2762. Inhibition of murine myeloid suppressor cells increases CD8$^+$ T cell activation *in vitro*

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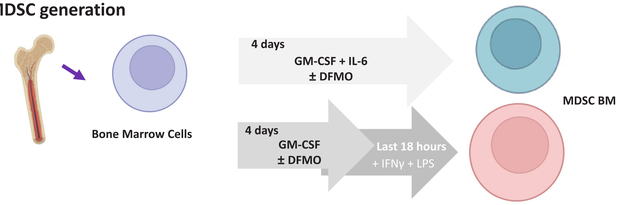
Introduction

- Tumor microenvironment (TME) is rich in cells that potentially inhibit T cell function, such as regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs).
- The purpose of this study was to develop an *in vitro* assay system to assess the ability of novel drugs to overcome MDSC-mediated immune suppression by monitoring changes to T cell function.
- Arginase 1 (ARG1), an enzyme that catalyzes L-arginine into L-ornithine and urea, is highly expressed in MDSCs. An ARG1 target, currently in clinical trials (INCB001158, CB-1158 from Calithera Biosciences), was used as a target of interest in this study.
- MDSC generation from bone marrow cells (MDSC BM) was compared using GM-CSF + IL-6 or GM-CSF + IFNγ + LPS.
- Therapeutics used in this study, α-Difluormethylornithine (DFMO) or N$^\omega$-hydroxy-nor-arginine (nor-NOHA), are 2nd generation ARG1 inhibitors that irreversibly bind ARG1, which leads to increased availability of L-arginine known to modulate T cell activity. DFMO was tested during MDSC generation from BM cells.
- Cytokines and other growth factors produced by MDSC BM have the potential to alter the activation state of CD8+ T cells; therefore, the effect of a suppressive TME on CD8+ T cells was monitored by co-culturing CD8+ T cells with MDSCs *in vitro*.

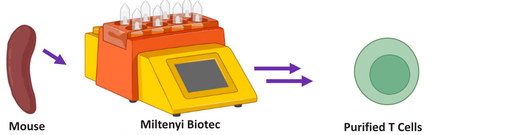
Methods

- Bone marrow cells and spleens were harvested from naïve BALB/cAnNHsd or C57BL/6NHsd mice.
- All animal work was performed in an AAALAC-accredited facility, in alignment with applicable animal welfare regulations.

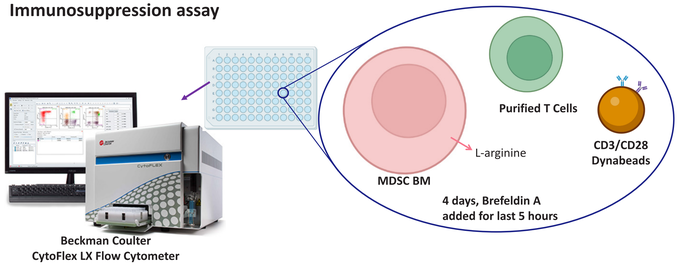
MDSC generation



T cell isolation



Immunosuppression assay



Myeloid-derived suppressor bone marrow cell characterization

- Flow cytometric analysis of MDSCs provided evidence that (1) GM-CSF + IFNγ + LPS treatment yielded higher ARG1 and iNOS expression compared to GM-CSF + IL-6 treatment, (2) DFMO reduced ARG1 expression and (3) this characterization was mouse strain independent for both C57BL/6NHsd and BALB/cAnNHsd mice.

