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Fetal spleen stroma drives macrophage commitment

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The role of the fetal spleen in hematopoeisis remains largely unknown. In this particular environment, we show that hematopoietic stem cells do not proliferate, but that they lose multipotency and differentiate exclusively into mature macrophages. B lymphocytes in the spleen derive from committed B cell precursors that are likely to have immigrated from the fetal liver. We developed fetal spleen stromal cell lines that are unique in their capacity to expand myeloid precursors, resulting in large numbers of mature macrophages. These lines secrete high levels of anti-inflammatory molecules. By phenotype, fetal splenic macrophages are reminiscent of their adult counterparts found in the red pulp. We postulate that F4/80$^+$ splenic macrophages participate in fetal erythropoiesis, as well as in the formation of the splenic architecture.

KEY WORDS: Hematopoiesis, Myeloid differentiation, Organogenesis

INTRODUCTION

The adult spleen is considered a secondary lymphoid organ because of its prominent role during immune responses. It is organized into red and white pulp areas. The red pulp, enriched in monocytes and erythrocytes, is separated from the white pulp by the marginal zone, which is composed of a special subset of B cells and macrophages (for a review, see Mebius and Kraal, 2005). Little is known about the ontogeny of this regionalization, but it was shown that white pulp lymphoid foci are detectable in newborns one week after birth (Morris et al., 1991). Defective adult splenic organization is observable in mice deficient for the lymphotoxin and TNFR-ligand pathways, and for the transcription factor Nkx2.3, highlighting the importance of hematopoietic cell interactions with the environment for the formation and maintenance of the spleen (for reviews, see Cyster, 2005; Fu and Chaplin, 1999).

Few studies have examined the role of the fetal spleen (FS) during fetal life. The FS forms at embryonic day (E) 11.5 as a condensation of mesodermal mesenchyme along the leftside of the mesogastrium, dorsal to the stomach (Green, 1967). The first step concerns the formation of a splanchnic mesodermal plate (SMP), as shown by mice deficient for the dominant hemimelia gene, and for the transcription factors Bapx1, Wt1 and capsulin, where the disruption of SMP leads to an asplenic phenotype. Mice deficient for the factor homeobox 11 have a progressive regression of the spleen at around E13.5 as a result of the incapacity of the mesenchymal cells to proliferate. Moreover, development of the FS is also dependent upon interactions between stromal and hematopoietic cells (for reviews, see Cyster, 2005; Fu and Chaplin, 1999; Mebius and Kraal, 2005).

During fetal life, most hematopoietic processes occur in the fetal liver (FL), which is colonized between E10 and E11.5 by hematopoietic stem cells (HSCs) emerging from the aorta-gonads-mesonephros region (AGM) (Bertrand et al., 2005a). The FS does not autonomously generate HSCs but rather is seeded by cells in circulation. The colonization of the FS by HSCs was shown to take place between E12.5 and E13, presumably from the FL, as FS explants cultured in vitro produce a hematopoietic progeny after this time (Godin et al., 1999). The spleen remains hemopoietic until the first two weeks after birth (Metcalf and Mas, 1971) and has been considered to be an important contributor to hematopoietic homeostasis (Wolber et al., 2002).

Hematopoiesis is a process that leads to the differentiation of mature blood cells from a pool of multipotent HSCs. In hematopoietic organs, FL and bone marrow (BM), HSCs are maintained through a process called self-renewal. They are also capable of differentiating into any of the hematolymphoid system. Both self-renewal and multipotency are strictly dependent upon the interactions between HSCs and the stromal cells of their microenvironment.

Differentiation towards multiple lineages depends on gene expression programs driven by transcriptional regulators. In the adult BM, an early decision concerns the lymphoid versus myeloid lineage commitment through the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP), respectively (Akashi et al., 2000; Kondo et al., 1997). In FL, the lineage restriction is less clear, as the fetal CLP counterpart possesses some myeloid potential (Mebius et al., 2001). Similarly, the fetal CMP possesses significant B, but not T, lymphoid potential (Traver et al., 2001). Transcription factors are key regulators in lineage commitment. The best example is Pax5, which is exclusively implicated in driving the early development of the B cell program. Pax5 stabilizes the commitment to the B cell fate by inhibiting genes associated with other lineages and by activating B cell-specific genes (for a review, see Busslinger, 2004). However, in respect to other pathways of differentiation, the pattern of gene expression required for lineage specification is more complex. No single major regulator was described in determining the myeloid fate, but rather a combination of a few key transcription factors appears necessary (Shivdasani and Orkin, 1996). PU.1 (Sfpi1 – Mouse Genome Informatics) is one of the regulators of lymphoid and myeloid development. *PU.1*$^{-/-}$ mice are embryonic lethal and are devoid of FL B lymphocytes, granulocytes and macrophages (McKercher et al., 1996; Scott et al., 1994). Low levels of PU.1 are associated with B-cell development, whereas higher levels result in macrophage differentiation (DeKoter and Singh, 2000), suggesting a dose-

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dependent effect of PU.1 on developmental outcome. Moreover, transcription factors could influence differentiation by modulating the response to environmental signals. PU.1 regulates the expression of numerous lymphoid and myeloid cytokine receptor genes, such as the receptors for the interleukin 7 (Il7rα) and the macrophage colony-stimulating factor (M-CSFR; Csf1r – Mouse Genome Informatics) (DeKoter et al., 2002).

Fetal hematopoiesis has been extensively studied at the level of the FL. Here, we examined the contribution of the FS to fetal hematopoiesis, and its capacity to sustain hematopoietic development from HSCs or other progenitors. Based on previously described Fetal Liver Organ Culture (FLOC) (Ceredig et al., 1998), we developed a Fetal Spleen Organ Culture (FSOC) to assess the microenvironmental capacities necessary to sustain hematopoietic differentiation in the absence of circulating cells. HSCs are not maintained after 4 days of FSOC and lymphocytes did not develop under these conditions. B cell production in the FS is thus likely to emerge from B cell precursors that arise in the FL at approximately E13-E14. To enable extended studies on the differentiation capacities of the stroma, we developed fetal spleen stromal (FSS) cell lines. They were used as supportive feeder layers, and their supernatants as supportive media for liquid cultures. Co-culture of HSCs on FSS lines led to myeloid differentiation with exclusive differentiation and proliferation of F4/80$^+$Mac1$^+$ macrophages. Proliferation and differentiation of hematopoietic progenitors using FSS supernatants was more efficient than that driven by various colony-stimulating factors, either alone or in combination, revealing a unique array of factors produced by these cells. In addition to its role in driving myeloid differentiation, the FS stroma could not allow the lymphoid commitment. The FS does not provide the environmental conditions to support HSC self-renewal or multi-lineage differentiation, but rather is oriented towards the production of a homogeneous population of macrophages.

MATERIALS AND METHODS

Animals and dissections

Two C57BL/6 congenic lines (*H-2^b* haplotype), bearing the *CD45.1* and *CD45.2* alleles of the pan-hematopoietic marker CD45, and their F1 progeny were used. *Rag*γ*c*$^{-/-}$ B10BR mice (*H-2^k* haplotype) were backcrossed to C57BL/6 bearing *CD45.1* in order to obtain *Rag*γ*c*$^{-/-}$ (*H-2^k* haplotype, *CD45.1*) mice, which were used as recipients for hematopoietic reconstitution assays (Colucci et al., 1999). Rag2^{GFP} and Actin^{GFP} transgenic mice, expressing the gene coding for green fluorescent protein (GFP) under the control of the *Rag2* and *Actin* promoting sequences, were used (Yu et al., 1999). Mice were bred in the animal facilities of the Pasteur Institute and reconstituted mice housed in filtered, positive pressured, cabinets. OVA 323-335 specific MHCII restricted TcR transgenic OT-II C57BL/6 mice were used to analyze APC functions (Barnden et al., 1998).

