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Clonal analysis of human dendritic cell progenitor using a stromal cell culture

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ABSTRACT

Different dendritic cell (DC) subsets co-exist in humans and coordinate the immune response. Having a short life, DCs must be constantly replenished from their progenitors in the bone marrow through hematopoiesis. Identification of a DC-restricted progenitor in mouse has improved our understanding of how DC lineage diverges from myeloid and lymphoid lineages. However, identification of the DC-restricted progenitor in humans has not been possible because a system that simultaneously nurtures differentiation of human DCs, myeloid and lymphoid cells, is lacking. Here we report a cytokine and stromal cell culture that allows evaluation of CD34$^+$ progenitor potential to all three DC subsets as well as other myeloid and lymphoid cells, at a single cell level. Using this system, we show that human granulocyte–macrophage progenitors are heterogeneous and contain restricted progenitors to DCs.

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1. Introduction

In humans, there are three subsets of DCs, CD1c$^+$ classical DC (cDC), CD141$^+$cDC and plasmacytoid DCs (pDCs) (MacDonald et al., 2002; Robbins et al., 2008; Ziegler-Heitbrock et al., 2010); collaboratively, they initiate and orchestrate innate and adaptive immune responses. DCs are short-lived and must be constantly replenished from their bone marrow progenitors through hematopoiesis (Liu et al., 2007, 2009). Descending from the hematopoietic stem cells (HSCs), hematopoietic lineages progress through distinct progenitor stages where differentiation decision occurs and lineages diverge from each other. Several human hematopoietic progenitor populations have been reported to produce DCs, including LMPP, CMP, CLP, GMP and MLP, but they also produce other cells of myeloid and lymphoid lineages (Akashi et al., 2000; Doulatov et al., 2010; Galy et al., 1995; Ishikawa et al., 2007; Kohn et al., 2012). Whether DCs and cells of other lineages arise from the same progenitor or from distinct progenitors within these populations is unknown. To answer this question, one must evaluate the potential of progenitor to simultaneously produce myeloid, lymphoid cells and DCs; importantly the evaluation must be performed at a single cell level. So far, a proper culture for this purpose has not been established. The widely used GM-CSF culture produces monocyte-derived DC that differs from the authentic DCs (Cheong et al., 2010; Crozat et al., 2010; Naik et al., 2006; Xu et al., 2007); a two-step cytokine cocktail culture produces large amount of cDCs but no pDCs (Doulatov et al., 2010; Poulin et al., 2010); several stromal cell cultures have been used to produce pDCs but their production of two cDC subsets, myeloid or lymphoid cells was not evaluated (Chicha et al., 2004; Proietto et al., 2012). Here we report how we establish and optimize a culture system of stromal cell and cytokines that enables simultaneous differentiation of three DC subsets, monocytes, granulocytes and lymphocytes at a single cell level. Using this culture to examine 144 single granulocyte–monocyte progenitor (GMP) cells from human cord blood, we show that human GMPs are heterogeneous and exhibit distinct clonal potential.

2. Materials and methods

2.1. Human CD34$^+$ HSPC isolation

Cord blood samples and peripheral blood samples were purchased from the New York Blood Center and processed according to protocols approved by the Institutional Review Board at Columbia University Medical Center. Immediately after sample arrival, mononuclear cells were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences), at 25 °C, 1500 rpm, swing bucket, with no brake. To obtain HSPCs, CD34$^+$ cells were first enriched from cord blood through positive selection using the CD34 Microbead Kit and LS MACS magnetic columns (Miltenyi Biotec, Auburn, CA); the CD34$^+$ enriched fraction was then stained with an antibody mix prior to sorting cells (Table 1). The optimal concentration for each antibody was determined by titration before its use. For every 1 × 10^6 cells, we used 10 μl of antibody mixture and

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Table 1
List of monoclonal antibodies to sort CD34+ cells.

