

Optimal Generation of Mature Human Dendritic Cells from CD14⁺ Monocytes in a Serum-Free Culture System

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Abstract

The unique ability of Dendritic Cells (DC) to efficiently present antigens and thus play a central role in the orchestration of the adaptive immune response has made these cells a major focus of interest in the conception of immunotherapeutic vaccine strategies. Few advances have been made in the engineering of a therapeutic DC product specifically optimized for this purpose. At present, several serum-free formulations exist which exhibit a limited potential for the generation of mature DC. To this end, we have developed a serum-free medium exclusively formulated to generate large numbers of mature DC that have been characterized morphologically, phenotypically and functionally. Stemline™ Dendritic Cell Medium (Sigma-Aldrich, St. Louis, MO) was compared with two commercially available DC media for their ability to generate mature DC from CD14⁺ peripheral blood monocytes. Briefly, purified CD14⁺ cells were incubated in the presence of GM-CSF and IL-4 for 6 days to produce immature DC. These immature DC were then driven to maturation by the addition of TNF- α and PGE₂ and cultured for an additional 2 days. While total nucleated cell numbers for competitor products were 10-15% higher, absolute numbers of cells expressing a panel of DC specific maturation markers including CD1a, CD25, CD80, CD83, CD86 and HLA-DR were 3.5 to 5.5-fold higher when CD14⁺ monocytes were cultured in Stemline™ Dendritic Cell Medium. These DC were functionally competent as determined by their ability to express IL-10 and IL-12 and stimulate allogeneic T-cells in mixed lymphocyte culture. It is proposed that both research and therapeutic applications may benefit greatly from the generation of optimal numbers of mature DC through the use of Stemline™ Dendritic Cell Medium.

Introduction

Dendritic Cells (DC) are considered to be the most potent of antigen presenting cells. Consequently, they play a pivotal role in the generation of primary T-cell responses through their ability to process and present antigens, while delivering co-stimulatory signals and secreting cytokines necessary for T-cell triggering. Traditionally, cell culture media have been supplemented with serum to support the *in vitro* growth of DC. However, specific cytokines in serum, such as TGF- β , and many other undefined components may affect the differentiative potential of DC precursors and the functional capacity of mature DC. The ability of these DC to efficiently present antigens and thus play a central role in the orchestration of the adaptive immune response have made these cells a recent focus of interest in the conception of vaccine strategies. Few advances have been made in the engineering of a DC product specifically optimized for this purpose. At present, there are several serum-free formulations available which exhibit a limited potential for the generation of mature DC. To this end, we have developed a serum-free medium, Stemline™ Dendritic Cell Medium (D 4192, Sigma-Aldrich, St. Louis, MO), specifically designed to generate large numbers of mature DC. These DC have been characterized morphologically, phenotypically

and functionally. We evaluated the ability of various serum-free media to support the generation of mature DC from CD14⁺ cells isolated from the mononuclear fraction of leukopheresis products. CD14⁺ cells were induced to differentiate into immature DC in the presence of GM-CSF and IL-4 and then driven to maturation by the addition of TNF- α and PGE₂. Mature DC in each culture system were characterized by flow cytometry, mature DC yield and morphology. Functionality of these DC as assessed in autologous and allogeneic mixed lymphocyte reactions and by their ability to express IL-10 and/or IL-12 under appropriate stimulatory conditions.

Materials and Methods

Cell Preparation

CD14⁺ cells were isolated from four separate donors through positive selection of leukopheresis material (BRT Laboratories, Baltimore, MD) using colloidal super-paramagnetic microbeads conjugated with monoclonal mouse anti-human CD14 antibodies (Miltenyi Biotec, Auburn, CA). Resultant CD14⁺ cells were frozen at 25×10^6 cells/ml in freezing medium (IMDM + 15% FBS + 7.5% DMSO) and transferred to the vapor phase of a liquid nitrogen dewar for future use.

