[Frontiers in Bioscience 14, 3814-3824, January 1, 2009]

IL-3 plays a critical role in development of dendritic cells from murine hematopoietic progenitor cells of fetal liver

Qiaoli Gu[1], Shan He[1], Yuhua Qiu[2], Tongyu Zhu[3], Tong Zhou[4], Jianqing Mi[5], Min Jin[1], Hailiang Ge[1], Jingwei Xu[1], Yanyun Zhang[1,6]

[1]Shanghai Institute of Immunology, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine and Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, [2]Department of Immunology, Soochow University, Suzhou, China, [3]Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China, [4]Department of Nephrology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [5]Shanghai Institute of Hematology, Department of Hematology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [6]Key Laboratory of Stem Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences

TABLE OF CONTENTS

1. ABSTRACT

We previously showed that hematopoietic progenitor cells (HPCs) with the (Lin)$^-$*c-kit*$^+$ phenotype isolated from day 13 postcoitus murine fetal liver (FL) can develop into dendritic cells (DCs) *in vitro* in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), Flt3 ligand (Flt3L) and tumor necrosis factor (TNF)-alpha. This differentiation is dependent upon the presence of PA6 stromal cells. In the present study, we show that after intravenous transfer into lethally irradiated mice, the FL Lin$^-$*c-kit*$^+$ HPCs generate E-cadherin$^+$ Langerhans cells, and splenic DCs that express high levels of Ia, CD11c, DEC205 and intermediate levels of CD40. These cells are capable of stimulating a T cell response. Interestingly, neutralizing anti-interleukin (IL)-3 antibody suppresses generation of FL HPC-derived DC *in vivo*. Moreover, addition to FL HPCs culture of IL-3 along with GM-CSF, SCF, Flt3L and TNF-alpha results in DC development from without the support of PA6 cells. This study provides the first evidence that IL-3 plays a critical role in DC development from FL Lin$^-$*c-kit*$^+$ HPCs.

2. INTRODUCTION

Dendritic cells (DCs), accepted as the most potent antigen presenting cells, can take up, process, transport and present antigens to T cells. DCs are not only crucial for the initiation of innate and adaptive immune response, but are also important for the maintenance of immunological balance within tissues (1,2,3). Allogeneic DCs can induce non-responsiveness in T cells in fetal thymic organ cultures, a phenomenon that suggests that DCs may be involved in the induction of central tolerance during fetal development (4). DCs are a highly heterogeneous population with a variety of tissue distribution patterns, surface phenotypes, and immune functions (5,6). In addition, Langerhans cells (LCs) are the only DCs which present in the epidermis and constitute the first immunological barrier against pathogens and environmental insults (7).

It is well known that DCs have their ultimate origin in hematopoietic progenitor cells (HPCs) (8). During embryonic development of hematopoiesis, HPCs

successively appear in yolk sac, para-aortic splanchnopleura, aorta-gonad-mesonephros region (AGM), fetal liver (FL), spleen, and bone marrow (BM) (9). FL is thought to be the principle hematopoietic organ during murine embryonic development, and serves as a pool of founder hematopoietic cells generated at early hematopoietic sites within the conceptus (10,11). Previous studies revealed that murine FL-derived progenitor cells differentiate *in vitro* into T cells, NK cells, B cells, and macrophages (12,13). We have successfully generated DCs from FL-derived progenitor cells *in vitro* (14). Interestingly, generation of DCs from FL HPCs *in vitro* requires the support of PA6 stromal cells, a condition different from that of BM HPC-derived DCs. Since BM is formed relatively late in gestation and is involved in the production of highly differentiated hematopoietic cells (10,15), DC development from FL HPCs may be expected to require culture conditions distinct from those of adult BM HPCs.

Accumulating studies have revealed that HPCs derived from BM or cord blood can develop into DCs *in vitro* in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-alpha and interleukin (IL)-4 (16,17,18). Stem cell factor (SCF), however, is required for BM HPC differentiation of cells with (Lin)$^-$*c-kit*$^+$ phenotype (19). CD44$^+$CD25$^-$FcR$^-$ progenitors present in fetal thymus generate DCs with the support of IL-3 and IL-7 (20). However, the identities of factors which allow development of DCs from FL HPCs are still unknown.

Transfer of mouse BM HPCs into irradiated recipients leads to the reconstitution of DCs in the spleen and thymus (21,22,23). In addition, CD8$^+$CD11c$^-$Lin$^-$ splenic cells differentiate into CD8$^+$ DCs *in vivo* (24). In the present study, we show in mouse chimera models of hematopoiesis reconstitution utilizing day 13 postcoitus (13 dpc) murine FL-derived Lin$^-$*c-kit*$^+$ HPCs. We find that Lin$^-$*c-kit*$^+$ HPCs derived from 13 dpc FL develop *in vivo* into DCs in the spleen and into LCs in the skin. These DCs possess an intact capacity to initiate T cell immunity. Moreover, we find that IL-3 is able *in vitro* to induce DC generation from FL HPCs without the support of PA6 cells and is also crucial for FL HPC-derived DC development *in vivo*.

3. MATERIALS AND METHODS

3.1. Experimental animals

C57BL/6 (B6, Ly5.2) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and were kept in a specific pathogen-free facility at the Animal Center of Shanghai Jiao Tong University School of Medicine. Congeneic B6 (Ly5.1) mice were a kind gift of Peking University Health Science Center (Beijing, China), and were purchased from The Jackson Laboratory. FL was obtained from 13 dpc fetuses born to female B6 mice mated with male B6 mice. The presence of a postcoital plug was used to determine day 0 of pregnancy Animal care and use were in compliance with institutional guidelines.

3.2. Cytokines and antibodies

Recombinant mouse GM-CSF, TNF-alpha, SCF, Flt3 ligand (Flt3L), IL-3, IL-7 and IL-15, and neutralizing monoclonal antibody (MoAb) to IL-3 (MP28F8) were purchased from R&D systems (Minneapolis, MN). Anti-*c-kit* antibody (ACK-2) was kindly provided by Dr T. Sudo (Basic Research Institute of Toray Co., Kanagawa, Japan). The neutralizing antibody to IL-7 was obtained from PeproTech EC (London, UK). Unless otherwise indicated, antibodies used for immunofluorescence staining were obtained from BD PharMingen (San Diego, CA). DEC205 (NLDC-145) MoAb was obtained from Serotec (Oxford, UK). The MoAb against mouse E-cadherin was purchased from Dainipon Pharmaceutical Corporation (Osaka, Japan). Alkaline phosphatase-conjugated goat anti-rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and alkaline phosphatase-conjugated goat anti-hamster IgG was purchased from Cedarlane (Ontario, Canada). Streptavidin-peroxidase (Histofine) was purchased from Nichirei Corporation (Tokyo, Japan).

3.3. Purification and transfer of Lin$^-$*c-kit*$^+$ HPCs

BM Lin$^-$*c-kit*$^+$ HPCs were obtained as previously described (19,25). FL Lin$^-$*c-kit*$^+$ HPCs were sorted from 13 dpc FL mononuclear cells (MNCs) using a FACSAria cytometer from BD Biosciences as previously described (14). In brief, FL MNCs were subjected to indirect staining using a biotin-conjugated anti-*c-kit* MoAb and phycoerythrin (PE)-labeled streptavidin, followed by treatment with a set of fluorescein isothiocyanate (FITC)-labeled MoAbs to CD3e (145-2C11), CD4 (H129.19), CD8a (53-6.7), B220 (RA3-6B2), Gr-1 (Ly-6G), CD11a (2D7), and CD11b (M1/70). Cellular purity was consistently more than 98% as reanalyzed with the cell sorter.

