Hematopoietic Supportive Functions of Mouse Bone Marrow and Fetal Liver Microenvironment: Dissection of Granulocyte, B-Lymphocyte, and Hematopoietic Progenitor Support at the Stroma Cell Clone Level

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We dissected the functions of the microenvironment of bone marrow (BM) and fetal liver (FL) at the cellular level by cloning individual stromal cells and characterizing their phenotypical and functional features. Stromal cell clones derived from FL are large in size (mean forward light scatter intensity (mFSC) of 450), express the surface antigen Thy-1 but not Sca-1 and 6 out of 6 are able to differentiate into fat accumulating adipocytes. BM derived stromal cell clones are either small (mFSC of 250) or large (mFSC of 450), express Sca-1 but not Thy-1 and only 2 out of 7 differentiate towards adipocytes. Heterogeneity in terms of vascular adhesion molecule-1, intracellular adhesion molecule-1 and heat stable antigen expression was found among the different cell clones. Functional assays using long- and short-term cocultures of stromal and hematopoietic cells revealed: (1) the capacity of 8 out of 12 stromal cell clones to support the expansion of primitive hematopoietic progenitors (colony forming unit spleen day 12) more than 10 weeks. Fat accumulation but not expression of stem cell factor by stromal cells did correlate with this supportive function. (2) Better support of granulocyte maturation and proliferation by BM compared to FL-derived stromal cell clones. However, stromal cell clones from both organs expressed macrophage-colony stimulating factor. (3) The ability of 4 out of 12 stromal cell clones (derived from both, FL and BM) to support the expansion of interleukin-7 dependent pre-B cells from the BM. Pre-B cell growth stimulating factor was not restricted to support B-cell or granulocyte expansion. Experiments comparing the support of BM- and FL-derived hematopoietic progenitors showed identical responses of late (B220+/c-kit+) but strikingly different responses of early (B220+/c-kit+) pre-B cells, revealing different proliferation requirements for FL- versus BM-derived early pre-B cells in vitro.

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MATURÉ hematopoietic cells are continuously replenished via differentiation and proliferation from a small number of undifferentiated, pluripotent hematopoietic stem cells (HSC). Commitment, proliferation, and differentiation of HSC takes place simultaneously and within the same hematopoietic organ, ie, the yolk sack and the liver during embryonic development and the bone marrow (BM) in adulthood. A supportive microenvironment consisting of stromal cells is essential for the generation of hematopoietic cells. Using long-term BM cultures (LTBMC) as model systems, mechanisms have been studied and molecules identified, which regulate hematopoietic cell proliferation and differentiation. Antibodies against the adhesion molecules phagocytic glycoprotein-1 (Pgp-1, CD44), vascular cell adhesion molecule-1 (VCAM-1, CD106), very late antigen-4 (VLA-4, CD49d), VLA-5 (CD49e) and the stem cell factor (SCF) receptor c-kit have been shown to impair severely in vitro hematopoiesis. Different supportive functions of stromal cells concerning the self-renewal, expansion, and differentiation of hematopoietic cells have been suggested. However, it has not been possible so far to correlate these functions with particular cell types of the microenvironment, which consists mainly of macrophages, endothelial cells, fibroblasts, and adipocytes. Because of different proliferation rates of particular stromal cell types in vitro, heterogeneous stromal cell cultures bear the risk of misrepresentation of certain cell types and might be biased in terms of their function. Hence, the establishment of stromal cell clones has gained importance in the dissection of microenvironmental functions. Limited knowledge about stromal cell growth requirements has probably biased the establishment of stromal cell clones in terms of cell types that were cloned so far.

Cloning efficiencies for stromal cells are low, retroviral transfection with oncogenes in vitro is limited by low transfection efficiencies and the establishment of stromal cell lines from primary cell cultures relies on the rare event of spontaneous transformation. To overcome these limitations and to generate a wide array of stromal cell clones that represent the microenvironment in both fetal liver (FL) and BM, we used a temperature sensitive SV40 large T antigen (TAg) transgenic mouse system. The transgene (tsAS8) is expressed under the control of the mouse major histocompatibility complex H-2Kb promoter and can be induced in vitro by interferons (IFN). The establishment of immortalized cell lines does not require transfection or retroviral-mediated oncogene insertion and, in addition, all cells have the same transgene integration site.

