environment within solid tumors is typically inhospitable for many cells due to the presence of reactive oxygen species (ROS) [8], hypoxia [9], and limited quantities of nutrients [10]. Tumor cells thrive in the TME because they have adapted to these harsh conditions. One mechanism used by tumor cells to survive is the activation of the transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2). Under non-stress conditions Nrf2 is bound to the Kelch-like ECH-associated protein (Keap1) in the cytoplasm where it is polyubiquitinated and subsequently degraded in the 26s proteasome [11-13]. Under conditions of oxidative stress, cytosolic Nrf2 is stabilized because cysteine

residues in Keap1 are oxidized resulting in conformational changes to Keap1 and the release of Nrf2. Stabilization of Nrf2 is also mediated by direct phosphorylation by kinases, including myc, Kras, PKC, ERK, and p38MAPK. Stabilized Nrf2 translocates to the nucleus where in conjunction with other transcription factors it binds to the antioxidant response elements (ARE) in the regulatory region of more than 200 genes [14-17]. Most of these genes encode proteins that protect against oxidative damage; however, genes that facilitate proliferation [18, 19] and autophagy [20, 21] are also activated. As a result, tumor cells are protected against oxidative stress and they proliferate.

Autophagy is another key mechanism used by tumor cells to thrive in the harsh TME. Cells undergoing so-called "macroautophagy" (henceforth called "autophagy") degrade non-essential cytosolic components in their lysosomes and recycle the constituents into molecules essential for survival [22]. Autophagy has multiple effects within the TME and differentially impacts different tumors. However, its main effect is to alter tumor cell metabolism under conditions of nutrient stress [23] and hypoxia [24], a process that is regulated by the AMP kinase and mTOR pathways [25, 26]. Since radiotherapy and chemotherapy induce intracellular tumor stress, autophagy also facilitates tumor cell survival in cancer patients undergoing these treatments [27].

High mobility group box protein 1 (HMGB1), a damage associated molecule pattern molecule (DAMP) is an established inducer of autophagy, and is ubiquitously present in the TME [28-30]. HMGB1 is also a potent inducer of MDSC and a driver of MDSC suppressive activity [31]. Given the known protective effects of autophagy and Nrf2 on cancer cells, we hypothesized that MDSC may use these same mechanisms to survive in the nutrient-depleted and hypoxic environment of solid tumors. This article reviews our studies supporting this hypothesis.

Nrf2 decreases the survival time of tumor-bearing mice

Some studies have demonstrated that host cell-expressed Nrf2 reduces carcinogenesis [32-34],

while other studies indicate Nrf2 supports tumor progression [35, 36]. Therefore, we first determined how Nrf2 expression impacted tumor progression by comparing the survival of wildtype and Nrf2-deficient BALB/c mice carrying the syngeneic 4T1 tumor and wild type and Nrf2-deficient C57BL/6 mice carrying the MC38 colon carcinoma. For both tumors, Nrf2-deficient mice had significantly longer survival times ($p<0.01$) compared to their wild type counterparts, indicating that in the setting of the current studies Nrf2 enhances tumor progression [37].

To ascertain that Nrf2 is not activated in the MDSC of Nrf2-deficient mice, quantitative RT-PCR was used to detect the expression of four genes known to be upregulated by activated Nrf2 (glutamate-cysteine ligase, heme oxygenase-1, catalase, and NADPH dehydrogenase) [38-40]. Circulating MDSC ($Gr1^+CD11b^+$ cells) from the blood of tumor-bearing Nrf2-deficient mice treated with tert-butylhydroquinone (tBHQ), a stressor that activates Nrf2 [41], did not contain detectable mRNA for any of these genes, while MDSC from wild type mice contained mRNA for these genes.

Nrf2 increases the frequency of tumor-infiltrating MDSC and the suppressive potency of MDSC

MDSC are potent suppressors of activated $CD4^+$ and $CD8^+$ T cells and prevent the activation of naïve T cells. To determine if Nrf2 impacts the frequency of tumor-infiltrating MDSC (TIMDSC), primary 4T1 tumors were resected from the breast tissue of BALB/c wildtype and Nrf2-deficient mice and the number of $CD45^+$ host hematopoietic cells was quantified. Significantly higher levels of $Gr1^+CD11b^+$ MDSC were detected in the tumors of the wild type mice relative to the Nrf2-deficient mice ($p<0.01$). No differences in the frequency of dendritic cells, $CD4^+$ or $CD8^+$ T cells, or B cells were found, although the tumors of Nrf2-deficient mice contained significantly higher levels of macrophages ($p<0.01$) [37]. The macrophage increase could be due to the reduced number of MDSC since macrophages and MDSC are derived from a common progenitor cell and fewer MDSC may result in differentiation of more macrophages.

