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Project Title: In vitro effects of G-CSF, GM-CSF, and M-CSF treatments on expansion and suppressive function of murine bone marrow-derived myeloid-derived suppressor cells

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SUMMARY: (no more than 250 words single spaced)

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that can suppress the adaptive and innate immune responses via multiple mechanisms, including the suppression of T cell proliferation and activation.

This immunosuppressive function may have an application in the setting of hematopoietic stem cell transplantation (HSCT). Graft-versus-host disease (GVHD) is a common complication following allogeneic HSCT that occurs when the donor T cells recognize the recipient's tissues as foreign and launch an immunological treatment with growth factors. Clinically, peripheral blood stem cell grafts are collected following donor treatment with growth factors G-CSF or GM-CSF; these growth factors are known to expand MDSCs. MDSC infusions have been shown to alleviate GVHD in the murine model.

We aimed to identify a growth factor that optimally expands and licenses bone marrow-derived MDSCs for inhibition of T cell proliferation and suppression of T cell function in a murine model, comparing the growth factors G-CSF, GM-CSF, and M-CSF.

Here we report a population of murine MDSCs induced in vitro with growth factor M-CSF with the ability to strongly suppress T cell proliferation through an iNOS pathway. These M-CSF-induced MDSCs have stronger suppressive functions than those induced by the clinically used G- and GM-CSF. Exploration of the use of M-CSF in the post-HSCT setting may reveal a new tool for the prevention and treatment of GVHD.

Student Signature **Supervisor Signature**

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Introduction & Background

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that can suppress the adaptive and innate immune responses and augment downregulatory elements of the immune system via multiple mechanisms, including the suppression of T cell proliferation and activation.[1] These cells have also been found to induce differentiation and expansion of regulatory T cells (Tregs).[2]

As a cell population that has been prevented from fully differentiating, MDSCs share cell surface markers with intermediate myeloid progenitor cells, and are thus defined by the presence of these surface markers as well as the demonstration of their immunosuppressive functions.[1] In mice, MDSCs are commonly defined as co-expressing markers CD11b and Gr-1. However, it has been noted that challenges exist in defining this "in-between" heterogeneous population using standard techniques such as flow cytometry.[3] Myeloid-derived suppressor cells are known to have granulocytic (G-MDSCs) and monocytic (M-MDSCs) subsets based upon different expression of the Gr-1 epitopes Ly-6G and Ly-6C. In mice, these are defined by markers $CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{low}$ and $CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{high}$ in the granulocytic and monocytic subsets, respectively.[1] These subsets have different mechanisms of T cell suppression and one or the other may predominate, depending on the inflammatory conditions.[4,5]

There are several known mechanisms through which MDSCs suppress T cell function. The main mechanisms include arginine depletion through arginase-1 (Arg-1) activity, nitric oxide (NO) activity produced by inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS).[6] In mice, it has been found that G-MDSCs act mostly through ROS and some Arg-1 activity, whereas M-MDSCs act through the iNOS mechanism (Figure 1).[5,7,8] Upregulation of prostaglandin E2 (PGE2) through the enzyme cyclooxygenase-2 (COX-2) has also been implicated in the suppressive function of this population.[7,9] Tryptophan depletion through the enzyme indoleamine 2,3-dioxygenase (IDO-1) has been found to mediate suppressive activities of MDSCs.[10] MDSCs have been found to exert their suppressive function through cell-to-cell contact.[11] Treg proliferation is thought to occur by MDSC secretion of IL-10 and TGF-β in the presence of IFN-γ.[12,13]

In the cancer setting, MDSCs have been found in the tumour microenvironment, implicated in aiding and abetting the cancerous cells in their escape from the immune attack. Tumour factors lead to the expansion of MDSCs, inhibiting T cell proliferation and cytotoxic T cell activation.[2] The recruitment and differentiation of MDSCs in the tumour microenvironment have been identified as therapeutic targets in the treatment of cancer. Because of their ability to target the inhibitory MDSC function, inhibitors of reactive nitrogen species and phosphodiesterase-5 inhibitors have been studied; tyrosine kinase inhibitors have been studied to evaluate their ability to inhibit MDSC expansion.[2] Tumour factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor, PGE2, COX-2, vascular endothelial growth factor, and macrophage colony-stimulating factor (M-CSF) have been linked to MDSC expansion in the cancer setting (Figure 1).[2]

Despite being implicated in the pathogenesis of cancer and panoply of inflammatory conditions including sepsis (where lipopolysaccharide was found to induce MDSCs) and autoimmune conditions,[1,14] MDSCs may have a role to play in the treatment of conditions that require immune tolerance – namely, transplantation.[15]

Recently, MDSCs have been identified as a regulatory cell population in solid organ transplantation tolerance. In a rat model of kidney transplantation, MDSC numbers were found to be significantly higher in tolerant recipients.[16] In a mouse model of allogeneic cardiovascular transplantation, an M-MDSC population was found to induce allograft survival by migrating to the graft and the preventing initiation of adaptive immune responses.[17]

Similarly, MDSCs have been found to play a role in recovery and prevention of complications following hematopoietic stem cell transplantation (HSCT), which replaces a patient's hematopoietic system. Allogeneic HSCT is used to treat hematological malignancies such as acute lymphoblastic leukemia and chronic myeloid leukemia, bone marrow failure, and hemaglobinopathies such as sickle cell anemia.[18] Stem cells are collected either from the bone marrow of the donor, or the peripheral blood after the stem cells are mobilized from the bone marrow to the blood by treating the donor with a growth factor. Graft-versus-host disease (GVHD) is a common complication following allogeneic HSCT that occurs when the donor T cells recognize the recipient's tissues as foreign and launch an immunological attack against the host.[19] Acute and chronic forms have been identified, with the former occurring in the first 100 days post-transplant with inflammatory components, and the latter occurring after, with a closer resemblance to autoimmune and fibrotic processes. The acute GVHD cascade is thought to begin with tissue damage from the pre-transplant conditioning regimen, leading to the activation of antigen-presenting cells and innate immune cells, causing CD4+ T cell activation and the precipitation of a cytokine storm causing a greater activation of this inflammatory environment, leading ultimately to damage of the skin, lungs, liver and gut by effector T cells, natural killer cells, macrophages and cytokines. Chronic GVHD is dependent on the T helper 2 population, with features of thymus damage and the production of pro-fibrotic cytokines leading to tissue fibroblast activation, B cell dysregulation, and low numbers of Tregs.[20] These fibrotic changes affect the mucosa, skin, lungs, kidneys, liver and gut.[19,20] However, in the clinical HSCT-setting, a balance must be achieved between GVHD and the graft-versus-leukemia (GVL) effect, which relies on the donor's alloreactive T cells in the graft to destroy the remaining rogue cancer cells in the recipient following myeloabalative, or in some cases, non-myeloablative preparative regimens. Thus, an ideal treatment for GVHD will not diminish the GVL effects, despite both being T cell mediated.[21]