Fluorochrome	Marker	Manufacturer	Clone	Dilution factor	Volume per 1×10^6 cells (μl)
AlexaFluor (AF) 700	CD45	Biolegend	HI30	200	0.05
Qdot-655	CD14	Invitrogen	Tuk4	800	0.01
Brilliant Violet (BV) 650	CD19	Biolegend	HIB19	200	0.05
BV650	CD3	Biolegend	OKT	200	0.05
BV650	CD56	Biolegend	HCD56	200	0.05
BV650	CD16	Biolegend	3G8	200	0.05
APC-Cy7	CD34	Biolegend	581	200	0.05
Additional PBS					9.69
Final volume					10.00

incubate the cells on ice for 40 min. The stained population was then sorted as $CD45^+Lin(CD3/19/56/14/16)^-CD34^+$ for culture.

In order to isolate GMPs, $CD34^+$ cells were stained with additional antibodies (Table 2) and incubated on ice for 40 min. GMPs were sorted as $CD45^+Lin^-CD34^+CD38^+CD10^-CD45RA^+CD123^{+/hi}$ (Doulatov et al., 2010).

All fluorescence-activated cell sorting was performed on the BD FACS Aria or Influx using HeNe and Argon lasers at Columbia Center of Translational Immunology.

2.2. Stromal cell culture conditions

S17 and MS5 stromal cells were maintained in complete alpha MEM medium supplemented with L-glutamine, ribonucleosides and deoxyribonucleosides (Invitrogen) with 10% heat inactivated FCS and penicillin/streptomycin (Invitrogen); the cells were passed when they reached 90% confluency. 24 h prior to coculture with $CD34^+$ HSPCs, stromal cells were plated at 1.5×10^5 cells per 0.5 ml in a 24-well plate or 3.75×10^4 cells per 50 μl in a 96-well plate. $CD34^+$ HSPCs and cytokines were added in 0.5 ml (final 1 ml) or 50 μl (final 100 μl) for either a 24-well plate or 96-well plate, respectively. For mitomycin C treatment, MS5 stromal cells were incubated with 10 μg/ml of mitomycin C (Sigma) for 3 h at 37 °C and washed with PBS before being plated onto 24-wells or 96-wells.

Cytokines used for culture are FLT3L (CellDex) at 100 ng/ml, SCF (Peprotech) at 20 ng/ml or GM-CSF (Peprotech) at 10 ng/ml. Cells were cultured for two weeks with half medium change every 7 days of culture.

2.3. Flow cytometry analysis

In order to analyze the lineage output from $CD34^+$ HSPCs, cells were harvested from each well. After washing in PBS, cells were resuspended in a dye plus antibody mix (Table 3) and incubated for 40 min on ice.

Table 2
List of monoclonal antibodies to sort GMPs.

Fluorochrome	Antigen	Company	Clone	Dilution factor	Volume per 1×10^6 cells (μl)
AlexaFluor (AF) 700	CD45	Biolegend	HI30	200	0.05
Qdot-655	CD14	Invitrogen	Tuk4	800	0.01
Brilliant Violet (BV) 650	CD19	Biolegend	HIB19	200	0.05
BV650	CD3	Biolegend	OKT	200	0.05
BV650	CD56	Biolegend	HCD56	200	0.05
BV650	CD16	Biolegend	3G8	200	0.05
APC-Cy7	CD34	Biolegend	581	200	0.05
PE	CD10	Biolegend	HI10a	100	0.10
FITC	CD45RA	Biolegend	HI100	80	0.13
BV421	CD38	Biolegend	HIT2	80	0.13
BV510	CD123	Biolegend	6H6	80	0.13
Additional PBS					9.21
Final volume					10.00

10 μl or 4 μl of antibody mix were used for each well either from bulk $CD34^+$ HSPC cultures or from the clonal assay, respectively. Absolute cell counts from each well were determined relative to a well with a known number of human $CD45^+$ cells (i.e. 1×10^5 cells) added to an empty well as a counting control. All flow cytometry analysis was performed on BDSLRII cytometers (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA) using HeNe and Argon lasers.

