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Burn injury is associated with an infiltration of the wound site with myeloid-derived suppressor cells

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) have been identified in the burn wound, however their characterization is incomplete. To study this, mice were subjected to a major burn and skin cells were isolated 3 days thereafter for analysis. Significant infiltration of the burn wound with MDSCs was observed as compared with uninjured skin. The skin of naïve mice did not contain MDSCs. Characterization of the cells showed that 33% of MDSCs in the wound were monocytic (M)-MDSCs, which was significantly less than that found in uninjured skin (52%). In contrast, polymorphonuclear (PMN)-MDSCs were greater in the burn wound as compared with uninjured skin. Burn wound TLR expression by both MDSCs subsets was decreased as compared with uninjured skin. Wound MDSCs produced pro- and anti-inflammatory cytokines and iNOS was present in both MDSC subsets, whereas ARG1 was only present in M-MDSCs. In conclusion, both M- and PMN-MDSCs infiltrate burn wound with after injury, however, they displayed decreased TLR expression, suggesting receptor down-regulation.

1. Introduction

Major injury, such as burn can induce a profound pathological response that has a marked inflammatory component [1,2]. Preclinical studies from our laboratory have demonstrated that the burn wound is infiltrated with a large number of myeloid-derived suppressor cells (MDSC) [3]. MDSCs are a cell population that is heterogeneous and is capable of expanding during various pathological conditions, such as inflammation, injury, infection, and cancer [4,5]. These unique cells are a component of the immune system regulating immune responses in both healthy individuals and in the context of various diseases [4]. A common feature of MDSCs is their myeloid origin, immature state and an ability to suppress *T*-cell responses and regulate cytokine production. The suppressive activity of MDSCs is associated with L-arginine metabolism, which serves as a substrate for inducible nitric oxide synthase (iNOS) and arginase, which can inhibit *T*-cell function [6,7]

Murine MDSCs are defined as cells expressing both CD11b and GR1, however, subpopulations have been shown to exist [8]. These subpopulations are defined as polymorphonuclear-MDSCs (PMN-MDSCs) and monocytic-MDSCs (M-MDSCs). PMN-MDSCs are $CD11b^+$ $LY6G^+$ $LY6C^{lo}$, whereas M-MDSCs are $CD11b^+$ $Ly6G2^-$ $Ly6C^{hi}$. Immunosuppressive activity is a major characteristic of MDSCs. It has been proposed that criteria for their definition include changes in at minimum 2 transcription factors/regulators (i.e., IRF8, phospho-STAT3, c/EBPβ, S100A8/A9) and upregulation of at least one critical cytokine/receptor or immune-regulatory molecule (i.e., IL-10, TGFβ, ARG1, iNOS, PGE_2) [8].

The Danger Theory provides a key concept by which traumatic injury may lead to the activation of immune cells [9,10]. The theory proposes that the function of the immune system is to prevent and recognize attack from harm in the context of "Danger" signals or damage-associated molecular patterns (i.e., DAMPs). The theory states that the mechanism by which a cell dies governs whether an immune response is initiated, in that DAMPs generated by necrotic cells activate the system via pattern recognition receptors, such as toll-like receptors (TLRs) which represent a key link between tissue injury, infection, and inflammation. Our studies have shown that DAMP levels are elevated post-burn in mice and are capable of activating immune cells, thus providing a key link between injury, inflammation and the systemic inflammatory response [11].

While previous work from our laboratory and others have identified MDSCs in the response to injury, these studies have been incomplete

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[3,12]. We propose that influxing MDSCs are critical effector cells in the transition from an inflammatory to a healing and tissue remodeling response and that this process in TLR-dependent. The current studies were undertaken to better characterize this unique cell wound cell population.

2. Methodology

2.1. Mice

In all experiments, C57BL/6 male mice (25–30 g, Charles River Laboratories) were used. Mice were acclimatized for one week or more prior to experimentation. They were maintained in ventilated cages under specific pathogen-free conditions. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas Health Science Center at San Antonio and the procedures were performed in accordance with the National Institutes of Health guidelines for the care and handling of laboratory animals.