Steroids are the current mainstay of GVHD treatment, but they come along with ample side effects including increased risk of infection, and some cases may ultimately be steroid-refractory;[20] therefore, identification of a targeted immunosuppressive therapy with minimal side effects would greatly improve morbidity and mortality in patients post-HSCT.[22]

T cells have been identified as the prime targets in GVHD therapy and prophylaxis. Clinical trials of antibodies and small molecules, for example, have attempted to dampen T cell responses through depletion with anti-thymocyte globulin and inhibit T cell proliferation with mTOR inhibitors such as rapamycin.[23,24] Cellular therapies for GVHD have also been considered, including mesenchymal stem cell infusions and regulatory T cell infusions.[20] Yet, MDSCs present themselves as an ideal target for therapeutic manipulation in the HSCT setting. As immature myeloid cells, they are found in the collected stem cell graft (either bone marrow or peripheral blood) that is administered to the patient.[14,25] Infusing or expanding in the recipient an MDSC

population with immunosuppressive activity may help to inhibit the alloreactive T cell responses in acute GVHD and increase numbers of Treg cells in chronic GVHD.

The impact of MDSC on GVHD outcomes has been studied in murine models of HSCT. Adoptive transfer of embryonic stem cell-derived MDSCs was found to prevent GVHD.[15] In a different study, when functional granulocyte colony-stimulating factor (G-CSF)-induced MDSCs isolated from donor spleens were added to the administered graft, GVHD was alleviated; when MDSCs were depleted *in vivo* in transplant recipients, GVHD was aggravated.[26] These results suggest that MDSC therapy in the setting of HSCT is a promising treatment for GVHD, raising the question of how to optimally expand and license an MDSC population to inhibit the development of GVHD while preserving GVL effects.

In order to discuss an MDSC population that would have a clinically significant impact on GVHD, it is important to examine what is currently known about MDSCs in the clinical HSCT setting. Our group has previously reported on MDSC recovery post-allogeneic HSCT, and showed that functional MDSC recovery occurs early (between 2 and 4 weeks), before lymphocyte recovery, demonstrating that this population is present and could be a target for GVHD therapy.[27] Clinically, peripheral blood stem cell grafts are collected from donors following treatment with growth factors granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), both of which are known to expand the MDSC population.[28] In a study of G-CSF mobilized PBSC donors, G-MDSC and M-MDSC were found to be expanded in the peripheral blood of donors and were able to suppress T cell proliferative *in vitro*, with the sorted M-MDSC showing a greater suppression rate than the G-MDSC.[25] Bone marrow grafts are not typically treated with growth factor before transplantation; however, patients may receive growth factor post-transplantation in order to promote engraftment and reconstitution of myeloid immune cells.[29] Recipients of peripheral blood stem cell transplants (PBSCTs) may also receive growth factor post-transplant. The treatment of donors with G-CSF before bone marrow harvest has been shown to reduce the incidence of acute (grades III-IV) and chronic GVHD in some instances, with similar rates of overall and disease-free survival.[30,31] This may be due to the expansion of an immunosuppressive MDSC population following the growth factor treatment, resulting in the attenuation of GVHD development. This presents an opportunity in the clinical setting of HSCT to treat either the donor or the recipient with a growth factor that will not only promote engraftment and immune recovery, but also stimulate an immunosuppressive MDSC population that can help to attenuate or prevent GVHD.[10]

We aimed to identify a growth factor that optimally expands and licenses bone marrow-derived MDSCs for inhibition of T cell proliferation and suppression of T cell function in a murine model, comparing the clinically used growth factors G-CSF and GM-CSF, as well as M-CSF, which is not currently used in the setting of HSCT in North America. G-, GM-, and M-CSF are hematopoietic growth factors that act on the granulocytic and monocytic myeloid progenitors. GM-CSF is thought to act on more primitive progenitor cells, committed precursor cells, and lineage committed cells, whereas G-CSF and M-CSF are thought to act on committed precursors and lineage committed cells; more than one growth factor may act on a lineage.[18,32,33] These growth factors have multiple functions beyond the induction the proliferation of new cells, including promoting survival, inducing differentiation and maturation, and functional stimulation,[33] and have unique features that may induce MDSC populations with distinct suppressive abilities. GM- and M-CSF receptors have different structures; however, both

target the macrophage lineage and lead to an expansion of this cell population.[34] Both have also the ability to induce granulocyte proliferation.[33] Most notably, G-CSF increases neutrophil counts, for which it is used clinically in neutropenia and post-transplant to encourage engraftment and white cell recovery.[29] These growth factors can be produced in peripheral organ tissue by many different cell types including endothelial cells, fibroblasts, and smooth muscle cells.[33–35] *In vivo,* there is a proposed cross-talk between these three hematopoietic growth factors in inflammatory states due to the communication between the different cell types that produce and respond to these growth factors, such as macrophages, neutrophils, immature progenitor cells, and non-hematopoietic cells.[34]

Materials & Methods

An approved animal protocol was used for the experiments listed below (Ethics # HS16600)

1. Growth factor dose titration: Bone marrow was harvested from the tibias and femurs of female C57BL/6 mice (aged 6 weeks; source: GMC). Following red cell lysis (BD Pharm Lyse, BD Biosciences), cells were counted and plated ($2x10^6$ cells per well) in 1 ml complete RPMI (RPMI 1640 with HEPES and L-glutamine, Lonza, Walkersville, MD; penicillin/streptomycin, Amresco LLC, Solon, OH; fetal bovine serum). Triplicate wells were treated with one growth factor (murine G-CSF, murine GM-CSF, or murine M-CSF; Peprotech, Rocky Hill, NJ) in two-fold serial dilutions from 80 ng/ml to 10 ng/ml alongside a control group that received no growth factor treatment. Cells were placed in incubator (37^oC/5% CO_2) on day 0, counted and tested for viability via trypan blue exclusion assay, stained with fluorescent antibodies for surface markers CD11b, Ly6G, and Ly6C (BioLegend, San Diego, CA) and Gr-1 (eBioscience, San Diego, CA) and analyzed by flow cytometry, on days 2, 3, 4, and 5.

2. Carboxyfluorescein succinimidyl ester (CFSE)-labeled bone marrow in vitro proliferation assay: Bone marrow was harvested from the tibias and femurs of female C57BL/6 mice (aged 6-8 weeks; source: GMC). Following red cell lysis, cells were counted and stained with CFSE. Triplicate wells were treated with murine growth factors according to previously titrated doses (20 ng/ml G-CSF, 10 ng/ml GM-CSF, 20 ng/ml M-CSF) alongside a control group that received no growth factor treatment in completed RPMI-1640. Cells were incubated for 4 days (37^oC/5% CO_2), then stained with fluorescent antibodies to surface markers CD11b and Gr-1, and analyzed by flow cytometry.

