M-CSF and GM-CSF Influence Naïve Murine Alveolar Macrophage Differentiation and Function *in vitro*

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Abstract and Introduction

Alveolar macrophages (AMs) and pulmonary dendritic cells (DCs) are crucial to the immune response against *Mycobacterium tuberculosis* infection. Both types of cells require the presence of colony stimulating factors (GM-CSF and M-CSF) for their terminal cell differentiation. A recent study reported that during *M. tuberculosis* infection there is a progressive decrease in pulmonary M-CSF levels with a paralleled increase in the levels of GM-CSF*. It is well known that pulmonary GM-CSF is essential for alveolar macrophage and dendritic cell differentiation and functions; however the role of M-CSF activity in the lungs is not clear.

The objective of this study was to compare the effect of M-CSF and GM-CSF in their abilities to affect the functional and phenotypical characteristics of AMs.

In this study, we harvested naïve uninfected murine alveolar macrophages by bronchoalveolar lavage (BAL) and cultured in the presence of M-CSF, GM-CSF or both. GM-CSF stimulated cell proliferation at a higher rate when measured by relative loss of CFDA-SE dye and had higher phagocytic capacity than M-CSF cultured cells. In contrast, alveolar macrophages cultured in the presence of M-CSF exhibited a dendritic-cell-like morphology and upregulated expression of dendritic-cell-associated markers like CCR7, MHC II and DEC205. The dendritic cell character of M-CSF-treated AMs was also manifested in their higher capacity to stimulate CD4⁺ T cell proliferation in a mixed lymphocyte reaction (MLR). This study demonstrates that M-CSF has a strong ability to differentiate AMs into DC-like cells.

Common abbreviations used in this poster: **M-CSF** = macrophage-colony stimulating factor; **GM-CSF** = granulocyte macrophage-colony stimulating factor; **BAL** = bronchoalveolar lavage; **AM** = alveolar macrophage; **DC** = dendritic cell

*This research has been published in *Relative Levels of M-CSF and GM-CSF Influence the Specific Generation of Macrophage Populations during Infection with* Mycobacterium tuberculosis by Higgins, D et al. in *Journal of Immunology*, 2008, 180: 4892-4900. Many figures and data here are duplicated from this paper.

Methodology

To harvest alveolar macrophages, a **BAL** was performed on C57BL/6 mice after sacrifice by CO_2 asphyxiation. The cells from 5 mice were pooled and cultured for 5 days in complete RPMI medium containing 20ng/ml of **GM-CSF, M-CSF**, **both cytokines**, or **media alone** (**Figure 1**). Most cells (90-95%) obtained from the BAL were alveolar macrophages [1].

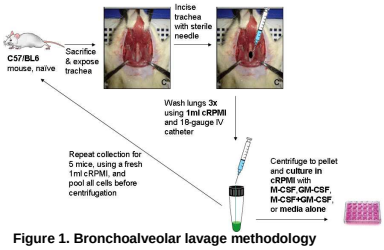


Figure 1. Bronchoalveolar lavage methodology

*To determine **morphology***, BAL cells were cultured for 5 days and then viewed under 240x total magnification.

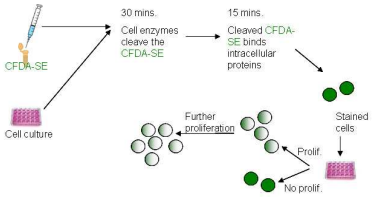


Figure 2. CFDA-SE staining methodology

*To determine **proliferation** of BAL-harvested AMs*, carboxy-fluorescin diacetate succinimidyl ester (CFDA-SE) dye was used to stain BAL-harvested AMs before culture. Proliferation after 5 days was measured as relative loss of CFDA-SE fluorescence. (**Figure 2**).

