

Myelopoiesis in spleen-producing distinct dendritic-like cells

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Abstract

Dendritic cells (DC) represent a heterogeneous class of antigen presenting cells (APC). Previously we reported a distinct myeloid dendritic-like cell present in spleen, as an *in vivo* counterpart to cells produced in murine spleen long-term cultures (LTC-DC). These cells, named 'L-DC', were found to be functionally and phenotypically distinct from conventional (c)DC, plasmacytoid (p)DC and monocytes. These results suggested that spleen may represent a niche for development of L-DC from endogenous progenitors. Adult murine spleen has now been investigated for the presence of L-DC progenitors. Lineage-negative (Lin)⁻ckit^{lo} and (Lin)⁻ckit^{hi} progenitor subsets were identified as candidate populations, and tested for ability to produce L-DC; *in vitro* upon co-culture with the spleen stromal line STX3, and *in vivo* after adoptive therapy into mice. Both subsets colonized STX3 stroma *in vitro* for L-DC production, indicating that they contained either a common or two distinct progenitors for L-DC. However, only the (Lin)⁻ckit^{hi} subset gave progeny cells after adoptive transfer into lethally irradiated mice. *In vivo* development was however multilineage and not restricted to L-DC development. Multilineage reconstitution reflects long-term reconstituting haematopoietic stem cells (LT-HSC), suggesting a close relationship between L-DC progenitors and LT-HSC. L-DC were however produced *in vivo* in much higher number than monocytes/macrophages and cDC, indicating the presence of a specific L-DC progenitor within the (Lin)⁻ckit^{hi} subset. A model is advanced for development of L-DC directly from haematopoietic progenitors in spleen and dependent on the spleen microenvironment.

Keywords: spleen • haematopoiesis • dendritic cells • myelopoiesis

Introduction

The mouse is a well-recognized model for studying haematopoiesis. At birth, HSC migrate from foetal liver to bone where they remain for the life of the animal. HSC also appear in mouse spleen soon after birth [1] and are maintained there for life. They also exist in extramedullary sites and small numbers mobilize through blood and lymph into tissues like spleen, liver, lung, brain and intestine in the steady-state [2, 3]. The number of haematopoietic stem/progenitor cells (HSPC) in blood, spleen and liver also expands noticeably following acute inflammation or drug treatment [4, 5]. The small number of progenitors in extramedullary tissues in the steady-state should not discount their importance or potential contribution to the immune response. Findings from this laboratory indicate that spleen in the steady-state does contain HSC detectable by their long-term reconstitution ability upon adoptive

transfer to irradiated host mice [6]. The importance of bone marrow (BM) in haematopoiesis is clear however since neonatally splenectomized mice can maintain normal BM haematopoiesis [1, 6]. The relative haematopoietic contribution of HSC from spleen and BM of adults is however not known, and it has long been assumed that spleen fills the role of an emergency or backup site in times of stress or disease.

Spleen is also a central organ for development of DC that take up and present antigen to lymphocytes. Multiple subsets have been identified including the CD8 α ⁻ and CD8 α ⁺ conventional (c)DC and plasmacytoid (p)DC [7], and the less well-defined regulatory DC [8–10]. Monocyte-derived DC (moDC) are also found in spleen but only under conditions of inflammation [11]. An essential element of DC biology is the definition of progenitors and precursors since this underpins the formation of lineages of cells with distinct function. Initially, cDC and pDC progenitors were defined as a Flt3⁺ subset amongst common myeloid and lymphoid progenitors (CMP/CLP) [12]. Current evidence points to a common monocyte/dendritic progenitor (MDP) in BM which gives rise to all monocyte/macrophage and dendritic-type cells [13, 14], and a more committed common dendritic progenitor (CDP) for cDC and pDC in BM [15, 16]. While CDP and MDP are

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not present in spleen or blood [17], spleen does harbour cDC precursors, which have a high turnover and are replaced by blood-borne precursors [18]. There is also evidence from parabiotic mouse studies to suggest that some splenic DC might arise from endogenous progenitors [19], although others have questioned that result [20].

In this laboratory, a novel dendritic-like antigen presenting cell was discovered in spleen on the basis of similarity with dendritic-like cells developing in splenic long-term cultures (LTC) [21, 22]. 'LTC-DC' have a characteristic immature phenotype as CD11c^{lo}CD11b^{hi}MHC-II⁻CD8 α ⁻ cells, distinguishing them from cDC, pDC and monocytes [22, 23]. They are also distinguishable as large, endocytic cells specialized in cross-presentation of antigen to CD8⁺ T cells [24], a function usually attributed to CD8 α ⁺ cDC [25]. They are also distinct in their very weak ability to activate CD4⁺ T cells, consistent with their MHC-II⁻ phenotype. Since they can be derived from *GM-CSF*^{-/-} mice [26], they are distinct from moDC [11] or 'Tip-DC' [27] that develop in response to inflammatory factors like GM-CSF/TNF- α . The *in vivo* equivalent 'L-DC' subset is readily distinguishable from cDC, pDC and monocytes on the basis of CD11b and CD11c expression, as well as many other markers including CD8 α , MHC-II, CD205 and myeloid markers like Ly6G and Mac3 [24]. L-DC show similar antigen cross presenting function as LTC-DC [24] and are functionally distinct from described subsets of regulatory DC which inhibit T cell proliferation [8–10]. The ontogeny and lineage origin of this subset appears to be distinct from other known DC and myeloid subsets in spleen.

It is hypothesized that spleen maintains a lineage of dendritic-like cells, which arise from endogenous haematopoietic progenitors maintained in spleen. Such tissue-specific production of DC has been previously reported for Langerhans cells in skin which are continuously renewed from radio-resistant, skin-derived progenitors [28], only being replaced by blood-borne progenitors under inflammatory conditions [29]. Splenic stromal cells which support haematopoiesis of L-DC *in vitro* have been shown to have an endothelial origin [30, 31], and L-DC have been shown to arise in co-cultures of BM progenitors or spleen subsets over a splenic stromal cell line [32]. Both neonatal and adult splenocytes contain progenitors that produce L-DC when co-cultured over STX3 spleen stroma [33]. This study identifies and characterizes L-DC progenitors in adult spleen in terms of capacity to produce L-DC in stromal co-cultures and to undergo haematopoiesis for L-DC production upon transplantation into irradiation chimeras.

