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Murine myeloid-derived suppressor cells are a source of elevated levels of interleukin-27 in early life and compromise control of bacterial infection

Madeline Gleave Parson¹, Juanita Grimmer¹, Jordan K. Vance², Michelle R. Witt², Brittany G. Seman², Travis W. Rawson², Logan Lyda¹, Christopher Labuda¹, Joo-Yong Jung³, Shelby D. Bradford², and Cory M. Robinson²

¹Department, Biomedical Sciences West Virginia School of Osteopathic Medicine, Lewisburg, West Virginia

²Department of Microbiology, Immunology, & Cell Biology, West Virginia University School of Medicine, Morgantown, West Virginia

³Department of Biology, Briar Cliff University, Sioux City, Iowa.

Abstract

Microbial infections early in life remain a major cause of infant mortality worldwide. This is consistent with immune deficiencies in this population. Interleukin (IL)-27 is suppressive toward a variety of immune cell types, and we have shown that production of IL-27 is elevated in humans and mice early in life. We hypothesize that elevated levels of IL-27 oppose protective responses to infection during the neonatal period. In the present study, we extended previous findings in neonatal mice to identify a population of IL-27 producers that express Gr-1 and were further identified as myeloid-derived suppressor cells (MDSCs) based on expression of surface markers and functional studies. In neonates, MDSCs are more abundant and contribute to the elevated pool of IL-27 in this population. Although the ability of MDSCs to regulate T lymphocyte activation has been well-studied, sparingly few studies have investigated the influence of MDSCs on innate immune function during bacterial infection. We demonstrate that macrophages are impaired in their ability to control growth of *E. coli* when co-cultured with MDSCs. This bacterium is a significant concern for neonates as a common cause of bacterial sepsis and meningitis. The suppressive effect of MDSCs on macrophage function is mediated by IL-27; inclusion of a reagent to neutralize IL-27 promotes improved control of bacterial growth. Taken together, these results suggest that the increased abundance of MDSCs may contribute to early life susceptibility to infection and further highlight production of IL-27 as a novel MDSC mechanism to suppress immunity.

Corresponding author: Cory M. Robinson, Ph.D., West Virginia University School of Medicine, Department of Microbiology, Immunology, and Cell Biology, One Medical Center Drive, 2067/2070 Robert C. Byrd Health Sciences Center, P.O. Box 9177, Morgantown, WV 26506-9177; Telephone: 304-293-2433; cory.robinson1@hsc.wvu.edu.

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Keywords

Myeloid-derived suppressor cells; interleukin-27; macrophages; infection; immune suppression

INTRODUCTION

The neonatal period is a vulnerable time in which newborns have the highest risk of death from infection. There were 2.6 million deaths in the first month of life across the globe in 2016, representing 46% of all under age-five child deaths¹. This data signifies a 40% increase over the last three decades¹. Moreover, the proportions of under age-five deaths that occur during the neonatal period is increasing as under age-five mortality declines¹. Approximately 26% of neonatal mortality is the result of infection^{1,2}. Differences in neonatal innate and adaptive immune function compared with those of older populations are thought to contribute to increased susceptibility to infection^{3,4}.

We have reported elevated expression of interleukin (IL)-27 early in life in mice and human cells. IL-27 is a heterodimeric cytokine of the IL-12 family composed of the subunits p28 and Epstein Barr virus-induced gene 3 (EBI3)^{5,6}. IL-27 was first identified for its role in differentiation of naïve T cells to a Th1 phenotype and the ability to augment IL-12-induced IFN- γ production^{6,7}. Although inflammatory activity toward human monocytes and dendritic cells, as well as antiretroviral activity have been reported^{8–13}, a multitude of reports point to immune suppressive influences that may dominate IL-27 biology. Collectively, IL-27 has been shown to limit Th1, Th2, and Th17 responses while inducing T regulatory cells^{14–18}. Similarly, IL-27 can diminish innate immune-mediated inflammation. Neutrophil migration and oxidative burst were diminished in response to IL-27 during bacterial sepsis¹⁹. Our laboratory along with others have shown that inflammatory cytokine production by human and murine macrophages is limited following exposure to IL-27^{15,20–23}. Negative regulation of inflammatory cytokine receptor expression and downstream signaling is also impaired in macrophages^{22,23}. In human macrophages, IL-27 signaling interferes with lysosomal acidification and compromises control of *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*^{24,25}. In experimental models of tuberculosis, mice unable to respond to IL-27 exhibit decreased bacterial burdens but suffer from excessive inflammation during chronic disease^{15,26}. These observations support the anti-inflammatory nature of IL-27 and detrimental effect on control of bacteria

