Splenic stromal niches support hematopoiesis of dendritic-like cells from precursors in bone marrow and spleen

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Objective. The aims of this study are to test the ability of stromal cells from murine spleen to support hematopoiesis, to define the tissue source of precursors that seed these hematopoietic niches, and to determine the type of cells produced.

Materials and Methods. Cloned isolates of murine spleen stroma have been developed that support hematopoiesis. Analysis has been investigated in terms of tissue source of progenitors. Type and number of cells produced were analyzed by flow cytometry.

Results. Hematopoietic precursors that seed cocultures exist in spleen and bone marrow (BM), but not thymus. Cell production is highest if overlay cells are enriched for hematopoietic precursors. BM contains more precursors than spleen, but the cell types produced are different. Cocultures established from spleen maintain a high proportion of a distinct class of dendritic-like cells produced in only low numbers in BM cocultures. These reflect the immature myeloid dendritic cell (DC) produced continuously in long-term spleen cultures established previously in this laboratory. Stroma-conditioned medium alone does not support DC development, but does support early outgrowth of myelomonocytic cells from precursors in both spleen and BM.

Conclusion. The outcome has been development of a coculture system that supports hematopoiesis of immature myeloid dendritic-like cells in vitro. Although production of monocytes can occur in the presence of stroma-conditioned medium alone, production of DC is dependent on stromal cell interaction. Results presented here raise questions about the role of spleen as a site for DC hematopoiesis from endogenous precursors. © 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.
cDC. Monocyte (mo)-derived DC represent a very different cell type, developing under inflammatory conditions and dependent on cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor – α, which drive cells from blood into lymph nodes for antigen presentation [6]. To date, macrophages and monocyte subsets have not been characterized as fully as DC subsets in terms of lineage and antigen-presenting function as DC.

In vitro cultures have been commonly used to expand DC from precursors and can be readily produced in cultures of bone marrow (BM) supplemented with inflammatory cytokines like GM-CSF and tumor necrosis factor – α [7]. However, this type of culture system is self-limiting because it supports the maturation or activation of already existing DC precursors. Recently, in vitro proliferation of resting cDC and pDC was achieved using Fms-like tyrosine kinase 3 ligand (Flt3L) [8]. While the concentrations used were much higher than physiological levels, the cells produced do reflect in vivo counterpart cell populations [9].

Long-term culture (LTC) methods have been used extensively for studying hematopoiesis. The establishment of a stromal cell layer is essential for providing a microenvironment that supports stem cell survival, self-renewal, and differentiation [10]. Stromal cell interactions and cytokines lead to hematopoiesis and cell development [11]. BM LTC were originally shown to support granulopoiesis [10] and later, an LTC system was reported to support early B-cell development [12]. LTC established from murine spleen have now been shown to support continuous hematopoiesis of DC in vitro [13,14]. These comprise an adherent stromal layer that maintains a population of progenitors as well as foci of developing dendritic-like cells. Murine spleen LTC produce a homogeneous population of dendritic-like cells (LTC-DC), phenotypically distinct as immature myeloid DC [14].

The STX3 splenic stromal cell line was isolated from one LTC, which ceased DC production because of loss of hematopoietic progenitors upon passage [15]. It can, however, support DC development from overlaid BM cells [15]. LTC also maintain a population of progenitors that, when sorted and overlaid on to the STX3 stromal line, generate this distinct LTC-DC subset [16]. Further analysis of the STX3 stromal line has involved cloning to determine its constituent cell components [17], as well as gene profiling to define supportive stroma as immature endothelial cells [18–20]. In a previous study, cloned stromal cell lines derived from STX3 were tested for capacity to support hematopoiesis of overlaid BM cells [19], however, those experiments did not fully investigate the type of cell produced or its progenitors. This study now examines the tissue source of precursors and progenitors in terms of cell subsets produced in stromal cocultures. It focuses on development of cells given the tentative designation of L-DC, which resemble LTC-DC.