B6 (Ly5.2) Lin$^-$*c-kit*$^+$ cells (5×10^4) derived from FL or BM were intravenously injected into gamma ray-irradiated B6 (Ly5.1) recipient mice (10.5 Gy), along with 5×10^4 unfractionated BM cells from B6 (Ly5.1) mice to ensure mouse survival. In some experiments, neutralizing antibodies to IL-3 and/or IL-7 were intraperitoneally injected into the recipient mice every 3 days for 6 weeks after implantation.

3.4. *In vitro* generation of DCs from Lin$^-$*c-kit*$^+$ FL HPCs

Purified FL Lin$^-$*c-kit*$^+$ HPCs were incubated at a cell concentration of 3×10^4 cells/mL in Iscove's modified Dulbecco's medium (Gibco, Rockville, MD), supplemented with 20% fetal calf serum, penicillin G (100 U/mL), and streptomycin (100 micrograms/mL) as complete medium, in the presence of GM-CSF (4 ng/mL), SCF (10 ng/mL) and Flt3L (10 ng/mL). In some experiments, combined IL-3 (10 ng/mL), IL-7 (10 ng/mL) and IL-15 (20 ng/ml), or IL-3, IL-7 and IL-15 alone was added to the culture system. Fresh medium containing cytokines was added after medium change every 2 or 3 days. After 12 to 14 days of culture, GM-CSF (4 ng/mL) and TNF-alpha (50 ng/mL) were added to the culture for an additional 3 to 5 days.

3.5. Immunofluorescence analysis

Immunofluorescence staining was performed as previously described (14,19,25). Cells (4×10^5) were

stained with PE-conjugated anti-Ia (AF6-120.1) and FITC-conjugated anti-CD11c (HL3), anti-CD40 (3/23), anti-CD86 (GL1), anti-CD8a (53-6.7), anti-NK1.1 (PK136), anti-Thy1.2 (30-H12) MoAbs. In some experiments, cells were stained with purified rat anti-DEC205 or anti-E-cadherin MoAbs, followed by FITC-conjugated goat anti-rat IgG $F(ab')_2$ antibody (Caltag, Camarillo, CA). In three-color analysis, cells were incubated with biotin-conjugated anti-Ly5.2 MoAb (104), followed by allophycocyanin-conjugated streptavidin. The instrument compensation was set in each experiment by using single-color and/or two-color stained samples.

3.6. Immunohistochemical staining

Double immunostaining was performed by indirect immunoalkaline phosphatase or immunoperoxidase method. Mouse ears were resected and torn into ventral and dorsal pieces. Dorsal ear pieces were then incubated in 0.5 M, pH 8.0 EDTA solution at a temperature of 37 degrees centigrade for 1 hour. The epidermis sheets were then spread on silanized slides (Dako-Japan Co.), rinsed twice in isotonic saline solution and fixed in cold acetone for 10 minutes. The sections were sequentially incubated with optimal dilutions of biotin-conjugated anti-Ly5.2 followed by streptavidin-peroxidase. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA) exhibiting a red color. The sections were then incubated with hamster anti-mouse CD11c (N418) followed by alkaline phosphatase-conjugated goat anti-hamster IgG. Sections were also separately stained with rat anti-DEC205, anti-CD8a, anti-E-cadherin, anti-Ia, or anti-Thy-1.2 followed by goat-anti-rat IgG complexed to alkaline phosphatase. Alkaline phosphatase activity was developed with the Vector Blue substrate (Vector Laboratories) revealing a blue color. Levamisole (0.024%; Sigma-Aldrich, MO) was added to the reaction mixture to block endogenous alkaline phosphatase activity.

3.7. DC isolation

DCs were isolated from spleens and lymph nodes (combined axillary, cervical and inguinal lymph nodes) as previously described (26) with slight modifications. Briefly, spleens and lymph nodes were digested with collagenase D (1 mg/mL; Sigma-Aldrich), and MNCs were isolated using Lymphoprep^{TM} gradient centrifugation. To enrich DC populations, these MNCs underwent magnetic selection for isolation of $CD11c^+$ or $DEC205^+$ cell subpopulations. In some experiments, the corresponding cell subpopulations were further purified utilizing a cell sorter.

3.8. Mixed leukocyte reaction (MLR)

Splenic DCs and cultured DCs were treated with mitomycin C (MMC; 15 micrograms/mL), and used as stimulators (27). Purified $CD4^+$ T cells were obtained from splenic MNCs in allogeneic mice by magnetic cell sorting. Indicated stimulator cells (from 3 to 100×10^4 cells) were added to the T cells (3×10^5) in 96-well round-bottom plates. After 5 days of culture at a temperature of 37^o C , cell proliferation was assessed using 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). In brief, 15 microliters of MTT (5 mg/mL in PBS) was added into each well and the plates were incubated at 37^o C for 4 hours. The absorbance at 550 nm was read using a microplate reader.

3.9. Contact hypersensitivity (CHS) response

Four hundred microliters of a 0.5% solution of FITC (Sigma-Aldrich) dissolved in acetone/dibutylphthalate (1:1) was applied to the shaved abdomens of mice. Twenty hours later, 1×10^5 donor-derived DCs were sorted from lymph nodes of FITC-sensitized mice, and then transferred into footpads of naive syngeneic mice. One week later, CHS was elicited by painting the dorsal and ventral ear surfaces with 5 microliters of 0.5% FITC. Ear thickness was measured 24 hours after the challenge and compared with that before challenge. Specific swelling was calculated by subtracting the background swelling from mice that were not sensitized but were challenged.

3.10. Statistical analysis

Data are presented as mean +/- SD of the results from multiple experiments. Student's *t*-test or one way ANOVA was used to determine the statistical significance. *P* value less than 0.05 was considered to be statistically significant.

4. RESULTS

4.1. FL $Lin^-c\text{-}kit^+$ HPCs can develop into splenic DCs *in vivo*

To study the development of DCs from FL HPCs *in vivo*, a murine chimera model of reconstituted hematopoiesis was established with 13 dpc FL and adult BM $Lin^-c\text{-}kit^+$ HPCs. Purified 5×10^4 FL or BM $Lin^-c\text{-}kit^+$ HPCs from Ly5.2 B6 mice were injected into lethally irradiated congeneic mice (Ly5.1) via the tail vein. The ability of these cells to reconstitute hematopoiesis and to initiate DC development *in vivo* was examined. The $c\text{-}kit^+$ donor cells occupied approximately 85% of all $c\text{-}kit^+$ hematopoietic cells of BM from the chimera reconstituted with FL HPCs, but only 65% of those reconstituted with BM HPCs at the third week after implantation. This difference indicates that FL-derived $Lin^-c\text{-}kit^+$ HPCs possess stronger self-renewal hematopoietic ability than their adult BM counterparts *in vivo*. In addition, donor-derived splenic $Ia^+DEC205^+$ cells were detected in preparation for DC generation. DEC205 was chosen as a marker for DC development to avoid the mistaken exclusion of $CD11c^{-/dull}$ DCs. This was necessary because of the previously observed marginal expression of CD11c antigen on DCs generated *in vitro* from 13 dpc $Lin^-c\text{-}kit^+$ FL HPCs in our coculture system with PA6 stromal cells (14). Donor-derived splenic DCs were undetectable in chimeras reconstituted with adult BM $Lin^-c\text{-}kit^+$ HPCs until 3 weeks after implantation. However, splenic DCs from FL HPCs were found only 4 weeks after hematopoietic reconstitution and their number increased to a plateau after 6 weeks (Figure 1), implying that the development of DCs *in vivo* from 13 dpc $Lin^-c\text{-}kit^+$ FL HPCs requires different conditions than that from adult BM HPCs.

