A serum-free medium for differentiation of monocytes to macrophages

Nooshin Tabatabaei-Zavareh[1], Tinya Wang[1], Kent Fawcett[1], Fareeha Khan[1], Evan A. Karas[1], Tim A. Le Fevre[1], Stephen J. Szilvassy[1], Terry E. Thomas[1], Allen C. Eaves[1,2], and Albertus W. Wognum[1]

[1] STEMCELL Technologies Inc., Vancouver, BC, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

Introduction

Macrophages play an important role in defense against pathogens and in tissue homeostasis. Monocytes enter tissues and differentiate into macrophages. The presence of various stimuli in the tissue environment creates various states of "macrophage activation or polarization" influencing macrophage phenotype and function. Macrophages are classified into two main groups depending on how they are activated: 1) classically activated or M1 (activated by IFN-γ and LPS) and 2) alternatively activated or M2, which include M2a (activated by IL-4 or IL-13), M2b (immune complexes with IL-1β or LPS) and M2c (IL-10, TGF-β, or glucocorticoids) subsets.

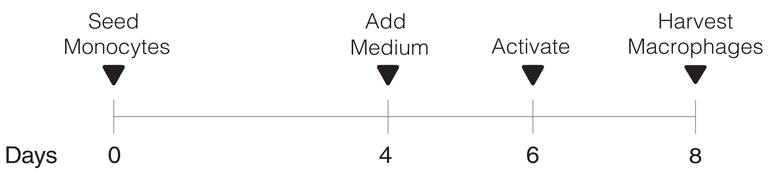
It is difficult to control macrophage differentiation and activation in cultures containing serum, in which variable amounts of M1- and M2-specific cytokines and other factors may be present. Described here is a culture system based on the use of serum-free ImmunoCult™-SF Macrophage medium that selectively supports the differentiation of human peripheral blood (PB) monocytes into either M1 or M2a macrophages. PB monocytes were first cultured for 6 days in ImmunoCult with 50 ng/mL M-CSF. Cells were then stimulated by adding 10 ng/mL LPS plus 50 ng/mL IFN-γ for M1 or 10 ng/mL IL-4 for M2a macrophage polarization and cultured for 2 more days. Total cell yields were 51 ± 18% in M1 and 58 ± 13% in M2a macrophage cultures (mean ± SD, n=57 - 59). M1 macrophages were $CD80^{+}CCR7^{+}CD206^{-/lo}CD209^{-/lo}$. In contrast, M2a macrophage marker expression profile was the opposite of M1 macrophage marker expression and was $CD206^{+}CD209^{+}CD80^{-/lo}CCR7^{-/lo}$. M1 macrophages produced TNF-α and IL-12 (mean ± SEM: 2821 ± 396 and 656 ± 86 pg/mL, n=24). M2a macrophages produced small amounts of IL-10 (29 ± 6 pg/mL, n=21). Both M1 and M2a macrophages were able to phagocytose fluorescently-labeled *E. coli* particles. By selecting appropriate stimuli this culture method can be easily adapted to generate other macrophage subsets as well and should prove useful for the study of macrophage biology.

Methods

Culture Protocol

$CD14^{+}$ monocytes were isolated from human PB mononuclear cells using the EasySep™ Human Monocyte Isolation Kit. The isolated monocytes (day 0) were plated at $1x10^{6}$ cells/mL in ImmunoCult™-SF Macrophage medium supplemented with 50 ng/mL M-CSF. On day 3 or 4, half the original volume of medium containing M-CSF was added and the cells were cultured for an additional 2 or 3 days. On day 6, 10 ng/mL LPS and 50 ng/mL IFN-γ were added for M1 activation while 10 ng/mL IL-4 was added for M2a activation. After two additional days of culture, M1 and M2a macrophages were harvested by adding appropriate dissociation reagents, i.e. accutase for M1 and 2.5 mM EDTA for M2a macrophages.