Fetal liver and fetal spleen organ cultures (FLOC and FSOC)

E13 FL and FS were explanted, placed on Nucleopore membrane, and kept for 4 days on complete medium (OptiMEM, 10% FCS, 1% penicillin/streptomycin). Cell suspensions were obtained from FLOC and FSOC, and analyzed by flow cytometry or injected into recipient mice.

We adapted this system to study the differentiation of progenitors in the FL and FS environment, as previously described for fetal thymus (Jenkinson et al., 1992). 250-rad γ-irradiated E13.5 fetal liver lobes and fetal spleens from *CD45.2*×*CD45.2* or *CD45.1*×*CD45.1* embryos were seeded with E10.5 AGM cells, all explanted from *CD45.2*×*CD45.1* embryos. After two days in hanging drop, FLOC and FSOC were transferred onto filters for 7-8 days. FLOC and FSOC were analyzed by flow cytometry for the presence of donor-derived cells.

Long-term reconstitution (LTR) experiments

Cell suspensions (CD45.2$^+$ H-2^b) from FL and FS, either freshly explanted, or cultured in toto (FLOC and FSOC), or cultured in toto after reconstitution with three embryo equivalents of AGM cells (CD45.1$^+$ H-2^b), were injected independently in 600-rad sub-lethally irradiated *Rag*γ*c*$^{-/-}$ B10BR mice (CD45.1$^+$ H-2^k). For long-term reconstitution analysis, bone marrow, spleen, intestines and thymus were analyzed, after 6 months, by flow cytometry for the presence of donor-derived B cells and myeloid cells (granulocytes); donor-derived cells were scored through the expression of donor marker (either CD45.2, CD45.1 or H-2^b).

Flow cytometry analyses and cell sorting

Flow cytometry analyses were performed in a LSR (Becton Dickinson) with the CellQuest software (Becton Dickinson). The following antibodies (biotinylated or coupled with different FITC, PE or APC) were used: CD4 (clone L3T4), CD16/32 (clone 2.4G2), CD45.1 (clone A20), CD45.2 (clone 104), Kit (clone 2B8), Mac1 (clone M1/70), Gr-1 (clone RB6-8C5), CD11c (clone HL3), CD45R/B220 (clone RA3-6B2), CD19 (clone 1D3), IgM (clone R6-60.2), CD43 (clone S7), H-2K^b (clone AF6-88), Sca-1 (clone E13-161.7) and TER-119. Biotinylated antibodies were revealed with streptavidin coupled with FITC, PE, PE-Cy7 or APC. All antibodies and streptavidin were from Becton Dickinson-Pharmingen. We also used the F4/80-PE antibody (clone CI#3-1, Caltag). Propidium iodide (PI) was used to exclude dead cells during the analysis. Cell sorting was performed with a MoFlo cell sorter (Cytomation Inc.).

Reverse transcriptase-polymerase chain reaction

Stromal cells from E13 FL and FS, and adult thymus were sorted as CD45$^-$ Ter119$^-$. Cells were lyzed in TRIzol (GibcoBRL) and total RNA extracted according to the manufacturer's protocol. Oligo (dT)-primed cDNA was prepared from 6×10^3 to 25×10^3 cells using AMV Reverse Transcriptase (GibcoBRL) in a reaction volume of 20 μl. cDNA from OP9 stromal cells was used as a control.

PCR reactions were performed in the GeneAmp®9700 PCR system (Applied Biosystems) in a final volume of 25 μl. The primers used were:

Hprt-for, 5′-GAC TGA AAG ACT TGC TCG AG-3′;
Hprt-rev, 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′;
Tgfb1-for, 5′-AAC AGC ACC CGC GAC CG-3′;
Tgfb1-rev, 5′-AAC GCC AGG AAT TGT TGC-3′;
Il7-for, 5′-TGC TGC TCG CAA GTT GAA G-3′;
Il7-rev, 5′-TCC TTA CTT GTG CAG TTC ACC A-3′;
Sdf1-for, 5′-TCT GCA TCA GTG ACG GTA AAC CAG T-3′;
Sdf1-rev, 5′-TCC TCC TGT AAG TTC CTC GGG CGT CT-3′; and
Sdf1-ri, 5′-TCT CCA GGT ACT CTT GGA TCC-3′.

Other primers used have already been described, *E2A*(E47) (Bain et al., 1994), *Ebf* and *Pax5* (Kawamoto et al., 2000).

Fetal spleen-derived stromal cell lines

E14.5 FS were explanted and prepared as cell suspensions, which were cultured in gelatin-coated (0.1%) six-well plates in complete medium (OptiMEM, 10% FCS, 1% antibiotics, 0.1% β-mercaptoethanol). After 5 days, non-adherent cells were removed, and adherent cells were trypsinized and cultured without gelatin, in complete medium. After three passages, cells were cloned in 96-well plates to establish fetal spleen stromal cell lines. Ten different cell lines were obtained and three of them were used for the assays.

In vitro potential of HSCs cultured on FS stromal cell lines

FSS cells were plated in 24-well plates, at 10^5 cells.ml^{-1} (500 μl). HSCs from the AGM region, or sorted from E14 FL or adult bone marrow, were then plated in complete medium, without cytokine complementation. After a 7 to 10-day culture period, wells were scored for the presence of hematopoietic cells.

Immunohistochemistry/fetal spleen histology

FS explanted from E15.5 *Rag2*^{GFP} embryos were embedded in OCT (Tissue-Tek, Sakura) and frozen on dry ice. For immunohistochemistry, tissue sections (30-40 microns) were treated with ethanol for 5 minutes and incubated overnight at 4°C with anti-GFP (Rabbit, Invitrogen) and F4/80 (rat IgG2b, clone Cl:A3-1, Caltag) antibodies. Antibodies were diluted in 2% bovine serum albumin and 2% fetal calf serum in PBS. The tissue sections were then rinsed in PBS and incubated with the appropriate secondary antibodies (goat-anti-rabbit IgG H+L-FITC, affinity purified, Rockland; goat-anti-rat-IgG H+L-TRITC, Chemicon International) and Hoechst 33342

(5 mg/m; SIGMA). Coverslips were mounted in Vectashield (Vector Laboratories), and sections observed with an upright microscope (Zeiss Axioplan 2 imaging), an Axiocam Hrc camera, and Apotome Zeiss and Axiovision 4.2 software. This system provides an optical section view reconstructed from fluorescent samples, using a series of 'grid projection' (or 'structured illumination') acquisitions, and thus allows a *z*-stack series to be produced.

Generation of bone marrow-derived dendritic cells (DCs) and macrophages

Total bone marrow cells from adult C57BL/6 mice were plated at 10^5 per ml and cultured for 6 days in the presence of 6.6 pg/ml of GM-CSF (R&D systems) or 10 pg/ml of M-CSF (R&D Systems) to generate, respectively, DCs and macrophages. Alternatively, bone marrow cells were cultured with in RPMI, 5% FCS, 1% antibiotics, 0.1% β-mercaptoethanol with FSS supernatant or S17 supernatant. After 7 days, DC and macrophage differentiation was analyzed by FACS (F4/80, CD11c, Mac1 antibodies). Cells were used as antigen-presenting cells (APC) to stimulate OT-II T cells, or were stimulated with CpG (Proligo) to produce cytokines. Il10, Il12p40, GM-CSF, M-CSF and Tgfβ1 secretion was measured by a standard sandwich ELISA with appropriate antibody pairs.