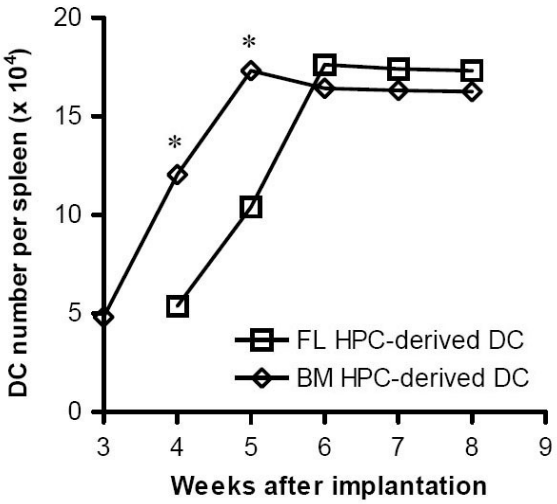

Figure 1. The kinetics of splenic DC generation *in vivo* from FL or BM Lin$^-$*c-kit*$^+$ HPCs. Purified 13 dpc FL or adult BM Lin$^-$*c-kit*$^+$ HPCs (5×10^4) isolated from Ly5.2 B6 mice were intravenously injected into lethally irradiated Ly5.1 B6 mice, accompanied by 5×10^4 Ly5.1-type BM cells to rescue the recipient mice from lethal irradiation. Ly5.2$^+$Ia$^+$DEC205$^+$ spleen cells isolated from chimeras were measured to assay donor-derived DC generation *in vivo* from FL or adult BM Lin$^-$*c-kit*$^+$ HPCs at the indicated time points after implantation. Each point was based upon a pool of 6 spleens obtained from the chimeric mice. The data represent mean +/- SD of number of donor splenic DCs from 3 independent experiments.

To further investigate the phenotypic characteristics of the DCs generated *in vivo* from FL Lin$^-$*c-kit*$^+$ HPCs, we isolated CD11c$^+$ and DEC205$^+$ splenocytes from chimeras. Immunophenotyping analysis were performed on these isolated splenic CD11c$^+$ and DEC205$^+$ cell subsets generated *in vivo* from FL or adult BM HPCs by gating on Ly5.2$^+$ donor cells. As shown in Figure 2A, the FL HPC-derived donor CD11c$^+$ cells expressed a high level of Ia and intermediate levels of CD40, DEC205 and CD8a molecules. The donor FL HPC-derived DEC205$^+$ splenic cell population also showed a high level of Ia and an intermediate level of CD40 molecules. Indeed, there were high levels of both Ia and CD11c expression observed in DEC205$^+$ splenic cell populations derived from donor FL and BM HPCs. Few of the freshly isolated donor cells from FL and BM HPCs expressed the CD86 molecule (Figure 2A). However, the expression of CD86 was greatly increased after culture overnight with GM-CSF and TNF-alpha (data not shown). The phenotypic characteristics of DCs generated *in vivo* from 13 dpc FL Lin$^-$*c-kit*$^+$ HPCs were comparable with those of adult BM HPCs. Remarkably, some of the donor FL HPC-derived CD11c$^+$ cells were also highly positive for the Thy1.2 marker.

We next examined the stimulatory ability of donor FL HPC-derived DCs. As shown in MLR (Figure 2B), FL HPC-derived splenic Ly5.2$^+$Ia$^+$DEC205$^+$ DCs stimulated the proliferation of allogeneic CD4$^+$ T cells to as potent a degree as did splenic DCs derived from BM HPC-reconstituted and wild type control mice. These findings suggested that FL HPC-derived donor splenic DCs could efficiently stimulate T cell proliferation *in vitro*.

4.2. FL Lin$^-$*c-kit*$^+$ HPCs can develop into skin LCs *in vivo*

LCs, the only DCs present in the epidermis, have been considered to represent immature DCs that differentiate into mature DCs when they migrate to the T cell areas of the draining lymph nodes after antigenic stimulation (1). As shown in Figure 3A, donor-derived LCs could be observed on the epidermal sheets of recipient mice receiving 13 dpc Lin$^-$*c-kit*$^+$ FL HPCs. These cells clearly displayed stellate, dendritic forms of slender processes that radiated from the cell body and extended to form a network with the keratinocytes. These donor-derived LCs partially expressed Ia, DEC205, E-cadherin and Thy1.2 while they expressed very little CD11c and were negative for CD8a.

CHS responses can be used to assess the function of LCs *in vivo*. We induced CHS responses in FL or BM HPC-reconstituted chimeras by applying FITC as a sensitizer on the ear skin. There were comparable levels of CHS elicited in both sets of mice, presumably as a consequence of epidermal LCs emigrating and carrying antigens to the T cell areas of draining lymph nodes (Figure 3B). These results indicate that LCs generated *in vivo* from 13 dpc Lin$^-$*c-kit*$^+$ HPCs can initiate immune responses by taking up, processing and presenting antigens to T cells.

4.3. Generation of DCs from FL Lin$^-$*c-kit*$^+$ HPCs *in vitro* without stromal cell support

Although a cytokine cocktail including GM-CSF, SCF, Flt3L and TNF-alpha is sufficient to generate mature DCs from BM Lin$^-$*c-kit*$^+$ HPCs, development of FL Lin$^-$*c-kit*$^+$ HPCs into mature DCs has been found to be dependent upon the presence of PA6 stromal cells *in vitro* (14). Several cytokines were tested for their abilities to generate DCs from FL Lin$^-$*c-kit*$^+$ HPCs in a stromal cell-free system. Addition of IL-3, IL-7 and IL-15 to FL HPC cultures in the presence of GM-CSF, SCF and Flt3L resulted in DC precursors, which subsequently further differentiated into mature DCs upon stimulation with GM-CSF and TNF-alpha. Flow cytometric analysis (gating on Ia$^+$ cells) indicated that these cells expressed high levels of CD86, CD11c, CD40, DEC205 and intermediate level of E-cadherin molecules. However, CD8a and NK1.1 were marginally expressed on these cells (Figure 4A). Their phenotypic characteristics were similar to those of DCs generated from FL HPCs *in vivo.* Functionally, these cells are able to stimulate allogenic T cell proliferation just as are DCs generated with PA6 cells in MLR (Figure 4B). These results indicate that combined stimulation of cytokines including IL-3, IL-7 and IL-15 are able to promote DC development from FL-derived HPCs independent of stromal cell support.