We generated a large number of conditionally immortalized stromal cell clones and analyzed them under identical conditions. Here we describe their different morphologies, different surface phenotypes, their cytokine expression and different hematopoietic supportive functions. We found preferential support of either myeloid or lymphoid differentiation from progenitor cells by individual stromal cell clones. Best myeloid support was found among BM compared to FL.
derived stromal cell clones. Our stromal cell clones also revealed different requirements for interleukin-7 (IL-7) dependent expansion of BM- versus FL-derived pre-B cells. Eight out of 13 stromal cell clones were able to support the maintenance and expansion of primitive hematopoietic progenitors over a period of 10 weeks as assessed by colony forming units-spleen day 12 (CFU-S12).26

MATERIALS AND METHODS

Animals. Mice transgenic for a temperature sensitive SV40 Tag (H-2K-ts A58)27 and (C57BL/6 x DBA/2)F1 mice (BDF1) were purchased from Charles River Laboratories (Wilmington, MA). Mating were performed in our virus antigen-free mouse facility. Embryonic age was determined by defining the day of vaginal plug detection as day 0.5 of gestation. Lethal irradiation of animals was performed in a Marc II irradiator with a 137Cs radiation source (J.L. Shepherd, San Fernando, CA). Two doses of 680 cGy and 620 cGy were administered with a 3-hour interval.

Heterogeneous stromal cell cultures. Femurs and tibiae from a 15-week-old female H-2K-ts A58 mice were flushed with a 25 μg/mL of the overgrowth of fibroblasts, but not miss them in the cloning procedure, duplicate cultures were set up, one of them containing D-valine, in GIBCO-BRL instead of L-valine. D-valine inhibits the overgrowth of fibroblasts.24

Cloning and expansion of stromal cells. Confluent heterogeneous stromal cell cultures were treated with 0.25% Trypsin, 1 mmol/ L EDTA (GIBCO-BRL) for 5 minutes and suspended in ice cold IMDM, 10% FCS. After being washed in phosphate buffered saline (PBS), cells were resuspended in culture medium with 20 U/mL IFN-γ (Genentech, San Francisco, CA). Cells were cultured in a humidified incubator at 5% CO2 and 33°C, the permissive temperature for the TAg. After 2 weeks confluent cultures were treated three times for 72 hours with 5 mg/mL mycophenolic acid (mpa) with 72 hours of recovery after each treatment. Mpa preferentially kills rapidly dividing cells, leaving behind a culture of stromal cells depleted from hematopoietic cells.27 FL were taken from embryos at 12.5, 14.5, and 16.5 days of gestation. Single cell suspensions were prepared by mincing the organs through a 70 μm nylon strainer (Becton Dickinson, Franklin Lakes, NJ). Culture conditions and mpa treatment were the same as for the BM stromal cell preparation. To avoid the overgrowth of fibroblasts, but not miss them in the cloning procedure, duplicate cultures were set up, one of them containing D-valine (GIBCO-BRL) instead of L-valine. D-valine inhibits the overgrowth of fibroblasts.24

Transmission electron microscopy (TEM). Stromal cell cultures were washed briefly with PBS and then fixed with 2.0% formaldehyde (Fisher Scientific, Fair Lawn, NJ, 2.5% glutaraldehyde (Fisher Scientific) in 1.0% picric acid (Sigma) 0.1 mol/L cacodylate pH 7.6. Specimens were postfixed in 1% osmium tetroxide, which was followed by staining with 1% uranyl acetate in 0.1 mol/L maleate buffer pH 5.2. After dehydration cell layers on the plastic were cut with a razor blade into 5 to 10 mm2 squares, transferred to a 1.5 mL microcentrifuge tube, centrifuged at 13,000 rpm for 20 minutes, and embedded in epon-araldite. This procedure positioned the cell layer for sectioning so that the plane of section was perpendicular to the substrata. Sections were viewed on a JEOL 1,200 electron microscope (JEOL USA, Peabody, MA).