Materials and methods

Animals

C57BL/6J and C57BL/6.SJL-Ptprc^aPep^{3b}/BoyJ (B6.SJL) mice were bred at the John Curtin School of Medical Research (Canberra, Australia) under specific pathogen-free conditions and used at 4–6 weeks of age.

Antibody staining

Antibody staining and flow cytometry were performed to analyse cell surface marker expression as described previously [33]. Non-specific antibody binding *via* Fc receptors was blocked by incubating cells ($\leq 10^6$) with anti-CD16/32 (FcR block) (eBioscience, San Diego, CA, USA). Biotin- or fluorochrome-conjugated antibodies specific for CD11c (N418), CD11b (M1/70), ckit (2B8), IL-7R (A7R34), CD45.1 (A20), CD19 (1D3), B220 (RA3-6B2), Thy1.2 (30-H12) and CD34 (RAM34) were purchased from eBioscience. Antibodies specific for CD8 (53-6.7), Sca1 (E13-161.7) and MHC-II (25-9-17) were purchased from Becton Dickinson (San Jose, CA, USA). Isotype control antibodies were purchased from eBioscience. Propidium iodide (PI: 1 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) was added prior to flow cytometry for discrimination of live and dead cells. Flow cytometry was performed immediately on a BD LSRII flow cytometer (Becton Dickinson). Data collected included forward scatter (FSC), side scatter (SSC) and multiple fluorescence channels detecting FITC, CFSE, PE, PI, PE-Cy7, APC and APC-Cy7 (channels FL1-4, FL9-10). BD FACSDiva Software (Becton Dickinson) was used to acquire data. Data analysis involved post-acquisition gating using FlowJo software (Tree Star, Ashland, OR, USA). Cells sorting was performed using a FACSAria cell sorter (Becton Dickinson) as described previously [33].

Enrichment for spleen precursors

Whole splenocytes were enriched for precursors by negative depletion of T cell and B cell populations using antibody-coated magnetic beads as described previously [33], which are specific for CD19 (eBio1D3), Thy1.2 (30-H12) and TER-119, (eBioscience). Recovered cells were washed and then stained with antibody for subsequent isolation of subsets by sorting.

Co-culture assays to assess DC development

Spleen stromal line STX3 is a spleen stromal cell line derived from a long-term culture which ceased production of DC over time with passage [34]. STX3 grows as a confluent monolayer and is passaged by scraping and cell transferal. When spleen or BM cells are co-cultured over STX3 ($1-5 \times 10^5$ cells/ml), haematopoiesis is established and myeloid dendritic-like cells are produced. For co-culture maintenance, half medium is exchanged every 3–4 days and non-adherent cells are collected for flow cytometric analysis.

Production of murine chimeras

Chimeras were generated using CD45-allotype distinct donor and host mice as described previously [24]. Haematopoietic cells (CD45.1) were transferred intravenously into lethally irradiated hosts (9.5 Gy) to assess *in vivo* reconstitution potential of blood cell lineages. Recipients were also given host (CD45.2⁺) BM (10^5 cells) to ensure survival.

Statistical analysis

Data are presented as mean \pm S.E., $n = 3$. With only small sample sizes, a normal distribution cannot be assumed. The Wilcoxon Rank Sum Test was therefore used to assess significance ($P \leq 0.05$).

A Early haematopoietic marker analysis

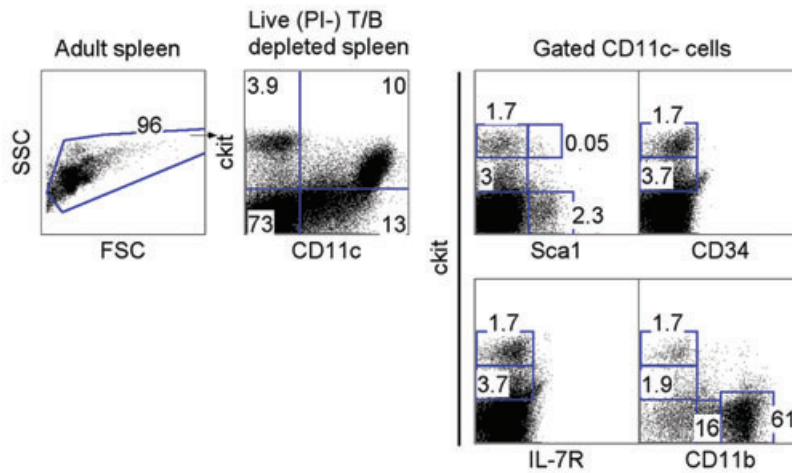
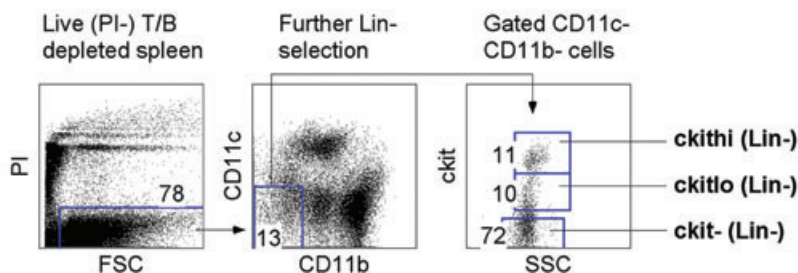


Fig. 1 Identification and isolation of haematopoietic progenitors in adult spleen. Spleen cell suspensions were prepared by depletion of red blood cells, and T and B cell depletion using magnetic beads. Cells were stained with fluorochrome-conjugated antibodies against ckit, Sca1, CD34, IL-7R, CD11c and CD11b. Prior to flow cytometry, propidium iodide (PI; 1 μ g/ml) was added to allow gating of live (PI⁻) cells. **(A)** Cells were gated based on forward scatter (FSC) and side scatter (SSC) parameters to exclude debris. Initial phenotype analysis involved ckit *versus* CD11c expression. Further marker analysis excluded CD11c⁺ spleen cells. Percent positive cells are indicated in quadrants and square gates. **(B)** For sorting progenitors, adult T and B cell depleted splenocytes were stained with fluorochrome-conjugated antibodies against ckit, CD11c and CD11b. Live (PI⁻) cells were gated to give Lin⁻ (Thy1.2⁻ CD19⁻ CD11c⁺ CD11b⁻) ckit⁻, ckit^{lo} and ckit^{hi} cell populations. Results are representative of multiple independent experiments.