Culture Conditions

Purified CD14⁺ cells from each donor were cultured in triplicate into each of four test media supplemented with GM-CSF (1000 U/ml, Immunex, Seattle, WA), IL4 (500 U/ml, BioSource, Camarillo, CA) and gentamicin sulfate (10 μ g/ml) in 6-well tissue culture plates (Corning-Costar, Corning, NY) at 1.0×10^6 cells/ml in a 5 ml final volume. Cells were incubated for 6 days at 37 °C, 5% CO₂ in a fully humidified incubator. After six days of incubation, PGE₂ (Sigma-Aldrich, St. Louis, MO) and TNF- α (BioSource, Camarillo, CA) were added to each culture at 5 μ M and 200 U/ml respectively. Cultures were incubated as above for two additional days. After day 8 of the protocol, each well was triturated to remove loosely adherent cells and to provide a single-cell suspension. Immediately after trituration, 500 μ l of each cell suspension was added to individually labeled Diluvials containing 20 ml Isoton II (Beckman Coulter, Miami, FL). The total nucleated cell count for each sample was determined using a Coulter ZM automatic cell counter. Photomicrographs of each culture condition were recorded prior to trituration with an Olympus CK40 inverted microscope fitted with a Hitachi KP-D50 Digital color camera.

Cytospins

One hundred microliters of each sample were added to the sample port of a cytospin slow absorption-pad chamber, with 100 μ l PBS added to the pre-wetting port. Cell suspensions were centrifuged at 500 or 1000 rpm for 5 minutes in a CytoPro 7620 cytocentrifuge (Wescor, Logan, UT). Slides (non-coated) were immediately fixed and stained with Diff-Quik (Dade Behring, Düringen, Switzerland)

Wright-Giemsa equivalent. Stained cells were evaluated at 20X and 100X magnification using an Olympus CX41 compound microscope fitted with a Hitachi KP-D50 Digital color camera.

Flow Cytometry

Cell populations from each respective sample were washed with PBS and centrifuged at 1500 g (3200 rpm) for 3 minutes. After removing all but 1 ml of the supernatant, cells were resuspended and 200 μ g normal mouse IgG (Caltag, Burlingame, CA) was added to the cell suspension for 15 minutes to prevent non-specific binding of reagents to Fc receptors. Following the incubation period, 100 μ l of cell suspensions were added to fluorochrome-conjugated reagents (FITC-anti CD14, PE-anti CD1a, PE-anti CD80, PP-anti HLA-DR, PE-anti CD83 and PE-anti CD86) and isotype control (Becton Dickinson, San Jose, CA). Again, cells were incubated in the dark for 15 minutes after light vortexing. Cells were washed and fixed with 200 μ l 2% formaldehyde and stored at 4 $^{\circ}$ C. Stained cells were analyzed at the Flow Cytometry Laboratory at Roswell Park Cancer Institute using a Becton Dickinson FACSort flow cytometer.

MLR

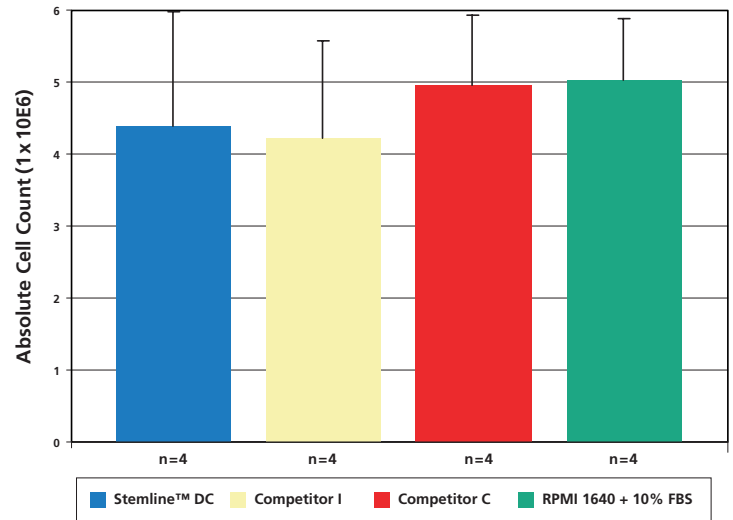
DC samples were assayed in mixed lymphocyte reactions at Aastrom Biosciences, Ann Arbor MI. Briefly, DC irradiated with 20 Gy were diluted so that 100 ml contained 25×10^3 , 5×10^3 , 1×10^3 , and 0.2×10^3 cells. One hundred μ l of DC at various concentrations were plated in triplicate in 96-well round bottom plates. One hundred thousand (100×10^3) responding cells in 100 μ l of medium + 4% FBS were added to the DC in appropriate wells. On day 5, 1 μ Ci of 3 H-thymidine was added to each well. Cells were harvested 18 hours later and 3 H-thymidine uptake quantitated using a Packard TopCount gamma counter.

