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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.

Keywords

Hematopoiesis; Dendritic cell; Single cell; Culture; Granulocyte–macrophage progenitor; Stromal cells

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

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Author contributions statements

J.L., G.B., M.C.N. and K.L. conceived and developed the original strategy of culture and clonal assay; J.L. modified the protocol and performed experiments; J.L. and K.L. wrote the manuscript; and all authors reviewed the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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 μ l of antibody mixture and 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 μ l in a 96-well plate. CD34⁺ HSPCs and cytokines were added in 0.5 ml (final 1 ml) or 50 μ l (final 100 μ l) for either a 24-well plate or 96-well plate, respectively. For mitomycin C treatment, MS5 stromal cells were incubated with 10 μ g/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. 10 μ l or 4 μ l 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 BDLSRII 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 μ M 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 MS5, mitomycin C pre-treatment increased the yield of bulk human CD45⁺ cells by 1.5-fold after 2 weeks of culture (Fig. 2A). In particular, pDCs increased by 1.5-fold, CD1c⁺ cDCs by 2-fold, CD141⁺ cDCs by 4-fold, and B cells by 3-fold. There was a less than 10% increase of granulocytes, monocytes and NK cells (Fig. 2A). The overall lineage composition was not affected (Fig. 2B). To optimize the output of multiple hematopoietic lineages, we added stem cell factor (SCF) and granulocyte–macrophage colony stimulating factor (GM-CSF) into the MS5 stromal cell culture. Comparing to FLT3L alone, the addition of SCF and GM-CSF increased the yield of total CD45⁺ leukocytes by 3-fold (Fig. 2C). In particular, both granulocytes and monocytes increased by 4-fold, pDCs, CD1c⁺ cDCs and CD141⁺ cDCs increased by 1.5-, 6- and 9-fold, respectively. NK cell yield was not affected, but there was a decrease of B cells, likely due to the GM-CSF's preferential driving of myeloproliferation (Basak et al., 2002; Ulich et al., 1990). Considering the dramatic increase of myeloid lineage and DCs and reasonable output of NK and B cell yield, we conclude among conditions tested, that mitomycin C pre-treated MS5 in combination with FLT3L, SCF and GM-CSF (MS5 + FSG herein) is optimal for simultaneous differentiation of all three DC subsets as well as myeloid and lymphoid cells.

Because different culture systems may skew the differentiation potential of a progenitor, consequently leading to the misinterpretation of its potential, we seek to verify whether MS5 + FSG culture allows unbiased differentiation. For this purpose, we examined how MS5 + FSG affects the lineage output of three human hematopoietic progenitors with known potential from cord blood: MEPs

(CD45⁺Lin(CD3/19/56/14/16)⁻CD34⁺CD38⁺CD10⁻CD123⁻CD45RA⁻) that should

produce only megakaryocytes and erythrocytes, B/NK progenitors

(CD45⁺Lin(CD3/19/56/14/16)⁻CD34⁺CD38⁺CD10⁺CD45RA⁺) that should produce lymphoid cells, and GMPs

(CD45⁺Lin(CD3/19/56/14/16)⁻CD34⁺CD38⁺CD10⁻CD123^{-/+}CD45RA⁺) that should produce myeloid cells, including DCs (Fig. 3A) (Doulatov et al., 2010). When cultured in MS5 + FSG, MEP did not produce any DCs, myeloid or lymphoid cells, B/NK progenitors produced only B cells, whereas GMPs produced all three DC subsets, myeloid cells including granulocytes and monocytes, and very few lymphocytes (Fig. 3B). We conclude that MS5 + FSG can differentiate all DC subsets, granulocytes, myeloid cells and lymphoid cells without skewing the intrinsic potential of the progenitors.

To determine whether MS5 + FSG culture can be used to analyze potential of single CD34⁺ human hematopoietic progenitor cells, we studied GMPs from cord blood using the culture (Fig. 3A). Single GMP cells were sorted into 144 wells containing MS5 + FSG, cultured for 2 weeks and then harvested for analysis. Out of 144 wells, 93 cells produced human CD45⁺ cells, thus 65% clones were productive. Analysis of their clonal potential revealed a hierarchical heterogeneity of progenitor potential. Among 93 productive clones, 3 clones produced undifferentiated cells, a low frequency of them (6/93) produced lymphoid and myeloid cells together, whereas most productive clones (84/93) lacked lymphoid potential and produced myeloid cells and/or DCs. Only 6 (6.5%) clones are multi-potent, simultaneously producing granulocytes, monocytes and DCs. Twenty five clones are bi-potent, including 11 clones (11.8%) that produced monocytes and DCs, 1 clone (1.1%) produced DCs and granulocytes, and 13 clones (14.0%) produced granulocytes and monocytes. The majority, 53 clones (57.0%), was uni-potential: 25 produced only granulocytes, 11 only monocytes and 16 only DCs. We conclude that MS5 + FSG can be used to interrogate the differentiation potential of human hematopoietic progenitors at a single cell level to granulocytes, monocytes, DC subsets and lymphoid cells.