B Sorting Lin⁻ckit⁺ subsets from spleen



Results

Characterization of L-DC progenitors in adult spleen

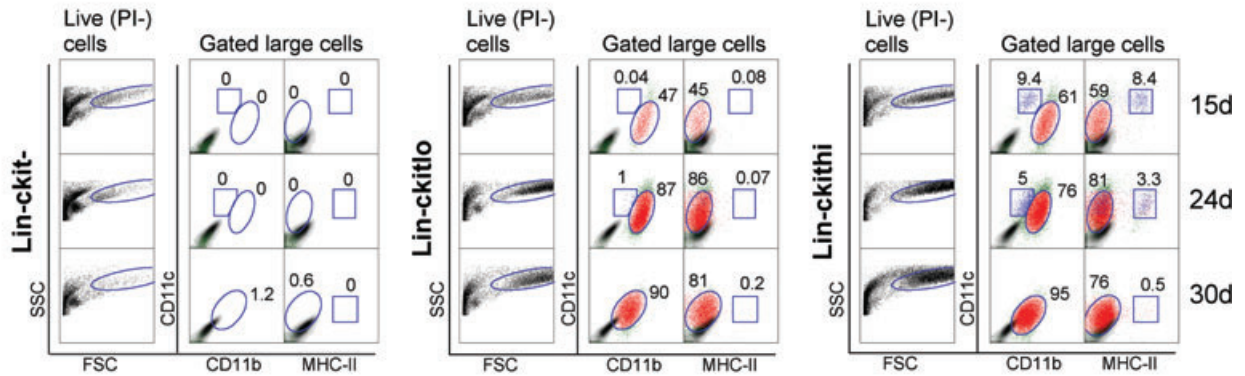
Candidate progenitor subsets were identified in adult spleen following staining with markers for HSPC (Fig. 1). A significant fraction of cells (10%) was found to be ckit^{lo}CD11c^{hi} cells, a population reflective of cDC [35]. CD11c⁺ cells expressing ckit were thus excluded from further analysis. The remaining Lin⁻ckit⁺ cells could be divided into subsets of Lin⁻ckit^{lo} and Lin⁻ckit^{hi} cells, representing ~3% and 1.7% of cells, respectively (Fig. 1A). These were analysed for expression of other known haematopoietic markers [36]. Sca1 was expressed on only ~3% of Lin⁻ckit^{hi} cells, and the HSPC markers CD34 and Flt3 (not shown) were not expressed on Lin⁻ckit⁺ cells. The absence of HSPC in spleen expressing CD34 or Flt3 has been confirmed by others [17, 20]. IL-7R, a marker of lymphoid progenitors in BM [37], was also

absent, while a large population of cells were found to be Lin⁻ckit⁻CD11b⁺ (61%), reflecting myeloid precursors.

Differentiation of splenic progenitors in co-cultures

In line with marker analysis shown in Figure 1A, splenocytes depleted of T and B cells (Thy1.2⁻CD19⁻) were gated by flow cytometry to exclude CD11c⁺CD11b⁺ cells and sorted to give Lin⁻ckit⁻, Lin⁻ckit^{lo} and Lin⁻ckit^{hi} subsets (Fig. 1B). Each of these populations was then co-cultured over STX3 stroma to assess differentiative potential. Lin⁻ckit⁻ adult spleen precursors failed to produce progeny cells (Fig. 2A and B). In contrast, sorted Lin⁻ckit^{hi} progenitors generated a high yield of large (FSC^{hi}) cells, reflecting two distinct populations of myeloid DC, including CD11b^{hi}CD11c^{lo}MHC-II⁻ L-DC (round gates) and CD11b^{lo}CD11c^{hi} MHC-II⁺ cDC-like cells (square gates). At 15 days after establishment of co-cultures with Lin⁻ckit^{hi} cells (Fig. 2A), MHC-II⁺

