1 **A method for the generation of large numbers of dendritic cells from CD34+ hematopoietic**
2 **stem cells from cord blood**
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4 Nicole Bedke, PhD[1], Emily J. Swindle, PhD[1],Camelia Molnar, MSc,[1],Patrick Holt,
5 PhD[2],Deborah Strickland, PhD[2],Graham C. Roberts, DM[1], Ruth Morris RSCN[1], Stephen T.
6 Holgate, DSc, MD,[1] Donna E. Davies, PhD[1], Cornelia Blume, PhD[1]
7

8 [1]: Academic Unit of Clinical and Experimental Sciences, Faculty of Medicine, University of
9 Southampton, Southampton, UK
10 [2]: Telethon Institute for Child Health Research, Centre for Child Health Research, University of
11 Western Australia, Perth, Australia
12

13 *Corresponding Author:*
14 Dr. Cornelia Blume
15 Clinical and Experimental Sciences
16 Faculty of Medicine
17 University of Southampton
18 Southampton, United Kingdom
19 Tel: +44 2381 203308
20 Email: C.Blume@soton.ac.uk
21

Abstract ***(300 words max)***
Dendritic cells (DCs) play a central role in regulating innate and adaptive immune responses. It is well accepted that their regulatory functions change over the life course. In order to study DCs function during early life it is important to characterize the function of neonatal DCs. However, the availability of neonatal DCs is limited due to ethical reasons or relative small samples of cord blood making it difficult to perform large-scale experiments. Our aim was to establish a robust protocol for the generation of neonatal DCs from cord blood derived CD34+ hematopoietic stem cells. For the expansion of DC precursor cells we used a cytokine cocktail containing Flt-3L, SCF, TPO, IL-3 and IL-6. The presence of IL-3 and IL-6 in the first 2 weeks of expansion culture was essential for the proliferation of DC precursor cells expressing CD14. After 4 weeks in culture, CD14+ precursor cells were selected and functional DCs were generated in the presence of GM-CSF and IL-4. Neonatal DCs were then stimulated with Poly(I:C) and LPS to mimic viral or bacterial infections, respectively. Poly(I:C) induced a higher expression of the maturation markers CD80, CD86 and CD40 compared to LPS. In line with literature data using cord blood DCs, our Poly(I:C) matured neonatal DCs cells showed a higher release of IL-12p70 compared to LPS matured neonatal DCs. Additionally, we demonstrated a higher release of IFN-γ, TNF-α, IL-1β and IL-6, but lower release of IL-10 in Poly(I:C) matured compared to LPS matured neonatal DCs derived from cord blood CD34+ hematopoietic stem cells. In summary, we established a robust protocol for the generation of large numbers of functional neonatal DCs. In line with previous studies, we showed that neonatal DCs generated form CD34+ cord blood progenitors have a higher inflammatory potential when exposed to viral than bacterial related stimuli.

Keywords: neonatal dendritic cells, CD34+ hematopoietic stem cells, cord blood, maturation, Poly(I:C), lipopolysaccharide

Highlights: (3-5 bullet points, each max. 85 characters incl. spaces)

- A robust protocol for the generation of high numbers of neonatal dendritic cells
- IL-3 and IL-6 are crucial for the proliferation of cord blood $CD34^+$ progenitors

53 - Neonatal DCs have a higher inflammatory potential when exposed to viral stimuli
54 - LPS induces higher release of IL-10 in neonatal DCs compared to Poly(I:C)
55

56 **1. Introduction**

57 Dendritic cells (DCs) act as sentinels of the immune system and therefore play a key role
58 in triggering and priming immune responses to environmental agents. Through expression of
59 receptors they sense infections and are able to link innate and adaptive immune response at
60 epithelial interfaces (Hammad and Lambrecht, 2011). Through mediators including TSLP, OX40
61 ligand, GM-CSF, CCL20 and others released by the infected or damaged tissue, e.g. epithelial
62 surfaces, DCs are recruited to the site of infection (Gill, 2012). In response, DCs are activated,
63 migrate to draining lymph nodes and upregulate MHCII, co-activation receptors including CD80
64 and CD86 and secrete cytokines which can then instruct naive T-cells (Th0) to differentiate into
65 Th1, Th2-cells, Treg or Th17 cells.