3. Co-culture of generated MDSCs and CFSE-labeled splenocytes to assess T cell proliferation: Bone marrow was harvested from the tibias and femurs of female C57BL/6 mice (aged 6-8 weeks; source: GMC). Following red cell lysis, cells were counted, plated, and treated murine growth factors according to previously titrated doses (20 ng/ml G-CSF, 10 ng/ml GM-CSF, 20 ng/ml M-CSF) alongside a control group that received no growth factor treatment in complete RPMI-1640, and incubated for 4 days (37^oC/5% CO_2). On day four, splenocytes were harvested from a second group of female C57BL/6 mice (aged 6-8 weeks; GMC) and labeled with CFSE. To assess the suppressive function of the generated MDSCs on CD4+ T cell proliferation, the CFSE-labeled splenocytes were then co-cultured with the growth factor-treated bone marrow that was harvested at day 4; these cells were counted and viability was assessed using a trypan blue exclusion assay. Cells were co-cultured at ratios 1:1 and 1:0.5

(splenocyte:MDSC) and added to a plate pre-coated with stimulatory murine anti-CD3 antibody and anti-CD28 antibodies (Biolegend, San Diego, CA) and then incubated (37°C/5% CO_2) for 5 days. Cells were then harvested (day 9) and stained with fluorescent antibodies to surface antigens CD3 and CD4, and analyzed via flow cytometry. Controls consisted of CFSE-labeled splenocytes stimulated with anti-CD3/CD28 without co-culture with MDSC. Suppression was calculated using the following formula:

$$\% \text{ Suppression} = 100 - \left(\frac{\% \text{ CFSE proliferation of CD4+ cells in co-culture}}{\% \text{ CFSE proliferation of CD4+ cells in stimulated control}}\right) \times 100$$

4. Co-culture of generated MDSCs and splenocytes to assess Treg cell proliferation: Murine bone marrow was treated with growth factor for 4 days in vitro and then co-cultured with murine splenocytes with anti-CD3 and anti-CD28 stimulation as described above in part 3 in co-culture ratios of 1:0.8, 1:0.4, and 1:0.2. On day 9, cells were harvested and stained with fluorescent antibodies to surface markers CD11b, CD4, and CD25 (eBioscience, San Diego, CA) and intracellular protein FOXP3 (eBioscience), and analyzed by flow cytometry. The increase in the Treg populations in the co-cultures was measured in proportion to the number of Treg cells found in the splenocytes with anti-CD3/CD28 stimulation (control) in the absence of co-culture using the following formula:

$$\text{Relative increase in \% Treg in population} = \frac{\% \text{ CD4+CD25+Foxp3+ Treg in co-culture}}{\% \text{ CD4+CD25+Foxp3+ Treg in control}}$$

5. Co-culture of generated MDSCs and CFSE-labeled splenocytes with inhibitors to key mediators in suppressive mechanisms: Bone marrow cells were harvested from female C57BL/6 mice (aged 6-7 weeks; source: GMC) and incubated for 4 days with growth factor treatment as described above in section 3. On day 4, splenocytes were harvested from a second group of mice and labeled with CFSE. To assess the suppressive function of the generated MDSCs on T cell proliferation, the CFSE-labeled splenocytes were then co-cultured with the growth factor-treated bone marrow that was harvested at day 4; these cells were counted and viability was assessed using a trypan blue exclusion assay. Cells were co-cultured at ratios 1:0.5 (splenocyte:MDSC) and stimulated with plate pre-coated with anti-CD3/CD28 Mouse T cell Activator Dynabeads (Life Technologies, Burlington, ON) then incubated (37°C/5% CO_2). Co-cultures of each growth factor experimental group as well as no treatment and fresh bone marrow controls were treated with inhibitors to enzymes iNOS (Aminoguanidine HCl, Cayman Chemical Co., Ann Arbor, MI; high concentration: 1 mM, low concentration: 0.3 mM), Arg-1 (N-hydroxy-L-arginine, Bachem, Bubendorf, Switzerland; high concentration: 320 uM, low concentration: 90 uM), IDO (1-methyl-dl-tryptophan, Sigma-Aldrich, St. Louis, MO; high concentration: 0.95 mM, low concentration: 0.15 mM), and COX-2 (Celecoxib, Focus Biomolecules, Plymouth Meeting, PA; high concentration: 30 uM, low concentration: 3 uM). On day 9, murine recombinant interleukin-2 (Peprotech) was added to each well to a concentration of 0.3 ng/ml to induce cell proliferation. On day 11 of this experiment, cells were harvested and stained with fluorescent antibodies to surface antigen CD4 (eBioscience, San Diego, CA) and analyzed via flow cytometry. The suppression rate was calculated relative to the proliferation of CFSE-labeled splenocytes with anti-CD3/CD28 stimulation in co-culture with the no growth factor treatment control. Suppression was calculated using the following formula:

$$\% \text{ Suppression} = 100 - \frac{(\% \text{ CFSE proliferation of CD4+ cells in co-culture with inhibitor})}{(\% \text{ CFSE proliferation of CD4+ cells in co-culture control})} \times 100$$

6. Analysis of cell surface and intracellular markers of growth factor-treated murine bone marrow: Bone marrow was harvested from the tibias and femurs of female C57BL/6 (aged 6 weeks; source: Charles River). Cells were treated with 20 ng/ml G-CSF, 10 ng/ml GM-CSF, or 20 ng/ml M-CSF and incubated ($37^oC/5\%$ CO_2) for 4 days. Then, cells were harvested and stained with fluorescent antibodies to surface antigens Gr-1, PD-L1 (Biolegend), CD11b (eBioscience) and intracellular markers Arg-1 (R&D Systems, Minneapolis, MN) and iNOS (Santa Cruz Biotechnology, Dallas, TX), and analyzed by flow cytometry.

8. Morphologic analysis: Cytospin slides were prepared by spinning harvested growth factor treated cells generated using the method described above (section 3) and control cells onto glass microscope slides. Cells were stained using Hema 3 stain, a Wright-Giemsa analog, according to manufacturer's instructions (Fisher Healthcare, Pittsburgh, PA). Images were obtained using an EVOS XL Core Imaging microscope at 20x magnification.

Data capture and analysis**:** Flow cytometry data was captured using a BD FACSCanto-II Flow Cytometry Analyzer System (3 laser) with the exception of experiment 4, which was analyzed using a Beckman MoFloXDP Analyzer (4 laser). Flow cytometry data was analyzed using the cell analysis software FlowJo. Statistical analysis was completed with GraphPad Prism (version 6.0e) to run one-way ANOVA tests to detect differences among group means (G-, GM-, M-CSF, and no growth factor treatment) in conjunction with Tukey's range tests to compare pairs of means as a post-hoc analysis (CI 95%, results considered significant when $p < 0.05$). Two-way ANOVA tests in conjunction with Tukey's range tests were utilized when two independent variables were introduced to treatments in the case of co-culture experiments (growth factor treatment and splenocyte-MDSC ratio) (CI 95%, results considered significant when $p < 0.05$).