4.4. IL-3 is important for generation of FL Lin$^-$*c-kit*$^+$ HPC-derived DCs *in vitro* and *in vivo*

We next determined which cytokine was responsible for the development of Lin$^-$*c-kit*$^+$ HPC-derived

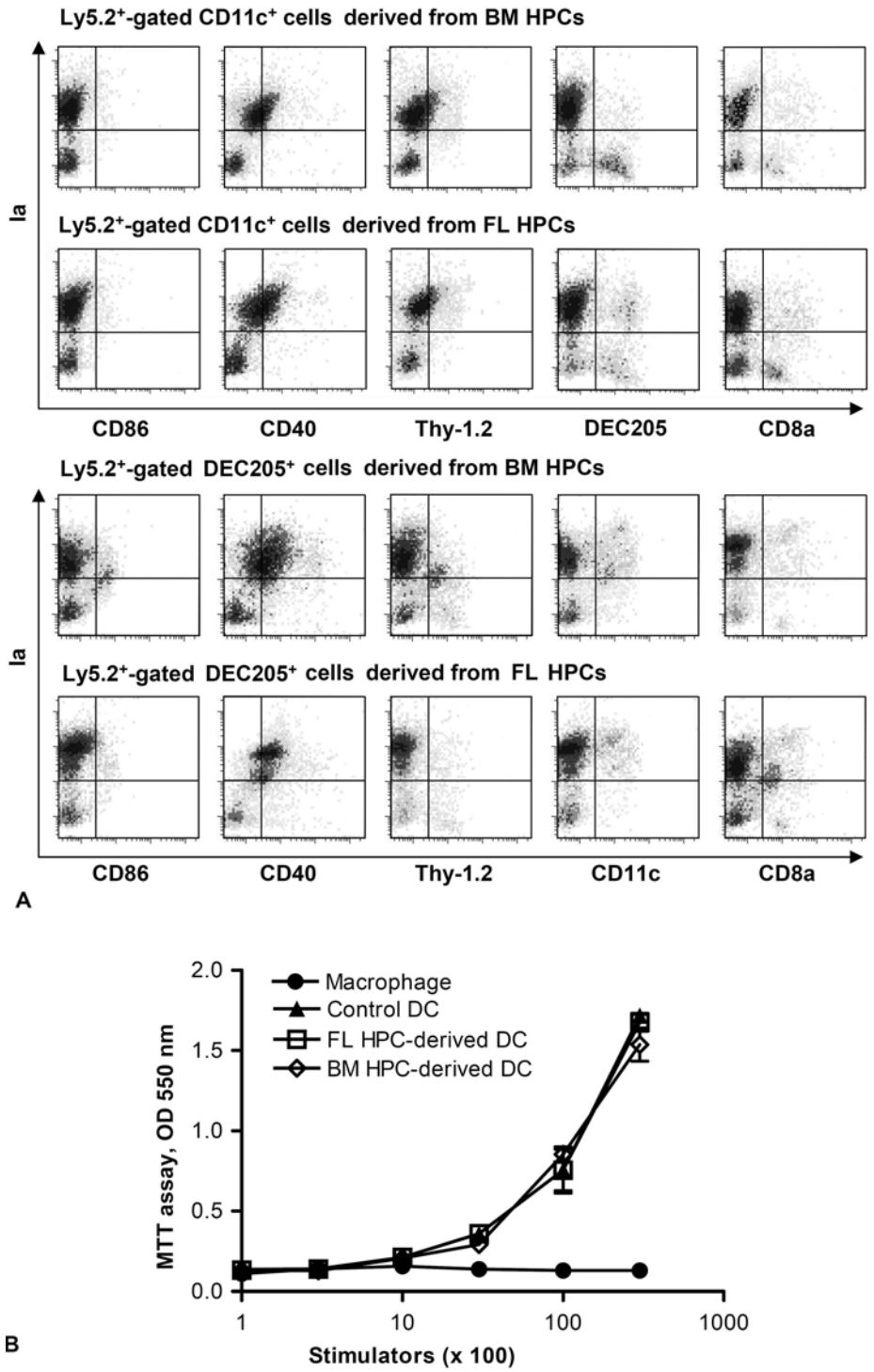

Figure 2. Characteristics of DCs generated *in vivo* from 13 dpc FL Lin$^-$*c-kit*$^+$ HPCs. After 28 days post implantation, splenocytes of these chimeras were isolated and CD11c$^+$ and DEC205$^+$ cells were enriched using magnetic selection. (A) The phenotypes of Ly5.2$^+$-gated donor CD11c$^+$ or DEC205$^+$ cells derived from FL or adult BM Lin$^-$*c-kit*$^+$ HPCs were analyzed using immunofluorescence staining as described in "Materials and Methods". Quadrants were applied to an isotype-matched control dot plot. (B) Allogeneic MLR was performed using purified CD4$^+$ T cells (3×10^5 cells/well in 96-round bottom plates) as responder cells. Donor splenic DCs derived *in vivo* from FL HPCs were obtained by FACS sorting with markers Ly5.2, Ia and DEC205, treated with MMC and used as stimulator cells. The proliferation of T cells was measured using MTT after 5 days culture. Donor splenic DCs derived *in vivo* from BM-derived HPCs, and splenic DCs and peritoneal macrophages isolated from naive Ly5.2$^+$ B6 mice were used as controls. Results are expressed as mean +/- SD of triplicate cultures.