Proliferation and differentiation of stromal cell clones. Proliferation and differentiation of stromal cell clones were performed under a variety of culture conditions. To remove IFNγ from cell cultures cells were trypsinized, washed three times in PBS, and then resuspended in the new culture medium. For differentiation, cells were transferred to Dextran type culture medium containing 12.5% FCS (JRH Biosciences), 12.5% horse serum (HyClone, Logan, UT), 5 × 10−5 mmol/L hydrocortisone (Sigma), but no 2-ME and no IFNg and maintained at 33°C incubation temperature.

Preparation of hematopoietic cells and their progenitors. To enrich BM cells for hematopoietic progenitors, 150 mg/kg body weight of the metabolically acting cytostatic 5-fluorouracil (5-FU; SoloPak, Franklin Park, IL) were injected into the tail vein of 10- to 12-week-old female BDF1 mice. Four days post 5-FU injection BM was drastically reduced in mature cells (5% of normal cell count in the BM of treated mice) and cycling late progenitors (30% reduction in CFU-granulocyte macrophage (CFU-GM) in the BM of treated mice), but enriched for earlier progenitors (threefold for CFU-S12 and 15-fold for narrow repopulating units [MRU]) which themselves are induced into cell cycle by this single dose of 5-FU.4,30 Four days after 5-FU injection femurs and tibiae were flushed and the cells suspended in culture medium. To assess the actual effect of 5-FU on the hematopoietic compartment, BM cells from each animal were counted. Only cells from animals with less than 1.5 × 106 cells/femur were used. We assessed the content of 4 days post 5-FU BM as 1 CFU-S12/105 cells. Total hematopoietic cells from BM and FL were obtained from untreated animals as previously described.

Coculture of stromal and hematopoietic cells. One day before the start of coculture individual stromal cell clones were seeded at 5 × 105 cells/cm2 on tissue culture plates (Becton Dickinson, Lincoln Park, NJ) previously treated with 0.1% gelatin (DIFCO, Detroit, MI). A stromal cell clone mixture was generated by mixing the 12 stromal cell clones that had been analyzed individually and seeding a total of 5 × 105 cells/cm2. The different doubling times of the cultures were taken into consideration, mixing increased numbers of slowly dividing cells with fewer fast dividing cells. 105 hematopoietic progenitor cells were added for short-term (3 weeks) and 5 × 105 (an equivalent of 500 CFU-S12) for long-term (10 weeks) cocultures. Culture conditions were either 37°C and culture medium containing 5% FCS (Whittlock-Nitte type (WnV)) or 33°C and culture medium containing 12% FCS and 12% horse serum (Dextran type) (HyClone Laboratories). Cultures at 37°C were also performed in the presence of IL-7, adding 5% JmIL-7.2 conditioned medium (JmIL-7.2 was kindly provided by Dr A. Rolink, Basel Institute of Immunologie, Basel, Switzerland). No IFNg was added to any of the cocultures. For flow cytometric analyses the cocultures were trypsinized, the cells counted and 90% used for analysis. Ten percent were further cocultured on a freshly established stromal layer. Long-term cultures at 33°C were trypsinized every 2 weeks, 70% to 90% of the cells used for CFU-S12 analysis and 10% to 30% of the cells returned to the original plate to avoid overgrowth of stromal cells. The removal of 70% to 90% of hematopoietic cells was taken into consideration for the determination of total CFU-S12 in culture.

Flow cytometry. Myeloid and B-lymphoid cells from cocultures were detected by their coexpression of GR-1/Mac-1 (CD11b) and B220 (CD45R) or B220/IlgM (CD117), respectively, using fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (MoAb) from PharMingen (San Diego, CA). Surface expression of intercellular adhesion molecule-1 (ICAM-1, CD54), Pgp-1 (CD44), and heat stable antigen (HSA), CD24 was determined with MoAb derived from hybridoma cell lines YN1/ 1.7.4, KM201, and M1/69.16.11.HL, respectively (all provided by