66 It is now well accepted that the characteristics of DCs change and mature during life
67 (Willems et al., 2009; Basha et al., 2014; Agrawal et al., 2017). In order to understand the role of
68 DCs over the life course, it becomes important to consider the source of DCs when studying their
69 responses to environmental agents. In particular, due to the limited availability of neonatal DCs,
70 it is challenging to investigate the characteristics of DCs at early life in detail. Hematopoietic
71 stem cells (HSCs) have increasingly become an important tool to generate large numbers of
72 immune cells of specific lineages due to their plasticity in being able to differentiate into a
73 desired cell type depending on the cytokines they are exposed to. Specifically, methods to
74 generate large numbers of DCs from $CD34^+$ HSCs from cord blood (CB) using different
75 cytokine cocktails have been developed, mainly for cancer immunotherapy (Balan et al., 2009;
76 Harada et al., 2011; Plantinga et al., 2019). Our aim was to establish a robust protocol for
77 obtaining large numbers of DCs from cord blood derived $CD34^+$ HSCs. To assess functionality,
78 we compared the responses of CB-derived neonatal DCs following exposure to the TLR3 ligand
79 polyinosinic:polycytidylic acid (Poly(I:C)), an analogue of viral double-stranded RNA, and to
80 the TLR4 ligand lipopolysaccharide (LPS), a component of the outer membrane of Gram-
81 negative bacteria.

82

83 **2. Materials and Methods**

84 *2.1. Isolation of cord blood mononuclear cells (CBMCs)*

85 CB samples were obtained following informed consent from mothers undergoing elective
86 C-section (ethics code 07/Q1704/21 and 10/H0502/11) at the Princess Anne Maternity Hospital,
87 Southampton and were processed within 30 minutes of collecting. CBMCs were isolated by
88 means of Ficoll-Hypaque (GE Healthcare Life Sciences, Buckinghamshire, UK) gradient
89 centrifugation and cryopreserved in 7.5 % DMSO / 50 % fetal bovine serum (FBS) until further
90 use. CBMCs were thawed rapidly in a 37°C water bath and media added. The cell suspension
91 was centrifuged and incubated in the presence of DNase I solution (Stemcell Technologies,
92 Grenoble, France) for 15 mins at room temperature. Cells were then counted and further used for
93 magnetic isolation of $CD34^{+}$ progenitor cells.

94 *2.2.Isolation and expansion of CD34+ progenitor cells*

95 $CD34^{+}$ progenitor cells were obtained by magnetic isolation using the EasySep™ Human
96 CD34 Positive selection kit (Stemcell Technologies, Grenoble, France) prior to plating in 24-
97 well or 6-well plates at a cell density of 5 x 10^{4} cells per ml. Progenitor cells were cultured in
98 IMDM (Life Technologies, Paisley, UK) containing 10% (v/v) heat-inactivated HI-FBS, 5
99 units/ml Penicillin G Sodium and 50 µg/ml Streptomycin sulphate, 0.1 mM β-mercaptoethanol
100 (IMDM complete) with either 25 ng/ml Fms-related tyrosine kinase 3-Ligand (Flt-3L), 10 ng/ml
101 Stem cell factor (SCF), 10 ng/ml Interleukin-3 (IL-3), 10 ng/ml IL-6 (IL-6) collectively called
102 FS36 or 25 ng/ml Flt-3L, 10 ng/ml SCF, 10 ng/ml Thrombopoietin (TPO), collectively called
103 FTS and incubated at 37°C, 5% CO_2 for a total of 28 days. Cells were cultured either for 1) 2
104 weeks in FS36, followed by 2 weeks in FTS; 2) 3 weeks in FS36, followed by 1 week in FTS; or
105 3) 4 weeks in FTS. After 7 days, cells were collected, centrifuged at 300 x *g*, 10 mins, RT,
106 counted and re-seeded at 5 x 10^{4} cells per ml. At days 14 and 21, the cell densities were
107 increased to 2 x 10^{5} cells per ml. At day 28, cells were seeded into a 6-well plate at 1 x 10^{6} cells
108 per ml without FBS and incubated for 10 mins. Non-adherent cells were removed by washing
109 twice with IMDM. Fresh media (IMDM complete) containing 20 ng/ml IL-4 and 50 ng/ml GM-
110 CSF was then added to the adherent cells and cultured for another 5-6 days. Cells were fed every
111 second day by semi-depletion with fresh IMDM complete containing GM-CSF and IL-4. All
112 cytokines and growth factors were purchased from Peprotech (London, UK). For analysis of cell
113 surface markers, cells were incubated and stained with fluorescent-conjugated antibodies to the
114 cell surface markers CD1a, CD14, CD33 and CD34, (BD Biosciences, Oxford, UK) according to
115 manufacturer's instructions. Whole cell populations were gated and analyzed by flow cytometry

