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Large-Scale Hematopoietic Differentiation of Human Induced Pluripotent Stem Cells Provides Granulocytes or Macrophages for Cell Replacement Therapies

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SUMMARY

Interleukin-3 (IL-3) is capable of supporting the proliferation of a broad range of hematopoietic cell types, whereas granulocyte colony-stimulating factor (G-CSF) and macrophage CSF (M-CSF) represent critical cytokines in myeloid differentiation. When this was investigated in a pluripotent-stem-cell-based hematopoietic differentiation model, IL-3/G-CSF or IL-3/M-CSF exposure resulted in the continuous generation of myeloid cells from an intermediate myeloid-cell-forming complex containing $CD34^+$ clonogenic progenitor cells for more than 2 months. Whereas IL-3/G-CSF directed differentiation toward $CD45^+CD11b^+CD15^+CD16^+CD66b^+$ granulocytic cells of various differentiation stages up to a segmented morphology displaying the capacity of cytokine-directed migration, respiratory burst response, and neutrophil-extracellular-trap formation, exposure to IL-3/M-CSF resulted in $CD45^+CD11b^+CD14^+CD163^+CD68^+$ monocyte/macrophage-type cells capable of phagocytosis and cytokine secretion. Hence, we show here that myeloid specification of human pluripotent stem cells by IL-3/G-CSF or IL-3/M-CSF allows for prolonged and large-scale production of myeloid cells, and thus is suited for cell-fate and disease-modeling studies as well as gene- and cell-therapy applications.

INTRODUCTION

Hematopoietic in vitro differentiation of pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced PSCs (iPSCs) holds great promise for disease modeling, drug testing, and the development of novel cell- and gene-therapy strategies. In the past, interest has been directed primarily toward reconstituting stem cells, a cell type that is difficult to generate from PSC sources. Recently, however, long-lived, mature myeloid cells have been described (Guilliams et al., 2013), and the organotropic transplantation of such cells may allow for new therapeutic scenarios (Happle et al., 2014; Suzuki et al., 2014).

During embryonic development, hematopoietic cells are generated by two distinct but partly overlapping programs termed primitive and definitive hematopoiesis. Both are orchestrated by a highly complex interaction of regulatory molecules, including transcription factors, cytokine-induced and intercellular signaling, and niche factors (Lancrin et al., 2009; Nostro et al., 2008; Sturgeon et al., 2014). Primitive hematopoietic development originates from distinct multipotent precursors known as hemangioblasts, which are able to generate both vascular and hematopoietic progeny via an intermediate, hemogenic endothelial stage (Lancrin et al., 2009). Subsequently, further hematopoietic specification and differentiation result in mature cells that are primarily of an erythroid and, to a lesser degree, myeloid lineage (Palis, 2014; Schulz et al., 2012). In a separate process originating in the dorsal aorta, definitive hematopoiesis allows for the generation of transplantable hematopoietic stem cells (HSCs) that are capable of repopulating the entire lympho-hematopoietic system long term. In this context, an important role for the cytokine interleukin-3 (IL-3) (Donahue et al., 1988; Robin et al., 2006; Wiles and Keller, 1991) as well as wnt signaling (Sturgeon et al., 2014) has been reported by a

number of groups. Again, the fate of these repopulating HSCs, such as self-renewal, apoptosis, quiescence, and further differentiation and proliferation, is dependent on their exposure to other cells, matrix factors, or cytokines (Arai et al., 2004; Williams et al., 1991). For both programs, granulocyte-colony-stimulating factor (G-CSF) and monocyte-CSF (M-CSF) constitute the main driving forces for the generation and terminal differentiation of functional cells of a granulocytic or monocytic/macrophage lineage, respectively (Sengupta et al., 1988; Welte et al., 1985a, 1987).

G-CSF originally was identified by its capacity to promote the differentiation of human bone marrow progenitor cells toward neutrophils and is a critical component of this process (Welte et al., 1985b, 1987). However, the G-CSF receptor (*CSF3R*) is not exclusive to myeloid cells and has also been identified on HSCs, thus explaining the profound stem cell defects observed in congenital neutropenia patients suffering from defects in G-CSF signaling (Panopoulos and Watowich, 2008). In contrast, M-CSF, the crucial cytokine for generating mononuclear phagocytes or macrophages (MΦ) from HSC sources, appears to be primarily involved in terminal lineage differentiation (Yoshida et al., 1990). M-CSF was the first hematopoietic cytokine to be identified and cloned, and acts by activating its type III protein tyrosine kinase family receptor (*c-fms*) (Clark and Kamen, 1987; Sieff, 1987). Generating MΦ by M-CSF exposure, similarly to priming with IL-4/IL-10, results in alternatively activated M2-type Φ, in contrast to the classical pro-inflammatory M1Φ, which is differentiated from monocytes by GM-CSF or interferon-gamma (IFN−γ) exposure (Martinez et al., 2008; Sica and Mantovani, 2012).

