Myeloid-derived suppressor cells (MDSCs) are a naturally occurring immune regulatory population associated with inhibition of ongoing inflammatory responses. In vitro generation of MDSCs from bone marrow has been shown to enhance survival in an acute model of lethal graft-versus-host disease (GVHD). However, donor MDSC infusion only partially ameliorates GVHD lethality. In order to improve the potential therapeutic benefit and ultimately survival outcomes, we set out to investigate the fate of MDSCs after transfer in the setting of acute GVHD (aGVHD). MDSCs transferred to lethally irradiated recipients of allogeneic donor hematopoietic grafts are exposed to an intense inflammatory environment associated with sGVHD, which we now show directly undermines their suppressive capacity. Under a conditioning regimen and GVHD inflammatory settings, MDSCs rapidly lose suppressor function and their potential to inhibit GVHD lethality, which is associated with their induced conversion toward a mature inflammasome-activated state. We find even brief in vitro exposure to inflammasome-activating mediators negates the suppressive potential of cultured murine and human-derived MDSCs. Consistent with a role for the inflammasome, donor MDSCs deficient in the adaptor ASC (apoptosis-associated speck-like protein containing a CARD), which assembles inflammasome complexes, conferred improved survival of mice developing GVHD compared with wild-type donor MDSCs. These data suggest the use of MDSCs as a therapeutic approach for preventing GVHD and other systemic inflammatory conditions will be more effective when combined with approaches limiting in vivo MDSC inflammasome activation, empowering MDSCs to maintain their suppressive potential. (Blood. 2015;126(13):1621-1628)
MDSCs undergo rapid differentiation to CD11c<sup>+</sup> activated phenotype in the context of GVHD

MDSCs were generated from cultured fresh BM with GM-CSF and G-CSF in 4 days. Previously, we demonstrated that activation of cultured MDSCs 1 day prior to harvest with IL-4 or IL-13 stimulates expression of Arg1 and promotes functional suppression of T cells both in vitro and in the context of GVHD. To demonstrate that IL-13–activated MDSCs (MDSC-IL13) are effective at prolonging survival in a murine aGVHD model, lethally irradiated BALB/c recipients were given allogeneic C57Bl/6 BM plus CD25-depleted T cells to induce aGVHD, in the presence or absence of MDSC-IL13 cellular therapy, at day 0. T cells induce aGVHD, resulting in a mean survival time of 24.5 days. However, animals receiving MDSC-IL13 therapy had extended survival to 47.5 days (Figure 1A). Despite an improved clinical outcome, a majority of treated animals succumbed to GVHD-induced death by day 100, suggesting pathology was reduced or delayed, but not eliminated. To investigate how conditions associated with aGVHD might directly alter MDSC function, BM or BM plus T cell (GVHD conditions) with congeneric CD45.2<sup>−</sup>, MDSC-IL13 were given to CD45.1<sup>−</sup> animals that were then sacrificed at day 5 posttransplant to examine the phenotype, function, and fate of recovered MDSCs.

Phenotypically, MDSCs recovered from transplanted animals receiving only conditioning plus BM maintained an immature CD11c<sup>+</sup>, MHCI<sup>+</sup>, F4/80<sup>+</sup> appearance (Figure 1B). Here, we demonstrate that in a murine GVHD model, a single early-posttransplant MDSC infusion transiently suppresses but does not eliminate GVHD. Our data establish that the inflammation of GVHD drives MDSCs toward a state of inflammasome activation, which is counterproductive to MDSC suppressive function in GVHD mice. However, genetic alteration of donor MDSCs to disable inflammasome activation results in increased GVHD survival relative to control MDSC therapy. Furthermore, we find supporting evidence that the same pathways are active in human MDSCs. Taken together, this new information should be used to augment ongoing and proposed studies of MDSCs and their potential therapeutic application.

Methods

MDSC generation

Murine MDSCs were generated in complete Dulbecco’s modified Eagle medium plus 10% fetal calf serum, supplemented with 100 ng/mL G-CSF and 2.5 ng/mL mouse GM-CSF for 4 days. On day 3, 40 ng/mL recombinant murine IL-13 was added for Arg1 induction; alternatively, 40 ng/mL interferon-γ was added. MDSCs were harvested on day 4 using trypsin/EDTA and light scraping to recover adherent cells resulting in >92% CD11b<sup>+</sup> recovery. For inflammasome induction, lipopolysaccharide (LPS) (0.2 μg/mL) was added to prime cultures. After 3 hours, to stimulate the NLRP3 inflammasome, 2 mM ATP was added for 1 hour; alternatively, for AIM2 inflammasome activation, poly(dI) was added using Lipofectamine 2000 reagent.