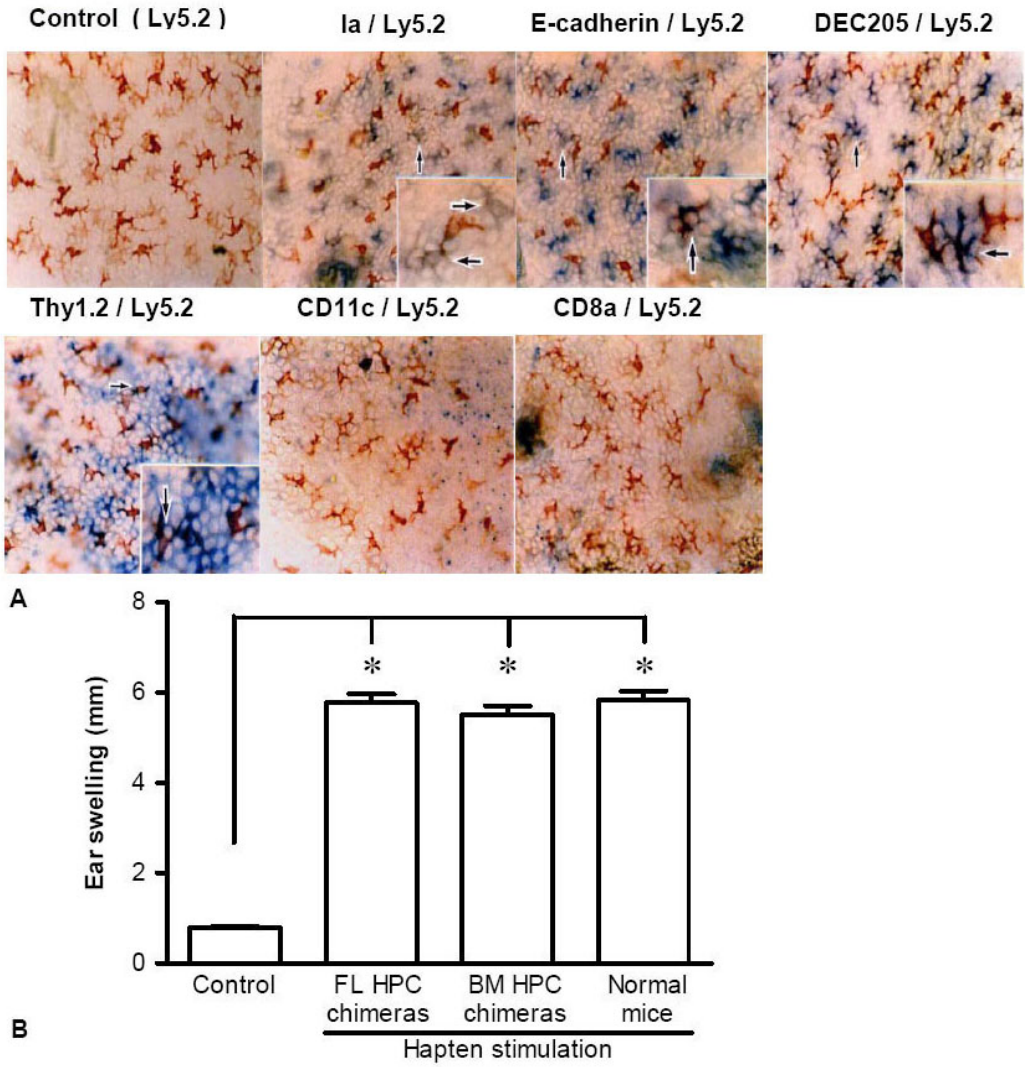

Figure 3. Generation of skin epidermal LCs *in vivo* from FL Lin$^-$*c-kit*$^+$ HPCs. (A) Skin sheets were prepared at day 28 after implantation with FL HPCs and exposure to UV light, and were stained by immunohistochemistry as described in "Materials and Methods". In these preparations, peroxidase activity yields a red product, whereas alkaline phosphatase activity appears blue. (B) Prepared FITC solution as a hapten was applied to stimulate the shaved abdomens of normal mice and of mice undergoing reconstituted hematopoiesis with FL or adult BM-derived HPCs. Twenty hours later, the donor DCs from draining lymph nodes of FITC-sensitized mice were isolated with markers Ly5.2, Ia and DEC205 using FACS. Sorted FITC-bearing DCs (1×10^5) were transferred into the footpads of naive syngeneic mice. One week later, a CHS response was elicited by painting the dorsal and ventral surfaces of the ears of the FITC-bearing DC-recipient mice with FITC solution. The magnitude of ear swelling was measured 24 hours after challenge and compared with ear thickness before challenge. Specific swelling was calculated by subtracting the background swelling of ears in mice that were not recipients of sensitized DCs but were challenged. Results are expressed as mean +/- SD (n = 6). These data are representative of three independent experiments. Asterisk represents *p* value less than 0.05.

DCs in the absence of stromal cells *in vitro*. Addition of IL-7 or IL-15 alone to the GM-CSF, SCF, Flt3L cocktail failed to induce DC precursors from FL HPCs. Interestingly, when IL-3 was added to the cocktail, DC precursors were generated, and subsequently differentiated into mature DCs after stimulation with TNF-alpha and GM-CSF. Moreover, a combination of IL-3 and IL-7 significantly increased the generation of FL HPC-derived DCs (Figure 4C). These induced cells were shown to enhance the proliferation of allogeneic T lymphocytes in MLR (Figure 4D). These results indicate that IL-3 and IL-7 can support DC development from FL-derived Lin$^-$*c-kit*$^+$ HPCs independent of stromal cell support. In particular, IL-3 appeared to be a key factor in the generation of DCs from FL HPCs.

Finally, we explored the effect of IL-3 and IL-7 in the development of FL Lin$^-$*c-kit*$^+$ HPC-derived DCs *in*