2.2. Burn procedure, skin collection, and cell isolation

Mice received a scald burn as described previously [13,14]. At 3 days after burn injury skin samples were collected. Normal non-injured skin was collected from the ventral surface and injured skin from the burn site. Skin samples from the burn site included injured skin and the wound margin. All skin samples were excised, down to the level of the musculofascia, including the submucosal layer, by sharp dissection.

Collected skin tissues were washed in PBS with 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO) in a 60 mm petri dish (Corning) and the skin was minced with scissors into small pieces of approximately 2–3 mm in size and placed into dispase II medium (0.05%, Roche) for overnight digestion at 4 °C on an orbital rocker. The following day the skin samples were further minced into smaller pieces and then digested by agitating in trypsin-GNK (0.3%, Glucose/dextrose, NaCl and KCl buffer, Sigma) for 30 min at 37 °C in a water bath shaker. Heat inactivated Fetal Bovine Serum (FBS, GIBCO) was added (10% total volume) to stop the digestion reaction and the dissociated cells were sieved through a 100 μm mesh. The cell suspension was centrifuged at 400 × *g* for 10 min and 4 °C and resuspended in RPMI culture medium [RPMI with 10% FBS, 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 μM non-essential amino acids (GIBCO), 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO)]. Cells were cultured overnight in a 12-well plate. The cultured cells were passed through a 70 μm mesh prior to staining for flow cytometry.

2.3. MDSC and TLR phenotyping by flow cytometry

The cells were washed in staining buffer (PBS with 0.2% BSA and 0.09% NaN_3) and treated with Fc-blocking antibody (anti-CD16/CD32, BD Biosciences) for 15 min. The cells were stained with the following directly conjugated antibodies: anti-CD11b (PAC), anti-Ly6G (PerCP), anti-Ly6C (AF700, anti TLR2 (PE) and anti-TLR4 (PE-Cy7). After 30 min of incubation on ice, the cells were washed and resuspended in staining buffer. For TLR9 analysis the cells were fixed after staining for CD11b, Ly6C, and Ly6G and permeabilized, using a Cytofix/Cytoperm™ solution (eBioscience), and stained with anti-TLR9 (PE). Appropriate isotype controls were used for all staining and gating was determined by The Fluorescence Minus One control, (FMO). FMO control is used to identify and gate due to the multiple fluorochromes in a given panel. All data were acquired using a FACS Celesta (BD Biosciences) and analyzed using FlowJo software (FlowJo, Treestar). MDSCs were identified according to the description of Bronte et al. [8]. In brief, after exclusion of debris and doublets, live $CD11b^+$ cells were gated and the proportion of $Ly6C^+$ and $Ly6G^+$ cells determined. Total MDSCs were identified as CD11b + $LY6C^+$, PMN-MDSCs were identified as are $CD11b^+$ $LY6G^+$ LY6Clo, and M-MDSCs are $CD11b^+$ $Ly6G^-$ Ly6Chi. A minimum of 100,000 events was collected.

2.4. Analysis of intracellular cytokines, iNOS, and ARG1 by flow cytometry

For measurement of intracellular cytokine expression, single cell suspensions were incubated for 3 h BD GolgiPlus™ (1 μg/ml) and BD GolgiStop (0.7 μg/ml) protein transport inhibitor (BD Biosciences, CA) followed by surface staining as described above. The cells were fixed, and permeabilized, using a Cytofix/Cytoperm™ solution (eBioscience), and stained with anti-IL-6 (PE), anti-IL-10 (PE-Cy7), anti-TGFβ (PE), anti-IL-12 (PE-Cy7), anti-ARG1 (PE) or anti-iNOS (PE-Cy7) or appropriate isotype controls. All data were acquired using a FACS Celesta (BD Biosciences) and analyzed using FlowJo software (FlowJo, Treestar).