Materials and methods

Animals

Specific pathogen-free C57BL/6 J female mice aged between 2 and 12 weeks were obtained from the John Curtin School of Medical Research (Canberra, ACT, Australia). ROSA–enhanced green fluorescent protein transgenic (C57BL/6 J) mice were kindly provided by Dr Klaus Matthaei (John Curtin School of Medical Research, Canberra, ACT, Australia). Mice were housed and handled according to protocols approved by the Animal Experimentation Ethics Committee at the Australian National University (Canberra, ACT, Australia).

Stromal cell cultures

Derivation of the STX3 spleen stromal line and the 2RL22 lymph node stromal line has been described previously [16,21,22]. Clonal isolates of STX3, namely SG3, 3B5, 7G10, 10C9, 2A8, and 8B11, have been partially characterized [19]. Stromal lines were maintained by scraping attached cells for passage into a new flask. Cells were cultured at 37°C in 5% CO₂ in air in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 5.10⁻⁴ M 2-mercaptoethanol, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mg/L glucose, 6 mg/L folic acid, 36 mg/L L-asparagine, 116 mg/L L-asparaginase hydrochloric acid (sDMEM). In order to maintain the stability of cloned lines, frozen stocks were established and cultures discarded after five passages. Cloned stromal cell lines were passaged using 0.25% trypsin-ethylene diamine tetracetic acid treatment to dissociate cells for transfer into a new flask.

Antibody staining

Both direct and indirect fluorescent antibody binding were used to detect cell surface marker expression. An LSRII flow cytometer was used to quantify fluorescence (Becton Dickinson, Franklin Lakes, NJ, USA). Antibodies used included: CD11c (N418: allophycocyanin-conjugated hamster immunoglobulin G [IgG]; eBioscience: San Diego, CA, USA), CD11b (M1/70: fluorescein isothiocyanate [FITC]-conjugated rat IgG₂b; eBioscience), and MHC-II (25-9-17: biotinylated mouse IgG₂a; Becton Dickinson). Fluorochrome-conjugated isotype control antibodies were used in all experiments to measure background binding. For staining, cells in fluorescein-activated cell sorting (FACS) buffer (DMEM, 0.1% sodium azide, 1% fetal calf serum) at 1 to 5.10⁵ cells/100 µL were incubated for 15 minutes on ice with 40 µg/mL “Fc block” -specific for FcγII/IIIIR (CD32/CD16) (eBiosciences), followed by addition of primary antibody for an additional 30 minutes on ice. Two washes with 150 µL FACS buffer were performed by centrifugation to remove excess antibody. When required, cells were resuspended in diluted secondary antibody or avidin conjugate, incubated on ice for 30 minutes, followed by two washes and resuspension in FACS buffer for flow cytometric analysis. In some experiments, propidium iodide (PI: 100 µg/mL) was added for discrimination of dead cells. To assess cell capacity for endocytosis, cells (10⁷/mL in sDMEM) were exposed to FITC-conjugated ovalbumin (2 mg/mL) for 45 minutes at 37°C. Control cells were held on ice over this time. Endocytosis was halted by addition of 5 volumes of ice-cold FACS buffer, and cells washed twice before assessment of FITC uptake flow cytometrically. BD FACSDiVa software (Becton Dickinson) was used to set voltage and event counts while running samples. For
multicolor analysis, single-color compensation controls were used to set compensation settings on the machine. FlowJo software (FlowJo, Ashland, OR, USA) was used to analyze data collected. Cell debris was gated out using a forward scatter (FSC) threshold of 100. Live (PI–) cells were then gated for further marker analysis. Isotype control antibodies were used to set gates to distinguish specific antibody binding. Postacquisition gating was used to obtain information on cell subsets.