T cell proliferation assay

$OVA_{323-336}$ peptide-specific T cells were purified from the lymph nodes and spleen of transgenic OT-II C57BL/6 mice using CD4 or CD90 microbeads (Miltenyi Biotech), following the manufacturer's instructions. OT-II $CD4^+$ (10^4 cells) were cultured with serial dilutions of various cell types as APC in the presence of $OVA_{323-336}$ peptide (pOVA), in RPMI 1640 supplemented with antibiotics, β-2 mercaptoethanol and 5% fetal calf serum for 4 days. Alternatively, 10^4 OT-II cells were cultured with 10^4 bone-marrow derived dendritic cells and pOVA in the presence of supernatants from previous assays, as indicated in the legend. To measure OT-II T cell proliferation, cells were pulsed with [^{3}H]-thymidine (ICN Biomedicals) for the last 6-18 hours of culture, harvested by an automated cell harvester (Skatron) and scintillation counted. Results are expressed as the mean of cpm of duplicates.

RESULTS

Fetal spleen does not maintain HSC activity

It has previously been described that E14.5 FS contains hematopoietic stem cells (Christensen et al., 2004; Godin et al., 1999; Kiel et al., 2005). We performed long-term reconstitution (LTR) experiments using E13 FS cells. Sub-lethally irradiated *Rag*$\gamma c^{-/-}$ recipients were injected with one, two, four, eight and 16 embryo equivalents of FS cells, or with one or two embryo equivalents of FL cells. Fig. 1A shows the myeloid reconstitution in the bone marrow (BM) of recipient mice 6 months after injection. One embryo equivalent of FL cells is sufficient to fully reconstitute the B lymphoid (data not shown) and myeloid compartments (chimerism was higher than 50%; data not shown), whereas a minimum of four embryo equivalents of FS cells is needed for hematopoietic reconstitution and LTR activity. Control dot plots are displayed to demonstrate that the donor-derived cells are phenotypically similar to those in the recipient (see Fig. S1 in the supplementary material). No reconstitution was obtained from only one or two embryo equivalents of FS cells, probably because of the limiting number of HSCs per organ. However, myeloid chimerism increased according to the numbers of fetal splenocytes injected, with values of between 2.5 and 4% for four to 16 embryo equivalents of FS cells. These results show that E13 FS has already been colonized by HSCs, but that each FS contains very few HSCs.

We then tested the capacity of the FS environment to maintain HSC activity. Considering that the FS is colonized as early as E13 by LTR-HSCs (Fig. 1A), we performed a 4-day organ culture of E13 FL and FS explanted from *CD45.2* embryos, during which explants are deprived of any hematopoietic progenitor input. Cell suspensions from the cultured explants (four embryo equivalents per recipient mouse) were tested for their LTR capacity in *CD45.1 Rag*$\gamma c^{-/-}$ recipients (Fig. 1B). BM and spleen were scored 5 to 6 months later for the presence of $CD45.2^+$ donor-derived progeny. B cells and granulocytes could be obtained from mice injected with cells recovered from FLOC, whereas mice injected with cells from FSOC could only provide B cell reconstitution with no donor granulocyte production, in the recipient BM. The absence of myeloid chimerism led us to conclude that mice injected with FSOC cells did not receive any HSCs as a result of the 4-day period of organ culture. It thus appears that the FS, although colonized by LTR-HSCs, cannot maintain LTR activity. To corroborate these results, and remove potential B cell committed precursors, E13 FL and FS (from *CD45.2 H-2*^{b} embryos) were irradiated, and each reconstituted by E10.5 AGM cell suspensions (*CD45.1 H-2*^{b}) obtained from a pool of three embryos. The AGM region at this stage contains roughly 120-150 HSCs (Bertrand et al., 2005a; Godin et al., 1999). The reconstituted organs were cultured in toto for 4 days, before their injection into *Rag*$\gamma c^{-/-}$ (*CD45.1 H-2*^{k}) recipients. Mice injected with FLOC cells were reconstituted by AGM-HSC donor-derived $CD45.1^+H\text{-}2^{b+}$ cells, which contributed to the B cell and myeloid compartments (Fig. 1C). AGM-HSCs co-cultured with FS could not reconstitute *Rag*$\gamma c^{-/-}$ mice. It thus appears that the FS, although colonized by LTR-HSCs, cannot maintain their LTR activity.

To address whether secreted molecules from the FL are able to maintain HSCs, FS and FL explants were reconstituted by the same number of donor HSCs (1000 per explant) and cultured in presence of FL supernatant (see Fig. S2 in the supplementary material). HSCs were sorted from E15 *actin*^{GFP} mice as Lin^- (B220, Gr-1, CD4, CD11c, CD19, Ter-119, F4/80, CD16/32, NK1.1, CD3ϵ) Kit^{hi} $Sca1^{hi}$ and used to reconstitute explants. After 4 days, explants were analyzed for the presence of donor HSCs. Analysis of the resulting progeny was not easy as myeloid progenitors that express different levels of Kit and Sca1 markers also developed in the explants. However, only FL explants display a typical HSC population. In FS explants, the population of HSCs is highly reduced and did not form a clear population per se. Moreover, if FL supernatant was added to the culture, even fewer cells were found. Hence, FL supernatant cannot counteract the effect of FS stroma on HSCs.

FS environment does not support the commitment of multipotent progenitors towards the B cell lineage

We then investigated the hematopoietic fate of HSCs in this organ. We performed an assay of FLOC and FSOC reconstitution, based on similar culture systems, used to study T or B cell development (Ceredig et al., 1998; Jenkinson et al., 1992). We used a congenic system where 250 rad γ-irradiated FL and FS were seeded with AGM-HSCs. AGM cells were collected at E10.5, when the number of multipotent HSCs reaches its maximum (Godin et al., 1999). After reconstitution, organ cultures were maintained for 7-8 days and scored by flow cytometry. Fig. 2 shows representative plots of 10 independent experiments. In FSOC, $CD19^+$ B cells could not be obtained (Fig. 2A) and HSC progeny consisted only of myeloid $Mac1^+$ cells. B cells were readily obtained when FSOC were seeded by E13 FL cell suspension (depleted of $Ter119^+$ erythrocytes) (Fig. 2B), or with E15 FL $CD19^+$ sorted cells (data not shown). As expected, AGM-HSCs can differentiate into B cells in the FL environment (Fig. 2C).

Thus, we conclude that FS stroma is permissive for myeloid commitment, but not for the engagement of HSCs towards the B lineage pathway. The presence of $CD45^+$ cells in E13 FS suggests an early colonization by hematopoietic cells. We thus tested whether