2.5. Statistical analyses

Data are expressed as mean ± SEM. Comparisons were analyzed using ANOVA Multiple comparisons were analyzed using the Tukey test, and student's *t*-test was used for comparisons between two groups. A p-value of < 0.05 was considered to be statistically significant for all analyses.

3. Results

3.1. MDSCs infiltrate both the wound site and uninjured skin after burn injury

Skin cells were collected from naïve mice and burn-injured and MDSC content was assessed. The data in Fig. 1 shows that MDSCs (as defined as $CD11b^+$ $Ly6C^+$ cells) were marginally present in the skin of naïve mice, but significant percentages were observed in uninjured and wound skin at 3 days after burn injury. The $CD11b^+$ population was approximately 4-fold greater in the wound than uninjured skin. MDSCs as a percentage of the total gated population was approximately 3-fold greater in the wound than uninjured skin (Table 1). However, as a percentage of the $CD11b^+$ population, MDSC levels were similar in both uninjured and wound skin.

Analysis of the MDSC subpopulations revealed a significant difference in the M-MDSC ($CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$) and PMN-MDSC ($CD11b^+$ $Ly6C^{lo}$ $Ly6G^+$) Overall MDSC content of the wound was greater than that of uninjured skin due to an increase in both the M-MDSC and PMN-MDSC populations. M-MDSC content of the burn wound was approximately twice that of uninjured skin, whereas PMN-MDSC content was 4-fold greater (Fig. 2). In uninjured skin, the MDSC population was equally distributed between M-MDSCs (52.2 ± 4.5%) and PMN-MDSCs (46.6 ± 4.8%). In contrast, in the burn wound PMN-MDSC predominated (66.5 ± 3.2%) over M-MDSC (33.5 ± 3.4%), which was statistically significant ($p < 0.05$, n = 5 mice/group)

3.2. Burn injury markedly alters MDSC TLR expression in the wound bed

TLR2, 4, and 9 expression was assessed on MDSCs from uninjured skin and the burn wound site (Fig. 3, Table 2). Significant percentages of Both M-MDSCs and PMN-MDSCs expressed TLR2, 4 and 9. Surprisingly, the percentage of TLR positive cells was significantly greater in the uninjured skin, irrespective of sub-population. The percentage of $TLR2^+$ cells was reduced by approximately 75% in the wound, whereas the percentage of $TLR4^+$ cells was reduced by 60–80%. TLR9 percentages were reduced by only 25% in both M- and PMN-MDSCs. The percentage of PMN-MDSCs positive for TLRs was approximately ½ of that for M-MDSCs in both uninjured skin and the burn wound.

Assessment of the TLR mean fluorescent intensity (MFI), a relative indicator of receptor number per cell, revealed a significant difference in the burn wound also (Fig. 4). In the M-MDSC population (Fig. 4A) TLR2 expression was reduced by over 50% in the burn group, TLR4 was reduced by 2/3rds and TLR9 was not significantly altered. TLR2 and 9