To determine if Nrf2 impacts MDSC suppressive potency, MDSC from the blood of 4T1 tumor-bearing BALB/c wildtype and Nrf2-deficient mice were analyzed for their production of hydrogen peroxide, one of the ROS used by MDSC to inhibit T cell function. The MDSC were also tested for their ability to inhibit the antigen-driven activation of $CD4^+$ T cells. The Nrf2-deficient MDSC produced significantly less ($p<0.01$) hydrogen peroxide and were less suppressive of T cell activation as compared to MDSC from wildtype mice. Levels of arginase, another molecule used by MDSC to inactivate T cells, did not differ between Nrf2-deficient and wildtype MDSC. These findings demonstrate that Nrf2 not only increases the numbers of TIMDSC, but also enables individual MDSC to be more suppressive through their production of ROS [37].

Nrf2 decreases intracellular oxidative stress in MDSC and reduces apoptosis of MDSC

MDSC have a half-life in vivo and in vitro of approximately 1-2 days. This relatively short half-life may be due to their high content of ROS. Nrf2 may contribute to MDSC survival by activating antioxidant genes that reduce intracellular ROS and thereby reduce intracellular oxidative stress. To test this possibility, circulating MDSC from 4T1 tumor-bearing wildtype of Nrf2-deficient mice, and MDSC differentiated in vitro from bone marrow progenitor cells were stained and analyzed by flow cytometry for their content of ROS. In vivo and in vitro generated PMN-MDSC from Nrf2-deficient mice contained significantly more ROS compared to MDSC from wildtype mice, indicating that wildtype MDSC are less oxidatively stressed than Nrf2-deficient MDSC. In addition, MDSC in the blood Nrf2-deficient mice were significantly more apoptotic than MDSC from wildtype mice ($p<0.05$). Therefore, activation of Nrf2 in MDSC sustains MDSC survival and reduces MDSC apoptosis by decreasing intracellular oxidative stress [37].

Nrf2 controls MDSC levels by regulating MDSC homeostasis

Although Nrf2 increases the number of TIMDSC and reduces MDSC apoptosis, tumor-bearing Nrf2-deficient and wildtype mice have the same levels of circulating MDSC in the blood. Given this apparent inconsistency, we speculated that Nrf2 may also regulate the rate of generation of MDSC in bone marrow. This hypothesis was tested by culturing bone marrow from Nrf2-deficient and wildtype BALB/c and C57BL/6 mice under conditions that induce MDSC differentiation. For both strains of mice, Nrf2-deficient bone marrow generated significantly more MDSC than wild type bone marrow. The increased number of MDSC was due exclusively to the production of more PMN-MDSC. Therefore, in the absence of Nrf2, MDSC turnover more quickly but are maintained at a constant level in the circulation by an increased rate of production in the bone marrow.

Collectively, these findings demonstrate that Nrf2 homeostatically regulates MDSC production in the bone marrow and facilitates MDSC survival in the inhospitable TME by reducing MDSC apoptosis [37].

HMGB1 promotes MDSC survival by promoting autophagy

To determine if autophagy facilitates survival, we compared MDSC that were induced to undergo autophagy by starving in nutrient-deprived medium vs. MDSC that were maintained in their normal culture medium. Following four hours of culture, MDSC were treated with autophagy inhibitors, and assessed for viability. Inhibition of autophagy with chloroquine or bafilomycin significantly reduced the number of viable MDSC as assessed by propidium iodide (PI) and annexin V staining, demonstrating that autophagic MDSC have enhanced survival [42]. Our previous studies established that HMGB1 is ubiquitously present in the tumor microenvironment and drives the accumulation and suppressive potency of tumor-infiltrating M-MDSC and PMN-MDSC [31]. These findings combined with the knowledge that HMGB1 is an established inducer of autophagy [28], suggested that HMGB1 may also promote MDSC survival. In vitro treatment of starved MDSC with the HMGB1 inhibitor ethyl pyruvate

significantly reduced MDSC viability ($p<0.01$) demonstrating that HMGB1 also regulates MDSC survival. Additional in vitro studies using the autophagy inhibitor bafilomycin and the HMGB1 inhibitor ethyl pyruvate indicated that HMGB1 sustains MDSC viability by promoting autophagy. Subsequent in vivo experiments using 4T1 mammary tumors showed that tumor-infiltrating MDSC are significantly more autophagic than MDSC circulating in the blood of either tumor-free or tumor-bearing mice ($p=0.02$). Collectively, these findings demonstrated that HMGB1 facilitates the survival of MDSC within the TME by inducing autophagy [42].