116 using a FACS Calibur. A Fixation and Dead Cell Discrimination Kit (Miltenyi Biotec, Surrey,
117 UK, #130-091-163) was used according to the manufacturer's instructions to gate out
118 populations of dead cells during analysis. The gating strategy is shown in Supplementary Figure
119 1.

120 *2.3.Stimulation and characterization of CBDCs*

121 After GM-CSF and IL-4 treatments, cells were plated at a density of 5 x 10^5 cells per ml
122 in a 12-well plate. High-molecular weight polyinosinic:polycytidylic acid (PolyI:C, 1.5–
123 8kb)(Invivogen, Toulouse, France) or lipopolysaccharide (LPS) (Sigma, Dorset, UK) were added
124 to cells at a concentration 10 µg/ml or 100-500 ng/ml respectively. Cells were incubated for 24
125 hrs at 37°C, 5% CO_2. For analysis of cell surface markers, cells were incubated and stained with
126 fluorescent-conjugated antibodies to the cell surface markers CD1a, CD14, CD33, CD34, HLA-
127 DR, CD40, CD80, CD86 (BD Biosciences, Oxford, UK) according to manufacturer's
128 instructions. Whole cell populations were gated and analyzed by flow cytometry using a FACS
129 Calibur. A Fixation and Dead Cell Discrimination Kit (MiltenyiBiotec) was used to gate out
130 population of dead cells during analysis.

131 *2.4.Cytokine assay*

132 Cell supernatants were analyzed for secretion of human IFN-γ, IL-1β, IL-6, IL-10, IL-
133 12p70 and TNF-α using a multi-array cytokines assay according to manufacturer's instructions
134 (Meso Scale Discovery, Rockville, USA).

135 *2.5.Statistical analysis*

136 Statistical evaluation was performed using the software SigmaPlot 12.5. If not stated
137 otherwise, related samples were analyzed for statistical significance using the Shapiro-Wilk test
138 for normality followed by a paired Student's t-test or Wilcoxon Signed Rank test. Differences
139 were regarded as significant when $P \leq 0.05$. For multiple comparisons, ANOVA followed by
140 Tukey test or the non-parametric Friedman followed by Dunn's was performed.

141

142 **3. Results**

143 *3.1.Establishment of expansion cell cultures from cord blood CD34^{+} hematopoietic stem*
144 *cells*

145 Our aim was to establish a robust method to expand precursors of DCs from CB CD34+
146 HSCs for the generation of DCs that can be used for analyzing neonatal immune responses.
147 Work published by Balan *et al*., (2009) used IMDM medium supplemented with a cytokine
148 cocktail containing 25ng/ml Flt3-L, 10ng/ml TPO and 20ng/ml SCF and 5% heat-inactivated
149 autologous cord blood plasma. However, using these conditions in our experiments, the CB stem
150 cells failed to survive beyond day 7. Therefore, we altered the culture conditions based on data
151 from the literature (Bontkes et al., 2002). After purification of CB CD34^{+} cells by magnetic
152 separation CD34^{+}, cells were cultured for a total of 4 weeks in either medium containing Flt3-L,
153 SCF, IL-3 and IL-6 (FS36) for 2 weeks followed by 2 weeks days in Flt3-L, TPO and SCF (FTS)
154 or were cultured for 3 weeks in FS36 medium followed by 1 week in FTS. As a comparison,
155 cells were also cultured for 4 weeks in FTS alone. We obtained the highest number of CD34^{+}
156 derived progenitor cells when cultures were grown in the presence of IL-3 and IL-6 (Figure 1A),
157 which is in line with previous observations (Bontkes et al., 2002). The ability of CD34^{+} HSCs to
158 proliferate was highest within the first 2 weeks of culture after which cell numbers plateaued or
159 decreased slightly (Figure 1A). This correlated with a decreased expression of the surface marker
160 CD34 indicating that cells had lost their pluripotency (Supplementary Fig. 2S). Cells cultured for
161 4 weeks in FTS showed very low proliferation demonstrating a key requirement for IL-3 and IL-
162 6 for growth. After 4 weeks in culture the progenitor cells were further characterized by flow
163 cytometry and both culture conditions showed comparable expression of cell-surface markers.
164 The majority of cells were positive for the myeloid lineage marker CD33 (88 – 94%). Between
165 25 – 30% were positive for the monocyte-specific marker CD14 (25 - 30%), and a low
166 percentage of cells were positive for the DC specific marker CD1a (2.5 – 3.5%) and the
167 hematopoietic stem cell marker CD34 (4.7 – 8.3%) (Figure 1B and C).