IL-10/IL-12 Luminex Assay

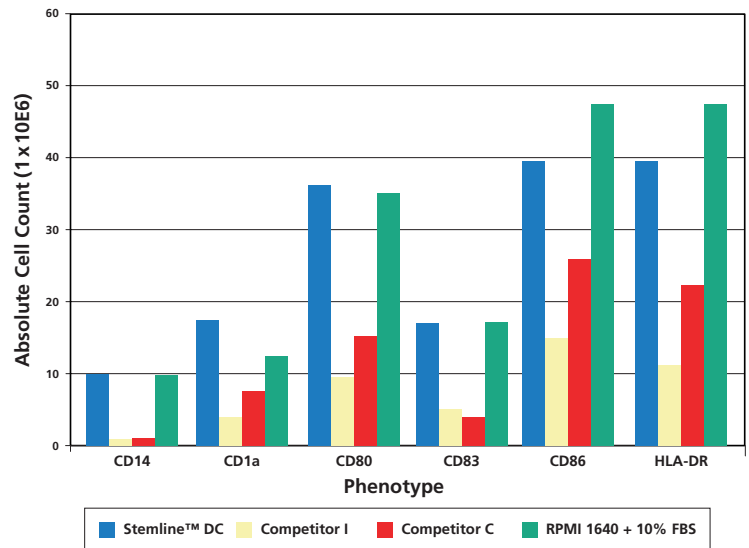
DC were evaluated for their ability to generate IL-10 and IL-12 *in vitro* using a Luminex soluble cytokine assay. Purified CD14⁺ cells were cultured in Stemline[™] Dendritic Cell Medium (Sigma-Aldrich, St. Louis, MO), Competitor I Medium, Competitor C Medium or RPMI 1640 +10% FBS at 1×10^6 cells/ml in 24 well polystyrene tissue culture plates. Cultures were supplemented with GM-CSF (500 U/ml) + IL4 (20 ng/ml) on Days 0, 3 and 6 to generate immature DC. On Day 6, samples were induced to mature using α CD40 (2 μ g/ml) + γ INF- β (500 U/ml). Supernatant aliquots were removed from each triplicate sample on Day 7 of the culture period and frozen at -80 $^{\circ}$ C for future analysis.

Thawed cell culture supernatants were assayed for soluble IL-10 and IL-12 using the Luminex fluorescent microsphere technology and compared to the fluorescence intensity values derived from IL-10 and IL-12 standard curves generated in each respective medium. Briefly, samples incubated with fluorescent microspheres coupled to α IL-10 and α IL-12 monoclonal antibodies were analyzed using a Luminex100 cytometer fitted with an XY Platform and Luminex Data Collector software (Luminex Corporation, Austin, TX). Fluorescent beads that had bound cytokine were differentiated from free beads through the use of a phycoerythrin-bound reporter antibody to the cytokine analyte. Liquid handling of the samples was performed through the use of a Tecan Genesis Workstation 150 with Gemini software programming (Tecan, Maennedorf, Switzerland).