**Preparation of BM and spleen cell suspensions**

Mice were sacrificed by cervical dislocation. BM was flushed from the femoral cavity using a 26-gauge needle connected to a 10-mL syringe filled with sDMEM. BM and spleen cell suspensions were dissociated by forcing tissue through a fine-wire sieve. BM/spleen cell suspensions were treated to remove red blood cells (RBC) using treatment with RBC lysis buffer (140 mM NH₄Cl, 17 mM Tris Base) for 5 minutes at room temperature. Five milliliters phosphate-buffered saline (PBS) was added and the cell suspension was centrifuged for 5 minutes at 300g. Cells were washed three times by centrifugation through PBS to remove cell debris and resuspended in sDMEM. Trypan blue staining (4% in 0.85% normal saline) was used to count viable cells. In some experiments, cell suspensions were separated by centrifugation above a Histopaque (Sigma-Aldrich, St Louis, MO, USA) cushion to isolate leukocytes.

**B-, T-, and myeloid-cell depletion**

Spleen was depleted of B and T cells, and bone marrow was depleted of B, T, and myeloid cells before use in some coculture assays. Antibodies used for depletion were specific for CD19 (MB19-1: biotinylated mouse IgA; eBioscience) on B cells, Thy 1.2 (30-H12: biotinylated rat IgG₂a; Becton Dickinson) on T cells and CD11b (M1/70: biotinylated rat IgG₂; eBioscience) on myeloid cells. Antibodies were diluted in labeling buffer (PBS [pH 7.2], 0.5% bovine serum albumin, 2 mM ethylene diamine tetraacetic acid) to concentrations giving minimum saturation binding, and absorbed to cells at 10⁶/mL in 100 µL on ice for 20 minutes. Cell suspension was washed twice with labeling buffer by centrifugation at 300g and 4°C for 5 minutes. Cells were resuspended in MACS anti-biotin microbeads (Miltenyi Biotech, Gladbach, Germany) 50 µL/mL in 100 µL labeling buffer and incubated on ice for a additional 30 minutes. Cells were washed once with labeling buffer and supernatant decanted. Cell pellets were resuspended in 500 µL labeling buffer and transferred to a MACS MS column (Miltenyi Biotech) for separation of labeled and unlabeled cells. The three antibodies used for B-, T-, and myeloid-cell depletion were used separately and in combination to achieve different depletions.

**Coculture assays**

The capacity of STX3 and STX3-derived stromal clones to support hematopoiesis was tested by overlay of hematopoietic cell populations above stromal cell monolayers followed by coculture for several weeks. Stromal cell lines were grown to 80% to 90% confluence, followed by addition of BM or spleen cells at 1 to 5,10⁵ cells/mL, prepared as described here by RBC lysis or depletion of specific cell subsets. Cocultures were left for 1 week before medium change. After 14 days for BM cocultures, and 17 days for spleen cocultures, nonadherent cells were collected by gently shaking the flask and removing the supernatant. For analysis of hematopoietic support capacity by stroma-conditioned medium, supernatant was collected from confluent splenic stromal cultures and stored at −20°C until use. Stromal alone or cocultures of BM or spleen above stroma in 25 cm² flasks were photographed under brightfield using an inverted Fluovert FS microscope (Leitz, Wetzlar, Germany) equipped with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Images were processed using SPOT RT software v3.5.1 (Diagnostic Instruments). To obtain photographs under phase contrast and fluorescence, a DM IRE2 inverted research microscope (Leica, North Ryde, NSW, Australia) equipped with DFC digital camera (Leica) was used. Images were processed using Leica IM software v4.0.

**Detection of dead and dying cells**

The Annexin-V – FITC apoptosis detection kit (Sigma-Aldrich) was used to identify early apoptotic cells and necrotic cells. Nonadherent cells were collected from overlay cultures above stroma and washed twice in PBS by centrifugation at 300g and 4°C for 5 minutes. Supernatant was decanted and cells resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of ~10⁶ cells/mL. Cells were then incubated with Annexin-V – FITC 0.5 µg/mL and PI 1 µg/mL and incubated in the dark at room temperature for 10 minutes before flow cytometric analysis.