In our previous report, monocyte-derived macrophages from cord blood were shown to express genes that comprise IL-27 at elevated levels compared with cellular counterparts from adult blood²⁷. This manifests as increased secretion of IL-27 in culture supernatants²⁸. In mice, expression of the EBI3 gene is increased in spleens from mice in the first week of life and maintained at a high level through 21 days of life before declining into adulthood²⁷. This is consistent with an increased frequency of IL-27-producing macrophages and a greater level of protein in young mice of the same age range as compared to older animals and adults²⁷. These findings established mice as a useful model to study the involvement of IL-27 in early life immunity. In the present study, we sought to build upon these

observations to further define the cellular sources of elevated IL-27 early in life in mice and further understand the impact on host responses to bacterial infection.

RESULTS

IL-27 levels are elevated in neonatal mice –

We previously reported elevated expression of EBI3 in the spleens of mice through 21 days that was accompanied by elevated production of IL-27 by macrophages over a similar time period²⁷. We wanted to determine if these observations manifest as elevated levels of IL-27 in circulation. To determine this, blood was obtained from mice at the indicated age ranges and serum isolated for analysis of IL-27 levels. As shown in Figure 1, IL-27 levels are significantly elevated through 8 days of life as compared to older animals. This finding was anticipated; however, it was unexpected that IL-27 levels did not continue to rise through 21 days as was observed in the spleen previously (Fig. 1)²⁷. Since macrophage production of IL-27 is highest in the third week of life, serum levels in the neonatal period could be reflective of an additional cell type that serves as an important source of IL-27. This cell type may be present in the spleen but also more abundant in circulation than macrophages.

Gr-1⁺ IL-27 producers are more abundant early in life –

We previously identified a large population of cells in mice through 17 days of life that stained positive for IL-27 but were negative for the murine macrophage marker F4/80 by flow cytometry²⁷. We hypothesized that MDSCs may represent a significant percentage of the non-macrophage IL-27 producers. These cells have been shown to be more abundant in umbilical cord blood and exert anti-inflammatory activity^{29–31}. To explore this possibility, we isolated the spleens from mice (n=8–9) at age intervals similar to our previous study and surface-labeled splenocytes for Gr-1 as a marker of MDSCs along with labeling the intracellular compartment for IL-27. Flow cytometric analysis revealed that in the first week of life, there was a significant increase in the frequency of Gr-1⁺ cells that were positive for IL-27 staining (Fig. 2). The frequency of cells positive for both markers declined with increasing age (Fig. 2). Although at the age range of 16–21 days, the number of Gr-1⁺ IL-27 producers was greater than in adulthood, the difference did not reach statistical significance (Fig. 2).

MDSCs are more abundant in neonates –

Murine MDSCs can be characterized by their expression of Gr-1 and CD11b and further subtyped as granulocytic (Gr-1^{HI}) or monocytic (Gr-1^{DIM}). Using commercially available reagents that allow for the separation of both MDSC subtypes, these cells were isolated by immunomagnetic selection from the spleens of mice at different ages. The isolated cells were subsequently labeled for Gr-1 and CD11b to determine purity and phenotype. Figure 3 demonstrates typical results obtained with neonatal mice. As expected, the Gr-1^{HI} population is more granular in nature as reflected by the increased side scatter and exhibits an approximately three-fold increase in Gr-1 staining (Fig. 3A and B). These cells are the most abundant subtype typically ranging from 80–90% of the total MDSCs (data not shown). This is visually reflected in the Gr-1 profile when the MDSC populations were combined and stained at the ratio from which they were obtained from the mice (Fig. 3B,

Combined). To determine the abundance at different ages, MDSCs were isolated from mice as described above. The percentage of double-positive cells following immunolabeling was applied to the total number of MDSCs isolated and reflected as a percentage of total splenocytes. This analysis demonstrates that MDSCs independent of their IL-27 production are more abundant in neonatal mice (Fig. 3C).