To determine the capacity of cells obtained from the BAL to stimulate T cell proliferation in a mixed lymphocyte reaction, BAL cells from C57BL/6 mice were cultured and added at a 1:10 ratio to a magnetically-separated and then CFDA-SE-labeled collection of CD4⁺ spleen cells from a Balb/c mouse. After five days of mixed culture, proliferation of CD4⁺ cells was determined. By flow cytometric analysis, each relative one-half reduction of 492/517nm fluorescence (excitation/emission) was assumed to indicate one cell division in lymphocytes (**Figure 3**).

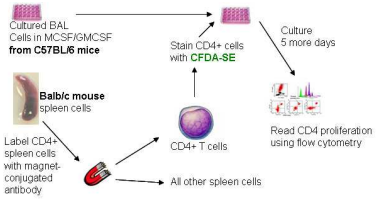


Figure 3. Mixed lymphocyte reaction methodology

*To determine **phenotype*** according to expression of the cell-surface markers CD11b, CD11c, CCR7, MHC class II, and DEC 205, fluorescent-conjugated antibodies directed at these surface markers were added to the culture, incubated for 30 minutes at 4⁰ C, washed to remove unbound antibody and read by flow cytometry.

*To determine **phagocytic** properties*, BAL cells were cultured for 5 days in the appropriate cytokine environments, followed by the addition of fluorescent-labeled latex beads in a 20:1 bead-to-cell ratio. After culture for 30 minutes, the number of beads per cell were counted using a fluorescent microscope.

Results: M-CSF is not a significant AM proliferative factor when compared to GM-CSF.

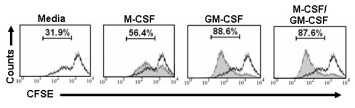
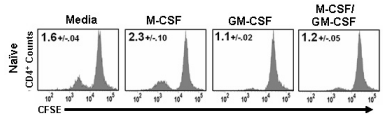


Figure 5. Relative loss of CFDA-SE dye in cultured AMs. Decreases in individual cell levels of CFDA-SE (referred to as "CFSE") beyond the "media" peak range indicates proliferation of AMs. Percentages listed represent the proportion of cells falling in this range. GM-CSF and M-CSF+GM-CSF show greater number of mitotic cells when compared to M-CSF. The unshaded overlays are of AMs cultured in media, whose main peak is assumed to represent the CFDA-SE intensity of undivided cells.

Results: M-CSF-cultured AMs display greater capacity to stimulate CD4⁺ proliferation in a MLR.

Figure 6. Relative loss of CFDA-SE dye in CD4+ T cells during a MLR. Numbers represent the total estimated number of mitotic events (x 10^5 +/- SD) during each MLR. These values were calculated using a previously-reported method, taking the sum of the number of daughter cells under each peak divided by 2^n, where n is the number of half-reductions from the initial peak CFDA-SE value [3]. M-CSF shows increased stimulation of CD4+ proliferation over GM-CSF and M-CSF+GM-CSF.



Results: M-CSF induces greater upregulation of DC-associated markers when compared to GM-CSF, and both CSFs increase CD11b expression.

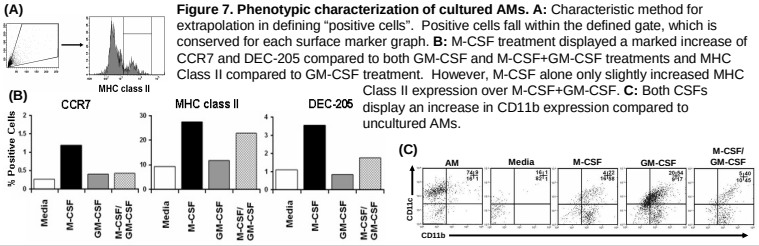


Figure 7. Phenotypic characterization of cultured AMs. A: Characteristic method for extrapolation in defining "positive cells". Positive cells fall within the defined gate, which is conserved for each surface marker graph. **B:** M-CSF treatment displayed a marked increase of CCR7 and DEC-205 compared to both GM-CSF and M-CSF+GM-CSF treatments and MHC Class II compared to GM-CSF treatment. However, M-CSF alone only slightly increased MHC Class II expression over M-CSF+GM-CSF. **C:** Both CSFs display an increase in CD11b expression compared to uncultured AMs.