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the American Type Culture Collection, Rockville, MD). VCAM-1 expression was determined by an MoAb derived from the hybridoma cell line 429.3.1 (kindly provided by Dr T. Springer, CBR, Harvard Medical School, Boston, MA). As a secondary reagent a PE conjugated goat-antirat IgG MoAb (Southern Biotechnology, Birmingham, AL) was used. Thy1.2, Mac-2, Mac-3, stem cell antigen-1 (Sca-1), and major histocompatibility complex-I (MHC-I) expression were determined with PE conjugated, BP-1 with biotin conjugated MoAb from PharMingen. Streptavidin-PE (Southern Biotechnology) or Avidin-FITC (Becton Dickinson) were used as secondary reagents for biotin conjugated MoAb. Stromal and hematopoietic cells from cocultures were discriminated by their light scatter characteristics, using freshly isolated cells from BM to define the hematopoietic cell gate. At light scatter detector settings required for BM hematopoietic cell detection stromal cell clones were off scale. Live cells were gated via propidium iodide (Sigma) exclusion. Data from fluorescent labeled cells were acquired with a FACScan flow cytometer (Becton Dickinson) and analyzed with CELLQUEST software from Becton Dickinson.

Reverse transcription and polymerase chain reaction (RT-PCR). Reactions were performed as described in the manual for the RNA PCR kit (Perkin Elmer, Branchburg, NJ). Briefly: 3 μg of stromal cell RNA were reverse transcribed. Two percent of the cDNA were used for each PCR reaction. Each PCR cycle consisted of 1 minute DNA denaturation at 95°C, 1 minute primer annealing at 52°C, and 1 minute of primer extension at 72°C. We performed different numbers of cycles in separate tubes. One third of each PCR reaction product was separated on an agarose gel and visualized with UV light. The expected size of amplified DNA fragments was an indication for specificity of the PCR reaction. The following fragments were amplified: 223 bp for pre B-cell growth stimulating factor (PBSF) with ctgcaatacagtgttaa and ctctcacatcttgagcct (kindly provided by Dr. Qing Me, CBR, Harvard Medical School, Boston, MA), 809 bp for SCF with gctgcctttccttatgaaga and aacaatgttgatacgtccac,51 651 bp for M-CSF with catacaggagacacacg and atgtgacccagagtctgccgt2 and 260 bp for β-actin with tgaatcttggtgcattagaga and taaacgcagctcagtaacagtccg.

CFU-S, assay. An estimation of the content of hematopoietic progenitors in the cocultures was made by determination of CFU-S, after injection into lethally irradiated, 10- to 12-week-old female BDF1 mice. Cocultures were trypsinized, 90% of the cells washed in PBS, resuspended in complete medium with 10% FCS, and incubated for 1 hour on a gelatin (DIFCO) coated tissue culture plate to remove adherent stromal cells. The floating cells were injected in a volume of 300 μL/mouse into lethally irradiated recipient BDF1 mice. Twelve days after injection, hematopoietic colonies in the spleen were scored under a stereoscopic microscope as described.36 Seven mice per coculture and injection time point were injected.

RESULTS

Growth, morphology, and ultra structure of stromal cell clones established from adult BM and FL. Heterogeneous stromal cell cultures were established from BM and FL of H-2K,ts A58 mice, as described in Materials and Methods. We derived a total of 8 clones from FL of embryos at day 12.5 of gestation (FLd12.5), 22 clones from FLd14.5, 16 clones from FLd16.5, and 52 clones from adult BM. They were established in culture medium containing D-valine. An additional set of 35 clones from adult BM was established in culture medium containing L-valine (see Materials and Methods). Cloning efficiencies were 8/100 for FL day 12.5 of gestation, 22/100 for FL day 14.5 of gestation, 16/100 for FL day 16.5 of gestation, 52/85 for BM cultured in D-valine, and 35/85 for BM cultured in L-valine containing medium. No conditioned medium or growth factors were used for the establishment of the heterogeneous cultures or for the cloning and maintenance of the stromal cell clones. The best culture conditions were found to be an incubation temperature of 33°C, which stabilizes the SV40 immortalizing protein and 20 U/mL IFNγ which induces its expression (Fig 1A). These conditions lead to doubling times of 35 hours and 100% viability. Increase in temperature or the lack of IFNγ lead to reduced proliferation rates (doubling times of 48 hours and more) and cause cell death after 2 to 3 weeks. Proliferation of a representative cell clone under the different culture conditions is shown in Fig 1A. Twelve clones were tested for their capacity to differentiate towards adipocytes when IFNγ was removed from the culture me-
Stromal Cell Clone & Fat Accumulation & Morphology Type

| CBR-BM,D#1 | +++ | B |
| CBR-BM,D#10 | — | B |
| CBR-BM,D#11 | ND | B |
| CBR-BM,L#17 | — | A |
| CBR-BM,L#19 | — | A |
| CBR-BM,L#21 | — | A |
| CBR-BM,L#23 | — | A |
| CBR-FL d13#2+ | +++ | C |
| CBR-FL d13#6+ | +++ | C |
| CBR-FL d15#4+ | +++ | C |
| CBR-FL d15#6- | +++ | C |
| CBR-FL d17#2- | + | C |
| CBR-FL d17#4+ | + | C |