168

169 *3.2.Generation of dendritic cells from expanded progenitor cells derived from cord blood*
170 *CD34+ hematopoietic stem cells.*

171 After 28 days of expansion culture using either 2W FS36 + 2W FTS or 3W FS36 + 1W
172 FTS, DC were generated from the progenitor cells following culture for 5-6 days in the presence

173 of GM-CSF and IL-4. Use of 2W FS36 + 2W FTS or 3W FS36 + 1W FTS expansion culture
174 resulted in a similar number of DCs (Figure 2A). After culture for 5-6 days in GM-CSF and IL-4
175 neonatal DCs derived from either protocol expressed high levels of CD1a, a marker of DCs,
176 HLA-DR and CD33, a marker of myeloid linage, whereas expression of CD14, a monocyte
177 specific marker CD14, was negligible (2W FS36 + 2W FTS: 1.42 ± 0.14 % positive cells, MFI
178 3.0 ± 0.69; 3W FS36 + 1W FTS: 2.42 ± 0.08 % positive cells, MFI of 3.65 ± 0.68 (mean ±
179 SEM)) (Figure 2B-D). Over 60% of the cells were $CD11c^{+}HLA\text{-}DR^{+}$ (Supplementary Figure 3S).
180 This marker profile identifies the cells as DCs. There was no difference in the ability of the DC
181 progenitor cells obtained from the two different expansion culture conditions to differentiate into
182 DCs. These data demonstrate that high numbers of neonatal DCs can be generated from $CD34^{+}$
183 HSC from cord blood.

184

185 *3.3.Maturation of DCs*

186 Next we investigated whether the CB-derived neonatal DCs generated from precursors
187 expanded using the 2 different culture conditions described above showed any differences in
188 their ability to mature. Following exposure to the TLR3 ligand Poly(I:C) the expression levels of
189 CD40, CD80 and B7H1 were significantly increased compared to the unexposed control in
190 CBDCs generated from 2W FS36 +2W FTS expansion cultures and the expression levels of
191 HLA-DR, CD80, CD86 and B7H1 were significantly increased compared to the unexposed
192 control in CBDCs generated from 3W FS36 +1W FTS expansion cultures (Figure 3). However,
193 the fold induction of these surface markers in Poly(I:C) exposed CBDCs compared to the
194 unexposed control was comparable between the two different expansion methods used for the
195 DCs precursor cells. After maturation, DCs release a variety of cytokines that are able to
196 modulate T cell function. Therefore, we compared the cytokine profile of mature CBDCs
197 generated from precursors that have been expanded using the two different culture conditions. As
198 shown in Figure 4, mature CBDCs showed an increased release of IFN-γ, IL-1β, IL-6, IL-10, and
199 TNF-α but only IFN-γ reached statistical significance. Release of IL-12p70 was unchanged.
200 Furthermore, there was no difference between the culture conditions in the levels of cytokines
201 generated. In summary, we did not observe any difference in the maturation of CBDCs derived
202 from precursors that have been expanded using the two different expansion culture conditions.