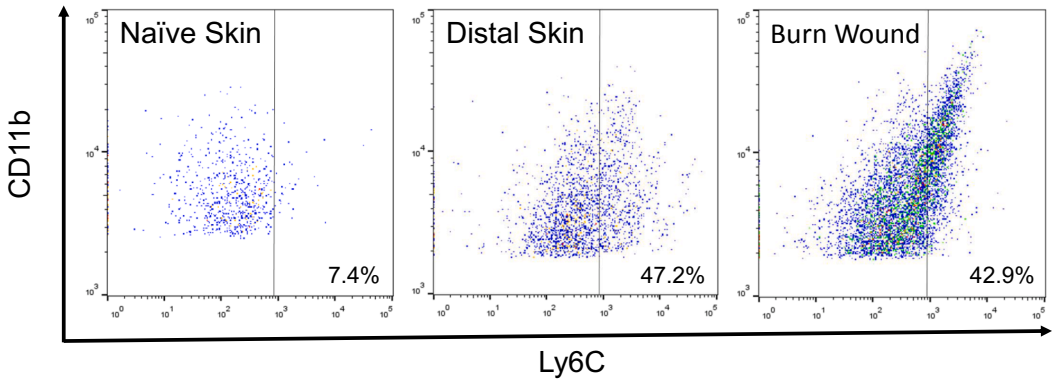

Fig. 1. MDSCs are present in the skin of burn-injured mice but not naïve mice. Skin samples were collected from naïve mice, uninjured skin of burn mice, and the burn wound and single cell suspensions were generated as described in the materials and methods for FACs analysis. Panels shown are gated on $CD11b^+$ cells stained with anti-Ly6C. Data are representative of 5 mice/group.

Table 1
Characterization of skin $CD11b^+$ cells.

	$CD11b^+$ (% of gated population)	$CD11b^+$ $Ly6C^+$ (% of gated population)	$Ly6C^+$ (% of $CD11b^+$ population)
Naïve skin	0.7 ± 0.1[a]	0.1 ± 0.1	7.4 ± 0.2
Uninjured skin	2.4 ± 0.6[b]	1.2 ± 0.3[b]	47.2 ± 7.5[b]
Burn wound	8.6 ± 0.8[b,c]	3.7 ± 0.4[b,c]	42.9 ± 2.4[b]

[a] Data are expressed as mean ± SEM for 5 mice/group.
[b] $p < 0.05$ as compared with respective naive skin group.
[c] $p < 0.05$ as compared with respective uninjured skin group.

expression by PMN-MDSCs (Fig. 4B) was also reduced in the burn group by 1/3rd. In contrast to M-MDSCs, TLR4 MFI was unaffected.

3.3. Burn wound MDSCs are activated for cytokine production and arginine metabolism

MDSCs isolated from the burn wound were assessed for constitutive cytokine production (Fig. 5). A significant number of M-MDSCs were positive for IL-6, IL-10, IL-12, and TGFβ. IL-10 and TGFβ were the most elevated. In contrast, PMN-MDSCs only produced significant amounts of IL-10 and IL-12, which was highly variable and lower than that observed in the M-MDSC population. Stimulation of the cells did not further increase cytokine production (data not shown).

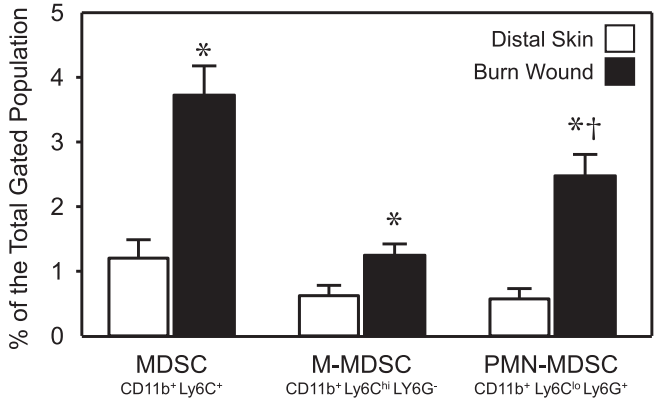

Fig. 2. Increased MDSCs in the burn wound are primarily of the PMN-MDSC population. MDSCs were phenotyped as MDSC ($CD11b^+$, $Ly6C^+$), M-MDSC ($CD11b^+$, $Ly6C^{hi}$, $Ly6G^-$) or PMN-MDSC ($CD11b^+$, $Ly6C^{lo}$, $Ly6G^+$) as described in the materials and methods. Data are expressed as mean ± SEM of the total gated population (n = 5 mice/group) $^*p < 0.05$ vs. uninjured skin, $^†p < 0.05$ vs burn M-MDSC.

Arginine metabolism was assessed by measuring iNOS and ARG1 levels (Fig. 6). Significant levels of ARG1 and iNOS were observed in the M-MDSC population. iNOS was also expressed by PMN-MDSC, but in approximately half as many cells than M-MDSCs. ARG1 was not expressed by the PMN-MDSCs.