Neonatal MDSCs produce IL-27 and exhibit function typical of MDSCs –

The enriched population of MDSCs isolated from neonatal mice were further evaluated for their functionality. These cells are homogenous population of IL-27 producers (Fig. 4A). This is consistent with expression of IL-27 genes and secretion of the protein heterodimer in culture supernatants (Fig. 4B and C). IL-27 p28 expression is comparable to actin (Fig. 4B). Several gene products have been linked with immune suppression in murine MDSCs. These include inducible nitric oxide synthase (NOS2), arginase (Arg)-1, and the NADH oxidase complex³². MDSCs isolated from neonatal mouse spleens express *nos2*, *arg1*, and the enzymatic subunit of the NADH oxidase complex, *cybB* (Fig. 4B). Expression of IL-27 subunits and MDSC effector genes in Gr-1^{HI} and GR-1^{DIM} cells separately is comparable (Supp. Fig. 1). Nitrite as a measure of NO production was detected in MDSC culture supernatants (Fig. 4D). Arginase-1 activity in MDSC cell extracts comparable to that of known amounts of purified enzyme is reflected in Figure 4E. Neonatal MDSCs produced ROS detected by dihydrorhodamine 123 that was induced to maximal levels following stimulation with PMA (Fig. 4F). To determine whether IL-27 influences expression of MDSC effector genes, MDSCs were treated with IL-27 (100 ng mL⁻¹) for 18 h and effector gene expression was measured. Although a modest increase in expression of *nos2*, *arg1*, and *cybB* genes was observed, this was not consistent and did not reach statistical significance (Supp. Fig. S2). Collectively, these data demonstrate that MDSCs isolated from neonatal mice produce IL-27 and further express genes typical of classical MDSCs with detectable levels of corresponding activity.

MDSCs are defined by the functional capacity to suppress T cell activity. Neonatal lymphocytes have been reported to be differentially programmed relative to those from adults displaying increased susceptibility to apoptosis and higher rate of cell turn-over^{33–35}. In addition, multiple studies have reported diminished expression of CD40 ligand on neonatal lymphocytes^{35–38}. Therefore, to investigate the ability of MDSCs isolated from neonates in our study to suppress T cell activity, CD4⁺ T cells were isolated from adult mouse spleens, labeled with CellTrace Violet, and expanded by stimulation of CD3 and CD28 for 4 days in the presence or absence of neonatal MDSCs. T cell proliferation was reduced during co-culture with MDSCs at a ratio of 1:1 (Fig. 5A). The mean fluorescent signal for the proliferated population increased more than two-fold (from 2,800 to 5,815) in the presence of MDSCs, indicative of reduced proliferation (Fig. 5A). This was accompanied by a significant reduction in IL-2 levels in culture supernatants during the first 2 days (Fig. 5B).

MDSCs compromise macrophage control of *Escherichia coli* –

MDSCs have been well characterized for their ability to negatively regulate T cell activity, but much less is known concerning their potential influence on innate immunity.

Furthermore, the innate response is critical for protection during infection in newborns as a result of several suboptimal features of the adaptive response⁴. As such, we evaluated the influence of MDSCs on the ability of macrophages to control bacterial infection. The neonatal innate immune response has been considered immature as a result of a variety of deficiencies relative to adults. These include decreased responsiveness to TLR ligands, impaired cell signaling in response to cytokine stimulation, and diminished inflammatory cytokine responses that are polarized toward anti-inflammatory. These observations in mind, we explored the interaction of neonatal MDSCs with adult macrophages to capture the full antibacterial potential of macrophages and subsequently assess the effect of MDSCs. A second advantage to this approach is that it minimizes the influence of macrophage-derived IL-27 and allows for a better dissection of the impact of MDSC-derived IL-27 since adult macrophages produce significantly less cytokine relative to neonates^{27,28}. *Escherichia coli* is a leading cause of neonatal sepsis³⁹. Bone-marrow-derived macrophages from adult mice were infected with *E. coli* for 1 h and then treated with gentamicin. The infected macrophages were incubated an additional 2 h to allow time for gentamicin to operate on residual extracellular bacteria. Exposure to gentamicin for this period of time is sufficient to completely kill extracellular bacteria prior to the addition of MDSCs; no viable bacteria are detected following 1 h exposure to gentamicin (Supp. Fig. 3). MDSCs isolated from neonatal mice were then added to the cultures at a ratio of 1:1 with macrophages. Following an additional 18 h incubation, the murine cells were permeabilized with saponin and viable bacteria were enumerated. The addition of MDSCs to the culture resulted in increased recovery of *E. coli* from the macrophages (Fig. 6A). *E. coli* were not recovered from MDSC cultures in the absence of macrophages (not shown) suggesting that these cells did not serve as a significant reservoir for the bacteria to evade gentamicin. The increased recovery of bacteria from macrophages required MDSCs to be present at an equivalent ratio to the macrophages; reducing the number of MDSCs eliminated the enhanced recovery of *E. coli* (Fig. 6B). This led us to hypothesize a role for IL-27 in the suppressive activity mediated by co-culture with MDSCs. To address this possibility, we included a soluble reagent to neutralize IL-27 (sIL-27R). Neutralization of IL-27 led to an intermediate reduction of bacteria recovered from the macrophages suggesting a partial role for IL-27 in the suppression of macrophage control of bacteria (Fig. 6C). IL-27 has been shown to limit inflammatory cytokine production by macrophages^{15,20–23}. However, there was no significant change in TNF- α production in the presence of MDSCs (Fig. 6D). Similar results were obtained with IL-6 (not shown). This suggests that the IL-27-dependent suppressive activity is mediated by directly reshaping the macrophage environment or effector functions and does not require a change in inflammatory cytokines.