Autophagic MDSC have decreased suppressive activity

Autophagy enables cells to maintain metabolic activity by repurposing amino acids that come from degraded non-essential cytoplasmic components [22]. Since autophagic survival eliminates some non-essential cellular functions, we speculated that autophagic MDSC may have diminished suppressive activity. MDSC treated with the autophagy inhibitors bafilomycin or chloroquine were more effective than control-treated MDSC at suppressing the antigen-specific activation of T cell receptor transgenic $CD4^+$ and $CD8^+$ T cells. Therefore, autophagy sustains MDSC survival, but also reduces MDSC suppressive potency [42].

Concluding remarks

The TME of solid tumors is an inflamed, hypoxic, and oxidatively stressed locale that is locally deprived of nutrients. This review highlights our work in understanding how MDSC survive and persist in the TME via Nrf2 signaling and HMGB1-induced autophagy (Figure 1A). Our data demonstrate that Nrf2 and autophagy independently regulate MDSC survival. However, there is also cross-talk between these two mechanisms. Nrf2 transcriptionally regulates the autophagy-related protein p62, which is an adaptor protein that recruits ubiquitinated autophagy substrates to the autophagosome. Subsequently,

p62 also interacts with KEAP1 and sequesters it away from Nrf2. Thus, activated Nrf2 not only directly protects MDSC against oxidative stress, but it also facilitates autophagy [43, 44], which further facilitates MDSC survival (Figure 1B).

MDSC are profoundly immune suppressive cells and may be a significant contributing factor in patients who are non-responsive to checkpoint blockade immunotherapy. Therefore, strategies to neutralize MDSC could have significant clinical benefit. Targeting Nrf2 activation may provide a reduction in tumor-infiltrating MDSC as shown here. However, Nrf2-deficiency in a mouse model has also been shown to increase in many of the proinflammatory mediators that drive MDSC accumulation [45]. The balance between these two apparently contradictory effects on MDSC levels may vary depending on the type of tumor and must be taken into account if considering Nrf2 attenuation as a therapeutic mechanism.

Strategies aimed at modulating HMGB1 may also be promising. Our previous studies demonstrated that neutralization of HMGB1 reduces tumor burden in mouse systems [31], and treatment with the autophagy inhibitor chloroquine has improved clinical outcome in patients with glioblastoma [46]. Neutralization of HMGB1 could also prevent the self-feedback loop whereby HMGB1 promotes autophagy and autophagy, in turn, promotes the release of additional HMGB1. This feedback loop was demonstrated in the mouse Lewis Lung carcinoma system in which HMGB1 drove tumor cell autophagy via the receptor for advanced glycation endproducts (RAGE) [47]. This HMGB1 release resulted in signaling through the mTOR pathway and enabled tumor cells to switch their energy source to glutamine obtained from skeletal muscle. Whether this metabolic change also occurs in MDSC remains unclear. However, neutralization of HMGB1 may have the dual benefits of impairing MDSC function, while simultaneously limiting cancer cell growth.

Regardless of what therapeutic strategy is ultimately successful, inactivation or elimination of MDSC is likely to benefit checkpoint blockade or other cancer immunotherapies that depend on the host's activated adaptive immune response.

Figure Caption

Figure 1. MDSC survival in the tumor microenvironment are facilitated by the activation of Nrf2 and by HMGB1-induced autophagy. (A) The TME is hypoxic, inflamed, and nutrient-deprived and includes reactive oxygen species (ROS) and high mobility group box protein 1 (HMGB1). Activation of Nrf2 in MDSC results in the production of many anti-oxidant proteins that (i) protect MDSC from oxidative stress, (ii) increase the number of tumor-infiltrating MDSC, (iii) facilitate MDSC survival, and (iv) protect MDSC from apoptosis. HMGB1, which is ubiquitously present in the TME increases MDSC (i) suppressive activity, (ii) accumulation, (iii) survival, and (iv) autophagy. **(B)** Nrf2 transcriptionally regulates the autophagy-related protein p62, which is an adaptor protein that recruits ubiquitinated autophagy substrates to the autophagosome. Subsequently, p62 also interacts with KEAP1 and sequesters it away from Nrf2. Thus, activated Nrf2 not only directly protects MDSC against oxidative stress, but it also facilitates autophagy, which further facilitates MDSC survival.