To date, most protocols for hematopoietic differentiation of PSCs in vitro have utilized a multitude of cytokines or small molecules to mimic the modulation of signaling pathways at various stages of embryonic development (Choi et al., 2011; Kennedy et al., 2012; Sturgeon et al., 2014). However, many of the factors involved in this process remain ill defined. Therefore, this excessive priming may have unwanted effects on the differentiation or functionality of the desired target cells, hampering their use in disease modeling or cell- and gene-therapy applications. Thus, the development of simple but robust protocols for generating nonbiased and fully functional hematopoietic cells appears to be highly warranted.

Given the (1) emerging role of IL-3 in early primitive as well as definitive hematopoietic specification (Donahue et al., 1988; Robin et al., 2006; Yang et al., 1986), (2) the fundamental importance of G-CSF and M-CSF in terminal granulocyte and monocyte/macrophage differentiation (Clark and Kamen, 1987; Sengupta et al., 1988; Sieff, 1987; Welte et al., 1987), and (3) the synergy reported between IL-3 and late-acting factors such as G-CSF, M-CSF, and granulocyte-macrophage CSF (GM-CSF) (Donahue et al., 1988; Wiles and Keller, 1991), we sought to investigate the combined use of IL-3 with either M-CSF or G-CSF, employing embryoid body (EB)-based hematopoietic in vitro differentiation. For this purpose, we developed an innovative protocol that allows for the prolonged and large-scale production of functional granulocytes as well as monocytes/macrophages. Generation of immature myeloid cells was mediated by an intermediate myeloid-cell-forming complex (MCFC) containing $CD34^+$ clonogenic progenitor cells, which upon continued G-CSF or M-CSF exposure generated terminally differentiated myeloid cells for ≥ 2 months. As generation of these cells was accomplished by exposure of PSCs to IL-3 and one additional cytokine only, this protocol can be expected to closely recapitulate many aspects of physiologic hematopoietic development. Thus, it may be particularly suitable for studying human hematopoietic development in vitro and generating mature functional cells for cell and gene therapy.

RESULTS

Hematopoietic Specification of Human iPSCs

To investigate the effect of IL-3/G-CSF or IL-3/M-CSF on hematopoietic in vitro differentiation of PSCs, we established a four-step differentiation protocol (Figure 1A) utilizing human iPSCs (hiPSCs) previously generated from nonmobilized peripheral blood (PB)-derived $CD34^+$ cells (hCD34iPSC11 and hCD34iPSC16) (Ackermann et al., 2014; Lachmann et al., 2014) or the human ESC line H9. To initiate differentiation, iPSCs were cultured on mouse feeder cells in the presence of basic fibroblast growth factor (bFGF), resulting in typical round-shaped colonies (Figure 1B, step 1). Induction of germ layer formation was induced within EBs for 5 days (d0–d5) by orbital shaking and reduction of bFGF concentrations by 4-fold. After 5 days, we obtained compact and spherical EB cells (Figure 1B, step 2), which showed a slight decrease in TRA1-60 expression (Figure 1C). Induction of mesoderm was confirmed by decreased expression levels of OCT4 (*POU5F1*) as well as increased expression of Brachyury (*T*) mRNA when compared with the original iPSCs (Figure 1D). From d0 onward, EBs were subjected to directed hematopoietic specification by using medium supplemented with IL-3 and either G-CSF or M-CSF. Culture of EBs under these conditions for 10–15 days resulted in the formation of endothelial-like stromal cells and MCFCs (Figure 1B, step 3). The stromal cells had endothelial structure (Figure S1A) and stained positive for CD31, CD309 (Flk1), and CD144 (VE-Cadherin), whereas cells isolated from MCFCs stained negative for CD31, CD309, and CD144 (Figure S1B). For the IL-3/M-CSF combination, hematopoietic specification within