DISCUSSION

In this report, we add to the current body of knowledge regarding IL-27 levels in early life. IL-27 gene expression and protein levels are increased in human neonatal macrophages and the spleens of mice through the first 3 weeks of life^{27,28}. In a previous report, macrophages producing IL-27 were more abundant in mouse spleens²⁷. Here we report that MDSCs contribute to the total pool of elevated IL-27 that is elevated in the spleen and blood serum. Moreover, to our knowledge, we are the first to report IL-27 production by MDSCs from

mice or humans. We do not expect that serum levels of IL-27 are solely reflective of MDSCs. Serum levels are most likely the result of production by all cellular sources, including unidentified low-level producers. However, given the abundance of MDSCs, their contribution to IL-27 production appears to be an important consideration for how the neonatal immune response is shaped.

Another important finding reported in this work was that the above observations manifest as higher levels of IL-27 circulating in serum of neonatal animals. We previously reported peak IL-27 levels in the spleen in the second and third weeks of life²⁸. Although speculative, the peak level of IL-27 in serum the first week of life could be explained by a shift from MDSCs as the dominant producer of IL-27 early to macrophages as the dominant producer in subsequent weeks. This idea is supported by a decline in the frequency of IL-27⁺Gr-1⁺ cells (Fig. 2) and an increase in the frequency of IL-27⁺ macrophages (~4%) over the same time frame²⁷. MDSCs, although present in the spleen, would be expected to circulate at higher levels than macrophages in neonates (as discussed below). Their presence in the spleen may in turn be a reflection of their circulation in the lymphatic and circulatory systems. Consistent with this trend is the possibility that monocytic MDSCs from circulation are deposited in the spleen and differentiate into splenic macrophages. They may retain the genetic programming to express high levels of IL-27 and contribute to the increased population of macrophage producers. One limitation of our study is that we do not get sufficient amounts of blood from neonatal pups to directly correlate IL-27 serum concentration with the frequency of circulating MDSCs and other immune cell types.

In addition to serving as a significant source of elevated IL-27 in the neonatal period, MDSCs are shown here to be more abundant in neonates. Reports have described a frequency of MDSCs in the spleens of normal mice to be 2–4% of splenocytes³². Our data from animals older than 9 days is consistent with this description. However, neonatal animals exhibit a significant increase in numbers. Our results are consistent with the finding of elevated numbers of MDSCs in human cord blood compared with older pediatric or adult blood donors²⁹. A separate study demonstrated that elevated numbers of MDSCs in human cord blood are independent of gestational age³¹. Our results are also in line with a recent report by He et al that establishes a greater abundance of MDSCs in the spleens of mice in the first two weeks of life⁴⁰. In our study, the MDSC population falls significantly immediately after the neonatal period. This is in line with a similar trend reported in human pediatric populations^{29,31}. That the number of Gr-1⁺CD11b⁺ cells (Fig. 3B) in neonates is lower than the number of Gr-1⁺IL-27⁺ cells (Fig. 2B) could be expected and suggests that some other cell types such as neutrophils may contribute to the total pool of Gr-1-derived IL-27. It is also possible that isolation of MDSCs and subsequent labeling is more selective than antibody labeling of total splenocytes.

Classically, three main effector functions have been described for MDSCs. These include nitric oxide generated by inducible nitric oxide synthase (NOS2), depletion of arginine mediated by arginase-1, and generation of reactive oxygen by the NADH oxidase complex³². The MDSCs that are abundant in neonatal mice align with this phenotypic description. These systems are expressed and active. A subtype-specific analysis demonstrates a comparable pattern of effector gene expression in GR-1^{HI} and Gr-1^{DIM}

MDSCs. A fourth, and until now undescribed effector function may involve the production of immune suppressive IL-27. Our gene expression analysis suggests that IL-27 does not significantly influence traditional MDSC effector gene expression. Although, it is important to emphasize that the neonatal MDSCs were isolated from an environment in which they were exposed to high levels of IL-27. As such, added levels of IL-27 in vitro may not be effective, but this does not necessarily rule out an influence of IL-27 on expression of MDSC effector genes. Future work that evaluates gene expression changes in MDSCs from mice that do not produce or respond to IL-27 will further address these possibilities. However, IL-27 independently has the potential to suppress immunity.