Human cell type and lineages were determined as follows: granulocytes ($CD45^+CD66b^+$), monocytes ($CD45^+CD14^+CD66b^-CD1c^-$), $CD141^+$ cDCs ($CD45^+CD141^+CLEC9a^+$), $CD1c^+$ cDCs ($CD45^+CLEC9a^-CD1c^+$), pDCs ($CD45^+CD1c^-CD303^+CD123^+$), B cells ($CD45^+CD19^+CD56^-$), NK cells ($CD45^+CD19^-CD56^+$) or undifferentiated ($CD45^+CD66b^-CLEC9a^-CD1c^-CD14^-CD303^-CD123^-CD19^-CD56^-$).

2.4. Single cell clonal assay

To determine the setting and efficiency for single cell sorting, human PBMCs were stained for 15 min with 5 μM of carboxyfluoresceindiacetatesuccinimidyl ester (CFSE; Molecular Probes) at 37 °C and $CFSE^+$ single cells were sorted into each well of a 96-well plate. 90.5% of wells contained 1 cell, 9.5% of wells had 0 cells and none had more than 1 cell as verified by microscopy.

Individual GMPs were sorted into each well containing mitomycin C-pretreated MS5 stromal cells and FLT3L, SCF and GM-CSF. After two weeks of culture with one media change at seven days, each well was analyzed for hematopoietic lineage potential by flow cytometry. A clone was considered positive when more than 10 human $CD45^+$ events were detected in the output; developmental potential of a given lineage was considered positive for a productive clone when more than 7 events were detected for the corresponding gate.

3. Results

Because Flt3L has been shown to be essential for DC differentiation in mouse and humans (Ding et al., 2014; Pulendran et al., 2000; Waskow et al., 2008), and also because stromal cells have been shown to facilitate differentiation of human pDCs and other hematopoietic lineages (Chicha et al., 2004; Olivier et al., 2006; Spits et al., 2000), we reason that the combination FLT3L with stromal cells may stimulate differentiation of all human DC subsets. We tested two murine bone marrow stromal cell lines, S17 (Collins and Dorshkind, 1987) and MS5 (Itoh et al., 1989), for their capacity to differentiate $CD34^+$ human hematopoietic progenitors (Fig. 1A) in combination with 100 ng/ml of FLT3 ligand (FLT3L). Culture output was evaluated at days 3, 7, 10 and 14 time points after culture; differentiated human hematopoietic cells were identified using flow cytometry. Comparison to culture with FLT3L alone, addition of either S17 or MS5 cultures significantly increased yield of human $CD45^+$ cells between 10 and 14 days of culture (Fig. 1B). Both S17 and MS5 cultures produced abundant granulocytes and monocytes. Compared to S17 culture, MS5 culture produced 6-fold more pDCs ($CD45^+CD66b^-CD14^-CD141^-CLEC9a^-CD1c^-CD303^+$ $CD123^+$), 2-fold more $CD1c^+$ cDCs ($CD45^+CD66b^-CD14^-CD141^-CLEC9a^-CD1c^+$) and 4-fold more $CD141^+$ cDCs ($CD45^+CD66b^-CD14^-CD141^+CLEC9a^+$) (Fig. 1C and D). In addition, MS5 cell culture produced B cells or NK cells, whereas S17 culture failed to do so. In contrast, FLT3L cytokine without stromal cells failed to expand and produce any of the DC subsets, indicating that stromal cells provide essential signals for human DC differentiation. We conclude that MS5 stromal cells in combination with FLT3L are superior in differentiating human $CD34^+$ HSPCs into multiple hematopoietic lineages including the three major subsets of DCs.

To improve the yield of human hematopoietic cells, we pre-treated MS5 stromal cells with mitomycin C to halt their proliferation and reduce their competition for nutrients. Comparing to the untreated

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