The degree of fat accumulation under Dexter culture conditions without IFNγ was determined according to Fig 1B. The morphology of stromal cell clones cultured in the presence of IFNγ was determined by phase contrast light microscopy and the stromal cell clones were classified according to Fig 2.

Abbreviation: ND, not determined.

The cell clones reported here have been maintained in culture for more than 1 year and display stable morphology. They can be grouped into three morphological types: Spindle-like, small cells (mFSC of 250) with low right angle light scatter properties (mSSC of 100) (Fig 2A), which organize side by side in “bundles” when the culture becomes confluent. They were exclusively found among BM derived clones established in the presence of L-valine (3 out of 35 clones). Large cells (mFSC of 450) with spread cytoplasm and high right angle light scatter characteristics (mSSC of 250) with cytoplasmic projections extending over neighboring cells when the culture is confluent (Fig 2B). This type represents most of the BM derived clones. Large cells (mFSC of 450) with highly spread cytoplasm and high right angle light scatter properties (mSSC of 250), which form well-organized confluent cell layers (Fig 2C). All FL derived cell clones and none from the BM display this morphology (Fig 2 and Table 1). TEM revealed long processes extended to contact adjacent cells in type A (Fig 3A). The second, type B, are flattened cells which seemed to be overgrowing neighboring cells. However, there is minimal significant cell to cell contact, with the exception of occasional distal processes, which exhibited a focal contact with the adjacent cell (Fig 3B). In contrast, all FL-derived stromal cell clones exhibit an epithelium-like morphology (Fig 3C) with extensive cell to cell contacts including numerous adherens junctions and forming junctional complexes although no definitive tight junctions were observed (Fig 3C insets).

Surface molecule expression on heterogeneous stromal cell populations and stromal cell clones. Flow cytometry revealed expression of ICAM-1 (CD54), VCAM-1 (CD106), Pgp-1 (CD44), HSA (CD24), and Thy-1 (CD90) on heterogeneous stromal cell populations derived from FL and BM maintained in culture for 8 weeks (Fig 4). No MHC-II, ICAM-2 (CD102), BP-1, Mac-1 (CD11b), CD3, GR-1, or B220 (CD45R) expressing cells were found in any of the heterogeneous stromal cell cultures (data not shown). Although VCAM-1 (CD106), ICAM-1 (CD54), and HSA (CD24) are expressed on both BM and FL derived stromal cells, Thy-1 (CD90), and Pgp-1 (CD44) are restricted to stromal cell cultures derived from FL (Fig 4). The proportions of positive cells in the heterogeneous cultures vary between different surface molecules (Fig 4), and double color analyses revealed subpopulations with different expression patterns (data not shown). Therefore, as expected, we found different expression patterns of surface molecules on particular stromal cell clones. Table 2 summarizes the frequency of stromal cell clones expressing individual cell surface molecules and shows the expression patterns of twelve particular clones. All stromal clones express MHC-I and most of them express high levels of VCAM-1 (CD106), whereas none of the analyzed clones express Pgp-1 (CD44) nor BP-1. ICAM-1 (CD54) and HSA (CD24) are expressed by the majority of BM and FL derived clones. While only BM derived stromal cell clones express Sca-1, Thy-1 is restricted to FL stromal cells (Table 2 and Fig 4). Surface molecule expression has been analyzed at various time points and was stable in all cell clones more than 1 year in culture.