FIGURE 1: Protocol



Assessment of Macrophages

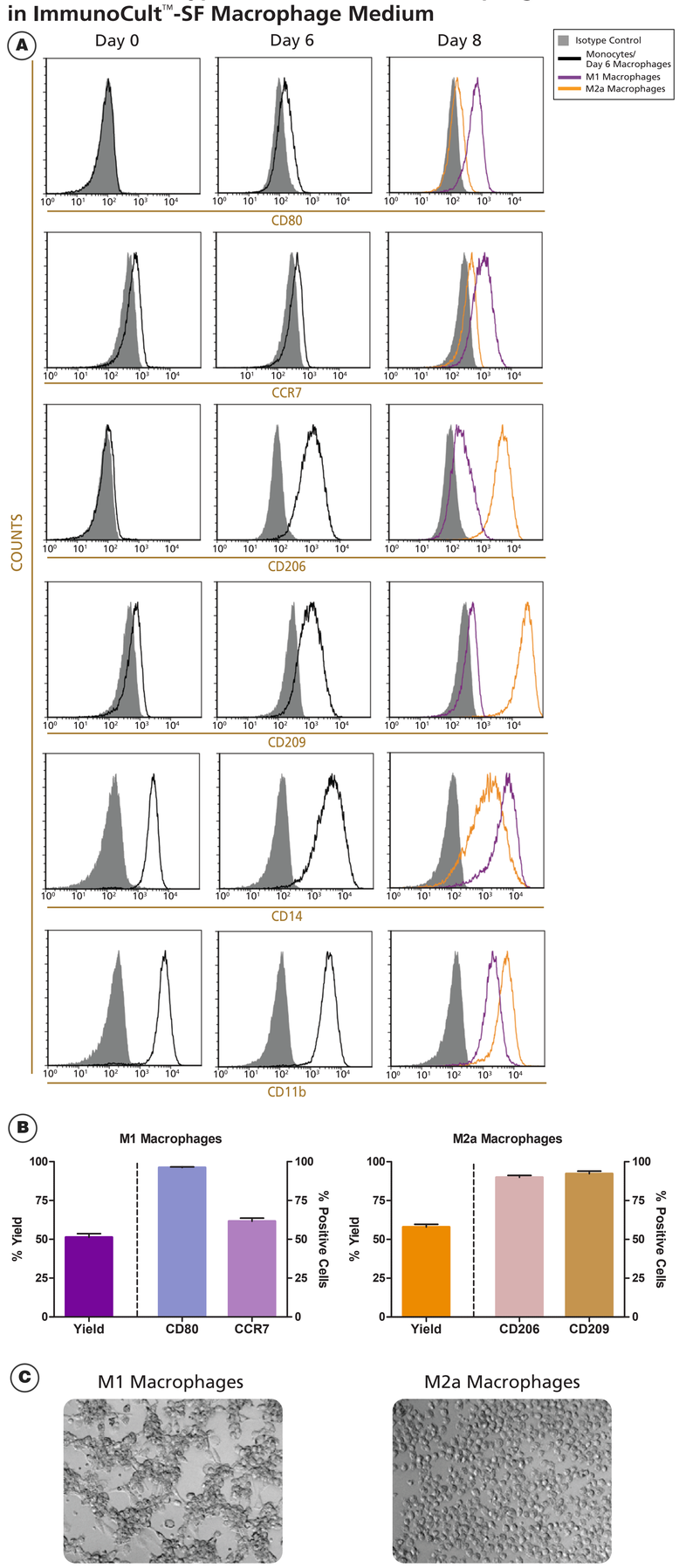
At Day 8, macrophages were harvested, counted and analysed by flow cytometry to assess the expression of macrophage markers CD80, CCR7, CD206, CD209, CD14 and CD11b. Dead cells and debris were excluded using 7-AAD staining and light scatter profile. Cell yields were calculated by dividing the macrophage counts on day 8 by the initial monocyte counts on day 0. TNF-α, IL-12p70 and IL-10 cytokine production was measured by ELISA on supernatants harvested on day 8.

Conclusions

- Immunocult™-SF Macrophage Medium supports the differentiation and activation of monocytes into macrophages in the absence of serum
- M1 and M2a macrophages express appropriate markers i.e. CD80 and CCR7 by M1 and CD206 and CD209 by M2a macrophages
- M1 macropahges produce pro-inflammatiory cytokines such as TNF-α and IL-12p70, M2a macropahges produce IL-10 not TNF- α
- M1 and M2a macropahges are functional and phagocytose *E. coli* particles

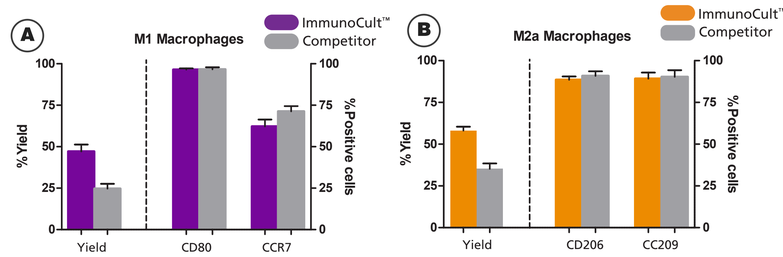
Results

FIGURE 2: Phenotype of Differentiated Macrophages Generated in ImmunoCult™-SF Macrophage Medium