Results

Growth factor doses were titrated to 10 ng/ml GM-CSF and 20 ng/ml G- or M-CSF following 4 days of in vitro culture of the murine bone marrow cells based upon the high numbers of live MDSCs generated and cell viability maintained at these doses (Table 1, data for other doses not shown). At these doses, GM-CSF treatment yielded the highest overall mean cell number and MDSC number, M-CSF treatment led to the greatest cell viability. G- and GM-CSF treatment lead to a significant increase in the percentage of the granulocytic CD11b+Ly-6G+ MDSC population; there was no significant difference found in between CD11b+Ly-6G+ percentages in the M-CSF and no growth factor populations (Figure 2, Table 2). M-CSF treatment led to the greatest significant increase in the monocytic CD11b+Ly-6C+ MDSC population of all growth factor treatments compared to no growth factor treatment, with a statistically significant difference found between the G- and GM-CSF treatments and M-CSF treatments as well (Figure 2, Table 2).

Light microscopy of stained prepared slides of the bone marrow-derived MDSCs appeared to show increased prevalence of immature granulocytes in following G-CSF and GM-CSF treatment, along with cultured no growth factor treatment control. GM- and M-CSF treatment appeared to show an increased prevalence of monocytic cells (Figure

3, Table 3). Cell proliferation after 4 days as measured by the dilution of intracellular CFSE upon cell division showed that growth factor treatment induced significant cell proliferation compared to no growth factor treatment (Figure 4). There was a statistically significant difference in % proliferation found between each growth factor treatment and the no growth factor treatment control. Taken together, 4 days of growth factor treatment expanded the number of MDSCs, maintaining high cell viability and proliferation, when compared to no growth factor treatment.

MDSCs induced by different growth factors can suppress the proliferation of CD4+ T lymphocytes in dose-dependent manner, of which M-CSF-induced MDSC showed greatest suppressive functions (Figure 5, Table 5). This was measured by CFSE dilution with successive cell divisions, as shown by the representative histograms of CFSE intensity for each treatment group; cell proliferation was evident in all treatment groups except for the M-CSF-induced MDSC co-culture which shows a single distinct peak (Figure 5). Table 5 lists the p values comparing groups using Tukey's multiple comparisons test revealing statistically significant differences between all pairs in both 1:1 and 1:0.5 co-culture ratios. GM-CSF-induced MDSCs exhibited stronger suppressive function compared to G-CSF-induced MDSCs which was found to be statistically significant (Table 5).

Co-culture of CFSE-labeled splenocytes plus M-CSF-induced MDSCs also showed a greater amount of proportional Treg expansion compared to G-, GM-CSF, and no growth factor treatment (Figure 6). No significant difference was found in Treg expansion in G- or GM-CSF-induced MDSCs compared to the no growth factor treatment control co-culture at either ratio (Table 6), however a statistically significant difference was found between M-CSF and no growth factor treatment groups at both ratios.

To evaluate whether MDSC expanded by growth factors suppress T cell proliferation via Arg-1, iNOS, IDO-1 or PGE2 mediated mechanisms, two different concentrations of inhibitors of these mediators were added to the splenocyte-MDSC co-cultures. The addition of inhibitors to enzymes iNOS, Arg-1, IDO-1, and COX-2 in splenocyte-MDSC co-cultures revealed some information about the suppressive mechanisms of the MDSC populations induced by different growth factors. Table 7 summarizes the results of the one-way ANOVA tests and Tukey's multiple comparisons tests for inhibitor treatments that appeared to have some reversal of suppressive function. The suppression rate was calculated compared to the suppression found in the splenocyte-no treatment MDSC control. Inhibition of Arg-1 appears to reverse the suppressive function of G-CSF-induced MDSCs, indicating that this mechanism is important to its suppressive function (Figure 7a); it is only statistically significant when comparing the high Arg-1 inhibitor dose to the no inhibitor group (Table 7). Inhibitors to iNOS appeared to decrease the suppressive function of GM-CSF-induced MDSCs in a dose-dependent manner, with a similar but less pronounced effect noted compared to that of the Arg-1 inhibitor, indicating that GM-CSF-induced MDSCs may exert their suppressive functions via both pathways, however only comparisons to iNOS inhibitor treatments and no inhibitor treatment revealed statistically significant results. As well, COX-2 inhibitor celecoxib, which inhibits the production of PGE2, appeared to reverse the suppressive effect of GM-CSF-induced MDSCs in a dose-dependent manner (Figure 7b) although it was not found to be statistically significant (Table 7). Addition of inhibitor of the enzyme iNOS, aminoguanidine, to splenocyte-MDSC co-culture appears to reverse the suppressive function of M-CSF-induced MDSCs in a dose-dependent

manner. Inhibitors to IDO-1 (1-methyl-dl-tryptophan), COX-2 production of PGE-2 (celecoxib) and Arg-1 (N-hydroxy-L-arginine) did not appear to have an effect on the suppressive functions of M-CSF-induced MDSCs (data not shown).

The expression of programmed death ligand-1 (PD-L1), an important surface molecule for T cell inhibition, was evaluated on MDSCs after 4 days of growth factor treatment through flow cytometric analysis. PD-L1 expression was dramatically increased in treatment group GM-CSF compared to all others (Figures 8a and 8b). G-CSF treatment also induced an MDSC population with PD-L1 expression that was higher than the M-CSF and no growth factor treatment groups (Figures 7a and 7b). However, there was also a statistically significant difference between the G- and GM-CSF treatment groups in their levels of PD-L1 expression, with expression being much higher in the GM-CSF treatment group. Additionally, flow cytometric analysis revealed an increase in iNOS expression in M-CSF induced MDSCs compared to other treatment groups and control groups (Figure 8c), which is consistent with the results of the inhibitor assay. A second peak of fluorescence is seen on the histogram of anti-iNOS dye conjugated antibody intensity in the M-CSF treatment group, which is not present in any others.