Expansion of CFU-S12 hematopoietic progenitor cells in long-term cocultures of post 5-FU BM hematopoietic cells with BM- and FL-derived stromal cell clones. Two sets of experiments were performed to assess the capacity of the stromal cell clones to maintain or expand CFU-S12 progenitors in cocultures. First, 5 × 10⁵ BM hematopoietic progenitors (4 days post 5-FU injection) were cocultured at 33°C in the presence of 10% FCS on pre-established layers of different stromal cell clones derived from the BM. After 2 and 8 weeks, the cocultures were trypsinized and analyzed for their CFU-S12 content. The results are shown in Table 3. All the BM derived stromal cell clones were able to prevent the fast decrease of CFU-S12 progenitors that occurs within 2 weeks in the negative control without a stromal cell layer. Clone CBR-BM,D#1, and to a lesser extent clones CBR-BM,D#10 and CBR-BM,L#23, were able to expand or maintain the pool of progenitors over a period of 8 weeks. In a second set of experiments stromal cell clones derived from the FL were analyzed. Progenitors were cocultured at 33°C in the presence of 12% FCS and 12% horse serum. None of these cocultures maintained more CFU-S12 than the negative control, probably because of the increased amount of serum in the culture medium. However, while later the level of CFU-S12 dropped to zero in the negative control most of the FL derived stromal cell clones were able to maintain CFU-S12 for up to 10 weeks. Considering the dilution of the cocultures due to removal of 90% of the cells every 2 weeks, the
Fig 2. Morphology of stromal cell clones in culture and light scatter properties in flow cytometry after trypsinization. Photographs were taken at 200× magnification under phase contrast conditions. Note that the cell density does not drastically affect the morphology. The flow cytometric characteristics forward light scatter (FSC) and right angle light scatter (SSC) give an indication of the size and the granularity of the stromal cells, respectively. The scales are linear and in arbitrary units representing the detection channels of the flow cytometer. The three morphology types are represented in (A through C).

calculated total number of CFU-S12 increases on some of the clones. We calculated an 86-fold increase of CFU-S12 within the last 6 weeks of coculture on CBR-BM,D#1 and a 430-fold increase of CFU-S12 within the last 4 weeks of coculture on CBR-FL d17#2−, which requires doubling times of 6.4 and 3.5 days, respectively.

Proliferation and differentiation of late myeloid progenitor cells in coculture with stromal cell clones from the FL and the BM. To compare the effect of BM- and FL-derived stromal cell clones on the proliferation and differentiation of late hematopoietic progenitors, we cocultured them with 10⁶ BM derived progenitor cells (day 4 post 5-FU injection, see Materials and Methods) at 37°C in the presence of 10% FCS for 3 weeks. At day 8 of coculture hematopoietic cells were counted, 90% analyzed for surface molecule expression by flow cytometry and 10% further cocultured on newly established stromal cell layers. Hematopoietic cells coexpressing 8C5 and Mac-1 (8C5+/Mac-1+) were considered granulocytes, cells expressing only Mac-1 macrophages, B220+ IgM negative pre-B cells were further dissected into early (c-kit+) and late (c-kit−) B-cell progenitors. After 8 days (Fig 5A, top panel) granulocytes were the most abundant cell type in all cocultures. Although their number increased to a maximum of threefold over input on FL stromal cell clones, there was a 3- to 15-fold expansion on BM derived stromal cell clones. Interestingly, no cell expansion at all was detected on CBR-BML#19. In all cocultures early and late B-cell progenitors were a minor frac-
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Fig 3. Morphology of stromal cell clones at ultrastructural level. (A) Long cell processes extending to contact points with neighboring cells (upper panel, large black arrow), a prominent actin cortex (white arrow), and an active secretory apparatus with Golgi (white arrowheads) and endoplasmatic reticulum (black arrow heads) were found in BM derived cell type (A). The small black arrows indicate the substrate layer the cells grow on in the tissue culture dish. (B) Minimal significant cell to cell contact was found in BM derived cell type (B). Obvious overgrowing of neighbor cells is shown in the upper panel where the round cell is lying on top of a flattened process of a neighboring cell (large black arrow). The lower panel shows two stromal cells one overlying the other. The small black arrows indicate the contact with substrate. (C) Epithelial-like morphology with extensive cell to cell contacts (black arrows) is shown by FL derived stromal cell clones. The bottom panels show examples of adhesion junctions found between these cells.