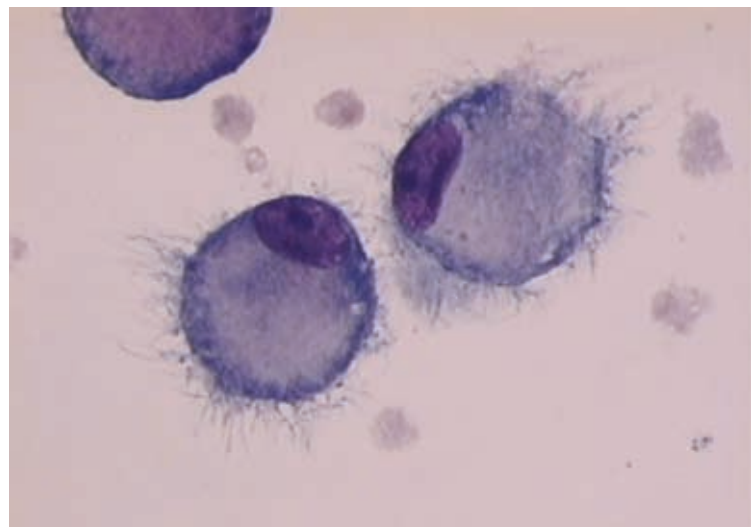
Average Total Nucleated Cell Counts



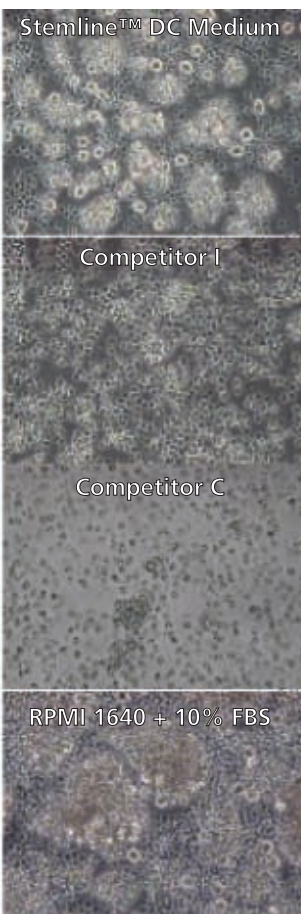
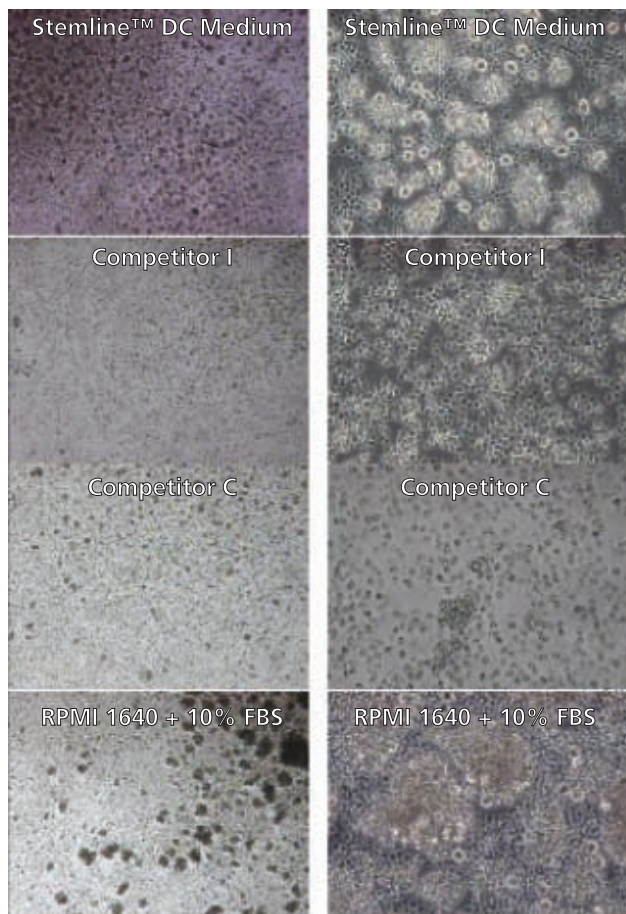
Flow Cytometric Analysis of Cell Populations Derived from CD14⁺ Monocytes After Incubation in Various Media