4. Discussion

MDSCs are a unique cell population capable of regulating the immune response under various conditions. Our findings presented here demonstrate that MDSCs likely play a critical role in the immune response to burn injury, as they express TLRs which can respond to DAMPs at the injury site, produce a range of cytokines, and express both iNOS and ARG1.

MDSCs were initially recognized for their role in suppressing immune responses in tumors, but it has also been shown that they expand under inflammatory conditions [15]. M-MDSCs are similar to monocytes, whereas PMN-MCSCs share characteristics with neutrophils [16]. In cancer, PMN-MDSCs have been shown to represent over 80% of the MDSC population [4]. Our findings herein are relatively consistent with this observation, as the MDSC population in the burn wound was predominately of the PMN-MDSC population.

MDSCs are primarily defined by their immunosuppressive activity [4,17]. M-MDSCs have been shown to suppress *T*-cell function in malignant tumors, whereas PMN-MDSCs are immunosuppressive in models of autoimmune diseases [18,19]. PMN-MDSCs can also inhibit the function of other myeloid and NK cells and have also been shown to promote tumor angiogenesis through the production of growth factors [8,16,20]. STAT3/ARG1 signaling has been shown to play a critical role in the expansion and activation of M-MDSCs and it is through this pathway that they mediate their *T*-cell suppressive activity [21]. While suppression of *T*-cell function after burn has been well established, whether MDSCs and specifically which MDSC subpopulation are involved is unclear [2]. Our previous findings, while demonstrating a marked infiltration of the burn wound with αβ *T*-cells, their CD69 expression was reduced, suggestive of a suppressed phenotype [22]. It can be speculated that the activation of infiltrating MDSCs are responsible for this suppressed *T*-cell phenotype.

Somewhat surprisingly, we observed are marked down-regulation of TLR expression on MDSCs found in the burn wound as compared with uninjured skin. This down-regulation may be due to prior exposure to DAMPs in the wound bed. DAMPs are a wide range of cellular factors released by necrotic cells that bind to pattern recognition receptor, such as TLR and initiate a sterile inflammatory response. DAMPs are associated with burn injury and tissue necrosis is also associated with this injury type. Previous findings have shown that inflammatory disease