Results: M-CSF induces DC-like AM morphology; GM-CSF does not induce morphological changes.

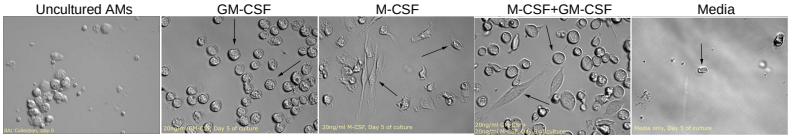
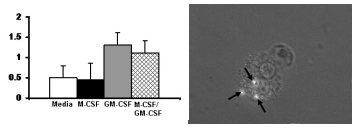


Figure 4. Microscopy of cultured BAL cells. Culture in M-CSF and M-CSF+GM-CSF resulted in BAL cells with a more DC-like, elongated morphology. This morphology was notably lacking for both GM-CSF cultured AMs and uncultured AMs. Many AMs cultured in media alone were apoptotic or exhibited similar morphology to uncultured AMs. These results support previous microscopy studies[2].

Results: GM-CSF increases phagocytic capacity of AMs, but M-CSF does not.

Figure 8: Average number of beads per cell according to culture treatment. The numbers of beads per cell were counted by two different researchers with a minimum sample size of n=25. The GM-CSF and M-CSF+GM-CSF treatments displayed at least a 2-fold increase in AM phagocytic capacity compared to media alone. M-CSF, however, displayed no ability to increase AM phagocytic capacity. Picture shown is representative of a cell with 3 beads inside.



Discussion

Our studies show that M-CSF has the ability to increase mature-DC-like phenotypical and functional characteristics of murine AMs *in vitro* whereas GM-CSF was a potent stimulator of proliferation and phagocytic capacity.

DEC-205 is a receptor involved in antigen presentation, found in high levels on DCs and thymic epithelial cells [4]. CCR7 is a receptor involved in DC trafficking to the lymph nodes [5]. The ability of M-CSF to upregulate DEC-205 and CCR7 in AMs indicates its role in inducing more DC-like phenotype and function of M-CSF-treated AMs.

Other features of M-CSF-treated AMs—increased levels of MHC class II and a higher T-cell-stimulating capacity in the MLR—are also commonly associated with mature myeloid DCs. In contrast, GM-CSF increases phagocytic capacity of AMs, a trait not attributed to mature DCs [6].

Though previously believed to be terminally-differentiated cells, our studies show that AMs have the ability to drastically alter their phenotypic and functional characteristics toward a DC-like state [2].

Our studies demonstrate the need to further characterize the effects of the cytokine environment on monocyte-derived cells—such as AMs and pulmonary DCs—to further understand the adaptive immune response to inhaled pathogens such as *M. tuberculosis*. In the context of the increasing rates of MDR- and XDR-TB, immunotherapy derived from such research could be vital to tuberculosis long-term prevention and control.

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References:

1. Fels AO, Cohn ZA. The alveolar macrophage. *J Appl Physiol*. 1986 Feb;60(2):353-69. Review.
2. Akagawa, K. S., K. Kamoshita, T. Tokunaga. 1988. Effects of granulocyte-macrophage colony-stimulating factor and colony-stimulating factor-1 on the proliferation and differentiation of murine alveolar macrophages. *J. Immunol.* 141: 3383-3390.
3. Wells, A. D., H. Gudmundsdottir, L. A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion: signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J. Clin. Invest.* 100: 3173-3183.
4. Jiang, W., W. J. Swiggard, C. Heufler, M. Peng, M. A., R. M. Steinman, M. Nussenzweig. 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature 375:151.-155.*
5. Jakubzick, C., F. Tacke, J. Llodra, N. van Rooijen, G. J. Randolph. 2006. Modulation of dendritic cell trafficking to and from the airways. *J. Immunol.* 176: 3578-3584.
6. Steinman RM, Inaba K. Myeloid dendritic cells. *J Leukoc Biol*. 1999 Aug;66(2):205-8.