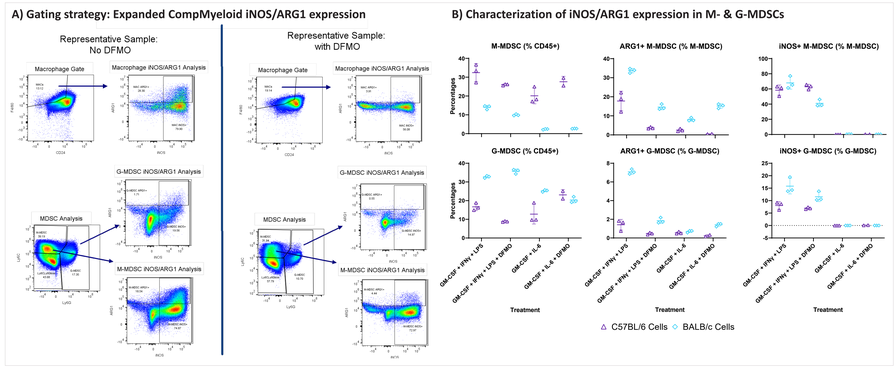


Figure 1. (A) Example gating strategy of GM-CSF + IFNγ + LPS treated naïve C57BL/6NHsd bone marrow cells analyzing macrophage, G-MDSC and M-MDSC iNOS and ARG1 expression with or without DFMO. (B) Graphs of iNOS and ARG1 expression in M- and G-MDSCs after treatment with either GM-CSF + IFNγ + LPS or GM-CSF + IL-6 in either naïve C57BL/6NHsd or BALB/cAnNHsd mice (n=2 or 3).

T cell activation (bead to T cell ratio optimization)

- CD4$^+$ and CD8$^+$ T cells were independently purified from C57BL/6NHsd or BALB/cAnNHsd splenocytes and characterized by flow cytometry. T cell activation was measured by proliferation (CellTrace™ Violet dilution), surface activation markers (CD69 and PD-1) and intracellular cytokines (IL-2, TNFα and IFNγ).
- T cell activation was dose dependent with DynaBeads® Human T-Activator CD3/CD28, where increasing amounts of beads resulted in increased activation of T cells.
- Activation of CD4$^+$ vs. CD8$^+$ T cells resulted in different profiles of activation for intracellular cytokines, with CD4$^+$ T cells releasing more IL-2, whereas CD8$^+$ T cells release more IFNγ.

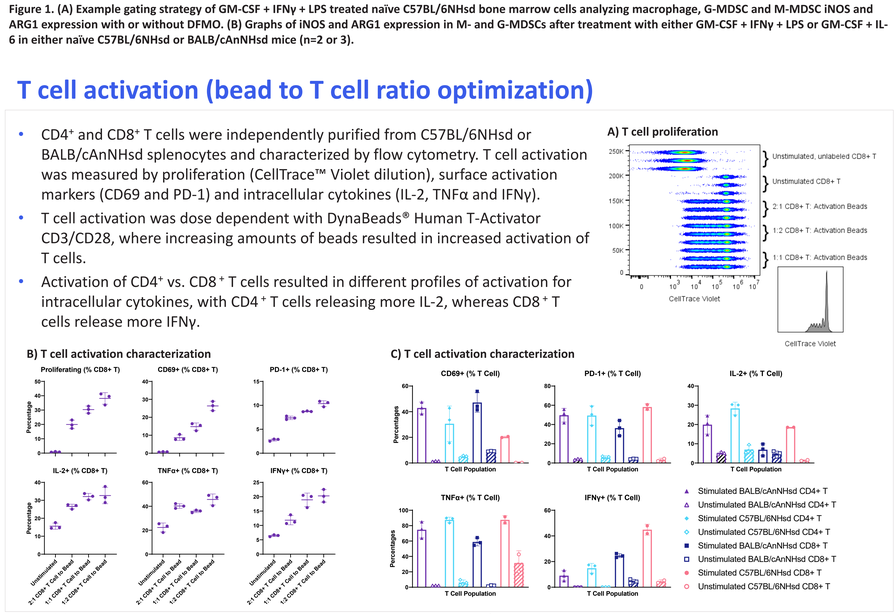


Figure 2. (A) Example CellTrace™ dilution in CD8$^+$ T cells stimulated for 4 days with anti-CD3/anti-CD28 antibody-coated beads at different bead to T cell ratios and (B) graphs characterizing CD8$^+$ T cells after stimulation. (C) Characterization of either unstimulated or stimulated CD4$^+$ or CD8$^+$ T cells.

Conclusions

- GM-CSF + IFNγ + LPS generated more reproducible MDSCs that express ARG1 than GM-CSF + IL-6.
- M-MDSC BM expressed 2 times more ARG1 as compared to DFMO cultured MDSC-derived BM.
- CD8$^+$ T cells co-cultured with DFMO-inhibited MDSC BM were more proliferative, had greater surface activation and greater intracellular cytokines as compared to when co-cultured with MDSC BM.
- Tregs did not inhibit CD8$^+$ T cell proliferation at the measured ratios, but CD8$^+$ T cell activation as measured by CD69 expression was inhibited.
- Overall, the ability to monitor changes in T cell activation following MDSC has broad applications in *in vitro* screening of novel drug compounds that alter the TME as well as sets the experimental framework for *ex vivo* analysis of cells isolated from TME following treatment.