A Cell production in co-cultures



B DC recovery from STX3 co-cultures

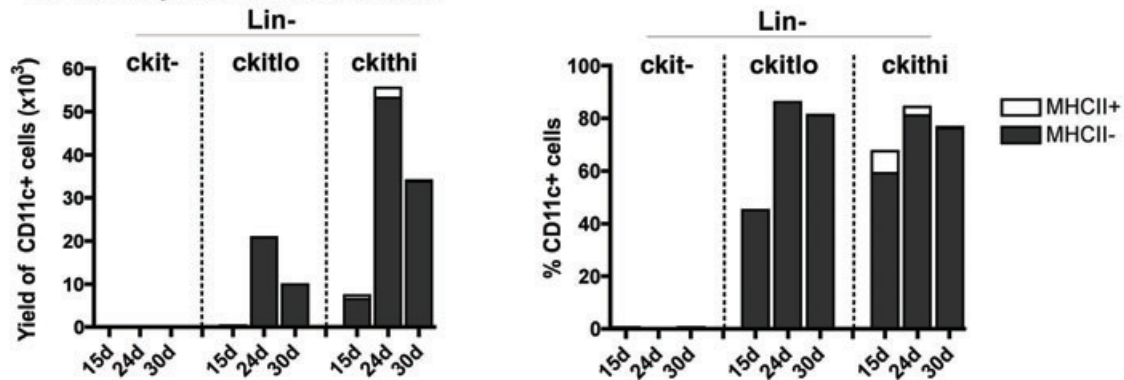


Fig. 2 *In vitro* development of L-DC from Lin⁻ckit⁺ spleen cells. Adult splenocytes were prepared as a Lin⁻ (Thy1.2⁻CD19⁻CD11c⁻CD11b⁻) subset and sorted into specific ckit⁻, ckit^{lo} and ckit^{hi} subpopulations as in Figure 1. Sorted cells were cultured over STX3 stroma to assess developmental potential for L-DC. (A) Progeny cells collected from co-cultures at 15, 24 and 30 days were stained with fluorochrome-conjugated antibodies specific for CD11c, CD11b, MHC-II or isotype control antibodies. Propidium iodide (PI; 1 μg/ml) staining prior to flow cytometry allowed gating of live (PI⁻) cells for analysis. Forward and side scatter parameters (FSC, SSC) were used to distinguish large-sized cells for estimation of cell yield, and multi-channel analysis. CD11c, CD11b and MHC-II positive staining gates were defined using relevant isotype controls (shown as a density plot overlay), with percent positive cells indicated within gates (shown as a dot plot). (B) DC production from 15, 24 and 30 day spleen Lin⁻ckit⁻, Lin⁻ckit^{lo} and Lin⁻ckit^{hi} co-cultures was scored in terms of CD11b^{hi}CD11c^{lo}MHC-II⁻ (L-DC) and CD11b^{lo}CD11c^{hi}MHC-II⁺ (cDC-like) cell populations, and represented as percent CD11c⁺ cells, and total yield of CD11c⁺ cells. Results are representative of multiple similar experiments.

cDC-like cells represented a minor population of cells (8.4%), while L-DC accounted for 59% of cells produced. Thereafter, the production of MHC-II⁺ cDC declined 16-fold over 30 days of co-culture (Fig. 2B). The development of MHC-II⁺ DC was thus transient, consistent with development from a preformed precursor, perhaps a ckit⁺CD11c^{lo} contaminant. In contrast, co-cultures established with Lin⁻ckit^{lo} precursors produced exclusively L-DC and no MHC-II⁺ cDC-like cells. Although Lin⁻ckit^{lo} splenocytes were restricted in their production of only L-DC, higher yields of L-DC were achieved in Lin⁻ckit^{hi} co-cultures (Fig. 2B). Production of L-DC in co-cultures established with Lin⁻ckit^{lo} and Lin⁻ckit^{hi} progenitors was minimal by 15 days and peaked at 24 days. Both Lin⁻ckit^{lo} and Lin⁻ckit^{hi} spleen fractions contained self-renewing

L-DC progenitors, with a few precursors amongst the Lin⁻ckit^{hi} subset able to develop MHC-II⁺ cDC-like cells transiently.

Absence of immediate L-DC precursors amongst spleen Lin⁻ckit⁺ progenitors

The presence of immediate L-DC precursors amongst the adult spleen Lin⁻ckit⁺ subsets was further investigated by adoptive transfer of 5 × 10⁴ sorted Lin⁻ckit^{hi} or Lin⁻ckit^{lo} cells into unirradiated hosts, with analysis of donor progeny after 7 and 14 days (Fig. 3). At these times, no donor-type CD11c⁺ dendritic or CD11b⁺ myeloid progeny cells were detected in spleens of recipient mice,

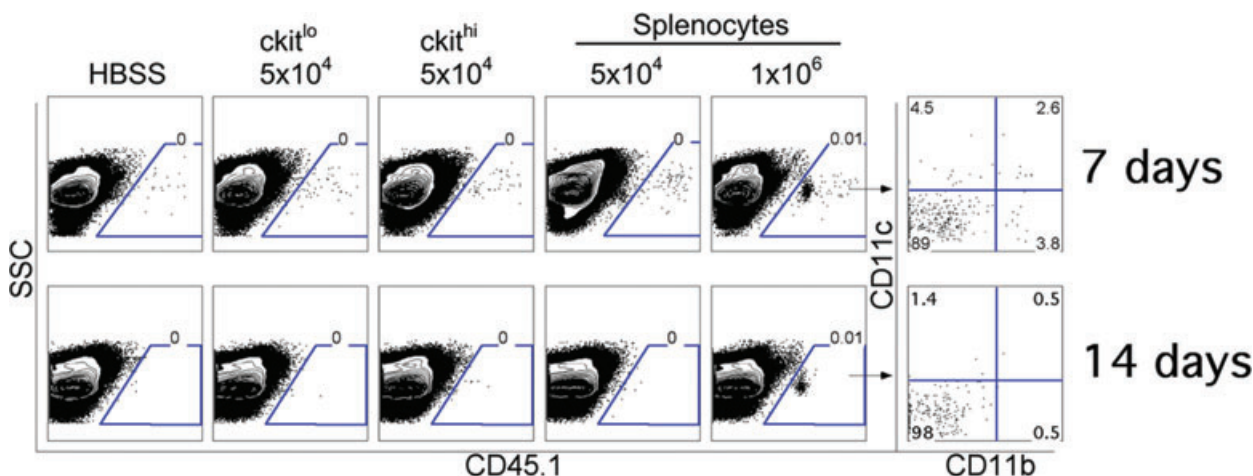


Fig. 3 *In vivo* developmental potential of isolated adult spleen $\text{Lin}^- \text{ckit}^+$ precursors. $\text{Lin}^- \text{ckit}^{\text{lo}}$ and $\text{Lin}^- \text{ckit}^{\text{hi}}$ spleen subsets were sorted as outlined in Figure 1 from adult B6.SJL mice (CD45.1^+). Cells (5×10^4) were transferred intravenously into unirradiated C57BL/6J (CD45.2^+) recipient mice. HBSS was injected as a 'nil' control, and 5×10^4 or 1×10^6 whole splenocytes were injected as positive controls. For analysis of cell development from pre-formed precursors, spleens were collected at 7 and 14 days. The presence of donor-type DC was investigated by prior T/B cell depletion of splenocytes and staining with CD45.1, CD11c and CD11b. Prior to flow cytometry, cells were incubated with propidium iodide (PI; $1 \mu\text{g/ml}$) for gating of live (PI^-) cells. Numbers in gates indicate percent positive cells. Two further experiments (not shown) involving irradiated mice (4.5 Gy) also gave negative results.

consistent with absence of immediate DC precursors amongst the $\text{Lin}^- \text{ckit}^{\text{hi}}$ and $\text{Lin}^- \text{ckit}^{\text{lo}}$ cell populations. Only control mice given 10^6 (and not 5×10^4) unfractionated spleen cells showed donor-type cells in spleen ($\sim 0.001\%$). These were found to be mainly $\text{CD11c}^- \text{CD11b}^-$. The transfer of 5×10^4 $\text{Lin}^- \text{ckit}^{\text{hi}}$ or $\text{Lin}^- \text{ckit}^{\text{lo}}$ cells represented a 1000-fold enrichment of progenitors over 5×10^4 unfractionated spleen cells, reinforcing the conclusion that these subsets contained no immediate precursors of L-DC.