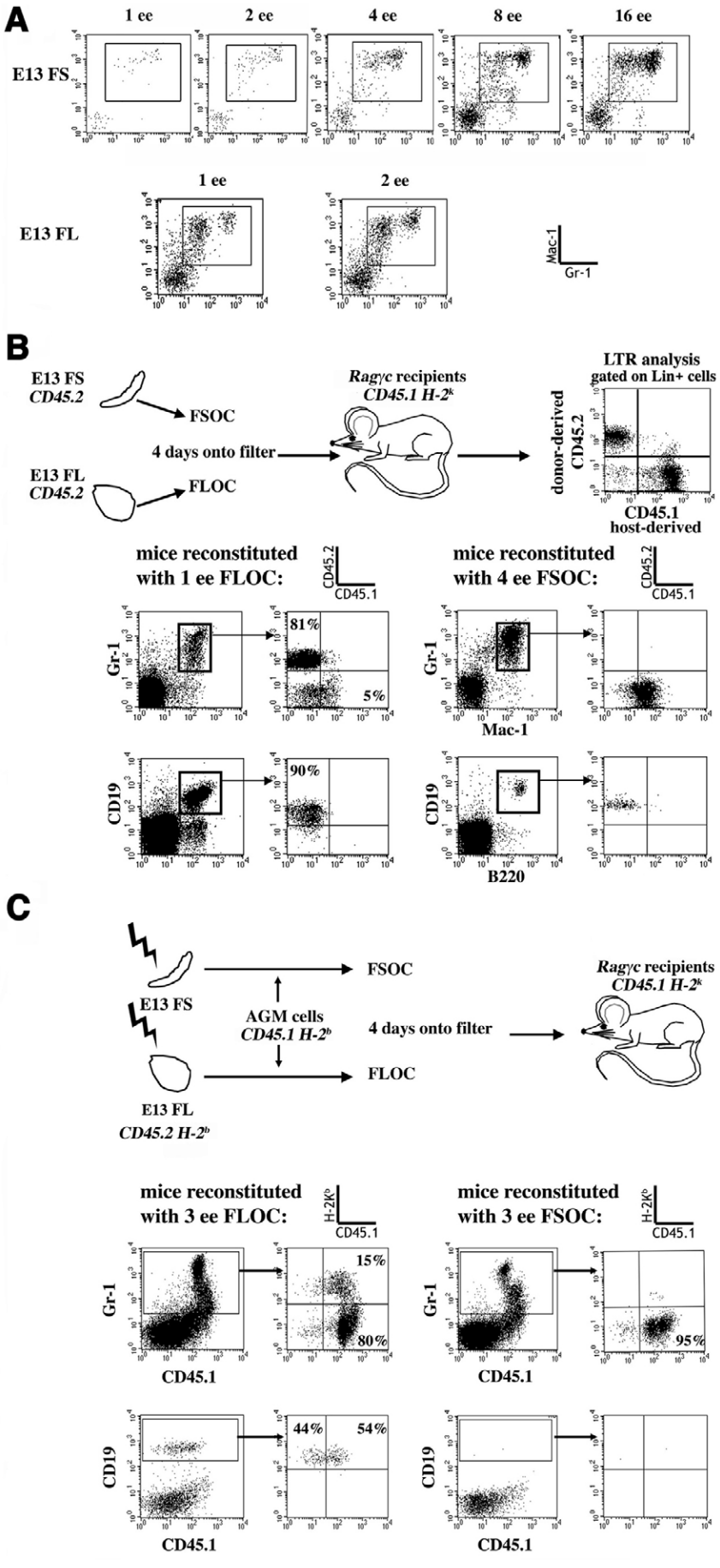

Fig. 1. Colonization and maintenance of LTR-HSCs in the FS. (**A**) Sub-lethally irradiated *Rag*γ*c*$^{-/-}$ mice (*H-2*^{k}) were injected with one or two embryo equivalents (ee) of E13 FL, or with one, two, four, eight or 16 embryo equivalents of E13 FS, explanted from C57BL/6 embryos (*H-2*^{b}). After 6 months, recipient BM was scored for the presence of H-2^{b+} donor-derived cells. Gr-1/Mac1 FACS plots are gated on live donor-derived H-2^{b+} cells to show the contribution of donor cells to the myeloid compartment. (**B**) E13 FL and FS explants (*CD45.2*) were cultured on filter for 4 days. FLOC and FSOC cell suspensions were injected into sub-lethally irradiated *Rag*γ*c*$^{-/-}$ recipients (*CD45.1*). After 5-6 months, recipient BM and spleen were scored for the presence of donor-derived (CD45.2^{+}) B and myeloid cells (*n*=6). (**C**) E13 FL and FS explants (*CD45.2 H-2*^{b}) were irradiated, reconstituted by the same number of E10.5 AGM cells (total of six embryo equivalents of *CD45.1 H-2*^{b}) and, after 4 days, FSOC and FLOC (3/injection) were dissociated and tested for LTR in *Rag*γ*c*$^{-/-}$ (*CD45.1 H-2*^{k}). The BM of recipients was scored for the presence of AGM-HSC-derived B (CD19) and myeloid (granulocytes, Gr-1) cells (*n*=6).

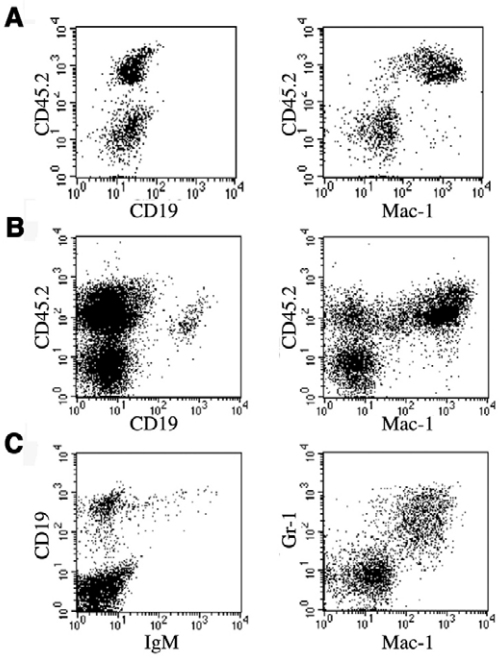

Fig. 2. AGM cell differentiation potential in FL and FS environments. (**A**) Differentiation of AGM-HSCs in the FS environment. CD45.1$^+$ FS explants were seeded with E10.5 CD45.2$^+$ AGM cells. After 8 days on filters, FSOC were scored for the presence of donor-derived B (CD19) and myeloid (Mac1) cells (n=12). Plots are gated on live PI$^-$ cells. (**B**) Differentiation of E13 FL precursors in the FS environment. CD45.1$^+$ FS explants were seeded with E13 FL Ter119-depleted cell suspension (CD45.2$^+$; n=12). Analyses are identical to A. (**C**) Differentiation of AGM-HSCs in the FL environment. CD45.2$^+$ FL explants were seeded with E10.5 CD45.1$^+$ AGM cells (n=12). In this microenvironment, AGM-HSCs could differentiate into CD19$^+$ B cells, a few of them reaching the mature IgM$^+$ stage by the end of the co-culture (7 days only). Plots show cells gated on live donor-derived (CD45.1$^+$ PI$^-$) cells.

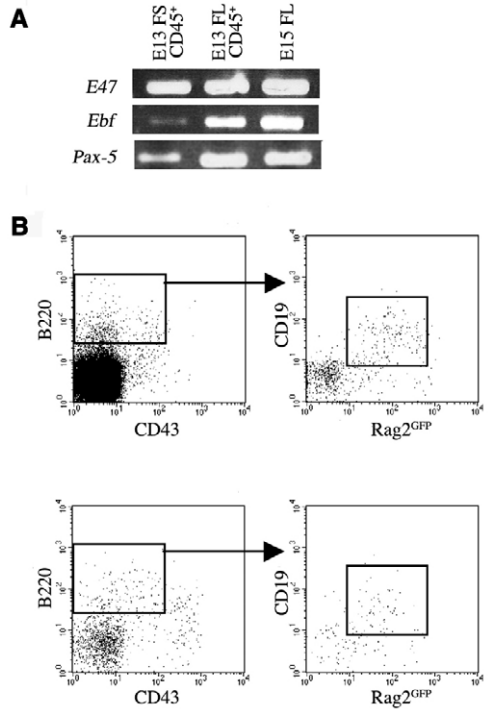

Fig. 3. FS colonization by B committed precursors at E13. (**A**) CD45$^+$ Ter119$^-$ cells from E13.5 FS and FL were sorted and analyzed by RT-PCR for the expression of transcription factors involved in B cell commitment: *E47*, *Ebf* and *Pax5*. E15 FL cells depleted of erythrocytes were used as a positive control. (**B**) FS and FL from E13.5 *Rag2*^{GFP} transgenic embryos were scored by FACS for B220 and CD43. We also obtained B220$^+$ CD43$^+$ B progenitors that express GFP.

committed B cell precursors could already be present among the first colonizing CD45$^+$ cells. The transcripts for *E2A* (*E47*), *Ebf* and *Pax5* were detected in the hematopoietic CD45$^+$ subset of E13.5 FS and FL, as well as in the whole of the E15 FL (Fig. 3A). Moreover, we could find B220$^+$CD43$^+$CD19$^+$GFP$^+$ cells when FS or FL were isolated from E13.5 Rag2^{GFP} transgenic embryos, supporting the hypothesis that committed B cell precursors are present in the first wave of FS colonizing progenitors (Fig. 3B). These colonizing progenitors are likely to be derived from the FL.