MDSCs have been most studied in the context of cancer, other chronic pathological states, and pregnancy. They are recruited to sites of tumor development and have a potent ability to suppress T cell activity and compromise immune surveillance^{32,41}. MDSCs expand during pregnancy and are associated with fetal maternal tolerance⁴¹. They have been less studied in the context of acute infection, although there are reports to be considered. MDSCs continue to accumulate during perinatal or postnatal infection beyond the already high levels found in cord blood³⁰. The MDSC population begins to expand at 3 days post-infection in the bone marrow and 5 days post-infection in the spleens in septic mice⁴². MDSCs isolated from these animals moderately suppressed CD4⁺ T cell proliferation but had a profound effect on suppressing IFN- γ production from CD8⁺ T cells, implicating them as players in post-sepsis immune suppression⁴². In a murine polymicrobial sepsis model, MDSCs were shown to increase post-infection⁴³. Adoptive transfer of the MDSCs from day 3 infected mice further compromised survival in recipient septic mice, and this was consistent with enhanced levels of inflammatory cytokines⁴³. Cystic Fibrosis (CF) patients chronically infected with *Pseudomonas aeruginosa* were found to have higher numbers of MDSCs in peripheral blood compared with non-CF controls and this correlated with impaired lung function⁴⁴. The MDSCs isolated from CF patients prevented proliferation of CD4⁺ and CD8⁺ T cells⁴⁴. During murine experimental tuberculosis MDSCs were found in the lungs and their depletion improved survival⁴⁵. Similarly, in a murine model of *Staphylococcus aureus* orthopedic biofilm infection, MDSCs accumulate at implant sites⁴⁶. Their depletion was associated with reduced bacterial burdens⁴⁶. Even considering these reports, we have a more limited understanding of how MDSCs may regulate the innate immune response. We showed that MDSCs compromised the ability of macrophages to control bacterial growth. This required a ratio of one MDSC per macrophage; diluting the number of MDSCs ten-fold diminished the suppressive effect on the macrophages (Fig. 3). This is consistent with a partial-dependence on IL-27 to mediate suppressive activity. Presumably, when the MDSCs are present at the lower ratio, the level of IL-27 does not rise to a concentration necessary for negatively regulating macrophage activity. Indeed, we have shown previously that IL-27 interferes with human macrophage control of gram-positive and gram-negative bacteria^{20,24,25}. However, our data also suggest that IL-27-independent MDSC-mediated mechanisms contribute to a suppressive influence on infected macrophages. It is important to point out the methodology associated with our approach. We are not assessing the antibacterial potential of MDSCs independent of the macrophage. By adding gentamicin for 2 h prior to the co-culture with MDSCs, any remaining extracellular bacteria following the media change are killed in this time period (Supp. Fig. 1). As such, while the MDSCs may

internalize dead bacteria, they do not serve as a permissive environment for intracellular growth and protection from the effects of gentamicin. Therefore, our findings presented here are not in conflict with those of He, et al⁴⁰. This group reported a slightly greater than two-fold reduction in bacterial viability when *E. coli* was incubated with directly with monocytic or granulocytic MDSCs, although the detailed methods were not provided⁴⁰. Separate studies will independently address the phagocytic potential and antibacterial capacity of MDSCs. There is also much to learn about whether exposure to or internalization of bacteria changes MDSC function, as well as the fate of bacteria within an MDSC. Taken together, our results help to explain some of the aforementioned reports in which the presence of MDSCs compromises survival during tuberculosis and promotes increased bacterial burdens during *S. aureus* colonization of orthopedic implants^{45,46}. A limitation of our study is that we do not routinely obtain sufficient numbers of Gr-1^{DIM} MDSCs to evaluate their full functionality independently. That said, there is value and relevance in studying the MDSCs at the ratio of each subtype present in vivo, which our study has done.

The observation that inflammatory cytokine production was not altered by the presence of MDSCs or IL-27 was a surprise. We have shown previously that during mycobacterial infection, neutralization of IL-27 elevates production of TNF- α , IL-6, and IFN- γ , albeit the latter two cytokines required 24 h for a change in levels to be detected^{20,21}. It is possible that for the findings presented here, the stronger stimulation of an inflammatory response by a greater number of faster growing bacteria counteracted negative regulation by IL-27 over a shorter time period. Overall, this result suggests a direct influence on macrophage effector functions mediated by MDSCs and IL-27. Previously, we reported that IL-27 negatively regulates lysosomal acidification and maturation in human macrophages^{24,47}. It is possible that the involvement of IL-27 in this context acts similarly to interfere with clearance of bacteria by the phagolysosomal pathway.