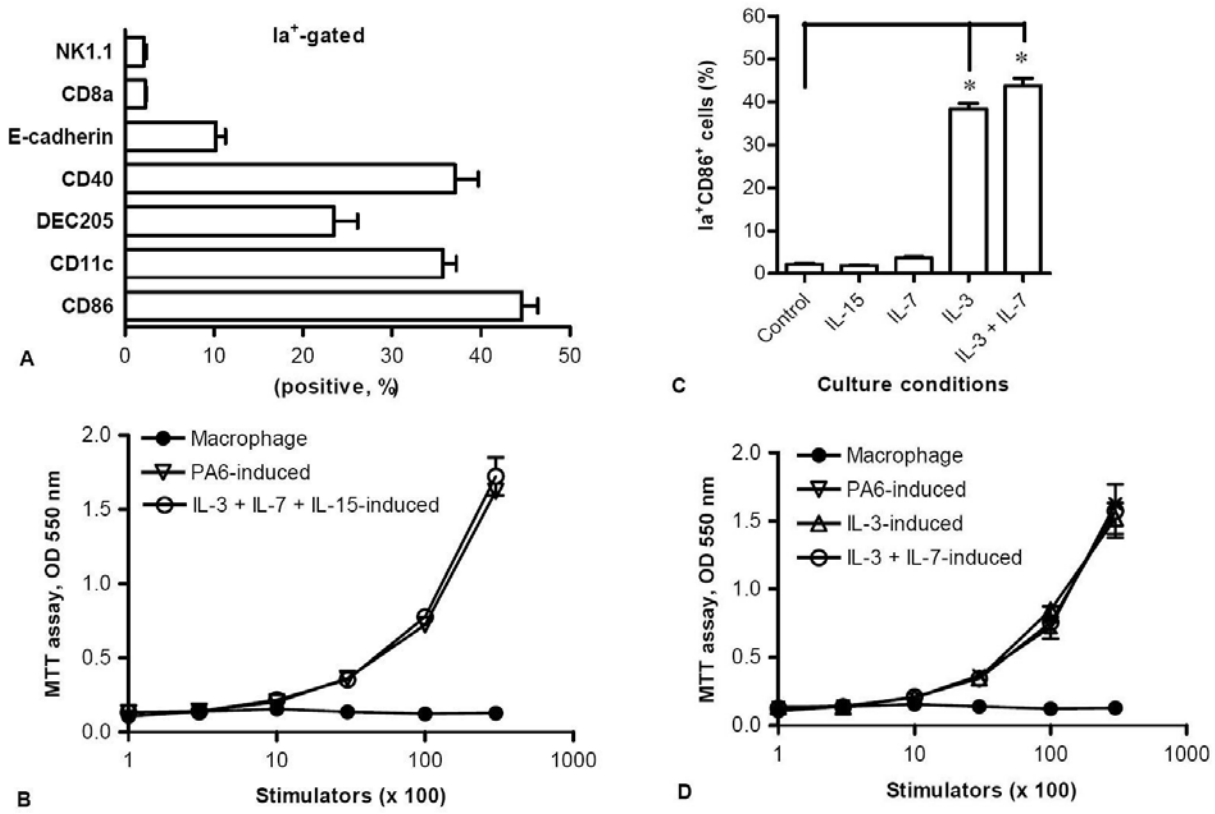

Figure 4. Effects of cytokines on development of DCs from 13 dpc FL Lin$^-$*c-kit*$^+$ HPCs *in vitro*. Individual or combined applications of IL-3, IL-7 and IL-15 were added to induce DC development from 13 dpc FL Lin$^-$*c-kit*$^+$ HPCs (3×10^4 cells/mL). Culture was accomplished in the presence of cocktail GM-CSF, SCF and Flt3L with additional IL-3, IL-7 or/and IL-15 for 12 to 14 days. The cultured cells were then restimulated with GM-CSF and TNF-alpha for 3 to 4 days. The phenotype of unfractionated nonadherent cells was analyzed by immunofluorescence staining as described in "Materials and Methods". The cells were stained with PE-conjugated Ia MoAb, and the indicated FITC-labeled MoAbs (CD8a, NK1.1, CD40, CD11c, DEC205 and E-cadherin, or/and CD86) were used to demonstrate the phenotypic characteristics of the generated DCs (A) and to show effects of the cytokine(s) on DC development from 13 dpc FL Lin$^-$*c-kit*$^+$ HPCs *in vitro* (C). In MLR, Ia$^+$CD86$^+$ cells derived from FL HPCs in the indicated cultures were isolated using FACS and were treated with MMC as stimulator cells, with stromal cell PA6-induced DCs derived from FL HPCs and peritoneal macrophages as controls, respectively. Allogeneic MLR was performed using MACS-isolated CD4$^+$ T cells (3×10^5 cells/per well in 96 well-plate) as responder cells. The proliferation of T cells was assayed using MTT after 5 days culture. Data are expressed as mean +/- SD of the triplicate cultures (B and D). These results are representative of 3 independent experiments. Asterisk represents *p* value less than 0.05.

vivo. Lethally irradiated Ly5.1 B6 mice were injected with anti-IL-3 antibody, anti-IL-7 antibody or IgG during reconstitution with FL HPCs. Interestingly, anti-IL-3 antibody treatment delayed the appearance of FL HPC-derived splenic DCs. Donor DCs were detected in the spleen up to 5 weeks after HPC transfer (data not shown). The number of FL-derived splenic DCs was significantly reduced when compared to that derived from IgG treated mice, while anti-IL-7 antibody could not suppress their generation (Figure 5). Interestingly, combined treatment with anti-IL-7 and anti-IL-3 antibodies could not inhibit FL HPC-derived DC generation to a greater extent than did anti-IL-3 antibody alone, a phenomenon which was contradictory to the expected synergetic effect of IL-7 in promoting IL-3-induced DC development *in vitro*. Moreover, antibody treatment did not significantly change the phenotype and function of donor-derived DCs as compared with those seen in IgG-treated mice (data not shown). All of these treatments had no obvious inhibitory effect on BM HPC-derived DC development (Figure 5). Thus, IL-3 plays an important role in DC development from murine hematopoietic progenitor cells of fetal liver.

5. DISCUSSION

In this study, we found that DCs develop from FL Lin$^-$*c-kit*$^+$ HPCs transferred into lethally irradiated mice. The donor derived splenic DCs were CD11c$^+$Ia^{hi}CD40^{int}, and could efficiently stimulate T cell proliferation. Donor FL Lin$^-$*c-kit*$^+$ HPCs also developed into skin LCs that expressed Ia, DEC205, E-cadherin and only marginally expressed CD11c. These LCs readily take up and present antigen as indicated by the CHS response. Interestingly, FL Lin$^-$*c-kit*$^+$ HPCs are shown to develop into DCs in the

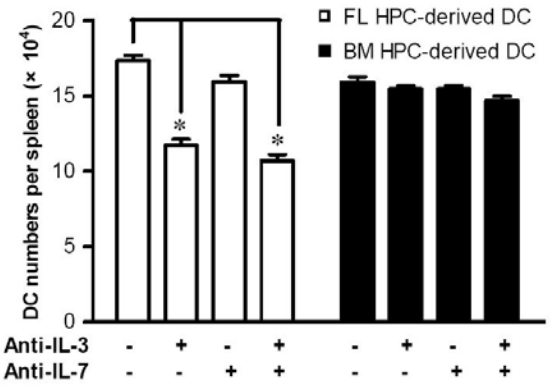

Figure 5. Effects of neutralizing antibodies to IL-3 or/and IL-7 on the development of DCs from Lin$^-$*c-kit*$^+$ HPCs *in vivo*. 5 × 10^4 purified FL or adult BM Lin$^-$*c-kit*$^+$ HPCs isolated from Ly5.2 B6 mice were injected into lethally irradiated Ly5.1 B6 mice via the tail vein. Neutralizing antibodies to IL-3 or/and IL-7 were intraperitoneally administered to the recipient mice every 3 days for 6 weeks after implantation. Ly5.2$^+$Ia$^+$DEC205$^+$ spleen cells isolated from recipient mice were measured to assay donor-derived DC generation *in vivo* from 13 dpc FL or adult BM Lin$^-$*c-kit*$^+$ HPCs. Each point was based upon a pool of 6 spleens from the recipient mice. The data represent mean +/- SD of number of donor splenic DCs from 3 independent experiments. Asterisk represents *p* value less than 0.05.

absence of stromal cells when IL-3 is added to a cytokine cocktail that includes GM-CSF, SCF, Flt3L and TNF-alpha. Although IL-7 alone did not appear to have a significant inductive effect, IL-7 significantly augmented this IL-3 induction. Furthermore, anti-IL-3 neutralizing antibody decreased DC generation from FL HPCs in chimeric mice. These findings suggest that IL-3 is important in DC development from FL HPCs *in vitro* and *in vivo*, a process that is notably different from DC development from BM HPCs.

We previously reported that BM HPCs, but not FL HPCs, differentiated into DCs *in vitro* in the presence of GM-CSF, SCF, Flt3L and TNF-alpha (19,25). Generation of FL HPC-derived DCs *in vitro* required the additional support of PA6 stromal cells (14), indicating that, as compared to BM HPCs, FL HPCs may require supplementary factors or different conditions to differentiate into DCs. Recent studies revealed that CD44$^+$CD25$^-$FcR$^-$ cells in fetal thymus differentiate into DCs in the presence of GM-CSF, SCF, Flt3L, TNF-alpha, IL-1alpha, IL-7 and IL-3 (20). However, the pivotal cytokine in this cocktail was not identified. In the present study, we investigated whether IL-3, IL-7 and/or IL-15 could induce DC generation from FL HPCs *in vitro* in place of stromal cells. We found that IL-3 was required to induce DC development from FL HPCs *in vitro* independent of PA6 stromal cells. Neither IL-7 nor IL-15 was able to induce DC development, whereas IL-7 enhanced the effect of IL-3 on induced DC development *in vitro*. Considering that IL-3 and IL-7 are not produced by PA6 stromal cells (28,29), it may be inferred that DC generation from PA6 coculture is independent of IL-3 and IL-7. We believe that IL-3-, or IL-3 + IL-7-induced DC development *in vitro* is, in some way, different from that induced by stromal cells. *In vivo* experiments showed that anti-IL-3 antibody treatment delayed the appearance of FL HPC-derived DCs in the spleen and significantly reduced the number of DCs generated from FL HPCs, whereas anti-IL-7 antibody treatment had little effect. These data indicate that IL-3 is pivotal in DC development from FL HPCs both *in vitro* and *in vivo*.