203

204 ### *3.4.TLR-dependent maturation of CBDCs*

205 In order to assess the potential of CBDCs to mature in response to different TLR stimuli
206 we analyzed their response to the TLR4 agonist LPS and to the TLR3 agonist Poly(I:C). For this
207 set of experiments, we used neonatal DCs that had been generated from 2W FS36 + 2W FTS
208 expansion cultures since expansion was slightly higher using this culture condition and the
209 ability of neonatal DCs to mature was not affected by the expansion culture condition. As shown
210 in Figure 5, Poly(I:C) at 10µg/ml induced a significantly higher expression of the maturation
211 markers CD80, CD86, and CD40 compared to LPS used at 100µg/ml and 500µg/ml. Expression
212 of HLA-DR showed no significant change. Since 100µg/ml LPS induced a slightly higher CD80
213 and CD86 expression than 500µg/ml LPS, we used this concentration of LPS to analyze the
214 cytokine release further. Additionally, we chose 10µg/ml Poly(I:C) to mimic virally induced
215 maturation as this concentration showed a significant effect on maturation marker expression by
216 neonatal DCs compared to LPS, which mimics bacterial induced maturation. Interestingly,
217 release of IL-10 was only significantly increased by LPS, whereas release of IL-6, TNF-α and
218 IL-1β were only significantly increased by Poly(I:C) compared to the control (Figure 6). These
219 results suggest that neonatal DCs show a higher inflammatory potential when maturation is
220 induced by virus-related compared to bacteria-related components.

221

222 ## **4. Discussion**

223 DCs play an important role in bridging the innate and adaptive immune response to
224 infection. It is known that immune responses change over the life course (Simon et al., 2015) and
225 DCs are thought to be centrally involved in the priming process. However, the availability of
226 neonatal human DCs for functional *in vitro* studies is limited resulting in an unmet need for a
227 source of large numbers of neonatal DCs that can be used in detailed mechanistic studies.
228 Therefore, our aim was to establish a robust protocol for the generation of high numbers of DCs
229 from CB-derived HSCs that have a more 'naïve' phenotype and can be used to investigate the
230 characteristics of DCs in the context of early life. Previous studies have used $CD34^+$ HSCs
231 cultured in a cocktail of growth factors to expand precursors and drive their differentiation into
232 DCs. While Chang et al. (2012) expanded cord blood derived CD34+ HSCs by using SCF only
233 for expansion cultures, Harada et al. (2011) used a combination of GM-CSF and SCF. Arrighi et

234 al. (1999) and Balan et al. (2009) expanded CD34+ HSC from cord blood using a cocktail of Flt-
235 3L, TPO and SCF. Using this cocktail for expansion, we only observed a moderate expansion of
236 DC precursor cells. Interestingly, when using heat-inactivated autologous serum instead of FCS
237 as described by Balan et al. (2009), cells failed to survive for more than one week in culture.
238 Therefore, we aimed to optimize the expansion culture conditions in order to increase the
239 proliferation rate of CD34+ HSC from cord blood that would result in a high number of
240 precursor cells that can be differentiated into functional DCs. We adapted an expansion protocol
241 that was previously used for CD34+ HSC from adult peripheral blood, where $CD34^+$ HSC using
242 Flt3L, TPO, SCF, IL-3 and IL-6 followed by treatment with GM-CSF and IL-4 to generate DCs
243 (Bontkes et al., 2002). As previously reported, we observed the highest increase in precursor
244 cells in the presence of IL-3 and IL-6, which made a large-scale experimental setup more
245 feasible. Ultimately, we were able to differentiate myeloid DCs that were
246 $CD1a^+CD11c^+CD40^+HLA\text{-}DR^+$ and $CD14^-CD123^-$, surface markers that are characteristic of
247 human myeloid DCs (Baharom et al., 2017). Although it has been reported that DCs with high
248 expression of CD11c can be obtained using only GM-CSF and SCF for expansion culture, these
249 cells also expressed high levels of the monocyte marker CD14 (Harada et al., 2011). As the
250 authors did not analyze expression of CD1a or CD123 it is difficult to compare the purity or
251 functionality of the cells. Furthermore, it has been shown that $CD34^+$ $CD117^+$ cord blood cells
252 are multipotent and able to develop into monocytes, granulocytes, B cells, pDCs, $CD1c^+$ and
253 $CD141^+$ cDCs when co-cultured with stromal cells and Flt-3L, SCF and GM-CSF (Breton et al.,
254 2015). This underlines the importance of the conditions used for expansion culture and
255 differentiation which can determine the characteristics of the resulting cell populations. Further
256 work will be required to fully characterize the surface marker expression for a in depth
257 comparison of DC subtypes generated using our protocol. Previously, concerns have been raised
258 about the prolonged presence of IL-3 and IL-6 in the expansion cultures leading to decreased
259 levels of IL-12p70 and increased levels of IL-10 (Bontkes et al., 2002; Ebner et al., 2002).
260 However, we did not observe any significant differences in the secretion of either cytokine from
261 mature CBDCs generated from expansion cultures using IL-3 and IL-6 for the first two weeks or
262 the first 3 weeks of expansion culture. A shorter GMP protocol, which has applicability in
263 cancer immunotherapy, consisting of one week expansion culture with Flt-3L, SCF, IL-3 and IL-
264 6 followed by one week differentiation into DCs using Flt-3L, SCF, GM-CSF and IL-4 has been