FS microenvironment drives macrophage commitment

We have shown that HSCs cannot differentiate into B cells when transferred in the FS microenvironment, but only give rise to a myeloid Mac1$^+$ progeny. Not enough cells are recovered from the FSOC reconstitution to perform a complete characterization of the cells produced. Thus, we derived 10 FSS lines to investigate HSC progeny. These cell lines were all clonally derived. They have a fibroblastic morphology and express surface markers (Sca1, Icam, CD90) and cytokines (Il7, Flt3 ligand, Kit ligand, Sdf1) usually expressed by stromal lines supporting hematopoiesis, such as OP9 or S17 (bone marrow-derived cell lines). The cultures were redundantly performed using three different FSS lines and could be considered as being independent triplicates. Cultures were performed in complete medium, without adding exogenous cytokines on FSS, closer representing the FS environment (data not shown).

E14 FL-HSCs cultured on FSS lines for 7-10 days could differentiate into CD45^{lo} F4/80$^-$ and CD45$^+$ F4/80$^+$ cells (Fig. 4A). Similar results were obtained when HSCs were derived from E10 AGM or from sorted adult bone marrow (data not shown). To establish a possible lineage relationship between these two subsets, we sorted CD45^{lo} F4/80$^-$ cells. After 7 days of culture, CD45$^+$ F4/80$^+$ cells were recovered, suggesting that the CD45^{lo} subset contains the precursors of F4/80$^+$ cells (Fig. 4B) and that CD45^{lo} F4/80^{int} cells are the transitional stage between CD45^{lo} F4/80$^-$ cells and F4/80^{hi} macrophages. Moreover, monocyte transcripts are expressed in these two populations (Fig. 4C). Both subsets express *PU.1*, *c/Ebpα* and myeloperoxidase (*Mpo*) transcripts, which are usually present during monocyte/macrophage development. Transcripts for toll-like receptors (*Tlr*) 4 and 9 and *M-csfr* were only detected in the F4/80$^+$ subset. No *Pax5* transcript could be detected, concordant with the absence of B cells in the HSC progeny cultured on FSS lines (data not shown). To compare FS F4/80$^+$ cells with other myeloid populations, macrophages from the peritoneal cavity were added as positive control. As expected, transcripts were observed for all genes tested, except *Mpo*, which is usually downregulated in mature macrophages.

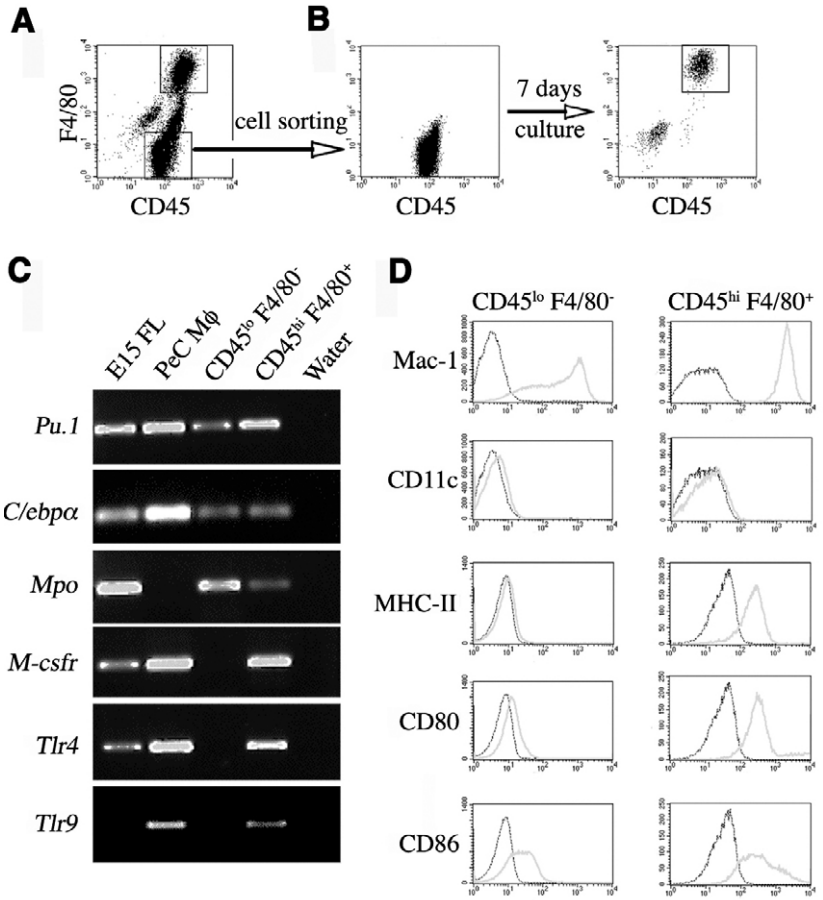

Fig. 4. Fetal spleen stroma drives HSCs to the sole macrophage lineage. (**A**) HSCs were sorted from E14 FL and cultured for 8-10 days onto three different FSS lines (n=5). (**B**) To understand the lineage relationship between these two cell subsets, sorted CD45^{lo} F4/80^{-} cells were cultured for 7 days on FSS cells. Only CD45^{+} F4/80^{+} macrophages were obtained. Both F4/80^{-} and F4/80^{+} populations were sorted and scored for the expression of myeloid/macrophage-related genes by RT-PCR. (**C**) For the RT-PCR, we used as positive control, two populations: E15 FL cells and macrophages from the peritoneal cavity (PeC). (**D**) Analysis of cell surface proteins by cytometry showing that macrophages are highly auto-fluorescent. The expression of indicated markers (gray line) was compared to a negative control (dashed line) that corresponds to the same unstained population (respectively, CD45^{lo} F4/80^{-} and CD45^{+} F4/80^{+}).

A phenotypic comparison between F4/80^{+} and F4/80^{-} subsets was done by FACS analysis (Fig. 4D). Both populations expressed Mac1 and the activation marker CD86, whereas they were negative for CD11c. Only the F4/80^{+} population expressed MHC class II and the co-stimulator CD80. Contrary to its precursors, the F4/80^{+} subset was also capable of latex bead phagocytosis and could produce Il10 and Il12p40 (data not shown). In contrast to BM-derived DCs, F4/80^{+} cells secreted high amounts of Il10 (600 pg/ml) and low amounts of Il12p40 (900 pg/ml) upon CpG stimulation, as measured by ELISA (data not shown). Thus, we conclude that HSCs that colonize the FS only differentiate towards a population of mature macrophages.