The spleen $\text{Lin}^- \text{ckit}^{\text{hi}}$ subset contains LT-HSC and L-DC progenitors

A unique characteristic of spleen LTC compared with other *in vitro* DC culture systems is that productivity is sustained for years. One explanation is that self-renewing stem cells maintained in culture differentiate to give L-DC perhaps *via* formation of an L-DC progenitor [32, 38, 39]. The nature of progenitors amongst the $\text{Lin}^- \text{ckit}^{\text{lo}}$ and $\text{Lin}^- \text{ckit}^{\text{hi}}$ subsets was therefore tested by assessing progeny produced following long-term reconstitution of mice with these cell subsets. Adult splenocytes from B6.SJL (CD45.1^+) mice were sorted to give $\text{Lin}^- \text{ckit}^-$, $\text{Lin}^- \text{ckit}^{\text{lo}}$ and $\text{Lin}^- \text{ckit}^{\text{hi}}$ populations which were transferred intravenously into lethally irradiated mice along with unfractionated host-type C57BL/6J (CD45.2^+) BM cells to ensure survival. Individual chimeras were assessed for lineage reconstitution of lymphoid, myeloid and DC subsets (including L-DC) at 15, 16 and 18.5 weeks. Control C57BL/6J mice were irradiated and given BM cells from CD45.1^+ B6.SJL mice.

Only the spleen $\text{Lin}^- \text{ckit}^{\text{hi}}$ subset produced progeny cells with multilineage long-term reconstitution in 7/7 mice consistent with

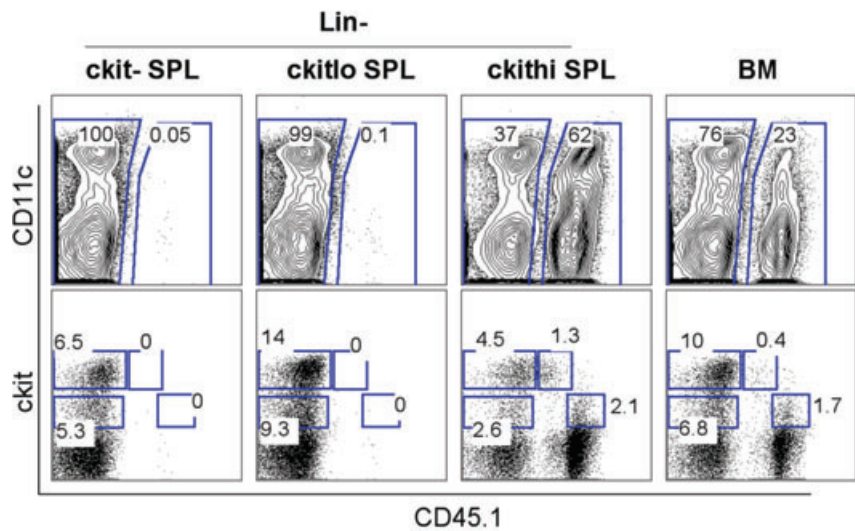
the presence of LT-HSC (Fig. 4). Multilineage reconstitution by HSC within the spleen $\text{Lin}^- \text{ckit}^{\text{hi}}$ subset was indicated by the detection of donor-derived DC, myeloid cells, T cells and B cells (Fig. S1). No progeny cell reconstitution was achieved with the spleen $\text{Lin}^- \text{ckit}^{\text{lo}}$ subset (0/3 mice), or with the $\text{Lin}^- \text{ckit}^-$ subset (0/4 mice), indicating absence of self-renewing progenitors or LT-HSC within this subset. Furthermore, in mice receiving spleen $\text{Lin}^- \text{ckit}^{\text{hi}}$ cells, donor-derived cells also reconstituted the corresponding $\text{Lin}^- \text{ckit}^{\text{hi}}$ stem cell compartment of spleen. Long-term, multilineage reconstitution of hosts by LT-HSC in the spleen $\text{Lin}^- \text{ckit}^{\text{hi}}$ subset in comparison with BM-derived HSC was also confirmed by analysis of chimeric mice at 54 weeks post-transplantation (Table 1). Three out of four chimeras given donor $\text{Lin}^- \text{ckit}^{\text{hi}}$ spleen cells showed complete reconstitution with donor myeloid cells, while a fourth chimera showed partial reconstitution. Control chimeras given donor-derived BM alone also gave complete long-term multilineage reconstitution with donor cells in three out of four mice, and also showed new HSC production.

Capacity of spleen $\text{Lin}^- \text{ckit}^+$ progenitors to produce DC *in vivo*

The distribution of APC subsets was compared in control mice and in haematopoietic chimeras reconstituted with donor $\text{Lin}^- \text{ckit}^{\text{hi}}$ spleen cells or donor BM cells. Progeny subsets analysed included $\text{CD8}\alpha^- \text{cDC}$ ($\text{CD11c}^{\text{hi}} \text{CD11b}^+ \text{CD8}^- \text{MHC-II}^+$), $\text{CD8}\alpha^+ \text{cDC}$ ($\text{CD11c}^{\text{hi}} \text{CD11b}^- \text{CD8}^+ \text{MHC-II}^+$), L-DC ($\text{FSC}^{\text{hi}} \text{CD11c}^{\text{lo}} \text{CD11b}^{\text{hi}} \text{MHC-II}^-$) and myeloid cells ($\text{FSC}^{\text{lo}} \text{CD11c}^- \text{CD11b}^{\text{hi}} \text{MHC-II}^-$) (Fig. 5A).