All other methods are described in detail in the supplemental files (available on the Blood Web site).

Results

MDSCs undergo rapid differentiation to CD11c<sup>+</sup> activated phenotype in the context of GVHD

One explanation for the loss of suppression by MDSCs in vivo could be compromised survival of the suppressive cells. MDSC viability is maintained by continuous suppression of the extrinsic caspase-8 and intrinsic mitochondrial death pathways. Manipulation of either of these pathways causes a rapid decline in MDSC viability and a concomitant decrease in suppression. Therefore, we investigated whether the number and viability of transferred cells was compromised in recipient spleens posttransfer. No significant differences were observed between BM-only and GVHD conditions when looking at the total CD45<sup>+</sup>CD11b<sup>+</sup> cell number or viability at day 5 (Figure 1E), though a differential fate might have been evident at later time points. Taken together, these data suggest that MDSC-IL13 reduce aGVHD for a limited period of time posttransplant and that MDSC-IL13 subsequently lose suppressor function under GVHD conditions, resulting in a failure to sustain a therapeutic benefit.
Recent data have shown host NLRP3 in associated conversion to IL-1β. To further investigate the limited suppressive function of MDSC in aGVHD conditions and in vitro induction of cultured MDSC-IL13. NLRP3 and AIM2 represent 2 families of inflammasome-associated serum caspase-1 and IL-1β. Bruchard et al found that MDSCs produce IL-1β when exposed to certain chemotherapeutic agents, resulting in an altered antitumor response. To investigate the pathway-dependent mechanisms causing the diminished suppressive activity of transferred MDSC-IL13 in aGVHD, we focused on the MDSC-intrinsic activation of inflammasomes. Cell lysates from MDSC-IL13 recovered from GVHD conditions on day 5 were probed for the processed p10 form of caspase-1, an upstream mediator of inflammasome activation. Western blot analyses showed increased amounts of caspase-1 p10 from MDSC-IL13 recovered from GVHD conditions relative to MDSC-IL13 recovered from BM controls (Figure 2A-B). Further evidence for MDSC conversion to inflammasome activation was found when recovered MDSC-IL13 were placed in complete media for an overnight culture; analysis of supernatants demonstrated increased IL-1β from GVHD conditions (Figure 2C). These data establish a correlation between MDSC-IL13 in the context of aGVHD conditions and inflammasome activity.

**MDSC-IL13 in vitro induction of IL-1β requires ASC**

IL-1β can directly interfere with Treg-mediated suppression and promotion of T-effector function. To investigate inflammasome activation and IL-1β production in our system, we examined in vitro inflammasome induction of cultured MDSC-IL13. NLRP3 and AIM2 represent 2 families of inflammasomes in which various signals can potentiate the inflammasome cascade. However, reports suggest a common component required for full activation is the adaptor protein ASC. Wild-type and ASC−/− (PyCARD−/−) BM was used to generate MDSC-IL13 to test for inflammasome activity. At transfer, ASC−/− MDSC-IL13
We observed that in vitro suppression assay of T-cell proliferation to anti-CD3

and CD4

hours, MDSC-IL13 were then washed extensively and plated in an

in IL13 responses independent of in induction of MDSC-IL13 might alter their suppressive capacity. Follow-

ing a diminished ability to promote survival in the setting of aGVHD. In vitro cultured MDSC-IL13 were treated as above for activation of the NLRP3 or AIM2 inflammasome, followed by extensive washing prior to transfer into our aGVHD model. As seen for in vitro suppression, inflammasome activation of MDSC-IL13 caused a diminution in the GVHD survival benefit compared with control MDSC-IL13 therapy, which significantly increased GVHD survival (P < .0001; Figure 3C).