**Statistical analysis**

Wilcoxon rank sum test was used to test significance (p ≤ 0.05).

**Results**

**Tissue source of hematopoietic precursors**

During many experiments, STX3 stroma has demonstrated unique capacity to support the development of dendritic-like cells from both overlaid BM and spleen (Fig. 1), but not thymus (data not shown). These cocultures showed production of cells with distinct dendritic morphology after 14 days for BM and 17 days for spleen, with higher cell recovery from BM than spleen (Fig. 1A and C). The length of coculture was determined as optimal for cell yield. By using BM and spleen derived from ROSA–enhanced green fluorescent protein transgenic mice as overlay cells, it was confirmed that nonadherent cells shed into medium after 14- to 17-day cocultures originated from enhanced green fluorescent protein–positive BM or spleen and not from stroma (Fig. 1B).

Continuous cocultures by nature maintain a subpopulation of dead or apoptotic cells. It was, therefore, necessary to identify live cells for analysis of cell type on the basis of cell surface marker expression. Nonadherent cells were collected from cocultures of BM and spleen and stained with Annexin-V to detect apoptotic cells, and PI to detect necrotic cells using flow cytometry. Distribution of live cells (Annexin–PI–), from apoptotic cells (AnnexinþPI–) and necrotic cells (PIþ) was analyzed in terms of FSC and SSC profile after gating to remove debris. These were then shown as colored overlays, which allowed...
identification of gates, based on FSC and SSC used to define live cells produced in subsequent cocultures (Fig. 2A).

Analysis of nonadherent cells collected from BM cocultures revealed populations of live cells having small and large size (Fig. 2A). Both populations contained significant numbers of CD11c−CD11b+ DC among live cells; 33% of small and 70% of large cells. A 20% population of CD11c−CD11b+ large cells were classified as monocytes/macrophages. In cocultures of splenocytes over STX3, nonadherent cells comprised three live cell populations, of small-, medium-, and large-sized cells (Fig. 2A). CD11c+CD11b+ DC were present only among the large cell population (40%), along with a 51% population of CD11c+CD11b+ myeloid cells reflecting monocytes/macrophages.

A large proportion of cells produced in spleen and BM cocultures phenotypically resembled the dendritic-like cells produced in LTC that maintain a stromal cell layer essential for long-term myelopoiesis. LTC support two populations of live small and large cells (Fig. 2B). The majority of both large and small cells are CD11b+CD11c−MHC-II− cells reflecting immature dendritic-like cells (Fig. 2B). By comparison with BM cocultures, almost no CD11c−CD11b+ cells were produced.

Optimization of assays for hematopoiesis of dendritic-like cells

Multiple cloned isolates of STX3 were then compared to optimize conditions for hematopoiesis of DC in BM cocultures. Cell production was measured at 14 days for each cloned line in terms of cell yield and cell surface phenotype. Six cloned lines (i.e., 2A8, 3B5, 5G3, 7G10, 8B11, and 10C9) were compared with STX3 for capacity to support hematopoiesis of DC identified as CD11c+CD11b+.
cells as in Figure 2. 2RL22 lymph node stroma was included as a control nonsupporting stromal line, and stroma- or BM-only cultures produced no progeny cells (not shown). The 2A8, 5G3, 10C9, and STX3 stromal lines were found to give the highest yield of nonadherent cells from overlaid BM, with cell recovery relative to input cell number as high as 75% after 14 days (Fig. 3A). These stromal lines were compared for capacity to support the outgrowth of colonies by 7 days during the assay, with later production of large nonadherent cells. In contrast, the 3B5 nonsupporter produced no colonies and released very few cells into medium (Fig. 3B).