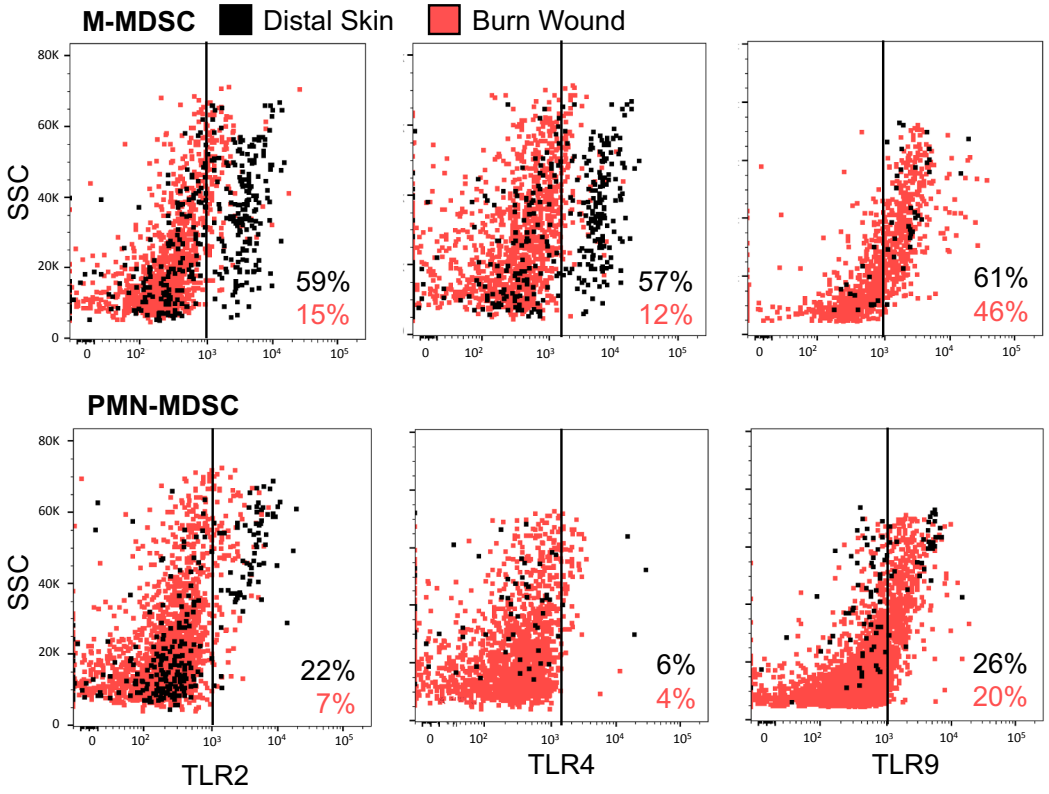

Fig. 3. MDSC TLR expression. The percentage of MDSCs positive for TLR2, TLR4, and TLR9 was determined as described in the materials and methods. M-MDSCs are shown in the top panels and PMN-MDSCs are shown in the lower panels. Data shown are representative of the evaluation of 5 mice per group.

Table 2
MDSC TLR expression.

	TLR2		TLR4		TLR9	
	M-MDSC	PMN-MDSC	M-MDSC	PMN-MDSC	M-MDSC	PMN-MDSC
Uninjured skin	58.9 ± 10.8[a]	22.1 ± 9.5[c]	56.5 ± 8.6	17.8 ± 3.7[c]	60.7 ± 3.7	26.0 ± 2.8[c]
Burn wound	15.4 ± 4.2[b]	6.5 ± 1.4[b]	12.4 ± 1.8[b]	7.3 ± 0.9[b]	45.7 ± 2.2[b]	19.9 ± 1.4[b]

[a] Data are expressed as mean ± SEM of % positive for 5 mice/group.
[b] $p < 0.05$ as compared with respective uninjured skin group.
[c] $p < 0.05$ as compared with respective M-MDSC group.

can be associated with suppressed TLR expression. Chang et al. demonstrated a significant reduction in the levels of TLR2 expression was observed on the neutrophils and monocytes of patients with active anterior uveitis compared with that of healthy control subjects [23]. Others have also shown that TLR2 and TLR4 expression are decreased at 1–3 days postoperatively [24]. Many receptors show attenuated responsiveness to agonists after prolonged or repeated activation, which can involve desensitization, sequestration, and down-regulation. Alternatively, following TLR activation there is a need for a checkpoint which returns the system to a homeostatic state to prevent further injury. A range of negative regulatory molecules has been identified including decoy factors, membrane-associated protein regulators, negative regulators of MyD88, ubiquitination/deubiquitination, feedback inhibition, and other mechanisms [25]. Recent findings have shown that targeting TLR can limit MDSCs suppressive activity. A combination of TLR7, −8 and −9 agonists enhanced anti-tumoricidal activity and reduced the presence of MDSCs [5,26]. Our findings indicate that there is a loss of cells expressing TLRs and a down-regulation of the number of receptors/cell as indicated by the reduced MFI. The decrease in TLR expression may serve to quell aspects of the early inflammatory response at the burn site. This concept is bolstered by our observation that burn wound MDSCs did not respond to TLR agonists by increasing cytokine production.