265 used by Plantinga et al. (2019) to expand CD34+ stem cells into a population consisting of about
266 34% $CD11c^{+}HLA\text{-}DR^{+}$ DCs. In contrast, our method resulted in over 60% $CD11c^{+}HLA\text{-}DR^{+}$
267 DCs. Additionally, Plantinga et al. demonstrated that CB-derived DCs originate exclusively from
268 CD115+ progenitor cells and resemble cDC2. In summary, we established a protocol that allows
269 the generation of high numbers of neonatal DCs from CB-derived HSCs that can be used to
270 analyze the DC immune response in an early life setting.

271 In our study, we analyzed the ability of CBDCs to respond to different stimuli. High
272 molecular weight Poly(I:C), which is an analogue of double-stranded RNA that is produced
273 during the replication cycle of certain viruses and has been shown to activate TLR3, RIG-
274 I/MDA5 and PKR, caused a more Th1 biased cytokine profile with high IL-12, IFN-γ, TNF-α,
275 IL-6 and IL-1β release. Using LPS to induce maturation, a bacterial membrane component of
276 Gram-negative bacteria, we observed a significantly induced release of IL-10. These findings
277 are consistent with previous studies using CB-monocyte derived DCs which showed that
278 Poly(I:C) induced maturation resulted in higher IL-12p70 release compared to LPS (Goriely et
279 al., 2001) and indicate that the immune responses specific for neonatal DCs are sustained in our
280 model system.

281 Our observation of significantly increased IL-6 release from neonatal DCs during
282 Poly(I:C) induced maturation, which mimics a viral infection, might have important modulatory
283 functions during the polarization of naive T cells in early life. It has been shown that exogenous
284 IL-6 in addition to TGFβ is an important factor during Th17 polarization (Swain et al., 2012;
285 Martinez-Sanchez et al., 2018). While the role of Th17 cells in viral infections is not fully
286 understood, there is evidence that Th17 cells can support neutrophil recruitment via release of
287 IL-17 (Ye et al., 2001; Mukherjee et al., 2011). Additionally, Th17 cells are thought to play an
288 important role in the anti-microbial host defense at mucosal surfaces (Rathore and Wang, 2016).
289 In order to demonstrate the direct T cell stimulating ability of these DCs further experiments
290 using DC-T cell co-cultures need to be performed. Through access to *in vitro* generated neonatal
291 DCs, the mechanisms of innate and adaptive immune responses to viral and bacterial infections
292 early in life can now be investigated in detail. This is of importance as this time in early life,
293 described as the neonatal window of opportunity, is linked to the susceptibility for many immune
294 disorders in later life (Torow and Hornef, 2017). In particular, this has the potential to identify

295 the mechanistic link between early life infections and the risk of developing chronic diseases
296 later in life. Furthermore, there is evidence that innate immune responses of neonates are
297 influenced by maternal factors in utero. For example, Hrdy et al. (2014) showed a higher IL-12
298 gene expression in neonatal CB-DCs derived from allergic compared to non-allergic mothers.
299 Interestingly, a reduced release of IL-6 was observed in CB-derived monocytes after stimulation
300 with LPS in neonates with maternal allergy compared to non-allergic controls (Saghafian-
301 Hedengren et al., 2008). However, further detailed mechanistic investigations are needed using
302 neonatal DCs in order to establish a functional link infections early in life, IL-6 production, Th17
303 cells function and the risk of developing chronic diseases later in life. In conclusion, we present
304 here a robust protocol for the generation of high numbers of neonatal DCs that are derived from
305 CD34+ HSCs from CB that can be used for mechanistic studies of early life immune responses.
306
307
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310