The high levels of circulating IL-27 and increased abundance of MDSCs in the neonatal period could have significant implications in susceptibility to infection by this age group. Indeed, IL-27 has recently been established as a novel biomarker for neonatal sepsis⁴⁸. In support of that report, neutralization of IL-27 in an adult mouse model of polymicrobial sepsis improved survival and control of bacteria¹⁹. As mentioned previously, the already high number of MDSCs continues to rise in septic neonates and it is tempting to speculate that cell numbers correlate with IL-27 levels³¹. Thus, increased levels of IL-27 may predispose the neonatal population to bacterial sepsis. We propose that IL-27 may represent a therapeutic target to reduce bacterial burden and improve outcomes. Antagonizing IL-27 levels specifically with neutralizing reagents or other small molecules and/or inhibitors of IL-27 expression may further help to mitigate the increased numbers of MDSCs early in life since they are now an established source of the immune suppressive cytokine. Approaches to reduce the abundance of MDSCs directly may prove challenging and could be deleterious. MDSC abundance early in life has been suggested to be valuable for limiting inflammation in the newborn during initial colonization by commensal bacteria⁴⁰. However, identification of the full range of effector mechanisms that MDSCs use to regulate immunity may offer the potential to antagonize specific immune compromising activities without eliminating the cells.

METHODS

Animals –

Breeding colonies of C57BL/6 mice were maintained at the West Virginia School of Osteopathic Medicine and West Virginia University School of Medicine. Male and female mice were used for experiments. Mice in this study were defined as neonates through 8 days of life, infants through two weeks, and adults at 6 weeks as described previously²⁷. Spleens, blood, or bone marrow were obtained from mice at the appropriate age by humane procedures. All procedures were approved by Institutional Animal Care and Use Committees.

Isolation and enumeration of myeloid-derived suppressor cells (MDSCs) –

Single-cell suspensions were prepared by crushing spleens from mice at the indicated age in mesh baskets (40 micron pore size). The splenocytes were collected with phosphate-buffered saline (PBS) and pelleted by centrifugation at 350xg for 5 min. The cell pellet was suspended in ACK red blood cell lysis buffer (5 mL) and incubated at room temperature for 5 min. MDSCs were isolated from the red blood cell (RBC)-depleted splenocytes by immunomagnetic selection using the Myeloid-Derived Suppressor Cell kit from Miltenyi Biotec (Bergisch Gladbach) according to supplier instructions. To enumerate percentage MDSCs per spleen, the immunomagnetic-separated cells were labeled for surface expression of Gr-1 and CD11b and analyzed by flow cytometry as described below. The percentage of double-positive cells was multiplied by the number of immunomagnetic-separated cells and divided by the number of total splenocytes.

Cell culture –

To generate bone marrow-derived macrophages (BMDM), femurs from adult mice were flushed with DMEM. The contents were pelleted and erythrocytes lysed with ACK lysis buffer (Lonza, Walkersville). Following a wash with phosphate-buffered saline (PBS), bone marrow cells were cultured in DMEM (GE Healthcare Life Sciences, Logan) that contained 2 mM glutamine, 25 mM HEPES, 10% FCS, and 10% L-cell-conditioned medium for 7 days at 37°C with 5% CO₂. MDSCs were cultured in DMEM that contained 2 mM glutamine, 25 mM HEPES, and 10% FCS.

Gentamicin protection –

For co-culture experiments to address bacterial recovery from macrophages, BMDMs were cultured with *Escherichia coli* O1:K1:H7 at a multiplicity of infection (MOI) of 20 and incubated as above for 1 h. The medium was then replaced with fresh supplemented with gentamicin (100 µg/mL) and the cultures incubated an additional 2 h. MDSCs were then added to cultures at the indicated ratio with or without soluble receptor to neutralize IL-27 (sIL-27R, R&D Systems, Minneapolis) and returned to incubation. At 18 h post-infection, the culture media was removed and the macrophages permeabilized with PBS that contained 1% saponin. Recovered bacteria were enumerated by serial dilution and plating onto Tryptic Soy agar (TSA, Becton Dickinson, Sparks).

Immunolabeling and flow cytometry –

Labeling of murine splenocytes was performed similar to as described previously²⁷. Splenocytes were suspended in flow stain buffer (PBS supplemented with 0.5% BSA and 0.1% sodium azide). For identification of Gr-1-expressing cells that produce IL-27, splenocytes or MDSCs were surface-labeled with phycoerythrin-conjugated anti-Gr-1 (0.25 µg, ThermoFisher Scientific, Waltham) or equivalent amount of isotype control, fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin for 10 min, and subsequently labelled with fluorescein-conjugated anti-IL-27 (0.5 µg, R&D Systems, Minneapolis) or isotype control. Following removal of unbound antibody, the splenocytes were again fixed with 4% paraformaldehyde. For phenotyping and enumeration of MDSCs, the cells were surface-labeled with phycoerythrin-conjugated anti-Gr-1 (0.25 µg, ThermoFisher Scientific, Waltham) and fluorescein-conjugated anti-CD11b (0.5 µg, ThermoFisher Scientific, Waltham) or equivalent amounts of isotype controls. Following removal of unbound antibody, the MDSCs were fixed with 4% paraformaldehyde. Cells were analyzed with a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes) or Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes).