The development of macrophage populations during embryogenesis was analyzed on FS from *Rag2*^{GFP} embryos. In these animals, GFP marks the lymphoid precursors (Yu et al., 1999). We first checked for the presence of Mac1^{+} F4/80^{+} macrophages in the E15 FS and determined that they represent 40% of the total splenocytes (Fig. 5A). The phenotype of macrophages evolves during development. Although most cells are F4/80^{+}Mac1^{+} during embryogenesis, a subset of F4/80^{+} Mac1^{-} cells appear around birth to become the main subset after the first week of age (Fig. 5A). Immunohistochemistry performed on splenic sections from E15.5 *Rag2*^{GFP} embryos show that the majority of the nucleated cells express F4/80. The abundant macrophage population is not organized into recognizable structures and appears evenly distributed throughout the sections. They are frequently found around the region where erythroblast enucleation can be detected. Interspersed, rare GFP-marked cells indicate the presence of committed lymphoid progenitors (Fig. 5B). This result shows that E15.5 FS is largely composed of monocytes/macrophages that appear homogeneously distributed throughout this organ.

Macrophage differentiation is driven by a signal secreted by the FS stroma

HSCs were then cultured by replacing FSS supportive stromal layers with their supernatants. Mac1^{+} F4/80^{+} progeny were also obtained when HSCs were cultured using FSS supernatant, indicating that the stromal signal driving myeloid differentiation towards macrophages is secreted.

No transcripts could be amplified for *G-csf* by RT-PCR on FSS lines. Moreover, the production of M-CSF and GM-CSF by FSS lines was examined at the protein level by ELISA (data not shown). Only M-CSF (6 ng/ml) is secreted by FSS lines, and the transcript was expressed in the whole of the CD45^{-} FS stroma (data not shown). We compared the proliferation and differentiation capacities of the BM hematopoietic progenitors in various conditions: FSS or S17 supernatant or media complemented with GM-CSF and M-CSF alone, or in combination (Fig. 6). The expansion of BM progenitors was more efficient in FSS supernatant than in S17 supernatant (3-fold less efficient) or the mix of cytokines (7-fold less efficient). As expected, bone marrow cells were mainly driven towards the dendritic cell (DC) fate in the presence of GM-CSF, where CD11c^{+} cells represent 80% of the progeny. In M-CSF media, mostly macrophages were obtained, as 91% of the progeny were Mac1^{+} cells. Both macrophages (57%) and DCs (7%) could be obtained when total BM cells were cultured with FSS supernatant. Thus, FSS

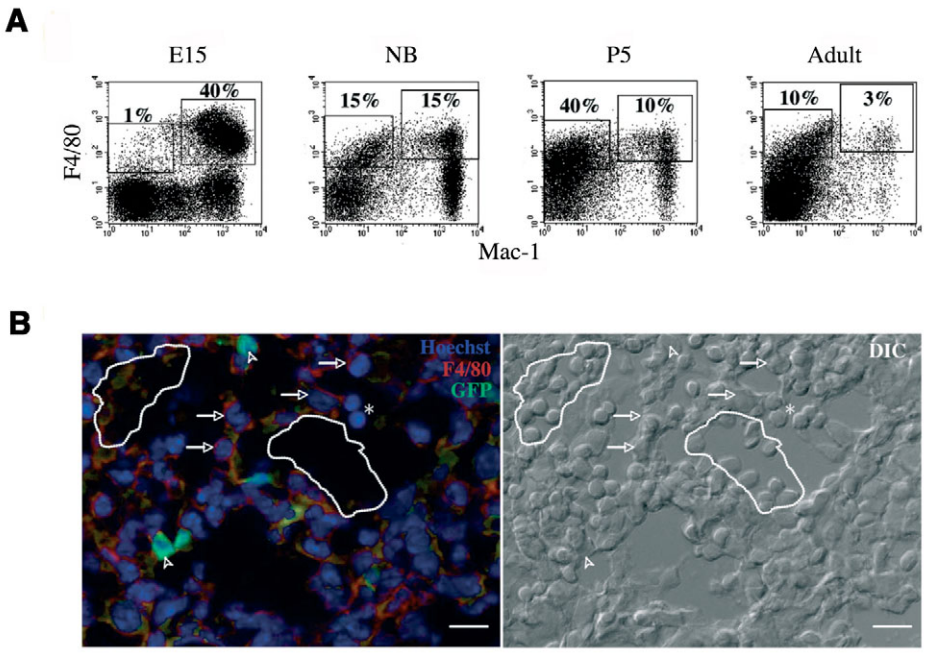

Fig. 5. Ontogeny of macrophage populations in the spleen. (**A**) CD45$^+$ FS cells were gated and then the presence of macrophages was scored at different time-points [E15, newborn (NB), 5 days after birth (P5) and adulthood], by the expression of F4/80 and Mac1 molecules. At E15, macrophages account for half of the FS cell suspensions. (**B**) E15.5 FS (explanted from *Rag2*^{GFP} embryos) was analyzed by immunohistochemistry for the presence and localization of F4/80 macrophages (red, arrows). Sections were observed with a Zeiss Axioplan 2 imaging microscope and the section shown is representative of many independent FS sections. We could determine no organization between macrophages (red), lymphoid progenitors (green, arrowheads) and erythrocytes – anucleated (delineated zones) or nucleated (asterisks) – although these latter seem to be in very close contact with macrophage. Scale bar: 10 μm.

supernatant allows DC differentiation to the same extent as GM/M-CSF. However, the high number of macrophages (8×10^6) obtained with FSS supernatant is suggestive of a factor combination driving the expansion of macrophage lineage precursors more efficiently than does M/GM-CSF.

The FS is an anti-inflammatory environment

The co-expression of MHC class II and the activation molecules CD80/86 in the F4/80$^+$ subset of macrophages prompted us to test their antigen-presenting capacities. We tested the capacity of FSS derived F4/80$^+$ cells to induce the proliferation of OT-II transgenic T cells. As expected, DCs derived from BM cultured with GM-CSF (BM-DCs) could activate OT-II cells, whereas low or no activation was detected when OT-II cells were co-cultured with M-CSF derived-macrophages, FSS-derived F4/80$^+$ cells or FSS cells (Fig. 7A). Thus, the F4/80$^+$ progeny of HSCs cultured in the FS microenvironment cannot activate T cells through MHC-TcR interactions. The supernatant from the culture reactions were collected and used on fresh DC/OT-II cultures. Fig. 7B shows that, although the supernatant from macrophages (BM or FSS derived) did not affect T cell proliferation, the supernatant from stromal FSS strongly inhibited DC-induced T cell proliferation. These results suggest that the supernatant from FSS cells contains an inhibitor of T lymphocyte proliferation and that spleen stroma can thus have a role in the control of peripheral T cell numbers.

To identify this inhibitor, we tested FSS supernatant for the presence of the anti-inflammatory cytokines Il10 and Tgfβ1 by ELISA (see Figs S3, S4 in the supplementary material). No Il10 was detected under these conditions. FSS stromal cells consistently produced Tgfβ1. To confirm the production of Tgfβ1 in the FS, we performed RT-PCR in mRNA from E15 FS. Tgfβ1 transcripts were readily detectable (data not shown). Experiments of DC-induced T cell proliferation were repeated in the presence of FSS lines or FSS supernatant using a neutralizing Tgfβ1 antibody. Th1 cell priming was tested by the detection of Ifnγ production (see Fig. S4B in the supplementary material). These results indicate that most of the T cell inhibition is due to Tgfβ1 production. In consequence, the inhibitory properties of the stroma could be partially due to the presence of this anti-inflammatory cytokine.