Discussion

Myeloid-derived suppressor cells are an interesting target for the inhibition and treatment of GVHD due to their presence in the patient pre-transplant, the harvested graft, and in the patient post-transplant.[27,36,37] Here we present a method of inducing murine MDSC proliferation and activation *in vitro* with M-CSF, a growth factor that expands the monocyte-macrophage cell population in normal physiological conditions. This MDSC population induced by M-CSF is more potent in suppressing CD4+ T cell proliferation and inducing Treg proliferation, at rates higher than the populations produced by the clinically used G- and GM-CSF. M-CSF is used clinically in Japan to treat neutropenia and post-HSCT, and has been shown in a retrospective analysis to attenuate the severity of chronic GVHD compared to patients who did not receive growth factor treatment post-bone marrow transplant (BMT).[38] Treatment with M-CSF pre-transplant in a mouse model has been found to ameliorate acute GVHD post-transplant through a proposed model of host macrophages engulfing allogeneic T cells.[39] MDSCs may then play a role in GVHD suppression in the murine model in the setting of M-CSF treatment based upon our results. Other settings where M-CSF treatment has been considered or investigated include in melanoma and fungal diseases.[40]

We also found that GM-CSF-induced MDSCs could more strongly suppress CD4+ T cell proliferation compared to G-CSF-induced MDSCs (Figure 5). In the clinical setting of HLA-matched sibling PBSCT, administration of GM-CSF to the donor prior to graft harvest showed a reduced risk of acute grade II-IV GVHD compared to G-CSF or G- and GM-CSF treatments, with similar rates of hematopoietic recovery and no difference in relapse risk or overall survival.[41] Perhaps this is due to a different MDSC population with stronger suppressive being present in the GM-CSF-mobilized graft.

The ability of the monocytic-predominant M-CSF-induced MDSC population to expand the Treg population is an important part of their overall immunosuppressive function. MDSCs have been found to induce Treg expansion *in vitro* and *in vivo* in mouse models.[42] In the renal transplant setting, monocytic MDSCs isolated from transplant recipients were able to expand Tregs in vitro, which corresponded to an

expansion of Tregs in vivo over time. These cells were also capable of suppressing CD4+ T cell proliferation in mixed lymphocyte reactions.[43] As well, high levels of Treg cells in hematopoietic stem cell grafts has been found to be associated with lower levels of acute GVHD and increased overall survival compared to grafts containing low levels of Tregs, in patients who received myeloablative conditioning.[44] The ability of this MDSC population to strongly suppress CD4+ T cell proliferation while promoting Treg expansion reveals that this population may be able to prevent the development of both acute and chronic GVHD, respectively.

Despite the ability of M-CSF to generate a strongly suppressive MDSC population *in vitro*, there may be some challenges to overcome concerning its use *in vivo.* Alexander et al. noted that M-CSF-dependent donor-derived macrophages are involved in mediation of chronic graft-versus-host disease in a mouse model. F4/80$^-$ macrophages with donor M2-like (alternatively activated) phenotype expressing the M-CSF receptor infiltrated the skin of mice receiving G-CSF-mobilized and non-mobilized grafts that developed cutaneous chronic GVHD. Treatment with M-CSF post-transplant exacerbated cutaneous GVHD in this murine model. Depletion of macrophages with a monoclonal antibody against the M-CSF receptor reduced cutaneous and pulmonary chronic GVHD.[45] This relationship between cutaneous chronic GVHD and M-CSF treatment would have to be further explored. Although the cells we studied are immature cells, they may have the capacity to further differentiate to macrophages *in vivo* with this M-CSF treatment leading to the development of cutaneous GVHD pathology. Blazar et al. showed in a mouse model that continuous infusion of recombinant human M-CSF after graft administration in lethally irradiated mice led to a significant decrease in donor cell engraftment compared to control mice receiving a continuous saline infusion, dependent on the presence of host natural killer cells with anti-donor activities.[46] These effects on chronic GVHD development and engraftment may be overcome by treating grafts or donors with M-CSF pre-transplant or avoiding continuous or chronic M-CSF treatment in the recipient; this might allow the development of a suppressive MDSC population but reduce the risk of chronic GVHD development and decreased in cell engraftment.

Growth factor treatment also affected the expression of cell surface marker PD-L1. PD-L1, a ligand of the programmed death 1 receptor, acts with its receptor as a co-inhibitory signal of T cell activation, promoting immune tolerance.[47] GM-CSF-induced MDSCs showed an increase in PD-L1 expression compared to all other treatment groups (that were also found to express comparatively small amounts of PD-L1, Figures 8a and 8b), indicating the PD-1/PD-L1 pathway may play a special role in the suppressive activity of this GM-CSF-induced MDSC population. Treatment of a splenocyte-GM-CSF-induced MDSC co-culture with a PD-L1 inhibitor in future experiments will reveal if it is a key player in suppression in this MDSC population. In a murine model, the programmed death-1 (PD-1) pathway was found to be upregulated in the development of chronic GVHD, and a blockade of the pathway with anti-PD-1, anti-PD-L1 or anti-PD-L2 monoclonal antibodies exacerbated the GVHD, whereas stimulation of the pathway alleviated chronic GVHD.[48] Therefore, this GM-CSF-induced MDSC population may have a role in stimulating the PD-1 pathway in the treatment of chronic GVHD.

Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is an inhibitory receptor mainly expressed on T cells that acts as a downregulator of T cell activation, though its expression is not solely restricted to the lymphoid lineage.[49,50] It was found to be present

in all MDSC groups studied here, however its expression was not increased in any growth factor treatment group over the others in a statistically significant manner (data not shown). CTLA-4 therefore may play a role in the suppressive functions of these MDSCs, but is most likely not responsible for the differences in suppressive functions among the different growth factor-induced populations. Neither CTLA-4 nor PD-L1 was expressed in a significantly greater amount in the M-CSF-induced MDSC population compared to the other treatment groups, suggesting that while they may play a role, they are not responsible for the increased suppressive activity of this population compared to the MDSCs induced by other growth factors.

The mechanism of suppression by intracellular enzymes in these MDSC populations was also analyzed. Flow cytometric analysis showed an increase in iNOS expression in M-CSF-induced MDSCs as a second peak of fluorescence of the stain to iNOS in the flow cytometry histogram, indicating that there might be one cell type in the mixed M-CSF-induced MDSC population that expresses higher levels of this enzyme that is not present in the other treatment groups (Figure 8c). The importance of this enzyme and pathway to this population's suppressive function was confirmed when there was a reversal of the suppressive function in a dose-dependent manner with iNOS inhibitor treatment, which is a main suppressive mechanism of M-MDSCs, which make up the majority of the M-CSF-induced MDSC population (Figure 7c). The reversal of suppressive activity with Arg-1 inhibition in the G-CSF-induced MDSC population is consistent with literature reports; Highfill et al. demonstrated that *in vitro* generation of a CD11b+Ly-6G^{low}Ly-6C+ MDSC population by G- and GM-CSF could inhibit GVHD in a mouse model via an Arg-1 dependent mechanism when infused at time of allogeneic HSCT, while preserving the graft-versus-leukemia effect.[51] In MDSCs isolated from two mouse T cell lymphoma models, distinct G-MDSC and M-MDSC populations were identified, with the M-MDSC population acting through a NO-dependent pathway.[8] Overall, the findings of the suppressive mechanisms of these two growth factor-induced MDSC populations are consistent with reports in the literature of the suppressive mechanisms of the G-MDSC and M-MDSC subsets in mice, given that G-CSF preferentially expands the granulocytic subset and M-CSF expands the monocytic subset.[8] GM-CSF-induced MDSCs were found to act through both an iNOS-dependent mechanism based on the reversal of suppressive activity with the addition of inhibitor in a dose-dependent manner that was found to be statistically significant (Figure 7b). This MDSC population's suppressive activity also appeared to be reversed in a dose-dependent manner with the addition of Arg-1 and PGE2-COX-2 inhibitors but this was not found to be statistically significant (Figure 7b). The use of both iNOS and Arg-1 mechanisms by GM-CSF-induced MDSCs is expected, because as the name of the growth factor implies, it acts to expand both granulocytic and monocytic subsets.