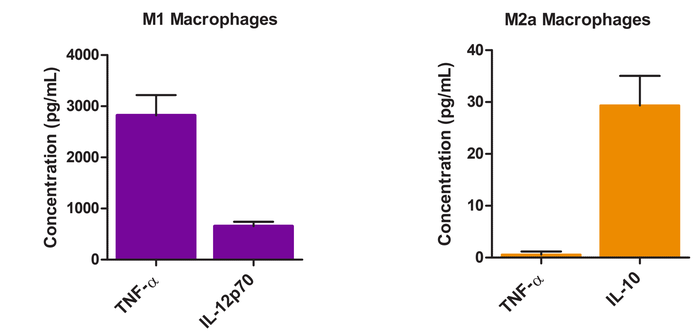
(A) Day 0 monocytes, day 6 macrophages and day 8 M1 and M2a macrophages were stained with fluorochrome-conjugated antibodies against CD80, CCR7, CD206, CD209, CD14 and CD11b. M1 macropahes expressed high levels of CD80 and CCR7 whereas M2a macropahges upregulated the expression of CD206 and CD209. CD14 epxression was slightly lower on M2a cells compared to M1 cells whereas CD11b expression was lower on M1 cells compared to M2a cells. **(B)** M1 and M2a macrophage yields after 8 days of culture were 51 ± 2% and 58 ± 2%. M1 macrophages were $CD80^{+}$ (96 ± 0.4%) and $CCR7^{+}$ (62 ± 2%) whereas M2a macrophages were $CD206^{+}$ (90 ± 1%) and $CD209^{+}$ (92 ± 2%). Data represents the mean ± SEM (n=57 - 59). **(C)** Bright field images of live day 8 M1 and M2a macrophages at 200x magnification.

FIGURE 3: Monocytes Differentiate Into M1 and M2a Macrophages in ImmunoCult™-SF Macrophage Medium with a Higher Yield Compared to a Competitor's Serum-Free Medium



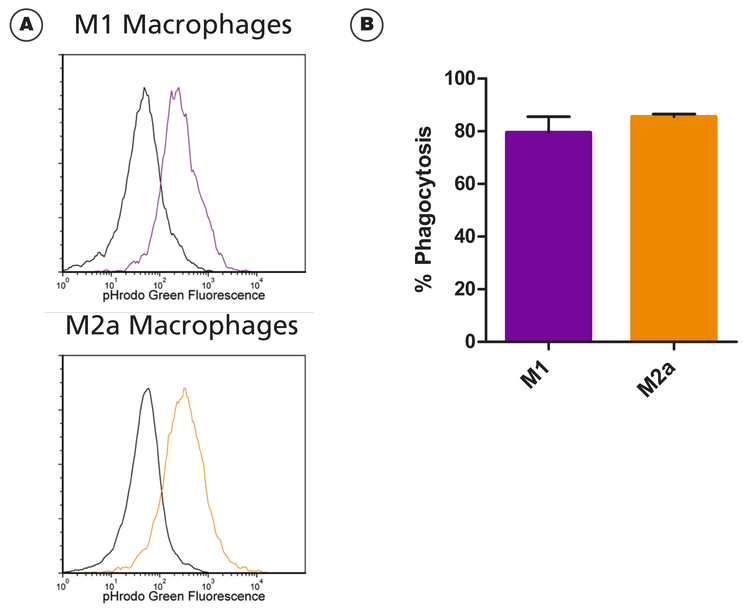
Monocytes were cultured in ImmunoCult™-SF Macrophage Medium or a competitor's serum-free macrophage medium and differentiated into **(A)** M1 or **(B)** M2a macrophages as described in Figure 1. Macrophage yields were significantly higher in ImmunoCult™ than in competitor's serum-free medium ($P < 0.05$, paired t-test; mean ± SEM; n = 18 - 19). M1 and M2a macrophages generated in ImmunoCult™ exhibited similar phenotype to M1 and M2a macrophages generated in competitor's medium.

FIGURE 4: Activated Macrophages Generated with ImmunoCult™ Medium Secrete Appropriate Cytokines



Macropnages were generated with ImmunoCult™-SF Macrophage Medium and activated using IFN-γ+LPS (M1 macrophages) or IL-4 (M2a) using the protocol described in Figure 1. At day 8, supernatants from M1 and M2a macrophage cultures were collected and the concentrations of TNF-α, IL-12p70 and IL-10 were determined by ELISA. M1 macrophages secreted 2821 ± 396 pg/ml TNF-α (n=24) and 656 ± 86 pg/mL IL-12p70 (n=25). M2a macrophages produced 29 ± 6 pg/mL IL-10 (n= 21) and did not produce TNF-α (below limit of detection, n=20). Data represents the mean ± SEM.

FIGURE 5: M1 and M2a Macrophages Phagocytose *E. coli* particles



Phagocytic activity of M1 and M2a macrophages was assessed using PH-sensitive pHrodo® *E. coli* Green Bioparticles (ThermoFisher). M1 and M2a macrophages were generated from monocytes according to the protocol described in Figure 1 and incubated with pHrodo *E. coli* Bioparticles in Live Cell Imaging Solution for 1 hour at 37˚C. Negative control plates were also set up and incubated at 4˚C. **(A)** M1 and M2a macrophages (purple and orange histograms, respectively) were then analysed by flow cytometry to detect the fluorescence from ingested *E. coli* particles as compared to their respective negative control (black histograms). **(B)** Shown is the average % phagocytosis from M1 and M2a macrophages (Mean ± SEM, n=3).