IL-3, a hematopoietic growth factor, is known to increase the number of AGM hematopoietic stem cell-enriched populations by promoting proliferation. IL-3$^{-/-}$ embryos display a decrease of AGM and yolk sac hematopoietic stem cells, suggesting that IL-3 is an important factor for the survival of embryonic HPCs (30). As compared to adult BM, FL is the principal hematopoietic organ during early embryonic development, and FL HPCs may need a complex set of factors for survival. Given the importance of IL-3 in embryonic HPC survival, it is possible that IL-3 may regulate the survival and/or differentiation of FL HPCs, a hypothesis that requires further investigation.

The role of IL-3 in the development of NK cells is controversial. IL-3 negatively regulates the development of NK cells from Sca-1$^+$*c-kit*$^+$ cells isolated from murine FL. In contrast, human FL progenitors differentiate into NK cells with the help of IL-3 (31). IL-15 and IL-7 have been reported to be important in NK cell development. Indeed, IL-15$^{-/-}$ or IL-7$^{-/-}$ mice displayed sharp reduction of NK cells (32,33). We previously showed that FL HPCs differentiatde into NK1.1 cells when cocultured with PA6 stromal cells in the absence of GM-CSF, suggesting that DCs derived from FL Lin$^-$*c-kit*$^+$ HPCs share common progenitor cells with NK cells (14). In the present study, IL-3, IL-7 and IL-15, alone or in combination, failed to induce FL HPC development into NK1.1$^+$ cells in the absence of PA6 cells (data not shown). These data indicate that FL Lin$^-$*c-kit*$^+$ HPCs, as common progenitors of DC/NK cells, may require distinct growth factors and conditions to differentiate into various lineages. This work also suggests that, although FL HPCs possess the potential to differentiate into NK cells, lineage specification can be regulated by extrinsic factors present in the cellular context in which they reside, the case in point being stromal cells.

LCs belong to the family of DCs specifically localized in the epidermis and play a major role in skin immunity. It has been reported that LCs can develop from blood Gr-1^{hi} monocytes as well as BM myeloid progenitors (34). The recruitment of BM-derived LC progenitors to the skin requires an inflammatory signal and depends upon inflammatory cytokines such as CCL20 produced in the skin (34,35). In our study, donor FL HPC-derived LCs can be detected in the skin after UV treatment of hematopoietically reconstituted mice, suggesting that FL Lin$^-$*c-kit*$^+$ HPCs contain LC precursors and may share similar migratory mechanisms with BM-derived HPCs. This finding is consistent with other reports (35). CHS responses depend upon intact DC functions to initiate T cell

immunity. During the induction phase of CHS, DCs are needed for antigen capture in the skin, antigen processing, transport to lymphoid tissues such as draining lymph nodes, and antigen presentation to naive T cells (36,37). To determine whether host LCs are a central participant in CHS responses in an animal model of reconstituted hematopoiesis, host LCs in chimeras were examined. Ly5.1$^+$ LCs were barely detectable on skin sheets of lethally irradiated and hematopoietically reconstituted mice (data not shown), indicating that host LCs were almost completely replaced by donor-derived LCs after lethal irradiation combined with UV treatment. These data suggest that LCs generated *in vivo* from Lin$^-$*c-kit*$^+$ HPCs are fully capable of antigen presentation to T cells during the CHS response.

LCs are CD11c positive, CD8 and LFA-1 negative and express intermediate levels of CD86 and CD40 (38). DCs generated from FL HPCs using an IL-3 supplemented cocktail regimen exhibited a high level of the CD11c molecule. A similar level of CD11c expression was found in splenic DCs generated *in vivo* from either BM or FL HPCs and in those generated *in vitro* from BM HPCs. In contrast, DCs generated from FL HPCs in coculture with PA6 barely expressed CD11c. Differential expression of CD11c was also observed between epidermal LCs derived from adult BM HPCs (high) and FL HPCs (low) in respective chimeras. However, the down-regulated CD11c expression on FL HPC-derived DCs did not affect their antigen presenting function. It is worthwhile to further examine whether a low level of CD11c expression may be a marker for DCs during murine embryonic development.

Overall, in this study we find that Lin$^-$*c-kit*$^+$ HPCs derived from 13 dpc murine FL develop into DCs *in vivo*. Further, IL-3 is crucial for the process of FL HPC-derived DC development both *in vivo* and *in vitro*, through a mechanism independent of stromal cell support. We believe that these insights will prove helpful for a deeper understanding of the mechanisms that drive the ontogenesis of DC.

6. ACKNOWLEDGEMENTS

We highly appreciate critical review of the manuscript by Drs Taylor B. Guo and Sheri M. Skinner (Baylor College of Medicine, Houston, USA). We also acknowledged help with statistical analysis by Dr. Hongmei Li (Soochow University, Suzhou, China). This work was supported by grants from the National Natural Science Foundation of China (NSF30670911 and NSF 30873045), 211 Project of Shanghai Jiao Tong University School of Medicine, the Programs of Science and Technology Commission of Shanghai Municipality (03JC14085, 074319102, 074119634 and 08JC1413300) and Shanghai Leading Academic Discipline Project (T0206).

7. REFERENCE

1. Banchereau J, R. M. Steinman: Dendritic cells and the control of immunity. *Nature* 392, 245-252 (1998)

2. Steinman R. M, M. C. Nussenzweig: Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* 99, 351-358 (2002)

3. Jonuleit H, E. Schmitt, K. Steinbrink, A. H. Enk: Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol* 22, 394-400 (2001)

4. Steinman R. M, D. Hawiger, M. C. Nussenzweig: Tolerogenic dendritic cells. *Annu Rev Immunol* 21, 685-711 (2003)

5. Ardavin C, H. G. Martinez del, P. Martin, F. Anjuere, C. F. Arias, A. R. Marin, S. Ruiz, V. Parrillas, H. Hernandez: Origin and differentiation of dendritic cells. *Trends Immunol* 22, 691-700 (2001)

6. Wu L, Y. J. Liu: Development of Dendritic-Cell Lineages. *Immunity* 26, 741-750 (2007)

7. Ginhoux F, F. Tacke, V. Angeli, M. Bogunovic, M. Loubeau, X. M. Dai, E. R. Stanley, G. J. Randolph, M. Merad: Langerhans cells arise from monocytes *in vivo*. *Nat Immunol* 7, 265-273 (2006)

8. Shortman K, S. H. Naik: Steady-state and inflammatory dendritic cell development. *Nat Rev Immunol* 7, 19-30 (2007)

9. Cumano A, I. Godin: Ontogeny of the hematopoietic system. *Annu Rev Immunol* 25, 745-785 (2007)

10. Bonifer C, N. Faust, H. Geiger, A. M. Muller: Developmental changes in the differentiation capacity of hematopietic stem cells. *Immunol Today* 19, 236-241 (1998)

11. Johnson G. R, M. A. Moore: Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature* 25, 726-728 (1975)

12. Kawamoto H, K. Ohmura, Y. Katsura: Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. *Int Immunol* 9, 1011-1019 (1997)