Inhibitor to enzyme indoleamine 2,3-dioxygenase (IDO-1) did not appear to reverse the suppressive effect of G-, GM-, or M-CSF induced MDSCs (data not shown), however, evidence in the literature suggests it plays some role in the suppressive effects of MDSCs. In the allogeneic HSCT setting, it was found that MDSCs from patients post-transplant inhibited the proliferation of T cells, which was reversed after blocking IDO-1 activity with 1-methyl-dl-tryptophan.[52,53] The addition of the COX-2 inhibitor celecoxib to reduce the production of its product PGE2 appeared to induce a small reversal in the suppressive function of the GM-CSF-induced MDSC population in a dose-dependent manner, although it was not found to be statistically significant. PGE2 has been shown to have a role in MDSC generation and PGE2-COX-2 interaction induces the expression of iNOS and IDO.[9,53] Further inhibitor dose titration is required in this case to definitively

rule out the importance of PGE2-COX-2 and IDO to the suppressive effects of our M-CSF-induced MDSC population since we may not have seen a reversal of suppressive function due to the improper does of inhibitor being used. It will be important to repeat the inhibitor assay with all four inhibitors tested here as we have had trouble inducing adequate T cell stimulation *in vitro* during this experiment in all wells and treatment groups, with the stimulation leading to inadequate T cell proliferation in our no co-culture control group. Thus we had to compare proliferation in our suppression rate calculations to the co-culture between the splenocytes with no additional growth factor treatment MDSCs; this still showed that the growth factor treatment group MDSCs had greater suppressive functions compared to no growth factor treatment and that the addition of some inhibitors reversed this function, but we cannot quantitatively compare the suppression rates in this inhibitor assay to the other suppression rates reported here.

Here we report a predominantly monocytic subset of murine MDSCs induced *in vitro* with growth factor M-CSF with the ability to strongly suppress CD4+ T cell proliferation through an iNOS pathway and induce Treg proliferation. This population of M-CSF-induced MDSCs has stronger suppressive functions than the populations induced by the clinically used G- and GM-CSF. Exploration of the use of this growth factor clinically in the post-HSCT setting may reveal a new tool for the prevention and treatment of GVHD, either as a drug, or in the generation of an MDSC population that could be administered as a cellular therapy product.

Since human and mouse granulocytic and monocytic MDSCs may have different mechanisms of function,[27] it will also be important to confirm that M-CSF treatment produces a similar suppressive MDSC population in human bone marrow samples, although there is already clinical data to suggest M-CSF treatment post-transplant can attenuate GVHD.[38] Investigation of the ability of other growth factors and cytokines either known to induce MDSC expansion or help to reduce or ameliorate GVHD may also be warranted in the search for a factor that maximally expands and licenses a suppressive MDSC population with minimal toxicities and side effects.

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Tables & Figures

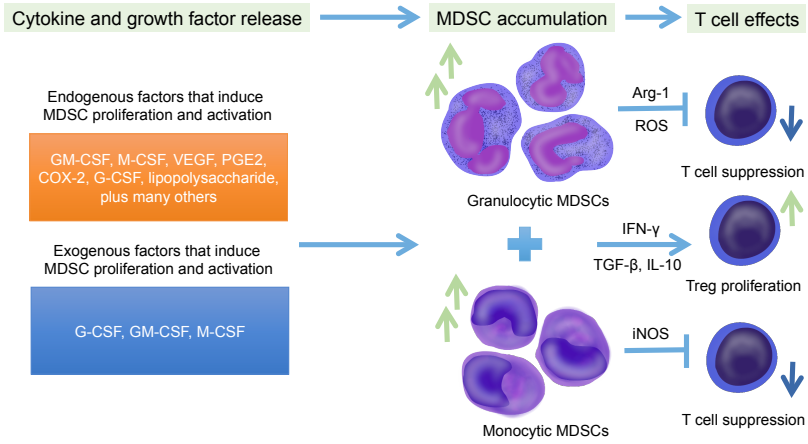

Figure 1. Induction of MDSC proliferation and activation in different settings via exposure to a variety of endogenous and exogenous cytokines and growth factors leading to cytotoxic T cell suppression and Treg proliferation

Table 1. Total cell number, viability, and MDSC (CD11b+Gr-1+) cell numbers following 4 days of *in vitro* growth factor treatment

	Mean cell number (x10^6)	Standard deviation	Viability (%)	Standard deviation	% MDSC	Standard deviation	MDSC number (x10^6)
G-CSF (20 ng/ml)	0.86	0.08	81.5	2.8	25.6	0.6	0.22
GM-CSF (10 ng/ml)	2.57	0.37	74.8	3.1	36.8	0.9	0.94
M-CSF (20 ng/ml)	1.07	0.18	89.1	1.0	34.8	1.6	0.37
No growth factor	0.65	0.11	63.7	4	39.0	1.2	0.25
Fresh bone marrow	--	--	--	--	43.0	11.1	--

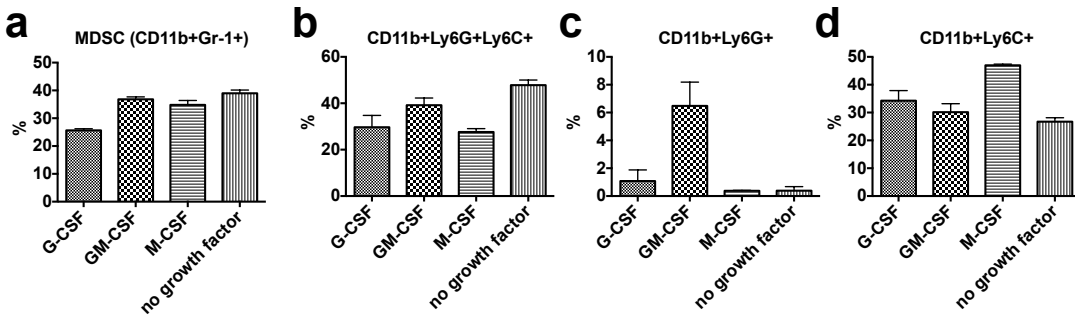

Figure 2. Characteristics of murine bone marrow after 4 days in vitro treatment with 20 ng/ml murine G-CSF, 10 ng/ml GM-CSF, or 20 ng/ml M-CSF, compared to no treatment (a) percentage of MDSCs (CD11b+Gr-1+) (b) percentage of CD11b+ cells that are Ly-6G+Ly-6C+ double positive (c) percentage CD11b+Ly-6G+ G-MDSCs (d) percentage of Ly-6C+ M-MDSCs. Results of statistical analysis of this data are summarized in Table 2.