Populations of small and large live cells were detectable in medium above cocultures after 14 days, each with significant numbers of both CD11c⁺CD11b⁺ dendritic-like cells and CD11c⁺CD11b⁺ myeloid cells. In terms of combined cell yield and proportion of CD11c⁺CD11b⁺ cells, 5G3 was the best producer among the cloned stromal lines (Fig. 3C). Small cells produced by productive stroma also contained minor but detectable populations of CD11c⁺ and CD11b⁺ cells, thought to reflect myeloid or DC precursors. Cells were also stained for c-Kit as a marker of hematopoietic progenitors/precursors. Very low numbers of c-Kit⁺ cells were detected among the small cell populations, and large cells produced by all stromal lines tested negative for c-Kit expression (data not shown), consistent with the absence of hematopoietic precursors among the large cell population.

These same stromal lines were compared for capacity to support the outgrowth of colonies by 7 days during the assay, with later production of large nonadherent cells. In contrast, the 3B5 nonsupporter produced no colonies and released very few cells into medium (Fig. 3B).
support hematopoiesis from spleen. The best producers were 2A8, 5G3, 10C9, and STX3 stroma after 17 days (Fig. 4A). As with BM cultures, 3B5 was a nonsupporter of hematopoiesis. Colonies of cells adhered to stroma were readily detectable in 5G3 cocultures, but not in 3B5 cocultures (Fig. 4B). The cell recovery from splenic cocultures was at best 5% of input cells and yields were significantly lower (~15-fold) than for BM cocultures (Wilcoxon rank sum test: \( p < 0.05 \)). This reduced cell yield could relate to either a lower precursor frequency in spleen by comparison with BM, or the presence of different types of precursors in the different tissues. 5G3 was a competent stroma for spleen cocultures, while 3B5 was a nonsupporting stroma. Again, no c-Kit\(^+\) hematopoietic cells were detected among large cell populations, and only few were noted within small cell subsets (data not shown). The population of large cells, although smaller than produced in BM cocultures, was consistently shown to contain a majority of CD11c\(^+\)CD11b\(^+\) dendritic-like cells, and almost no CD11c\(^-\)CD11b\(^+\) myeloid monocytic cells (Fig. 4C). In contrast, BM cocultures showed equal representation of both populations.

**Stromal cell-conditioned medium supports distinct myelopoiesis**

The capacity of stromal lines to produce soluble factors that influence hematopoiesis was tested by culturing BM or
spleen cells in conditioned medium collected from stromal cell lines grown to near confluence. Cell recovery and phenotype was assessed at 7 days using the same methods of analysis employed for stromal cocultures as mentioned. After 7 days, significant cell death occurred in cultures. Negative controls included BM or spleen cells cultured in medium only, or conditioned medium cultured without BM or spleen cells. No live cells were produced in any control cultures (data not shown) confirming that cell production was dependent on stromal cells present in overlaid BM or spleen.

Cultures of BM cells with stroma-derived conditioned medium gave lower cell recovery (12% to 25%) (Fig. 5A) than with stroma (50% to 75%) (Fig. 3A). This result was consistent for all stromal clones and for STX3. While 3B5 showed inability to support hematopoiesis of dendritic-like cells in cocultures (Fig. 3A), it did secrete factors that support myelopoiesis during a 7-day period to give a clear majority population of CD11b+CD11c− monocytes/macrophages, and very few CD11b+CD11c+ dendritic-like cells (Fig. 5C). This result was mirrored for all spleen stroma lines analyzed and identifies distinct myelomonocytic precursors in BM that respond to soluble stromal factors to produce myeloid monocytic cells within 7 days (Fig. 5C). When stroma-conditioned medium was added to spleen cells in culture, very little cell growth was evident. After a 7-day culture, live cell yield was <2% across all stroma (Fig. 5B). The presence of a lower number of progenitors/precursors in spleen compared with BM, would account for the reduced recovery rate of spleen cultures. Flow cytometry on overlaid splenocytes after 7 days showed clear subpopulations of CD11c+CD11b+ and CD11c−CD11b− cells developing on addition of condition medium derived from all stroma (data not shown). Despite the small numbers, it was also notable that the relative proportion of CD11c+ cells among CD11b− cells was higher than for cultures of BM in stroma-conditioned medium (data not shown).