Having implicated MDSC-IL13 conversion to a mature, inflammasome-activated state after therapeutic transfer in the setting of aGVHD, we hypothesized that using MDSC-IL13 genetically incapable of inflammasome activation would better maintain function and further enhance GVHD survival. Indeed, survival of recipients of ASC−/− MDSC-IL13 was further improved relative to wild-type MDSC-IL13 (P = .0006), and both had significantly better survival than the no-MDSC group (Figure 3D). Furthermore, ASC−/− MDSC-IL13 recovered from GVHD animals 5 days posttransfer had increased T-cell–suppressive capacity compared with wild-type MDSC-IL13 (Figure 3E). These findings together directly implicate MDSC-IL13 intrinsic inflammasome activation under GVHD conditions as playing a role in limiting the efficacy of MDSC cellular therapy.

Previously, we have demonstrated that the mechanism for MDSC-IL13–mediated suppression of GVHD is Arg1 activity, which directly undermines T-cell responsiveness and promotes GVHD survival.8 To determine whether arginase activity was reduced in concert with inflammasome induction we measured enzymatic bioactivity from MDSC-IL13 after inflammasome induction. A marked drop in arginase activity as measured by either mRNA (not shown) or bio-enzymatic activity was found when either NLRP3 or AIM2 inflammasomes were induced (Figure 4A-B). A similar drop was not evident in ASC−/− MDCSs, though there was a trend, suggesting that the pathways may not be directly linked. To further investigate Arg1 activity specifically, we used Arg1 reporter mice (YARG), in which Arg1 expression is linked to yellow fluorescent protein (YFP) expression, allowing us to quickly assay for an associated loss of expression.20 Using this system, we found IL-13 readily upregulated YFP fluorescence as expected, whereas interferon-γ (which induces inducible nitric oxide synthase–expressing MDSCs) did not increase YFP fluorescence (Figure 4C-D).

As above, inflammasome induction via NLRP3 or AIM2 pathways resulted in a concomitant loss of YFP fluorescence, indicating Arg1 expression had been arrested in association with inflammasome inducing conditions. Finally, MDSC-IL13 generated from YARG transgenic animals were applied to our transplant model, given either BM only or BM plus whole T cells for GVHD conditions. YFP fluorescence from MDSCs recovered from BM-only animals on day 5 was reduced relative to expression immediately after culture but still readily detected above background (Figure 4E). However, in MDSC-IL13 recovered from GVHD animals, YFP was no longer detectable, being reduced to background levels seen in unstimulated YARG transgenic BM. These results further support our conclusions and link an associated loss of Arg1 expression to inflammasome activity during GVHD.

Figure 2. Inflammasome activity evident in recovered MDSCs. (A) Western blot of cell lysates from recovered wild-type or ASC−/− MDSC-IL13 probed for the active p10 form of caspase-1 and β-actin. ImageJ software was used to convert to grayscale and straighten and crop the gel image to highlight lanes of interest according to size. (B) Caspase-1 p10 blot quantification relative to β-actin; GVHD vs all other groups (P < .05). Quantification was carried out on scanned blots by densitometric analysis from ImageJ software (National Institutes of Health). (C) IL-1β enzyme-linked immunosorbent assay (ELISA) of supernatants after day 5–recovered MDSC-IL13 were plated in complete RPMI media overnight; GVHD vs all other groups (P < .05). Dotted line indicates limit of ELISA detection. All data are representative of 2 independent experiments. wt, wild-type.
MDSC are often defined as heterogeneous, with 2 major subsets described in murine systems being granulocytic (Ly6G+) and monocytic (Ly6C\(^+\) G-). At the time of transfer, we noted that while >90% are Ly6C\(^+\), a majority of our cultured MDSC-IL13 have an Ly6G\(^{-}\) phenotype, with a minor subset (<30%) exhibiting increased Ly6G\(^{+}\) staining (supplemental Figure 4). To determine whether each subset had a similar capacity for inflammasome activation and how suppressor capacity might be differentially affected, MDSC-IL13 were sorted based on Ly6G expression (supplemental Figure 7). NLRP3 inflammasome-activating conditions (LPS + ATP) lead to equivalent levels of IL-1\(\beta\) production for both Ly6C\(^+\) G- monocytic and Ly6G\(^{+}\) granulocytic subsets (Figure 4F). However, when applied to an in vitro suppression assay independent of inflammasome activation, we found that suppressor capacity was contained virtually in its entirety within the Ly6C\(^+\) G- monocytic subset (Figure 4G), which is consistent with our recent report of MDSCs generated with IL-6 and GM-CSF\(^5\); furthermore, Arg1 bioactivity was associated with the monocytic subset (Figure 4H). Thus, although each subset appears to have an equal capacity for inflammasome-mediated production of IL-1\(\beta\), arginase activity and suppressive capacity are primarily associated with the monocytic (Ly6C\(^+\) G- ) subset. These results suggest that further enrichment of the monocytic product would likely enhance overall therapeutic potential.