Table 2. Results of one-way ANOVA tests and Tukey's multiple comparisons tests comparing MDSC populations induced by different growth factor treatments in Figure 2

	% MDSC (CD11b+Gr-1+)	% CD11b+Ly-6G+Ly-6C+	% CD11b+Ly-6G+	% CD11b+Ly-6C+
F value, one-way ANOVA	80.63	24.58	28.55	38.24
p value, one-way ANOVA	< 0.0001	0.0002	0.0001	< 0.0001
Adjusted p values (Tukey's multiple comparisons test)				
G-CSF vs. GM-CSF	< 0.0001	0.0300	0.0006	0.2577
G-CSF vs. M-CSF	< 0.0001	0.8620	0.8072	0.0011
G-CSF vs. no growth factor	< 0.0001	0.0006	0.8161	0.0244
GM-CSF vs. M-CSF	0.2123	0.0105	0.0002	0.0002
GM-CSF vs. no growth factor	0.1662	0.0457	0.0002	0.3833
M-CSF vs. no growth factor	0.0085	0.0003	> 0.9999	< 0.0001

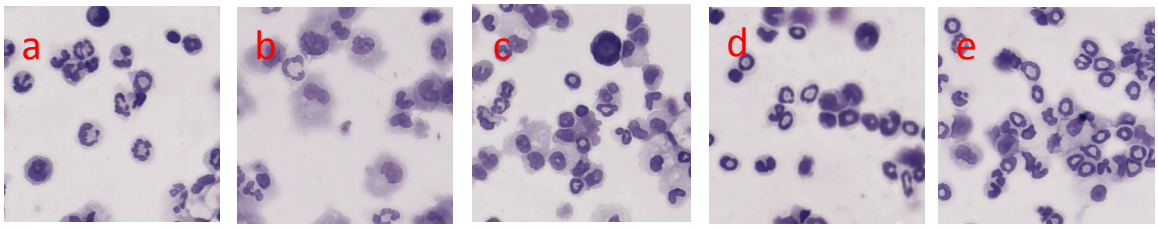

Figure 3. Cytospin preparation of murine bone marrow and bone marrow-derived MDSCs after 4 days of treatment, Wright-Giemsa stain, original magnification: 20x (a) G-CSF-induced MDSCs (b) GM-CSF-induced MDSCs (c) M-CSF induced MDSCs (d) no additional growth factor treatment (e) fresh bone marrow

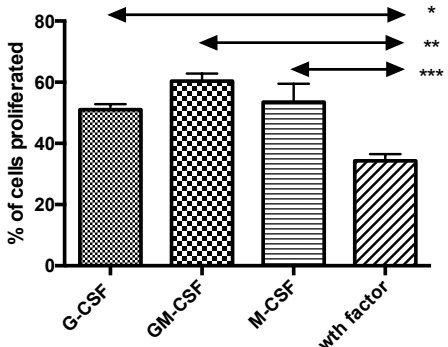

Figure 4. Proliferation of CFSE-labeled bone marrow cells following growth factor treatments. One-way ANOVA showed a statistically significant difference among the 4 groups ($F = 29.28$, $p < 0.0001$), Tukey's multiple comparisons test showed significant differences between all treatment groups and no growth factor treatment; * adjusted p value 0.0018; ** adjusted p value < 0.0001; *** adjusted p value 0.0007

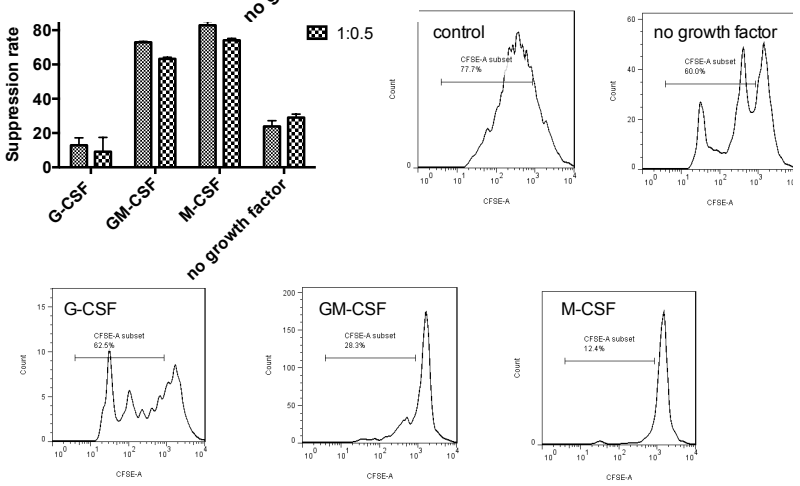

Figure 5. Suppressive functions at splenocyte-MDSC ratios of 1:1 and 1:0.5 with representative histograms of CFSE intensity in CD4+ lymphocytes after 5 days of co-culture at 1:1 ratio. Results of statistical analysis of this data are summarized in Table 5

Table 5. Results of Tukey's multiple comparisons tests (post-hoc following two-way ANOVA) comparing suppression rates depending on growth factor treatment and splenocyte-MDSC co-culture ratio

	1:1 splenocyte-MDSC	1:0.5 splenocyte-MDSC
Adjusted p values (Tukey's multiple comparisons test)		
G-CSF vs. GM-CSF	< 0.0001	< 0.0001
G-CSF vs. M-CSF	< 0.0001	< 0.0001
G-CSF vs. no growth factor	0.0104	0.0005
GM-CSF vs. M-CSF	0.0200	0.0373
GM-CSF vs. no growth factor	< 0.0001	< 0.0001
M-CSF vs. no growth factor	< 0.0001	< 0.0001

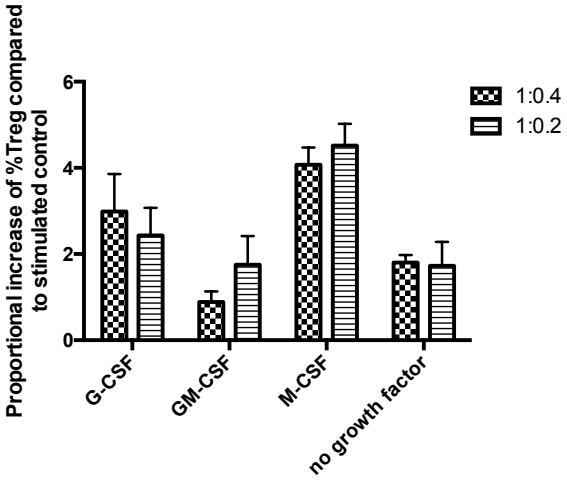

Figure 6. Treg (CD4+CD25+Foxp3+) expression in stimulated splenocyte-MDSC co-culture at splenocyte-MDSC ratios 1:0.4 and 1:0.2 reported as the relative increase in % Treg compared to the stimulated splenocyte control with no co-culture. Results of statistical analysis of this data are summarized in Table 6.