An important mechanism of action of MDSCs is the production of cytokines [27]. In our study, the MDSC (and in particular the M-MDSC population) in the burn wound were potent cytokine producers. IL-6 (which was strongly associated with the wound M-MDSC population) has been shown to enhance the expansion and activity of MDSCs [28]. IL-10 (which was produced by both wound M- and PMN-MDSCs) has been shown to be immunosuppressive, whereas TGFβ (an M-MDSC product) induces Treg cells, a Th2 profile, and suppresses NK cell activity [29,30]. IL-10 and TGFβ are consistent with an M2 MDSC phenotype which suppresses *T*-cell function, whereas an MDSC M1 phenotype is associated with IL-12 and iNOS [29]. Our findings indicate that both an M1 and M2 phenotype are present in the M-MDSC population and likely serve complementary roles. In contrast, the wound PMN-MDSC population was skewed more towards an M2 phenotype with elevated IL-10 production. Cytokines of both the M1 and M2 phenotypes are important in regulating the MDSC population. IL-10 has been shown to be important in regulating MDSC expansion via an S100A9 mediated pathway, M-MDSC expansion is TGFβ dependent, and IL-12 is involved in MDSC recruitment [31–33]. The dual phenotypes we observed may be in part due to the time we assessed MDSC activation and reflect the transition from an inflammatory milieu to one

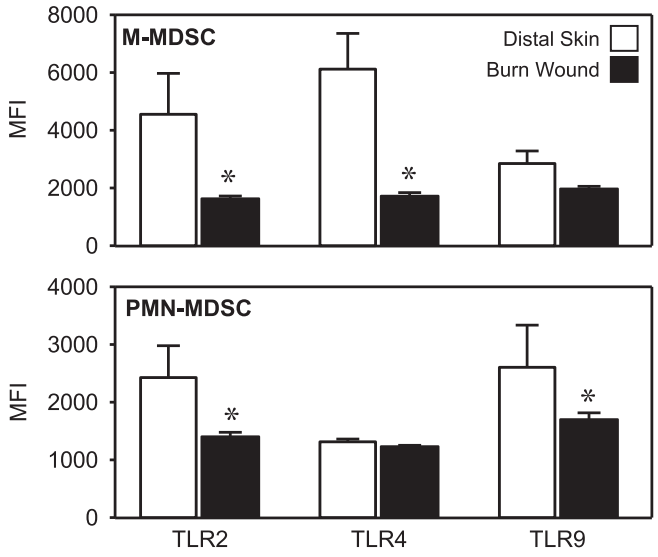

Fig. 4. TLR expression by MDSCs is suppressed in the burn wound. The relative expression of TLRs per MDSC (mean fluorescent intensity, MFI) was determined as described in the materials and methods. M-MDSCs are shown in the top panel and PMN-MDSCs are shown in the lower panel. Data are expressed as mean ± SEM for 5 mice/group $^{*}p < 0.05$ vs. uninjured skin.

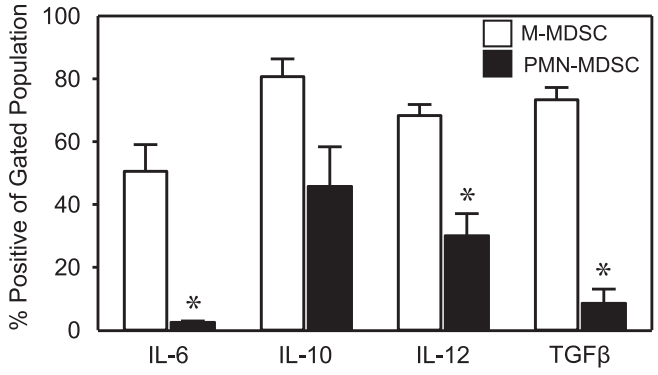

Fig. 5. Burn wound MDSCs constitutive produce cytokines. Intracellular cytokines were determined as described in the materials and methods. Data are expressed as mean ± SEM of % positive cells for 10–15 mice/group $^{*}p < 0.05$ vs. M-MDSCs.