4. Discussion

Despite the importance of human DC hematopoiesis, there were no good methods to analyze the differentiation potential of a single progenitor cell to all three DC subsets as well as other myeloid and lymphoid lineages. This is of high relevance in the field of human DC differentiation for two main reasons. First, DCs have been shown to descend from lymphoid progenitors as well as conventional myeloid progenitors; a restricted DC progenitor has been identified in mouse but its equivalent in humans was unknown. Therefore, to identify DC-restricted progenitors, candidate precursors must be tested for their DC differentiation potential in the context of other cell types. This can only be achieved in a culture that simultaneously nurtures myeloid and lymphoid lineages as well as DCs. Furthermore, the DC lineage has been difficult to distinguish from the monocytic lineage. Although GM-CSF

and IL-4 culture has been widely used to generate human DCs from CD34⁺ cells or blood monocytes, these GM-CSF-driven DCs have been shown to descend from monocytes and differ significantly from the authentic DCs in the steady state that depend on Flt3L (Ginhoux et al., 2009; McKenna et al., 2000; Waskow et al., 2008). Therefore, a culture that simultaneously supports the development of both monocytes and authentic DCs is necessary to discern monocyte and authentic DC lineages.

We show that MS5 stromal cells together with FLT3L, or with FLT3L, SCF and GM-CSF, can simultaneously support the development of all three subsets of DCs, monocytes, granulocytes and lymphoid cells from CD34⁺ hematopoietic progenitors. Especially, this method not only applies to CD34⁺ cells isolated from cord blood (Fig. 1C), but also from the human bone marrow (Lee et al., 2015) and peripheral blood (data not shown). The MS5 + FSG culture drives sufficient proliferation and differentiation for clonal assay. This indicates that signals from MS5 stromal cells are essential and sufficient for differentiation of human DCs, suggesting that the molecules mediating such effects must be highly conserved between mouse and human.

Interestingly, when a seemingly homogeneous GMP population was subject to clonal assay in MS5 + FSG, heterogeneous clones with distinct potential were revealed. Only a minor fraction of GMPs are multi-potent, whereas the majority has exhibited certain extent of lineage divergence. One fraction of (17.2%) GMP clones appear restricted to produce DCs alone but lack granulocyte or monocyte potential, resembling the common dendritic progenitor (CDP) in the mouse (Liu et al., 2009; Naik et al., 2007; Onai et al., 2007); while another fraction (~11.1%) of GMP clones have DC and monocytic potential but lack granulocytic potential, resembling the macrophage–dendritic progenitor (MDP) in the mouse (Fogg et al., 2006; Geissmann et al., 2010). Further, among bi-potent progenitors DC–granulocyte progenitors were very rare, implying the divergence between DC and granulocyte may take place first. Collectively these indicate considerable heterogeneity of progenitors in the GMP population, which contain human equivalent of MDPs and CDPs.

In conclusion, the MS5 + FSG culture system we describe can be used to interrogate the differentiation potential of single human hematopoietic progenitor cells to DC subsets and other myeloid and lymphoid cell lineages. Heterogeneity of GMPs suggests that restricted human DC and monocyte progenitors (MDPs and CDPs) may be further purified from this population.

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Fig. 1.

MS5 stromal cells support CD34⁺ HSPC proliferation and differentiation to human DCs. (A) Flow cytometry plots show gating strategy for sorting CD34⁺ HSPCs and post-sort purity. (B) Kinetic plot shows total human CD45⁺ cell counts from HSPC cultures without stroma (triangle), with S17 (square) or MS5 stromal cells (circle) together with FLT3L at days 3, 7, 10 and 14. Day 0 indicates the input number of CD34⁺ HSPCs, 3×10^3 . (C) Plots show representative flow cytometry analysis of human CD45⁺ composition from each culture at day 14. Granulocytes, brown; monocytes, orange; pDCs, green; CD1c⁺ cDCs, blue; CD141⁺ cDCs, red; B cells and NK cells, purple. (D) Bar plots show the absolute number of specified cell types at day 14 from cultures without stroma (white), with S17 (gray) or MS5 (black) stromal cells.