The combined results emphasize the importance of stromal cell contact rather than soluble stromal factors for
development of the CD11b⁺CD11c⁺MHC-II⁺ population of dendritic-like cells from hematopoietic progenitors in BM and spleen. In coculture assays, clear populations of these cells were most evident after 14 days for BM and 17 days for spleen cocultures, while myelomonocytic cells developed by 7 days in response to stromal cell–conditioned medium.

Confirmation that spleen stroma is a sufficient niche for DC hematopoiesis
The contribution of soluble factors produced by mature cells present in BM and spleen overlays was also tested. Coculture assays were therefore, established with the selected 5G3 and 10C9 stromal cell lines using overlay cells derived from spleen and BM depleted of mature myeloid and lymphoid cells. RBC lysed BM and spleen cell preparations were depleted of B cells, T cells, myeloid cells, or all of B, T, and myeloid cells. Spleen was depleted of B cells, T cells, or both. Depletion procedures effectively removed ~90% of T, B, and myeloid cells from BM and spleen. Whole BM or spleen was used as a positive control, and cultures of stroma alone, and BM and spleen cultured in medium were used as negative controls.

For BM cultures, similar results were obtained for both 5G3 and 10C9, and so only 5G3 data are shown. Large non-adherent cells with dendrites, medium round nonadherent cells, and scattered foci of small round adherent cells, were observed in all cocultures of lineage depleted (Lin⁻) BM, and not in negative controls (Fig. 6A). Depletion of myeloid cells, by Histopaque separation, had no effect on the level of hematopoiesis and gave similar cell yield as whole BM. Cocultures established with BM depleted of T and B lymphocytes, gave two- to threefold higher cell yields (Fig. 6B). A similar increase was seen in cell recovery from cocultures established with lineage (Lin⁻) BM (T-/B-/myeloid-cell depleted). Cell production in cocultures occurs independently of mature lymphoid and myeloid cells or any soluble factors they produce.
Cell production in stromal cocultures established with lineage-depleted spleen or bone marrow (BM) cells. Spleen and BM cell populations were exposed to a range of depletion techniques to remove T cells (T–), B cells (B–), T, and B cells (T/B–), myeloid cells (My–), or T, B, and myeloid cells (Lin–). These depleted populations were then compared with whole BM or spleen for production of cells following coculture above the 5G3 stroma at a concentration of 10⁶ cells/mL. Negative controls comprising stroma only or depleted cell populations cultured in medium only were nonsupporting (not shown). (A) Cocultures were photographed at 14 days for Lin– BM and 17 days Lin– spleen under phase using an inverted microscope. Scale bar: 200 μm. (B) At 14 days for BM cocultures and 17 days for spleen cocultures, nonadherent cells were collected and percent cell recovery calculated based on total input cell number using trypan blue exclusion to count viable cell number. (C) Collected cells were stained with antibody specific for CD11c, CD11b, and major histocompatibility complex class II, as well as propidium iodide (PI). Cells were also incubated with fluorescein isothiocyanate (FITC)–ovalbumin (OVA) (2 mg/mL at 37°C for 45 minutes) to determine capacity of cells to endocytose soluble antigen. Control cells were held on ice over this time. Cells were gated initially to remove debris and dead (PI+) cells. Live PI– cells were then gated on the basis of forward- and side-scatter (FSC, SSC) to reveal a major large cell population (shown) that was then analyzed for marker expression. Isotype control antibodies were used to set quadrant gates to identify positive staining cells. Numbers shown in quadrants represent percent cells among the total live cell population. Endocytosis was plotted relative to a nonendocytic control cell population (filled histogram). (D) Percent representation of subsets produced in Lin– BM and Lin– spleen cocultures. Cell subsets were designated on the basis of CD11c, CD11b, and major histocompatibility complex class II phenotype as conventional dendritic cells (cDC), immature DC, and monocytes.
To identify cell subsets formed in Lin– BM cocultures, five-color antibody staining was performed using antibodies specific for CD11c, CD11b, B220, CD8α, and MHC-II to distinguish dendritic and myeloid cell subsets. Large cells produced in cocultures comprised equal numbers of CD11b+CD11c+MHC-II+ cDC-like cells (42%), and CD11b+CD11c–MHC-II– cells (44%) resembling monocytes, with a minor (8%) population of CD11b+CD11c+MHC-II– cells resembling L-DC (Fig. 6C and D). Cells produced were also highly endocytic, a property common to both monocytes and DC. Large live cells stained negative for B220 and CD8α (data not shown). This confirmed the absence of T- and B-lymphocyte production, and excluded production of B220+ pDC or CD8α+ cDC.