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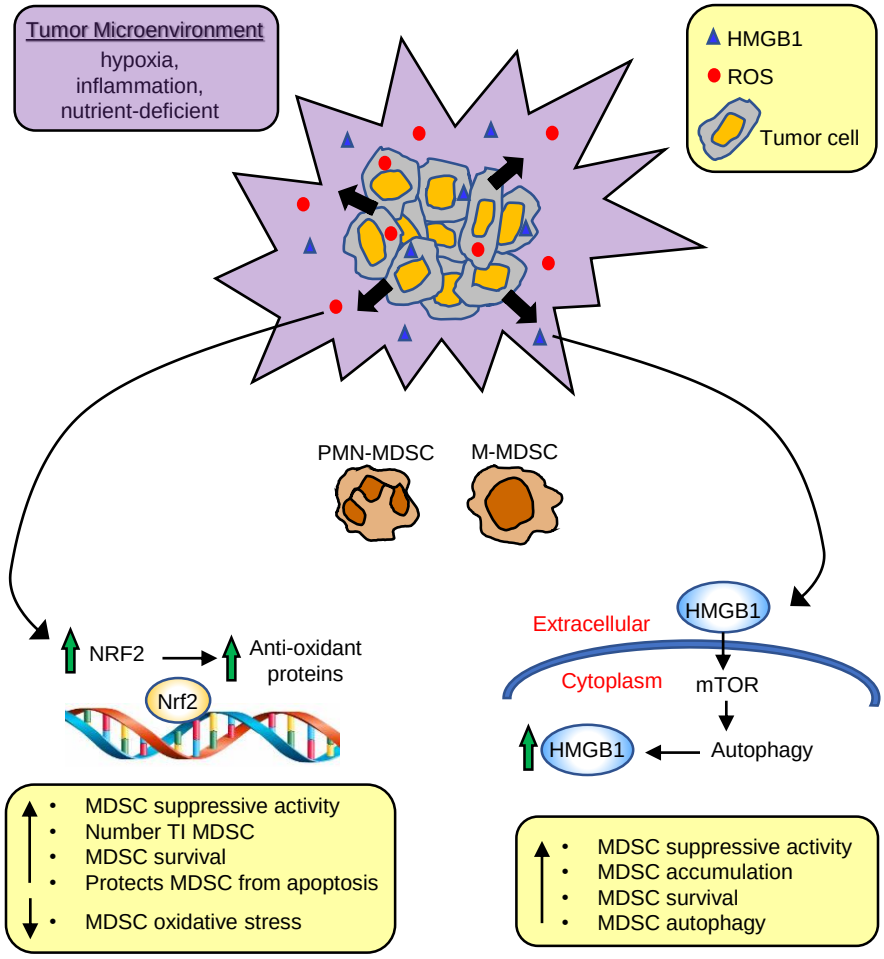
Tumor Microenvironment
hypoxia,
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M-MDSC
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Anti-oxidant
proteins
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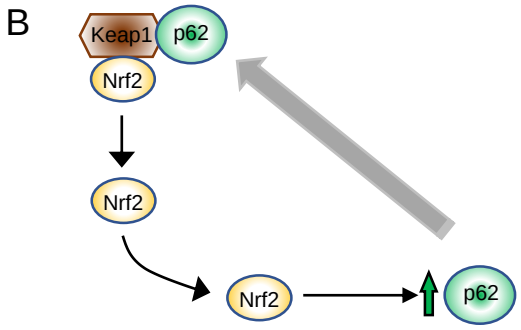
Extracellular
HMGB1
Cytoplasm
mTOR
HMGB1
Autophagy
MDSC suppressive activity
Number TI MDSC
MDSC survival
Protects MDSC from apoptosis
MDSC oxidative stress
MDSC suppressive activity
MDSC accumulation
MDSC survival
MDSC autophagy


B
Keap1
p62
Nrf2
Nrf2
Nrf2
p62