Quantitative real time PCR –

MDSCs were lysed with PureZol™ (Bio-Rad, Hercules), and RNA was isolated according to the commercial product protocol. iScript™ cDNA synthesis reagents (Bio-Rad, Hercules) were used to generate first strand cDNA according to manufacturer protocol. Real time cycling of reactions that included cDNA diluted ten-fold from above, gene-specific primer probe sets (Applied Biosystems, Foster City), and iQ™ Supermix (Bio-Rad, Hercules) was performed in triplicate using a Step One Plus (Applied Biosystems, Foster City) real time detection system. Gene-specific amplification was normalized to β-actin as an internal reference gene.

Detection of reactive oxygen and nitrogen –

Nitrite levels were measured in overnight MDSC culture supernatants using the Measure-iT™ High-sensitivity nitrite Assay kit (Invitrogen, Carlsbad) as described previously⁴⁷. Nitrite standards were measured in parallel. Fluorescence (λ_{ex} =365, and λ_{em} =450 nm) was measured using a Synergy HT Multi-Mode Microplate reader (Biotek, Inc., Winooski). For detection of reactive oxygen generated by NADH oxidase, MDSCs were cultured in 96-well dishes at 2×10^5 /well. Some cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 20 µg/mL, Fisher Scientific, Hampton) for 1 h. Dihydrorhodamine (DHR, 433 nM, Molecular Probes, Eugene) was added and cultures were incubated for 30 min at 37°C with 5% CO₂. Fluorescence (λ_{ex} =485, and λ_{em} =528 nm) was measured using a Synergy HT Multi-Mode Microplate reader (BioTek, Inc., Winooski).

Arginase activity assay –

The method to measure arginase activity was adapted from that described previously with modification⁴⁹. A whole cell extract of MDSCs (5×10^5) was prepared by subjecting them to 25 µL RIPA lysis buffer (Santa Cruz Biotechnology, Dallas) supplemented with PMSF (2 mM) and protease inhibitor cocktail (diluted 1:100, Santa Cruz Biotechnology, Dallas) for

10 min on ice. The lysate was clarified by centrifugation at 10,000xg for 10 min at 4°C. Cell lysate (25 µL) or recombinant arginase positive control (R&D Systems, Minneapolis) was combined with 0.9 mM thioarginine (Cayman Chemical, Ann arbor), 0.1 mM 5,5'-Dithiobis (2-nitrobenzoic acid, DTNB, St. Louis), and PBS to a final working volume of 100 µL and incubated 30 min at 37°C with 5% CO₂. Absorbance at 412 nm was measured and compared to purified standards.

T cell isolation and proliferation –

Single-cell suspensions were prepared by crushing spleens from adult mice in mesh baskets (40 micron pore size). The splenocytes were collected with PBS, pelleted by centrifugation, and RBCs lysed as described above. CD4⁺ T cells were isolated by immunomagnetic selection using the CD4⁺ T cell isolation kit from Miltenyi Biotec (Bergisch Gladbach) according to supplier instructions. T cells (2×10⁶/mL) were labeled with 5 µM CellTrace™ Violet (ThermoFisher Scientific, Waltham) for 20 min at 37°C with 5% CO₂. The staining was then quenched with 10% FBS in PBS and the T cells pelleted by centrifugation at 300xg for 10 min. The T cells were cultured in RPMI supplemented with 2 mM glutamine, 25 mM HEPES, 10% FCS, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol in 96-well plates (1.5×10⁵ cells/well) in the presence (stim) or absence (control) of Dynabeads® Mouse T-Activator CD3/CD28 (ThermoFisher Scientific, Waltham). MDSCs (1.5×10⁵ cells/well) were added at the time of T cell stimulation where indicated. At 4 days post-stimulation the cells were collected and analyzed with a Becton Dickinson LSRFortessa flow cytometer (Becton Dickinson, Franklin Lakes).