Human cultured MDSCs lose function when their inflammasome is activated

To investigate whether human MDSC might have a similar predisposition toward inflammasome-activated loss of function as found with murine MDSCs, we generated MDSCs from peripheral blood mononuclear cells (PBMCs) using a modified method from a published protocol\(^\text{21}\). Human PBMCs, enriched for the myeloid marker CD33, were cultured for 7 days with GM-CSF and IL-6. Under these conditions, human MDSCs suppressed anti-CD3\(\alpha\) mAb-driven proliferation of unrelated PBMC responders.\(^\text{22}\) We confirmed that human MDSCs are capable of responding to inflammasome-activating conditions by adding LPS followed by ATP to engage the NLRP3 inflammasome. As expected, both LPS and ATP are needed to drive MDSC production of IL-1\(\beta\) (Figure 5A). Next, inflammasome-activated MDSCs were added to the anti-CD3\(\alpha\) mAb-driven PBMC proliferation assay. As in the murine system, MDSCs exposed to inflammasome-activating components (Stim + Infl. T\(\xrightarrow{\text{d}}\) d) lost suppression concomitant with IL-1\(\beta\) production (Figure 5B-C).

Discussion

Alloreactive T cells are a major contributing factor to morbidity and mortality in clinical GVHD, and the use of regulatory cell therapy is gaining traction as a viable means to bring them under control. MDSCs can be generated from normal BM in a relatively short amount of time and have been shown to effectively suppress GVHD\(^\text{8,23}\) as well as autoimmunity\(^\text{24}\) and allograft rejection.\(^\text{25,26}\) In our studies, we have found that MDSCs activated by the cytokine IL-13 produce Arg1 that is in turn critical to their ability to suppress GVHD.\(^\text{8}\) However, in a stringent GVHD model with full major histocompatibility complex mismatching, MDSC therapy promotes extended survival but fails to ultimately protect a majority of animals from lethal GVHD. Here, we demonstrate that intrinsic inflammasome activation of adoptively transferred MDSCs limits their efficacy in vivo. We establish that...
cultured MDSCs are capable of rapidly responding in an ASC-dependent fashion to produce significant amounts of IL-1β, resulting in an associated loss of suppression in vitro. Furthermore, shortly after in vivo transfer, MDSCs in the context of aGVHD convert to a mature CD11c+ phenotype and demonstrate a loss of ex vivo suppressive capacity. These cells have increased amounts of an important indicator of inflammasome activation, caspase-1 p10, and secrete IL-1β when placed in culture overnight, unlike controls under non-GVHD conditions. Finally, when inflammasome activation is genetically ablated using ASC−/− MDSC-IL13, GVHD survival is further improved over wild-type MDSC-IL13 transplant recipients and recovered ASC−/− MDSC-IL13 maintain better ex vivo suppressive capacity.

Myeloid cells play a critical role in initiating and shaping immune responses in both pro- and anti-inflammatory directions and demonstrate remarkable plasticity. This adaptability may be both a blessing and a curse, in that it allows us to rapidly generate highly suppressive cells from normal BM in vitro yet permits the transient functionality seen upon transfer to a severe inflammatory environment such as aGVHD. Although MDSCs are described as heterogeneous in nature and phenotypic markers do not always translate between disease models or species, inflammasome-activation pathways appear to be highly conserved, and in this study, we find that MDSCs generated from mouse BM or human PBMCs readily activate inflammasomes, resulting in IL-1β production that then correlates to a loss of suppressor function. Our finding that the monocytic (Ly6C+G−) subset is primarily responsible for suppressive capacity of cultured MDSC-IL13 has implications for future studies aimed at improving the therapeutic potential of cultured MDSCs. Interestingly, the ratio of monocytic to granulocytic cells at the time of transfer (roughly 2:1; supplemental Figure 4) is inverted by day +5 posttransplant (Figure 1B). Due to the limited recovery, it was not feasible to further sort these cells; however, future studies will be aimed at addressing the tempo of phenotypic and functional changes for MDSCs in the context of GVHD as well as varied conditioning regimens.