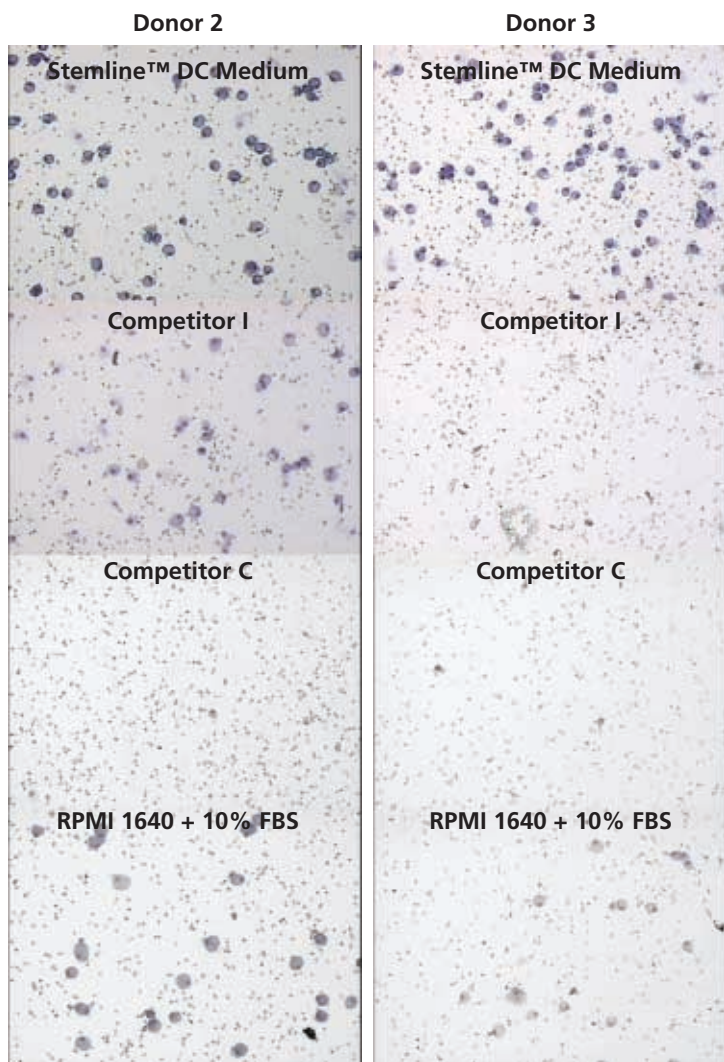
Dendritic Cells Cultured in Stemline[™] DC Medium (Cytospin, 100x)



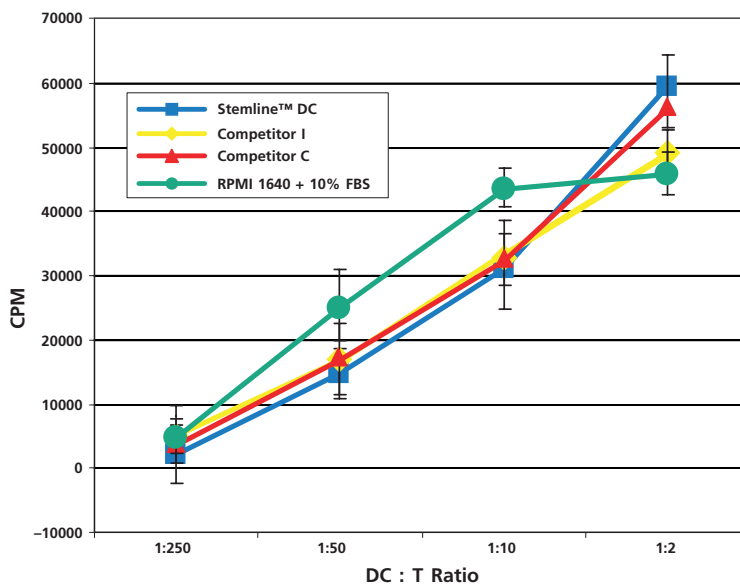
Culture Morphology in Stemline™ DC vs. Competitors