In order to assess the importance of stromal cell contact, Lin– BM was overlaid on to STX3 stroma for 24 hours and then nonadherent cells collected and overlaid on a second stromal layer of STX3. This experiment confirmed the importance of adhesion between stroma and overlaid precursor cells for subsequent hematopoiesis. When cocultures were compared after 11 days by flow cytometry, total cell yield differed, being noticeably less for cultures established with cells that had not adhered within 24 hours (Fig. 7). In each of the BM cocultures, the phenotype of subsets produced was the same, with equally high numbers of CD11b+CD11c+MHC-II+ cDC-like cells and CD11b+CD11c–MHC-II– monocytes, and a smaller population of CD11b+CD11c–MHC-II– immature DC resembling those produced in LTC.

Depletion of mature T and or B cells from spleen cell populations (Lin– spleen) had a similar enhancing effect on cell recovery from 17 day cocultures over 5G3 stroma (Fig. 6A). Increases in yield were detected following depletion of T or B cells, or both T and B cells (Fig. 6B). These cultures showed development of cells in clusters on stroma with large dendritic-like cells also evident (Fig. 6A). Flow cytometry revealed two major populations of large-sized cells resembling immature CD11b+CD11c+MHC-II– DC, along with a major population of CD11b+CD11c–MHC-II– monocytes (Fig. 6C and D). Spleen cocultures produced far fewer large cells overall, and a much smaller proportion of CD11b+CD11c–MHC-II– cells reflective of cDC (5%).

The fact that distinct myeloid subsets are produced in cocultures established from Lin– BM as opposed to Lin– spleen suggests that different precursors are maintained in these tissue sites (Fig. 6D). These same findings were repeated for cocultures established above both 5G3 and 10C9 stroma, although only 5G3 results are shown.

**Discussion**

This study assesses the potential of splenic endothelial stromal cell lines to support hematopoiesis. It identifies the production of unique immature myeloid dendritic-like...
cells from both BM and spleen overlaid on splenic stromal lines that resemble previously identified dendritic-like cells produced in LTC of spleen. Cocultures established in this way, therefore, represent an alternate method for production of L-DC, more readily achievable than the more difficult LTC method. BM was tested in cocultures because it is a rich source of hematopoietic stem cells, progenitors, and precursors [23]. Because stromal cell lines are spleen-derived, splenocytes were also tested to determine if spleen contains endogenous DC progenitors or precursors capable of differentiating into DC. This prediction was based on studies involving spleen LTC, which are known DC producers [13]. The capacity for stroma-produced soluble factors to induce production of myelomonocytic cells rather than DC from precursors in BM and spleen was also demonstrated. Overall, this study indicates the important role of spleen in DC development, particularly because the cell type produced is distinct from cDC and pDC as a CD11b^+CD11c^- cell. The possibility that the spleen microenvironment supports development of a DC with unique functional capacity is currently under investigation.