DISCUSSION

Whereas the FL is the major hematopoietic site during embryonic development, the spleen has hematopoietic activity from E13 to the second week after birth. However, ongoing hematopoiesis in the FS has been poorly documented. Previous studies have shown that E14-E15 FS contains HSCs and multipotent cells (Godin et al., 1999). We show here that FS is colonized by a low number of LTR-HSCs as early as E13. We used methods previously shown to promote the expansion of AGM-HSCs to amplify the number of HSCs in the FS. However, no LTR activity could be recovered from FSOC. It could be argued that the reconstitution assays using FSOC were not robust as so few HSCs are present in four embryo equivalents of FS when compared with one embryo equivalent of FL. To test our hypothesis that FS stroma cannot support HSCs, we tested the reconstitution of mice using FS and FL that were initially seeded by the same number of HSCs. No myeloid reconstitution was detected from HSCs that have been cultured in the FS environment, in accordance with our previous 4-day FSOC reconstitution results. To investigate the potential role of FL stroma-secreted molecules in maintaining HSC activity, we reconstituted E15 FS and FL explants with 1000 HSCs from E15 *Actin*^{GFP} FL. Explants were then cultured in toto in presence of FL supernatant or complete medium. The absence of an HSC population from FS explants compared with FL suggests that secreted molecules alone are not sufficient to maintain an HSC population in FS. Hence, either cell-cell contact is necessary to maintain HSC activity and it does not exist in the FS environment, or the signal provided by the FS microenvironment to induce myeloid differentation is too strong when compared with the FL-secreted signal.

Thus, in contrast to the FL, the FS stromal microenvironment is not capable of maintaining HSC fate. The LTR capacity previously detected in FS from E14 until birth (Christensen et al., 2004) is likely to originate from a constant seeding of HSCs

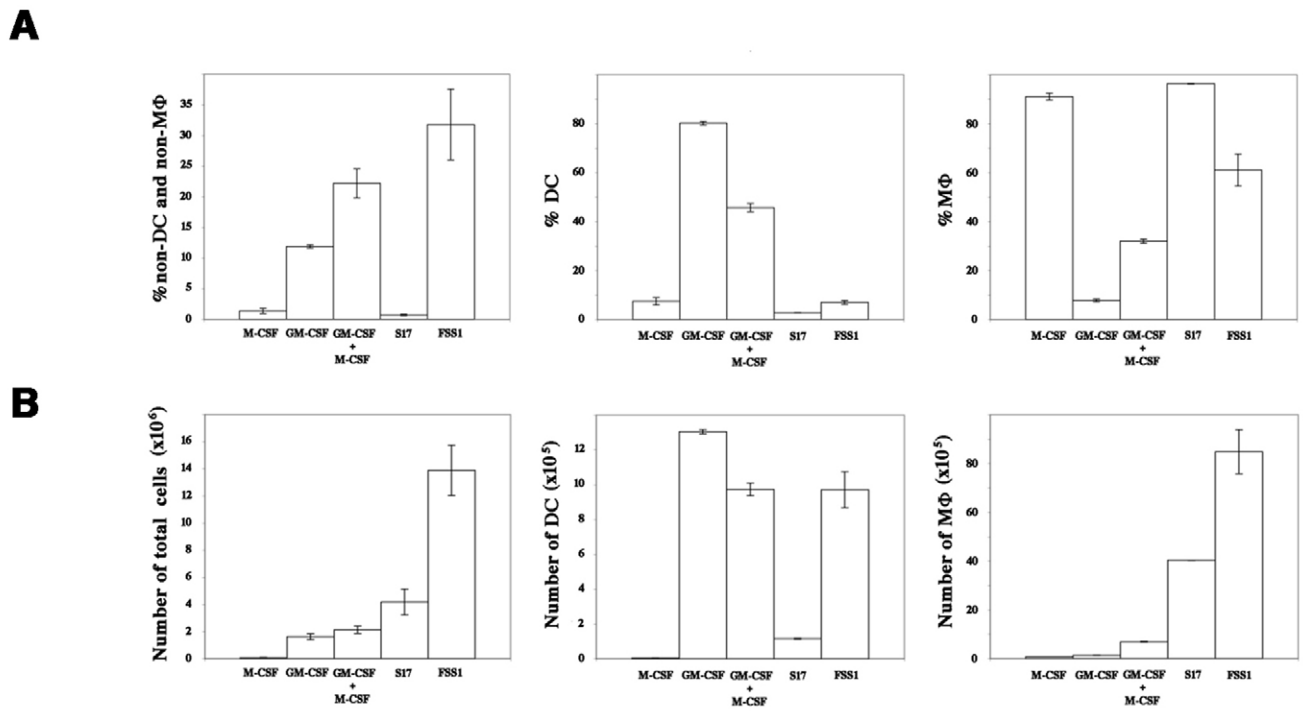

Fig. 6. Bone marrow precursor proliferation is highly enhanced by FSS supernatant. Total bone marrow cells (10^6) were cultured for 7 days with supernatant from either fetal spleen stromal cell lines (FSS) or a bone marrow stromal cell line (S17), or with recombinant growth factors, alone or mixed, as indicated. (**A**) Cell differentiation was analyzed by FACS for macrophages ($CD11c^-$ $Mac1^+$ $F4/80^+$ or $F4/80^-$) and DCs ($CD11c^+$); the percentage of each population is indicated. (**B**) Proliferation of total bone marrow precursors was evaluated for each condition by counting the absolute number of live cells (Trypan blue exclusion). To evaluate whether FSS supernatant selectively acts on the proliferation and differentiation of particular precursors, the number of each cell type obtained was determined. The percentage of macrophages and DCs in the various conditions of culture (obtained by FACS analysis in A) was multiplied by the absolute number of live cells.

through the circulation (Kiel et al., 2005). Our results suggest that the FS stromal environment differs from that of other hematopoietic organs, either by inducing commitment towards a given lineage or by lacking the signals responsible for the maintenance of LTR activity.

B cell precursors can be detected from both E13 and E15 FS explants (Godin et al., 1999), although no HSC-derived B cell progeny is obtained after FSOC reconstitution or co-culture with the splenic-derived stromal FSS lines. It thus seems that HSCs are unable to commit and engage in this developmental pathway. However, experimental evidence supports the notion that the FS environment is permissive for B cell differentiation: all signals necessary for B precursor survival and proliferation (such as Il7, and Sdf1) (Nagasawa et al., 1996; von Freeden-Jeffry et al., 1995; Zou et al., 1998) have been detected in FS stroma, and when FSOC are seeded with sorted $CD19^+$ cells, a mature B cell (IgM^+IgD^+) progeny is obtained (data not shown). Moreover, we show, by RT-PCR, that hematopoietic cells ($CD45^+Ter119^-$) present in the FS and the FL at E13 already express *E2A*, *Ebf* and *Pax5* transcripts. Furthermore, we detect, from FS of E13 transgenic *Rag2*^{GFP} embryos, a pro-B ($B220^+CD43^+$) cell fraction expressing GFP. In reconstitution assays where donor B cell production is observed from either 4-day FSOC injection or from HSCs colonizing 4-day FSOC injection, only the former was able to generate B lymphocytes. Together, these results are consistent with the conclusion that B cell development in the FS occurs from committed FL-derived B-cell precursors that reach the FS through the circulation (Delassus and Cumano, 1996). The observation that FS stroma is not capable of delivering the required signals for B cell commitment reinforces our previous conclusion that the stromal compartment in this organ has unique properties.