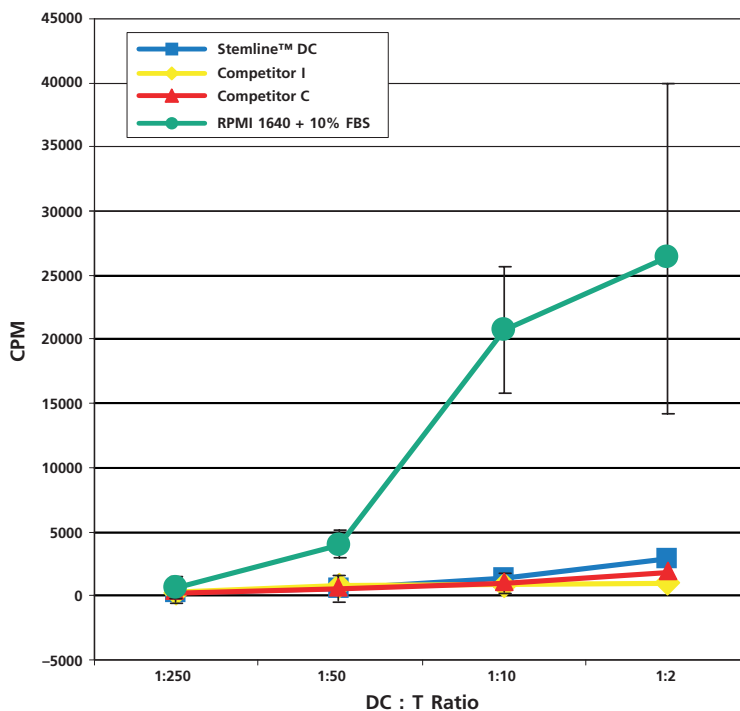
Cytospins, 20x Magnification



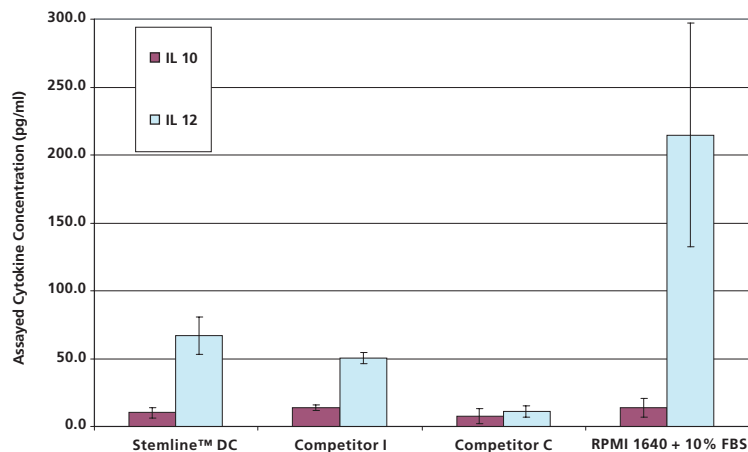
Proliferative Response of Allogeneic T-Cells to Mature DC Generated in Various Media



Proliferative Response of Autologous T-Cells to Mature DC Generated in Various Media



IL-10 & IL-12 Expression in Various Media



Conclusions

- Stemline™ DC Medium is a serum-free formulation designed for the *optimal generation of mature DC from peripheral blood CD14⁺ cells.*
- Stemline™ DC Medium *outperforms other commercially available serum-free media in the generation of mature DC as evidenced by:*
 - Numbers of mature DC as observed microscopically.
 - Absolute numbers of DC as determined by flow cytometry.
- DC *yield* for Stemline™ DC Medium is 3.5 to 5.5X higher than that of other commercially available media.
- *Percent conversion* from CD14⁺ cells to mature DC for Stemline™ DC Medium is 50 – 80%. For competitor products, the percent conversion to mature DC is 20-40%.
- Mature DC generated in Stemline™ DC Medium are *functionally active* as demonstrated by their ability to:
 - Efficiently induce a proliferative T-cell response in mixed lymphocyte culture.
 - Produce IL-10/12 when activated.
- DC derived using Stemline™ DC Medium *most closely reproduce DC generated in serum-containing medium* (with the exception of IL-12 production).