Table 6. Results of Tukey's multiple comparisons tests (post-hoc following two-way ANOVA) comparing % Treg proportional increases depending on growth factor treatment and splenocyte-MDSC co-culture ratio

	1:0.4 splenocyte-MDSC	1:0.2 splenocyte-MDSC
Adjusted p values (Tukey's multiple comparisons test)		
G-CSF vs. GM-CSF	0.0013	0.4498
G-CSF vs. M-CSF	0.1145	0.0014
G-CSF vs. no growth factor	0.0761	0.4203
GM-CSF vs. M-CSF	< 0.0001	< 0.0001
GM-CSF vs. no growth factor	0.2150	> 0.9999
M-CSF vs. no growth factor	0.0006	< 0.0001

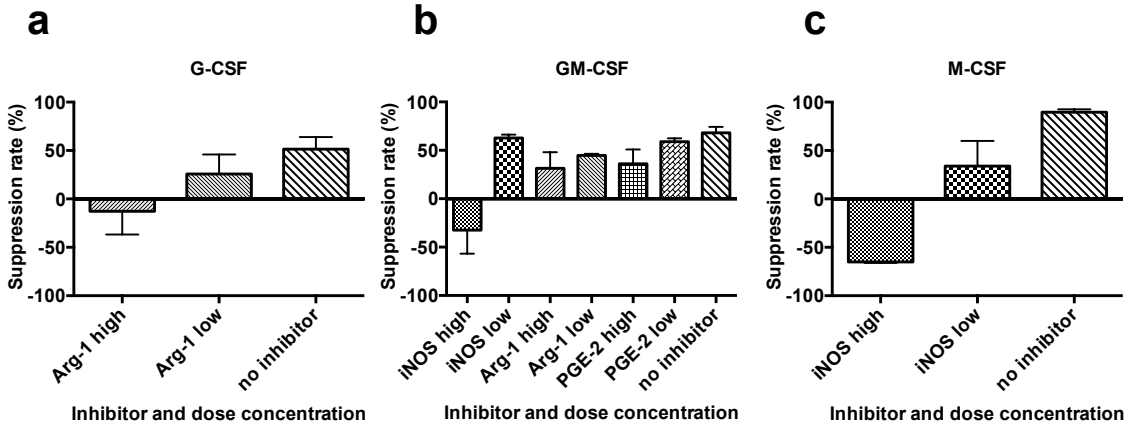

Figure 7. Suppression rates of CD4+ CFSE-labeled T cell proliferation following 7 days co-culture with growth factor-induced murine MDSCs compared to suppression of splenocyte-no treatment MDSC co-culture after treatment with inhibitors to important suppressive pathway enzymes (a) G-CSF-induced MDSCs show dose-dependent reversal of suppressive function with addition of Arg-1 inhibitor N-hydroxy-L-arginine (b) GM-CSF-induced MDSCs appear to show dose-dependent reversal of suppressive function with addition of iNOS inhibitor aminoguanidine, Arg-1 inhibitor N-hydroxy-L-arginine and COX-2/PGE2 inhibitor celecoxib (c) M-CSF-induced MDSCs show dose-dependent reversal of suppressive function with addition of iNOS inhibitor aminoguanidine. Results of statistical analysis of this data are summarized in Table 7.

Table 7. Results of one-way ANOVA tests and Tukey's multiple comparisons tests comparing % suppression after inhibitor treatments

	G-CSF	GM-CSF	M-CSF
F value, one-way ANOVA	8.395	15.90	131.5
p value, one-way ANOVA	0.0252	< 0.0001	< 0.0001
Adjusted p values (Tukey's multiple comparisons test)			
iNOS high vs. iNOS low	--	0.0002	0.0006
iNOS high vs. no inhibitor	--	< 0.0001	< 0.0001
iNOS low vs. no inhibitor	--	0.9995	0.0082
Arg-1 high vs. Arg-1 low	0.1655	0.9412	--
Arg-1 high vs. no inhibitor	0.0217	0.0988	--
Arg-1 low vs. no inhibitor	0.3857	0.5841	--
PGE-2 high vs. PGE-2 low	--	0.6087	--
PGE-2 high vs. no inhibitor	--	0.1788	--
PGE-2 low vs. no inhibitor	--	0.9897	--

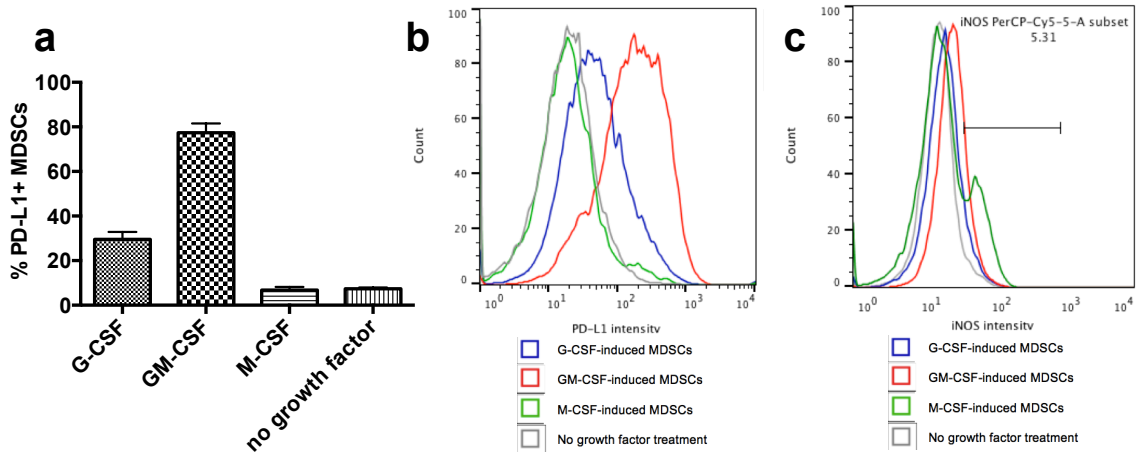

Figure 8. (a) % of CD11b+Gr-1+ MDSCs that express PD-L1 and histogram showing dye intensity of PD-L1 antibody in CD11b+Gr-1+ MDSCs; one-way ANOVA showed that there are statistically significant differences between treatment groups ($F = 429.1$; $p < 0.0001$) with Tukey's multiple comparisons test yielding adjusted p values < 0.0001 in all comparisons between 2 treatment groups except for M-CSF vs. no growth factor treatment (adjusted p value = 0.9947) (b) histogram showing fluorescence intensity of PD-L1 stain in CD11b+Gr-1+ MDSCs (c) histogram showing fluorescence intensity of iNOS stain in CD11b+Gr-1+ MDSCs