Fig. 2. Mitomycin C treatment of MS5 stromal cells increases human leukocyte proliferation. (A) Bar plots show absolute counts of human CD45⁺ cells from 1×10^3 CD34⁺ HSPC cocultures with mitomycin C untreated (gray) or treated (black) MS5 stromal cells. (B) Bars show the relative frequencies of (from the bottom) granulocytes (brown), monocytes (orange), pDCs (green), CD1c⁺ cDCs (blue), CD141⁺ cDCs (red) and B cells (purple stripes). (C) Bars show the absolute number of cells from 2×10^3 HSPCs and mitomycin C pretreated MS5 cocultures in FLT3L alone (gray) or FLT3L, SCF and GM-CSF (black).

Fig. 3.

MS5 + FSG supports myeloid lineage differentiation from single cell GMPs. (A) Flow cytometry plots show gating strategy for sorting cord blood derived human B/NK progenitors, MEPs and GMPs, as well as GMPs post-sort purity. (B) Representative flow cytometry analysis from cultures at day 14. 5 cells of each progenitor were incubated in MS5 + FSG cultures. (C) Bar graphs show the absolute number of cells for the indicated populations. White bar, MEP; gray bar, B/NK progenitor; black bar, GMP. (D) Single GMP cells were directly sorted into 96 well plates with MS5 stromal cells together with FLT3L, SCF and GM-CSF. Bar graphs show the cumulative number of wells giving rise to the specified potential out of the total number of positive clones. G, granulocyte; M, monocyte;

DC, pDCs and/or cDCs; L, lymphoid (B and/or NK cells); Undiff, undifferentiated; +, “and”; /, “or”.

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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	Biologend	HI30	200	0.05
Qdot-655	CD14	Invitrogen	Tuk4	800	0.01
Brilliant Violet (BV) 650	CD19	Biologend	H1B19	200	0.05
BV650	CD3	Biologend	OKT	200	0.05
BV650	CD56	Biologend	HCD56	200	0.05
BV650	CD16	Biologend	3G8	200	0.05
APC-Cy7	CD34	Biologend	581	200	0.05
Additional PBS					9.69
Final volume					10.00

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	Biologend	HI30	200	0.05
Qdot-655	CD14	Invitrogen	Tuk4	800	0.01
Brilliant Violet (BV) 650	CD19	Biologend	HIB19	200	0.05
BV650	CD3	Biologend	OKT	200	0.05
BV650	CD56	Biologend	HCD56	200	0.05
BV650	CD16	Biologend	3G8	200	0.05
APC-Cy7	CD34	Biologend	581	200	0.05
PE	CD10	Biologend	HI10a	100	0.10
FITC	CD45RA	Biologend	HI100	80	0.13
BV421	CD38	Biologend	HIT2	80	0.13
BV510	CD123	Biologend	6H6	80	0.13
Additional PBS					9.21
Final volume					10.00

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Table 3

List of Monoclonal antibodies for analyzing cultured cells.

Fluorochrome	Antigen	Company	Clone	Dilution factor	Volume per sample (24-well plate) (μl)	Volume per sample (96-well plate) (μl)
Live/Dead ®		Life Technologies		1000	0.01	0.01
AlexaFluor (AF) 700	CD45	Biologend	HI30	200	0.05	0.02
Qdot-655	CD14	Invitrogen	Tuk4	800	0.01	0.01
PE	CLEC9a	Biologend	8 F9	500	0.02	0.01
PerCP-Cy5.5	CD66b	Biologend	G10F5	100	0.10	0.04
PE-Cy7	CD1c	Biologend	L161	100	0.10	0.04
FITC	CD303	Biologend	201A	100	0.10	0.04
APC-Cy7	CD56	Biologend	HCD56	100	0.10	0.04
APC-Cy7	CD19	Biologend	H1B19	80	0.13	0.05
BV510	CD123	Biologend	6H6	80	0.13	0.05
APC	CD141	Miltenyi	AD5-14H12	80	0.13	0.05
Additional PBS					9.13	3.65
Final volume					10.00	4.00