Example 16-week chimeras

Fig. 4 Long-term reconstitution potential of adult spleen Lin⁻ckit⁺ cells for myeloid cell subsets. Lin⁻ckit⁻, Lin⁻ckit^{lo} and Lin⁻ckit^{hi} spleen (SPL) subsets were sorted as described in Figure 1 from spleens of adult B6.SJL mice (CD45.1⁺). Sorted cells (2.5 × 10⁴) were assessed for competitive reconstitution with 10⁵ host-type whole bone marrow cells by transfer into lethally irradiated (9.5 Gy) C57BL/6J (CD45.2⁺) recipient mice. Dissociated whole bone marrow (CD45.1⁺) was transferred intravenously alone as a positive control, and age-matched unirradiated C57BL/6J mice were analysed as negative controls. To assess long-term reconstitution, spleens were collected for subset analysis at 15, 16 and 18.5 weeks, enriched for DC by depletion of T and B cells, and stained with fluorochrome-conjugated antibodies to define donor and host cells and their lineage. Prior to flow cytometry, cells were incubated with propidium iodide (PI; 1 μg/ml) for gating live (PI⁻) cells. An example analysis is shown for 16-week chimeras stained to detect CD45.1⁺CD11c⁺ progeny cells (top row) and Lin⁻ckit⁺ spleen progenitors (bottom row). The frequency of donor cell reconstitution in chimeras after 15, 16 and 18.5 weeks is indicated.



Overall frequency of donor cell reconstituted chimeras (15, 16, 18.5 weeks)

Lin-ckit-SPL	Lin-ckitlo SPL	Lin-ckithi SPL	BM
0/4	0/3	7/7	7/7

Table 1 Representation of donor-derived haematopoietic cells in 54-week chimeras

Percent CD45.1 ⁺ donor-derived cells amongst each subset							
Animals* (#)	CD8 ⁺ cDC	CD8 ⁺ cDC	L-DC	Myeloid cells	B cells [†]	T cells	HSC [‡]
C57BL/6J control (1)	0	0	0.2	0	0	0	0
Lin⁻ckit^{hi} SPL chimera (1)	0	0.2	0.2	0.1	0	0	0
Lin⁻ckit^{hi} SPL chimeras (1)	78	84	100	90	17	28	97
(2)	99	97	100	92	98	92	56
(3)	37	28	61	28	85	82	49
(4)	96	97	100	96	73	82	92
BM chimeras (1)	8.2	10	23	5.7	10	77	13
(2)	99	99	100	99	74	92	97
(3)	94	94	100	98	95	87	100
(4)	100	100	100	100	90	83	81

*Chimeras were prepared as described in Figures 3 and 4. Analysis of all chimeras was performed at 54 weeks post-reconstitution. [†]B cells and CD8⁺ T cells were detected in lymph node. [‡]HSC were identified in spleen as a Lin⁻ckit^{hi}Sca1⁺ subset.

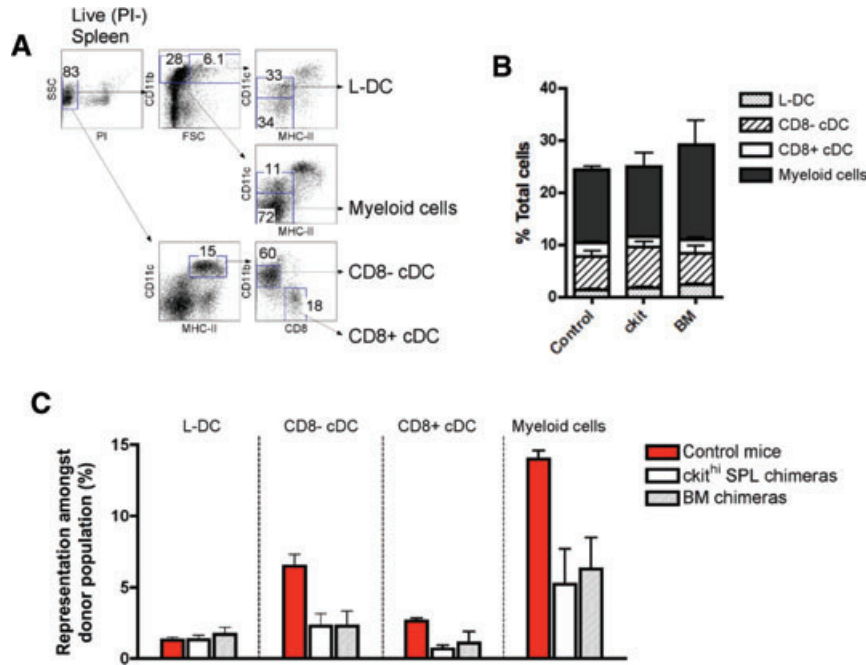


Fig. 5 Lin⁻ckit^{hi} cells in adult spleen give preferential reconstitution of L-DC. Chimeras established in Figure 4 with donor Lin⁻ckit^{hi} spleen cells and host-type bone marrow, or with host-type bone marrow alone, were analysed for the presence of different myeloid and DC subsets of both donor (CD45.1⁺) and host (CD45.2⁺) type. Control unirradiated host-type mice were given no bone marrow. **(A)** The procedure for delineation of myeloid and DC subsets is shown for a control mouse, in the absence of CD45.1/CD45.2 gating. The total population of CD11b⁺ myeloid/dendritic cells was gated from live (PI⁻) T and B cell depleted splenocytes, and then analysed for expression of CD11c, MHC-II and CD8 α . Cell subsets gated included: L-DC (FSC^{hi}CD11c^{lo}CD11b^{hi}MHC-II⁻), CD8 α ⁻cDC (CD11c^{hi}CD11b⁺CD8 α ⁻MHC-II⁺), CD8 α ⁺cDC (CD11c^{hi}CD11b⁻CD8 α ⁺MHC-II⁺) and myeloid cells (FSC^{lo}CD11c⁻CD11b^{hi}MHC-II⁻). **(B)** Prevalence of myeloid/dendritic cell subsets in irradiation chimeras reconstituted with either donor SPL ckit^{hi} (ckit) or donor bone marrow (BM) cells is

shown in terms of percent cells amongst total of T and B cell depleted splenocytes. Distribution of spleen myeloid cell subsets in age-matched, unirradiated mice is shown as a control. Results are shown as the mean \pm S.E. of three mice. **(C)** Representation of donor-derived (CD45.1⁺) cells (L-DC, CD8 α ⁻cDC, CD8 α ⁺cDC and myeloid cells) in spleens of ckit^{hi} SPL chimeras and BM chimeras compared with the myeloid subset composition of control donor-type B6.SJL (CD45.1⁺) mice.