Reports have demonstrated cleaved caspase-1 and increased IL-1β in patients with aGVHD, further supporting the conclusion that GVHD is associated with inflammasome activation. MDSCs are nearly ubiquitously associated with established tumors and actively perturb immune therapy interventions. An important distinction between tumor- and GVHD-induced inflammasome activation of MDSCs is that tumor-associated MDSC development occurs in the setting of chronic localized inflammation, in contrast to the intense, systemic inflammatory response of GVHD. It will be of interest to better understand the inciting signals for these divergent effects that are dependent upon the milieu in which MDSCs reside.

Because GVHD-activated MDSCs secrete IL-1β, it is possible that such MDSCs contributed to the GVHD lethality process. Earlier studies examining the IL-1 pathway in GVHD found an important role for IL-1 in the initiation of aGVHD. IL-1β has pleiotropic effects dependent on the cell producing it, the state of the surrounding microenvironment, and temporal expression, but it is generally understood to be proinflammatory and, in some instances, counterregulatory. When MDSC-IL13 recovered from day 5 GVHD transplant recipients are applied to an in vitro suppression assay, the proportion of T cells proliferating was not different from no-MDSC controls, although proliferating T cells underwent fewer cell divisions, suggesting...
some suppressive capacity remained. These data suggest that GVHD-activated MDSC-IL13 did not directly drive GVHD lethality, consistent with the finding that survival curves in MDSC-IL13–treated recipients paralleled those of no-MDSC controls after a 2- to 3-week delay. Although IL-18 can also be produced by activated inflammasomes and is also produced during clinical GVHD, several reports have found IL-18 actually attenuates GVHD. However, we have been unable to detect active IL-18 secretion from in vitro or ex vivo inflammasome-activated MDSCs (not shown). Thus, we do not favor a role of IL-18 in GVHD suppression by inflammasome-activated MDSCs. In contrast, Arg1 expression, which we have previously shown to be critical for the survival benefit conferred by MDSC-IL13 during adoptive transfer, was inhibited in inflammasome-activated MDSCs, potentially accounting for the loss of suppression by donor MDSCs.

Members of the NLR family of inflammasome mediators, such as NLRP3, are likely candidates for promoting inflammasome conversion under GVHD conditions, as both ATP and associated danger signals are found after conditioning and are known to play a role in enhancing GVHD.

In a preliminary study using a nonconditioning model (BALB/c Rag2−/−γc−/− recipients) of aGVHD, we found that wild-type and ASC−/− MDSC-IL13 performed similarly in enhancing survival (supplemental Figure 8). These findings implicate the conditioning regimen in concert with allo–T effectors as mediators of inflammasome-mediated loss of function, and future studies will be aimed at alternative induction protocols. NLRP3 inflammasome activation in the context of GVHD has been demonstrated for both radiotherapy and chemotherapy induction protocols, resulting in tissue damage and release of danger-associated molecular patterns. Our findings show that adaptively transferred MDSCs are also susceptible to inflammasome induction, and the same mediators are likely to contribute to in vivo activation. Reagents selectively targeting the NLRP3 inflammasome may be worth exploring for the dual purpose of inhibiting inflammasome activation in the host and infused donor MDSCs.

MCC950, a small-molecule inhibitor, and β-hydroxybutyrate, a ketone produced under metabolic stress, have demonstrated specificity toward suppressed NLRP3 and NLRP3-mediated diseases. Alternatively, viral and bacterial products, such as dsDNA, can also be potent drivers of both GVHD and the AIM2-like receptor family of inflammasomes. Although GVHD development is not dependent on host MyD88/TRIF pathway activity, the release of dsDNA by dead/dying cells from radiation and GVHD-induced injury may amplify lethality under some conditions.

Taken together, our data indicate that for the translational potential of donor MDSCs to be fully realized, inhibition of intrinsic inflammasome activation that occurs during the intense inflammatory environment of GVHD should be addressed. Until such a time as in vivo approaches for inhibiting inflammasome activation are available, multiple MDSC doses given during crucial early stages of allo–T-cell priming can be used to provide more continuous suppression by “replacing” MDSCs that have already experienced inflammasome activation.

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GVHD-associated, inflammasome-mediated loss of function in adoptively transferred myeloid-derived suppressor cells

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