FSOC colonized by HSCs generated only $Mac1^+$ myeloid cells and similar results were obtained when HSCs were isolated from AGM, FL or adult BM. RT-PCR analysis and cell-sorting experiments confirmed that $F4/80^+$ and $F4/80^-$ HSC-derived cells correspond to two stages of macrophage differentiation. F4/80 is a specific marker for murine macrophages and its expression is modulated during development (Austyn and Gordon, 1981; Gordon et al., 1992). Transcripts for *PU.1*, *c/Ebpα* and *Mpo* were present in both subsets, whereas those for *M-csfr*, *Tlr4* and *Tlr9* were found only in the $F4/80^{hi}$ population, confirming their later stage of maturation. We conclude that the FS is a hematopoietic organ specialized in macrophage differentiation.

The existence of a secreted signal that favors macrophage development is suggested from FSS supernatant cultures, the nature of which is yet undefined. Myeloid lineage development depends upon the availability of cytokines, and members of the colony-stimulating factor (CSF) family seem to be important (for a review, see Barreda et al., 2004). Only M-CSF is secreted by FSS, whereas GM-CSF and G-CSF are absent. The total number of cells recovered after culture is considerably increased in FSS medium. In conclusion, FSS supernatant contains growth factors, leading to the overproliferation of myeloid precursors, and M-CSF that might directs them towards the macrophage lineage. We are currently interested in determining whether other factors also drive macrophage development in the FS.

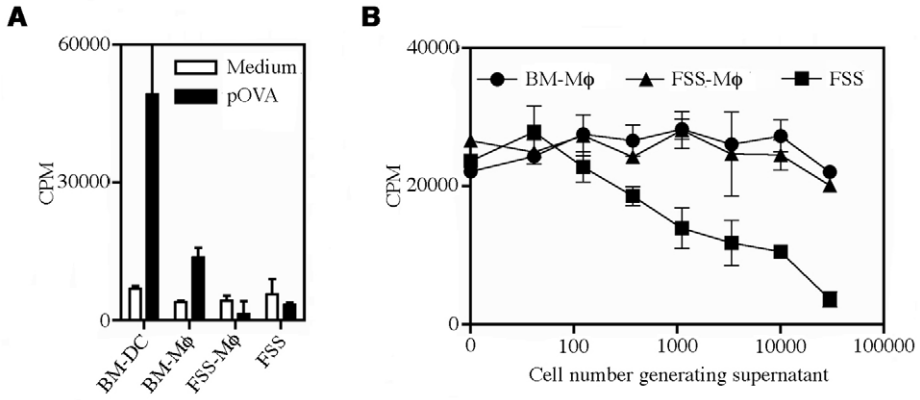

Fig. 7. FSS supernatant is a strong inhibitor of T cell proliferation. (**A**) T cell proliferation assays were carried out using 10^4 OT-II CD4$^+$ T cells stimulated with 10^4 of different putative APCs (BM derived macrophages and DCs, FSS derived macrophages and FSS) in the absence (white bars) or presence (black bars) of 1 μg/ml of $OVA_{323-336}$ peptide (pOVA) for 4 days. (**B**) Supernatants from serially diluted putative APC (10 to 10^5 cells), from the experiments shown in A, were added to a new proliferation assay performed with 10^4 OT-II CD4$^+$ T cells stimulated with 10^4 BM-derived DCs. T cell proliferation was measured by [^{3}H]-thymidine incorporation for a period of 18 hours and is expressed as the mean of cpm of duplicates.

Lymphoid commitment is inhibited from HSCs placed in FSOC, on FSS lines or with their supernatants. A recent study has shown that the differences in hematopoiesis between the FL and FS could not be due to intrinsic differences of spatially distinct HSCs, as they exhibit identical phenotype, function and gene expression (Kiel et al., 2005). Thus, two non-exclusive possibilities to explain the restricted HSC lineage differentiation in FS can be considered. The FS stroma might deliver either a signal blocking the lymphoid fate or an instructive signal for macrophage development, consequently inhibiting the other alternative fates. Mac1$^+$ progeny expresses both *PU.1* and *c/Ebpα* transcripts. It was shown that sustained high-level expression of PU.1 drives myeloid differentiation and favors macrophage development (McIvor et al., 2003). We are currently investigating the role of the FS stroma in the upregulation and maintenance of PU.1 expression levels.

During embryogenesis, the FS anlagen is colonized by macrophages at E12 (Morris et al., 1991), which likely originate in the yolk sac (Bertrand et al., 2005b). From E15 to birth, the phenotype of the macrophage population in the FS evolves from Mac1$^+$F4/80$^+$ to Mac1$^-$F4/80$^+$ cells. F4/80$^+$Mac1$^-$ macrophages are known to be M-CSF dependent and are mostly localized in the red pulp in the adult spleen (Stanley, 2000). They are considered important in organogenesis but not primordial for immune responses (Cecchini et al., 1994; Pollard and Stanley, 1996). Immunofluorescence assays have demonstrated that, in E15.5 spleen, F4/80$^+$ cells are dispersed throughout the organ and represent the major splenic population, as lymphocytes are rare. This high density of F4/80$^+$ macrophages is conserved in the newborn spleen, where small regions of white pulp just become discernible (Morris et al., 1991). FS macrophages are adjacent to clusters of nucleated erythroid precursors. Fetal F4/80$^+$ macrophages could be involved in embryonic erythropoiesis, such as in the adult red pulp spleen (Sadahira et al., 2000). Phagocytic capacities of FS macrophages were demonstrated in vitro by the ability of FSS-derived macrophages to incorporate fluorescent latex beads.

It has been shown that stromal cells isolated from neonatal or adult spleen can induce the maturation of BM-derived DCs into 'regulatory' DCs (expressing low levels of CD11c) that control the proliferation and activation of naïve T cells (Svensson et al., 2004; Zhang et al., 2004). In our experiments, no CD11c$^+$ or CD11c^{lo} cells were obtained, although FS macrophages might possess anti-inflammatory capacities, as they secrete higher levels of Il10 than do BM macrophages. This difference could reflect the particular properties of fetal and adult splenic stroma, even if the inducing anti-inflammatory characteristic seems conserved.

FS macrophages harbor a profile of activated mature APCs showing expression of MHC class II and co-stimulatory molecules. However, no T cell proliferation was obtained when they were tested for their ability to activate T cells in MLR assays, in reference to early studies which showed that macrophages are poor stimulators of T cells in vitro (Banchereau and Steinman, 1998; Steinman and Cohn, 1973). Fetal and neonatal splenic stroma secrete high amounts of Tgfβ (Svensson et al., 2004; Zhang et al., 2004), suggesting that the splenic stroma (fetal or adult) represents a non-favorable environment for lymphocyte expansion in non-immunogenic conditions. The effect of Il10 and Tgfβ in regulating T cell activity is well documented (for a review, see Coombes et al., 2005) and may control the spleen lymphoid compartment in the newborn. Thus, FS macrophages in association with the stroma could control the proliferation of newly generated lymphoid cells, avoiding oligoclonal expansion. A newborn spleen is devoid of T lymphocytes, a situation similar to that of lymphopenic mice. Thus, in contrast to the proliferation observed after T cell transfer in adult lymphopenic mice, the FS does not provide the conditions to sustain T cell expansion in the neonatal period (for a review, see Almeida et al., 2005). The combined action of stromal cells with the F4/80$^+$ macrophage population may also be important in the construction of the red pulp by inhibiting lymphocyte accumulation/proliferation while enhancing the myeloid cell development.

To conclude, we show that the FS is colonized early in ontogeny, probably by FL-derived HSCs. These HSCs are highly restricted in their fate outcomes by the FS microenvironment, generating only macrophages, while lymphoid commitment is inhibited. Macrophages and stroma in the fetal spleen have anti-inflammatory activities with a possible role in the control of lymphocyte homeostasis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/18/3619/DC1

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