Overlay of BM or spleen cells on to unirradiated stroma, followed by coculture for 2 to 3 weeks revealed the presence of distinct hematopoietic precursors/progenitors in those two sites. The productivity of cocultures was improved if overlay cells were depleted of mature lymphoid and myeloid cells. Additional ongoing studies have now verified that cell production in Lin^- BM cocultures like those described here can continue for up to a year with low but consistent production of cells with the phenotype of immature myeloid DC, identified here as L-DC (unpublished data). With a battery of cloned stromal lines derived from the original STX3 line, it was possible to determine the best stromal lines and to assess their reproducibility in supporting hematopoiesis. Indeed, all but the 3B5 line were good supporters and produced similar populations of cells. Even 3B5 produced cells of similar phenotype, although in much lower numbers.

Studies involving stroma-conditioned medium in place of stromal monolayers demonstrate the importance of soluble mediators in hematopoiesis. Previous studies identified the production of several known cytokines by STX3 stroma including stem cell factor, macrophage-CSF, and transforming growth factor – β, as well as interleukin-6 in low level [20,21]. It should be noted, however, that although STX3 supports development of both myeloid cells and DC in cocultures of BM and spleen, it does not produce inflammatory factors like GM-CSF, tumor necrosis factor – α, interleukin-4, or Flt3L, which are commonly used to induce DC development and proliferation from precursors in vitro [7,8,20]. The outgrowth of myelomonocytic CD11b^+CD11c^-MHC-II^- cells from both BM and spleen precursors presumably occur through production of macrophage-CSF by stromal cell lines [20].

Although soluble factors produced by stroma support development of CD11b^+CD11c^-MHC-II^- myelomonocytic cells from both BM and spleen, cell-to-cell contact with stroma produced different cell types. In BM cocultures after 14 days, myelomonocytic CD11b^+CD11c^-MHC-II^- cells were present in equal numbers with the CD11b^+CD11c^+ MHC-II^- cDC-like cells. Cocultures of spleen, however, showed very little production of cDC after 17 days, and myelomonocytic CD11b^+CD11c^-MHC-II^- cells and L-DC (CD11b^-CD11c^-MHC-II^-) were produced in roughly equal numbers. Both BM and spleen contain myelomonocytic precursors. However, spleen contains relatively more progenitors for L-DC than cDC, and BM contains relatively more progenitors for cDC than L-DC. This result is not unexpected because macrophage/DC progenitors (MDP) and common dendritic progenitors (CDP) are present in BM, but do not exist in spleen [24]. Production of the L-DC cell population is also dependent on the presence of stromal cells and non-stroma-conditioned medium. A prediction of these studies is that cDC-like cells derive from pre-cDC [9] present in BM, but that L-DC derive from a precursor that is distinct from pre-cDC and present in higher numbers in spleen than BM.

Both cDC and pDC in spleen are known to derive from CDP [24–26], however, another report claims that cDC recruited into spleen derive from MDP, more closely aligned with monocyte/macrophase development [27]. It is now clear that the MDP is the more primitive progenitor, giving rise to both CDP and monocytic cells [24]. At this stage, the precursor of L-DC is still under investigation and its lineage relationship with cDC, pDC, and monocytes is not known. However, it is distinct from monocytic precursors that respond readily to stroma-conditioned medium. It would also appear to be distinct from pre-cDC because cDC production can be distinguishable in both spleen and BM, and also distinct from CDP and MDP, which are not present in spleen. The relationship between precursors of myelomonocytic cells, L-DC, and cDC precursors is now under further investigation. At this stage, we predict the presence of a novel dendritic-like cell subset in spleen that derives from distinct splenic progenitors. These cells are expected to resemble progenitors maintained in LTC, which sustain long-term cell production.

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**Conflict of Interest**
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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