Cytokine measurements –

Supernatants were collected from 24 h cultures of MDSCs at a density of 10⁶/mL or 18 h MDSC-macrophage co-cultures as described above. IL-27, TNF-α, or IL-6 protein levels in culture supernatants were detected using Ready-SET-Go!™ ELISA kits (ThermoFisher Scientific, Waltham) according to manufacturer protocol. Protein standards were assayed in parallel. Blood was collected from mice at the indicated age and serum collected by standard techniques. IL-27 serum levels were measured using luminescent detection reagents according to manufacturer's protocol (MesoScale Discovery, MSD, Rockville) and analyzed using MSD Discovery Workbench software. Protein standards were assayed in parallel with samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Gr-1⁺ cells that produce IL-27 are more abundant in neonates. Splenocytes from C57BL/6 mice at the indicated age were labeled for surface-expressed Gr-1 and intracellular IL-27 and analyzed by flow cytometry. **(a)** Representative dot plots relative to isotype controls are shown. **(b)** The combined percent Gr-1⁺IL-27⁺ population from at least 8 mice at each age group are shown. Statistical significance was determined using an ANOVA and Tukey's multiple comparison's test.

Figure 3.

MDSCs are more abundant in neonates. MDSCs were isolated from C57BL/6 mouse splenocytes at the indicated age as described in the *Methods*. Distinct populations of granulocytic (Gr-1^{HI}), monocytic (Gr-1^{DIM}), or combined MDSCs were subsequently labeled with anti-Gr-1 and anti-CD11b to demonstrate purity and analyzed by flow cytometry. (a,b) Representative dot plots and live cell gates are shown for neonatal MDSCs. The dashed line was added to aid in visualization of the shifted populations. (c) The Gr-1⁺CD11b⁺ cells from the isolated MDSC fraction was expressed as a percentage of splenocytes from at least 9 mice at each age group. Statistical significance was determined using an ANOVA and Tukey's multiple comparison's test.

Figure 4.

Isolated MDSCs produce IL-27. MDSCs were isolated from neonatal C57BL/6 mouse spleens as described in the *Methods*. **(a)** The isolated cells were labeled for surface-expressed Gr-1 and intracellular IL-27 and analyzed by flow cytometry. A representative dot plot relative to isotype controls is shown. **(b)** MDSC expression of the indicated genes relative to a no template control was determined by real time PCR. Six independent measurements using MDSCs obtained from pups ranging 5–8 days of age are combined and shown. **(c)** IL-27 concentration was determined in 24 h MDSC culture supernatants by ELISA. Seven independent measurements using MDSCs obtained from pups ranging 5–8 days of age are combined and shown. **(d)** The nitrite concentration in 48 h MDSC culture supernatants was measured. Five independent measurements using MDSCs obtained from pups ranging 5–10 days of age are combined and shown. **(e)** Arginase activity was measured relative to purified standards. Eight independent measurements using MDSCs obtained from pups ranging 5–8 days of age are combined and shown. **(f)** Oxidative burst was measured in MDSCs using Dihydrorhodamine 123. Data using MDSCs obtained from six day old pups representative of three separate measurements is shown.

Figure 5.

MDSCs suppress T cell proliferation. CD4⁺ T cells were isolated from adult mice by immunomagnetic selection, labeled with CellTrace™ Violet (5 μ M), and activated by CD3 and CD28 stimulation (Stim) in the presence or absence of neonatal MDSCs at a ratio of 1:1. (a) T cell proliferation at 4 days was measured by flow cytometry. Histograms representative of four separate experiments are shown. Control indicates unstimulated T cells. (b) IL-2 production measured by ELISA from four combined experimental replicates is shown. Statistical significance was determined using unpaired *t* tests.

Figure 6.

MDSCs compromise macrophage control of *E. coli*. Bone marrow-derived macrophages were infected with *Escherichia coli* at an MOI of 20 in the presence or absence of MDSCs (5–13 days of age) at the indicated ratio. Recovery of intracellular bacteria was enumerated at 18 h and shown as mean log_{10} recovered colony forming units $\pm$ standard error (a). Statistical significance in a combined six experiments was determined using a paired samples *t test*. (b) MDSCs were added to cultures where shown at the indicated ratio to macrophages. A combined four experiments are shown. Statistical significance was determined using paired samples *t tests*. (c) Macrophages were cultured as indicated $\pm$ MDSCs (ratio of 1:1 with macrophages) in the presence or absence of a soluble receptor to neutralize IL-27 (sIL27R). Five combined experiments are shown. Statistical significance was determined using paired samples *t tests*. (d) The concentration of $\text{TNF-}\alpha$ in culture supernatants was determined by ELISA. The presence of bacteria (Ec), sIL-27R, and ratio of MDSCs to macrophages (0.1X or 1X) is indicated. Results from an individual experiment representative of multiple are shown.