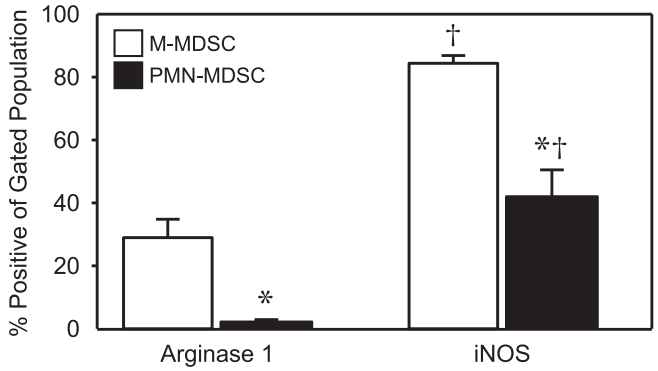

Fig. 6. Arginine metabolism by burn wound MDSCs. Intracellular staining for iNOS and ARG1 was conducted as described in the materials and methods. Data are expressed as mean ± SEM of % positive cells for 10–15 mice/group $^{*}p < 0.05$ vs. M-MDSCs.

that is angiogenic.

The vast majority of studies have examined the role of MDSCs in the suppression of *T*-cells in tumor biology. The evidence points to a suppressive pathway that involves either starvation of arginine via ARG1 or NO production by iNOS. Most studies have addressed the ability of MDSCs to suppress T cell activity in tumor-bearing hosts [5]. Findings also indicate that PMN-MDSCs are more effective in suppressing *T*-cells than M-MDSCs. PMN-MDSCs act via the generation of peroxynitrite, whereas M-MDSCs act via iNOS derived NO [5,34]. iNOS-mediated suppression of *T*-cell responses is well-established in the burn literature and previous findings have demonstrated that high levels of iNOS are found in the burn wound of injured mice, consistent with our findings herein [35,36]. It is likely that the MDSC iNOS is acting similarly in suppressing burn wound *T*-cell function, as iNOS levels were markedly greater than that of ARG1.

One of the main inflammatory mediators involved in angiogenesis is NO. Studies conducted with iNOS inhibition have provided evidence that inducible NO regulates tumor-induced angiogenesis [37]. iNOS also has been shown to modulate components of newly formed fibrovascular tissue (angiogenesis, inflammation, and collagen deposition). The absence of iNOS has been shown to reduce angiogenesis and exacerbated inflammation and fibrosis in the synthetic implants [38]. Thus, a potential role for iNOS as a regulator of inflammatory angiogenesis during the fibrovascular tissue formation exists which is in part similar to burn wound healing. In tumor biology, MDSC can also promote angiogenesis [39].

Our study has a number of limitations as we looked at only a single time (i.e., 3 days post-injury), rather than assessing MDSC profiles longitudinally after injury. The 3-day time point was based on our previous showing maximal cellular infiltration and iNOS presence in the wound at that time [3,22,35]. Additionally, we did not deplete the injured mice of MDSCs and observe healing responses, however, our previous studies with γδ *T*-cells suggests that depletion of MDSCs would attenuate healing [3].

The precise role of MDSCs in wound healing remains to be delineated. It can be speculated that the infiltrating wound MDSCs may help to quell the early inflammatory response at the wound site and begin the process of shifting the milieu towards a profile that supports angiogenesis and tissue remodeling. Alternatively, they may be deleterious, as an overabundance of MDSCs or prolonged activation might promote wound infection through their immunosuppressive properties. A more complete characterization of the MDSCs in the wound and uninjured skin and direct assessment of angiogenesis in the wound is warranted in future studies.

In conclusion, marked infiltration of the burn wound with MDSCs of both the myeloid and polymorphonuclear lineage occurs at 3 days post-injury. These cells expressed decreased TLR expression, which may be related to receptor down-regulation by prior exposure and activation by DAMPs earlier post-burn.

Acknowledgments

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Author contributions

MGS was responsible for the scientific conception, experimental design and interpretation and drafting of the manuscript.

SRS was responsible for in vitro experiments, flow cytometry, and data analysis. RKM assisted with experimental design and flow cytometry.

SEN and APC were responsible for interpretation and assisted in

drafting the manuscript.

All authors read and approved the final manuscript.

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