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Figure Legends

Figure 1: Expansion of cord blood CD34+ progenitor cells using different cytokine cocktails. A: proliferation of progenitor cells in the presence of different cytokine cocktails. B: Expression of surface markers after 28 days of culture. Mean±SEM. n=3-5 independent experiments each using different donors. C: Representative histogram overlays. (thin line: isotype control, grey infill: marker expression; 2W FS36+2W FTS: 2 weeks culture in Flt3-L, SCF, IL-3 and IL-6 followed by 2 weeks Flt3-L, TPO and SCF. 3W FS36+1W FTS: 3 weeks culture in Flt3-L, SCF, IL-3 and IL-6 followed by 1 weeks Flt3-L, TPO and SCF. 4W FTS: 4 weeks Flt3-L, TPO and SCF.

Figure 2: Expression of DC-specific surface markers of neonatal DCs generated from expanded CD34+ progenitor cells. After 4 weeks of expansion culture using cytokine cocktails detailed in Materials and Methods section, neonatal DCs were generated using IL-4 and GM-CSF. A: The number of DCs generated using the 2 different expansion culture conditions is expressed per CD34+ haematopoietic stem cell (HSC). B and C: Expression of surface markers was analysed by flow cytometry. Mean fluorescence intensity (B) and % positive cells (C) of neonatal DCs generated from 2 different CD34+ cell expansion culture conditions. Mean±SEM. n=4 independent experiments using different donors. D: Representative histogram overlays of surface marker expression by neonatal DCs generated from 2W FS36 + 2W FTS expansion cultures. (thin line: isotype control, grey infill: marker expression)

Figure 3: Expression of maturation markers by neonatal DCs. After expansion of CD34+ haematopoietic stem cells using 2 different cytokine cocktails and differentiation into DCs maturation was induced by incubation with Poly(I:C) for 24h. Expression of surface markers on immature and mature DCs was analysed by flow cytometry. A: Expression of maturation markers by neonatal DCs that have been generated from expansion cultures using 2 weeks Flt-3L,

408 SCF, IL-3 and IL-6 followed by 2 weeks in Flt-3L, TPO and SCF (2W FS36+2W FTS). B:
409 Expression of maturation markers by neonatal DCs generated from expansion cultures using 3
410 weeks Flt-3L, SCF, IL-3 and IL-6 followed by 1 weeks in Flt-3L, TPO and SCF (3W FS36+1W
411 FTS). C: Fold induction of maturation markers by mature neonatal DCs normalised to the level
412 of immature DCs generated from CD34+ expansion cultures using 2 different cytokine cocktails.
413 Mean±SEM. n=4 independent experiments using different donors. *: p≤0.05
414
415 **Figure 4: Release of cytokines by neonatal DCs after maturation**. Neonatal DCs were
416 generated from CD34+ progenitors using 2 different cytokine cocktails for expansion culture as
417 described in the Method section. DCs were matured by incubation with 10μg/ml Poly(I:C) for
418 24h and the release of mediators determined in cell-free supernatants by multiplex assay. A: IL-
419 12p70; B: IL-10; C: IFN-γ; D: IL-6; E: TNF-α; F: IL-1β. Mean±SEM. n=4 independent
420 experiments using different donors. *: p≤0.05
421
422 **Figure 5: Expression of maturation markers by neonatal DCs varies with different TLR**
423 **activation.** Neonatal DCs were incubated for 24h with the TLR4 ligand LPS or the TLR3 ligand
424 Poly(I:C) and the expression of surface markers analyzed by flow cytometry. A: CD80; B: CD86;
425 C: CD40; D: HLA-DR. Results shown are means ± SEM, n=4 independent experiments using
426 different donors. *: p≤0.05 (ANOVA followed by Tukey multiple comparisons test).
427
428 **Figure 6: Differential release of cytokines after maturation of neonatal DCs with TLR3 or**
429 **TLR4 ligands.** After incubation with LPS (TLR4 ligand) or Poly(I:C) (TLR3 ligand) for 24h the
430 release of mediators was analysed in the cell culture supernatants by multiplex assay. A: IL-
431 12p70; B: IL-10; C: IFN-γ; D: IL-6; E: TNF-α; F: IL-1β. Results shown are means ± SEM, n=4
432 independent experiments using different donors. *: p≤0.05 compared to control (Friedman test
433 followed by Dunn's multiple comparisons test).