The latter population was also identified in terms of granulocyte and macrophage subsets using specific antibody (data not shown). The population distribution of DC/myeloid subsets in spleens of chimeras was similar to control mice, indicating full haematopoietic reconstitution (Fig. 5B). Each DC or myeloid compartment in spleen was restored to homeostatic levels, with myeloid cells representing the largest population, followed by CD8 α ⁻cDC, CD8 α ⁺cDC and L-DC (Fig. 5B). However, only L-DC in spleen showed full reconstitution with donor-derived (CD45.1⁺) HSC present in spleen Lin⁻ckit^{hi} cells or in BM (Fig. 5C). The CD8 α ⁻cDC, CD8 α ⁺cDC and myeloid cell compartments were only partially replaced by donor-type cells. In chimeras of this type, donor-derived HSC do colonize BM in low number (data not shown). However, there is no clear evidence yet for L-DC development in BM, although a similar but distinct subset of cells is under further investigation.

A comparison of relative numbers of donor- versus host-derived cells confirmed differing levels of chimerism for each cell subset. A distinct trend in relative donor:host levels for different DC subsets was evident across chimeras analysed at 15 to 18.5 weeks, despite variance in overall donor cell reconstitution levels between individual mice. When the fold-increase in donor versus host cell numbers was calculated for each APC subset and standardized to CD8 α ⁻cDC (donor:host ratio = 1.0), L-DC consistently exceeded myeloid cells and cDC in terms of relative donor to host cell contribution (Fig. 6). By this analysis, donor-derived

progenitors reconstituted CD8 α ⁻cDC and CD8 α ⁺cDC subsets to equal levels, but gave ~2-fold more myeloid cells. These results are developmentally consistent with a common progenitor (CDP) for cDC subsets [16], and a common upstream progenitor (MDP) for cDC and macrophages [13]. In contrast, donor-derived progenitors gave rise to significantly higher numbers of L-DC (4–12 fold increase; mean = 8.7) compared with CD8 α ⁻cDC (Fig. 6), perhaps indicative of a separate developmental origin for L-DC compared with cDC and macrophages. Since this same trend was also observed for control mice given only donor BM intravenously (Figs 4 and 5), we concluded that the source of HSC was not important. The lodgement of donor-type progenitors into empty niches in spleen would appear to determine the development of L-DC in higher relative numbers than other myeloid/dendritic cell types in spleen.

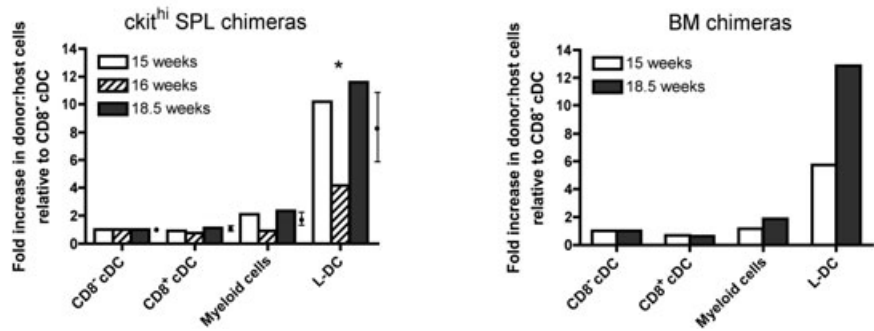
Discussion

This study addresses myelopoiesis in spleen leading to the development of a novel dendritic-like cell type, namely L-DC, which is unique in terms of its phenotype and immune functional potential [24]. Adult murine spleen is clearly an extramedullary haematopoietic site containing low numbers of multipotent HSC

Fig. 6 Lin⁻ckit^{hi} adult spleen cells give preferential reconstitution of L-DC in irradiation chimeras. The long-term reconstituting potential of Lin⁻ckit^{hi} spleen cells for L-DC, CD8 α ⁺ cDC, CD8 α ⁻ cDC and myeloid cells was assessed in irradiation chimeras described in Figure 4. These mice were given a mix of donor-type Lin⁻ckit^{hi} spleen cells and host-type bone marrow. Controls included two irradiation chimeras reconstituted with donor-type bone marrow. The individual myeloid subsets of donor (CD45.1⁺) versus host (CD45.2⁺) type were identified in spleen using flow cytometry as

shown in Figure 5. The relative prevalence of donor:host cells for individual subsets was calculated for each animal, based on a relative value of 1.0 for CD8 α ⁻ cDC. Mean \pm SE is shown for SPL chimeras ($n = 3$). Cell subsets having significantly higher representation of donor:host-type cells compared with CD8 α ⁻ cDC are indicated (*) [$P \leq 0.05$ (Wilcoxon Rank Sum Test)].

Prevalence of donor subsets in spleen of chimeras



[1]. The hypothesis that extramedullary haematopoiesis mediates the production of tissue-specific APC like L-DC with site-specific functions, is consistent with compartmentalism of the immune response to meet the needs of distinct tissue sites and their respective pathogens. While multiple extramedullary sites clearly contribute to haematopoiesis, the nature of cells produced and the conditions under which this occurs are not yet well defined.

In order to identify an APC subset as distinct it is necessary to show that the lineage origin and progenitor of those cells differs from that of other common dendritic and myeloid subsets. Marker analysis of adult spleen has led to the identification of minor subsets of Lin⁻ckit^{lo} and Lin⁻ckit^{hi} cells which lack markers like Flt3, CD34 and IL-7R associated with myeloid and lymphoid haematopoietic progenitor subsets in BM (Fig. 1) [17, 20]. There is already evidence that HSC in different tissue sites are different since the marker profile of foetal liver HSC differs from that of BM HSC [40]. Here we have localized the progenitor of L-DC within the Lin⁻ckit^{hi} subset of spleen, which also contains LT-HSC. The presence of LT-HSC in this subset was evident since this subset gave long-term multilineage reconstitution of chimeras out to 54 weeks (Fig. 6). This study therefore identifies a close, if not linked, relationship between LT-HSC and the L-DC progenitor in murine spleen. Further work is under way to obtain the full marker expression profile of splenic HSC, and to determine whether this cell type differs from that of BM HSC with the same differentiative capacity.