Immunosuppression assays

- Flow cytometric analysis showed differences in CD8+ T cell activation after 4 days of co-culture with MDSC BM.

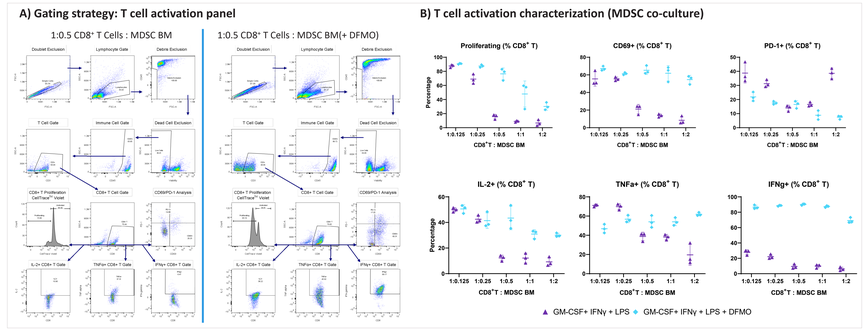


Figure 3. (A) Example gating strategy comparing 1:0.5 purified CD8+ T cell from naïve C57BL/6 mice to MDSC BM from naïve C57BL/6 mice (derived in GM-CSF + IFNγ + LPS ± DFMO, cytokines and drug were not included in immunosuppression assay). (B) Graphs characterizing T cell activation (n=3).

- Purified Tregs were co-cultured with purified CD8$^+$ T cells ± TNFα for 4 days and then CD8$^+$ T cell activation was measured.
- Flow cytometric analyses showed minimal increases in proliferation.
- Additionally, large increases in the activation marker CD69 were observed in both strains with the addition of TNFα. BALB/cAnNHsd CD8$^+$ T cells co-cultured with BALB/cAnNHsd Tregs generally were more activated as measured by CD69 compared to C57BL/6NHsd CD8$^+$ T cells co-cultured with C57BL/6NHsd Tregs.

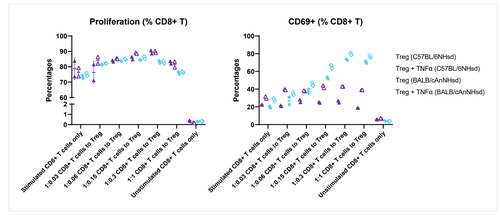


Figure 4. Graphs of CD8$^+$ T cell activation as measured by CellTrace™ violet dilution and CD69 expression when co-cultured with Tregs (n=2).

- IL-4 induces ARG1, whereas LPS + IFNγ induces iNOS expression in RAW 264.7 cells. CD8$^+$ T cell proliferation increased when co-cultured with RAW 264.7 cells treated with IL-4, whereas CD8$^+$ T cell proliferation decreased with IL-4 treated conditioned media.

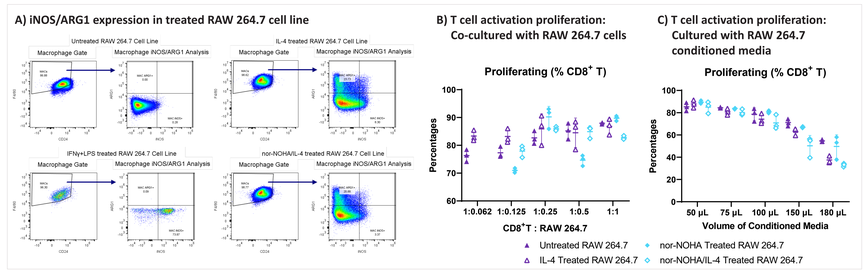


Figure 5. A mouse macrophage cell line, RAW 264.7 was cultured with LPS + IFNγ, IL-4 or IL-4 + nor-NOHA for 4 days. (A) iNOS and ARG1 expression was measured at the end of treatment. (B) Treated RAW 264.7 cells were co-cultured with purified BALB/cAnNHsd CD8$^+$ T cells for 4 days and CD8$^+$ T cell proliferation was measured. (C) Conditioned media from the various treatments were used to co-culture C57BL/6NHsd splenocytes for 4 days and the CD8$^+$ T cell proliferation was measured.

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