13. Aiba Y, M. Ogawa: Development of natural killer cells, B lymphocytes, macrophages, and mast cells from single hematopoietic progenitors in culture of murine fetal liver cells. *Blood* 90, 3923-3930 (1997)

14. Zhang Y, Y. Wang, M. Ogata, S. Hashimoto, N. Onai, K. Matsushima: Development of dendritic cells *in vitro* from murine fetal liver-derived lineage phenotype-negative c-kit$^+$ hematopoietic progenitor cells. *Blood* 95, 138-146 (2000)

15. Dzierzak E: Ontogenic emergence of definitive hematopoietic stem cells. *Curr Opin Hematol* 10, 229-234 (2003)

16. Zhou L. J, T. F. Tedder: CD14$^+$ blood monocytes can differentiate into functionally mature CD83$^+$ dendritic cells. *Proc Natl Acad Sci U S A* 93, 2588-2592 (1996)

17. Inaba K, M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, R. M. Steinman: Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc Natl Acad Sci U S A* 90, 3038-3042 (1993)

18. Caux C, B. Vanbervliet, C. Massacrier, C. Dezutter-Dambuyant, C. Jacquet, K. Yoneda, J. Banchereau: CD34$^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF + TNFα. *J Exp Med* 184, 695-706 (1996)

19. Zhang Y, N. Mukaida, J. B. Wang, A. Harada, M. Akiyama, K. Matsushima: Induction of dendritic cell differentiation by granulocyte-macrophage colony-stimulating factor, stem cell factor, and tumor necrosis factor alpha *in vitro* from lineage phenotypes-negative c-kit$^+$ murine hematopoietic progenitor cells. *Blood* 90, 4842-4853 (1997)

20. Shen H. Q, M. Lu, T. Ikawa, K. Masuda, K. Ohmura, N. Minato, H. Kawamoto: T/NK bipotent progenitors in the thymus retain the potential to generate dendritic cells. *J Immunol* 171, 3401-3406 (2003)

21. D'Amico A, L. Wu: The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. *J Exp Med* 198, 293-303 (2003)

22. Chicha L, D. Jarrossay, M. G. Manz: Clonal type I interferon-producing and dendritic cell precursors are contained in both human lymphoid and myeloid progenitor populations. *J Exp Med* 200, 1519-1524 (2004)

23. Shigematsu H, B. Reizis, H. Iwasaki, S. Mizuno, D. Hu, D. Traver, P. Leder: Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of the cellular origin. *Immunity* 21, 43-53 (2004)

24. Wang Y, Y. Zhang, H. Yoneyama, N. Onai, T. Sato, K. Matsushima: Identification of CD8alpha$^+$CD11c$^-$ lineage phenotype-negative cells in the spleen as committed precursor of CD8alpha$^+$ dendritic cells. *Blood* 100, 569-577 (2002)

25. Zhang Y, A. Harada, J. B. Wang, Y. Y. Zhang, S. Hashimoto, M. Naito, K. Matsushima: Bifurcated dendritic cell differentiation *in vitro* from murine lineage phenotype-negative c-kit$^+$ bone marrow hematopoietic progenitor cells. *Blood* 92, 118-128 (1998)

26. Vremec D, M. Zorbas, R. Scollay, D. J. Saunders, C. F. Ardavin, L. Wu, K. Shortman: The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* 176, 47-58 (1992)

27. Cohen P. J, P. A. Cohen, S. A. Rosenberg, S. I. Katz, J. J. Mule: Murine epidermal Langerhans cells and splenic dendritic cells present tumor-associated antigens to primed T cells. *Eur J Immunol* 24, 315-319 (1994)

28. Kodama H, H. Hagiwara, H. Sudo, Y. Amagai, T. Yokota, N. Arai, Y. Kitamura: MC3T3-G2/PA6 preadipocytes support *in vitro* proliferation of hemopoietic stem cells through a mechanism different from that of interleukin 3. *J Cell Physiol* 129, 20-26 (1986)

29. Hayashi S, T. Kunisada, M. Ogawa, T. Sudo, H. Kodama, T. Suda, S. Nishikawa: Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. *J Exp Med* 171, 1683-1695 (1990)

30. Robin C, K. Ottersbach, C. Durand, M. Peeters, L. Vanes, V. Tybulewicz, E. Dzierzak: An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Dev Cell* 11, 171-180 (2006)

31. Muench M. O, L. Humeau, B. Paek, T. Ohkubo, L. L. Lanier, C. T. Albanese, A. Barcena: Differential effects of interleukin-3, interleukin-7, interleukin-15, and granulocyte-macrophage colony-stimulating factor in the generation of natural killer and B cells from primitive human fetal liver progenitors. *Exp Hematol* 28, 961-973 (2000)

32. Kennedy M. K, M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charriera, L. Sedgera, C. R. Willisa, K. Brasela, P. J. Morrisseya, K. Stockinga, J. C. L. Schuha, S. Joyceb, J. J. Peschona: Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* 191, 771-780 (2000)

33. Feyen O, U. Gobel, D. T. Schneider, S. E. Burdach, T. Niehues: Long-term haematopoietic reconstitution and survival without interleukin-7 in a murine syngeneic bone marrow transplantation model. *Scand J Immunol* 64, 601-608 (2006)

34. Mende I, H. Karsunky, I. L. Weissman, E. G. Engleman, M. Merad: Flk2$^+$ myeloid progenitors are the main source of Langerhans cells. *Blood* 107, 1383-1390. (2006)

35. Merad M, M. G. Manz, H. Karsunky, A. Wagers, W. Peters, I. Charo, E. G. Engleman: Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* 3, 1135-1141 (2002)

36. Kripke M. L, C. G.Munn, A. Jeevan, J. M. Tang, C. Bucana: Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J Immunol* 145, 2833-2838 (1990)

37. Steinman R. M, L. Hoffman, M. Pope: Maturation and migration of cutaneous dendritic cells. *J Invest Dermatol* 105, 2S-7S (1995)

38. Anjere F, P. Martin, I. Ferrero, M. L. Fraga, G. M. del Hoyo, N. Wright, C. Ardavin: Definition of dendritic cell subpopulations present in the spleen, peyer's patches, lymph nodes, and skin of the mouse. *Blood* 93, 590-598 (1999)

Abbreviations: AGM: aorta-gonad-mesonephros region; BM: bone marrow; CHS: contact hypersensitivity; DCs: dendritic cells; 13 dpc: day 13 postcoitus; FITC: fluorescein isothiocyanate; FL: fetal liver; Flt3L: Flt3 ligand; GM-CSF: granulocyte/macrophage colony-stimulating factor; HPCs: hematopoietic progenitor cells; IL: interleukin; LCs: Langerhans cells; Lin: lineage phenotype; MMC: mitomycin C; MLR: mixed leukocyte reaction; MNCs: mononuclear cells; MoAb: monoclonal antibody; MTT: 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide; PE: phycoerythrin; SCF: stem cell factor; TNF: tumor necrosis factor.

Key Words: Dendritic cells, IL-3, Hematopoietic progenitor cells, Fetal liver, Development

Send correspondence to: Yanyun Zhang, Shanghai Institute of Immunology and Institute of Health Sciences, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China; Tel: 86-21-63844597, Fax: 86-21-63852705; E-mail: yyzhang@sibs.ac.cn