Both adult Lin⁻ckit^{lo} and Lin⁻ckit^{hi} adult spleen subsets were found to contain progenitors which differentiate to give L-DC in co-culture over the STX3 splenic stroma. However, only the Lin⁻ckit^{hi} subset and not the Lin⁻ckit^{lo} subset contains cells, which reflect self-renewing LT-HSC as demonstrated by their long-term multilineage reconstitution potential in irradiated mice. The differential function of these two subsets *in vivo* did not mirror their common *in vitro* differentiative capacity. A first explanation is that they contain a common progenitor, not yet identifiable

with available antibodies. The second explanation is that the Lin⁻ckit^{lo} subset contains L-DC progenitors that derive directly from progenitors within the Lin⁻ckit^{hi} subset of spleen, and that this differentiation occurs when Lin⁻ckit^{hi} cells are co-cultured over STX3 stroma. The transition of Lin⁻ckit^{hi} cells into Lin⁻ckit^{lo} cells within co-cultures has proven very difficult to test because of the small size of these subsets in spleen, and the difficulty of recovering enough cells from co-cultures to perform an analysis of phenotypic change.

Indeed, the L-DC progenitor in spleen is phenotypically distinct from other described myeloid and dendritic progenitors present in BM. The CDP and MDP are phenotypically Flt3⁺ cells [13], with no counterpart subset in spleen. The CDP has distinct differentiative capacity for cDC, and the MDP differentiates to give macrophages and DC [17]. Furthermore, neither the splenic Lin⁻ckit^{lo} nor Lin⁻ckit^{hi} subsets described here resemble the immediate cDC precursor or pre-cDC subset previously identified as ckit⁺CD11c^{lo} cells, which respond to Flt3L to produce mature cDC and pDC [15, 18]. This pre-cDC subset would have been excluded by our sorting protocol, which specifically gated out Lin⁺ cells including CD11c⁺ DC.

L-DC are a distinct CD11c^{lo}CD11b^{hi}MHC-II⁻ dendritic-like subset in spleen with strong cross presentation capacity for CD8⁺ T cell activation [24]. While these cells are phenotypically distinct from monocytes, which are CD11c⁻ and are also unable to cross present antigen [24], it is not yet known whether L-DC share a common lineage relationship with monocytes. Since monocytes, macrophages and cDC/pDC all originate from BM progenitors like MDP and CDP, one expectation is that these cell types might all be reconstituted to similar levels following HSC transfer. This prediction was in fact verified in radiation chimeras shown here, and multiple chimeras demonstrated equal long-term reconstitution of donor-derived splenic monocytes/macrophages, and the CD8 α ⁻ and CD8 α ⁺ cDC populations (Fig. 6). However, these same chimeras showed an average 9-fold increase in donor over host reconstitution of L-DC compared with CD8 α ⁻ cDC, and a 7-fold

increase of L-DC over myeloid cells (Fig. 6). Further analyses involving purified progenitors will be necessary in order to establish whether this result indicates independent development of L-DC from macrophage/monocyte lineage cells.

Both *in vivo* and *in vitro* evidence now supports the hypothesis that spleen endothelial cells represent a niche for haematopoiesis of L-DC from spleen endogenous self-renewing HSPC. The presence of HSC in spleen has been demonstrated firstly by their ability to reconstitute lethally irradiated hosts [1] (Fig. 4), and secondly since transplanted HSC can home to and engraft spleen [41]. In this laboratory, we have also shown that L-DC progenitors are maintained in spleen LTC, where they appear to continuously self-renew for several years, in a manner strictly dependent on contact with spleen endothelial cells [42]. This study therefore supports a role for spleen as a tissue site supporting haematopoiesis for subsequent production of L-DC. Indeed, a precedent already exists for organ-specific DC haematopoiesis in skin [28]. At this stage, it is not yet known whether L-DC development is an intrinsic property of HSC maintained in spleen, or whether spleen stromal niches can direct DC development from HSC originating from other tissues like BM. Since mice reconstituted with either spleen- or BM-derived HSC also show a strong bias towards donor-derived L-DC reconstitution over cDC and myeloid cells (Fig. 6), the spleen microenvironment rather than the tissue origin of HSC may be the critical factor in directing haematopoiesis of L-DC.

Indeed, an explanation for the bias in development of L-DC over cDC and monocytes can be found in terms of niche space. HSC transplanted intravenously into lethally irradiated mice seed spleen with 37% frequency and BM with 50% frequency [41]. BM contains multiple bone compartments [41] with endothelial and osteoblastic niches, which support HSC development [43]. In contrast, spleen has both lower HSC prevalence [40] and less endothelial niche space than BM, with no osteoblastic niches [43]. Therefore, based on HSC seeding frequency, and relative HSC niche space between BM and spleen, it is likely that intravenously transferred HSC engraft and saturate spleen HSC niches ahead of BM niches, since they are fewer in number but comparable in seeding frequency 1:1.3 ratio [41]. Saturation of niches in spleen by intravenously delivered donor HSC could lead to higher development of donor over host L-DC in spleen, if L-DC development is dependent on splenic endothelial cells.

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Evidence is advanced for a spleen-endogenous lineage of dendritic-like cells which develop under steady-state conditions. Both *in vivo* and *in vitro* studies support this conclusion that LTC established from neonatal spleen continuously produce L-DC, the adult spleen Lin⁻ckit⁺ subset contains L-DC progenitors, and adult spleen HSC can reconstitute L-DC to a greater extent than other DC or myeloid subsets. Such a model for spleen-specific DC haematopoiesis will require testing under conditions where cell development is monitored from HSPC engrafted into spleen, but in the absence of BM engraftment.

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Conflict of interest

The authors declare no commercial conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Shows evidence of long-term multi-potential reconstitution of irradiation chimeras given ckit^{hi} SPL and bone marrow cells.

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