tion (less than 5%) of the hematopoietic cells at the same time point of coculture (Fig 5A). Macrophages expanded best on most of the BM derived stromal cells (not on CBR-BM,L#19) and little on FL stromal cell clones derived from 16.5-day-old embryos (CBR-FL d17#2- and CBR-FL d17#4+). On stromal cell clones derived from the FL of younger embryos (12.5 days and 14.5 days old) macrophages and granulocytes did not expand at all (Fig 5A). Coculture of post 5-FU BM progenitor cells with a mixture of the 12 stromal cell clones resulted in a ninefold increase of granulocytes and a fourfold increase in macrophages. In the negative control (10^6 post 5-FU BM derived hematopoietic progenitors cultured without pre-established stromal cell layer) no live hematopoietic cells were detected after 8 days. No expansion of granulocytes was detected on any of the stromal cell clones after further coculture for a total of 3 weeks (data not shown).

IL-7 dependent proliferation and differentiation of late B-cell progenitors in coculture with stromal cell clones. To analyze the capacity of the established stromal cell clones to support the proliferation and differentiation of late pre-B cells (B220^-c-kit+) in the presence of IL-7, 10^6 BM derived hematopoietic progenitors (day 4 post 5-FU injection) were cocultured with 12 different stromal cell clones, adding IL-7 to the culture medium. After 8 days, stroma-adherent and floating hematopoietic cells of the cocultures were analyzed by flow cytometry (Fig 5A, bottom panel). Those clones that did not support the proliferation and maturation of granulocytes in the absence of IL-7 (CBR-BM,L#19, CBR-FL d13#8+, CBR-FL d13#2+, and CBR-FL d15#4+) allowed pre-B cells of the B220^-c-kit+ phenotype to expand up to fourfold within 8 days. No B-cell expansion was seen on other stromal cell clones, the negative control, nor the mixture of all 12 stromal cell clones.

Proliferation of IL-7 dependent early and late pre-B cells from FL and BM on stromal cell clones. To address the
microenvironmental influence of the stroma on the proliferation of pre-B cells from BM and FL we performed W/W type cocultures in the presence of IL-7. 10\(^6\) hematopoietic cells derived from BM or FL were cocultured on BM- and FL-derived stromal cell clones. After 1 week of coculture hematopoietic cells were counted and the pre-B cell content was determined by immuno flow cytometry (Fig 5B). Within the first 8 days of coculture pre-B cells decreased to 50% in all cocultures except on CBR-BM,L#19, which induced a 20-fold increase. Seventy-five percent of these pre-B cells were of the late phenotype B220\(^{-}\)/c-kit\(^{-}\). Control experiments without IL-7 did not lead to any pre-B cell proliferation at all (data not shown).

To determine the potential of our stromal cell clones to support the growth of early pre-B cells (B220\(^{+}\)/c-kit\(^{+}\)) which have long-term proliferative capacity in the presence of IL-7, 10% of the hematopoietic cells from the cocultures previously described (8 days) were transferred to a new stromal cell layer and assayed again after 22 days of coculture (Fig 5C). In the absence of IL-7 hematopoietic cells were so few that flow cytometry was not possible (not shown). In the presence of IL-7, however, BM- and FL-derived pre-B cells expanded on 4 out of 12 and 10 out of 12 stromal cell clones, respectively. Wherever pre-B cells expanded, their proliferation was 5 times higher for FL compared to BM derived cells (CBR-BM,L#19, L#23, CBR-FL, d13#8+ and d15#4+ in Fig 5C). Between 50% and 95% of these late (3 weeks) responding pre-B cells were of early B220\(^{+}\)/c-kit\(^{+}\) phenotype.

Correlation of mRNA expression of SCF, M-CSF, and PBSF and the hematopoietic supportive functions of different stromal cell clones. Potential correlations between hematopoietic supportive functions and cytokine expression of the stromal cell clones were addressed by analyzing with RT-PCR the expression of two lineage specific (M-CSF and PBSF) and one precursor specific (SCF) cytokine (Fig 6). The housekeeping gene \(\beta\)-actin was also amplified to control for the amounts of template RNA. Different numbers of DNA amplification cycles were performed for each stromal cell clone and each cytokine. With one exception, we found equal expression for SCF, M-CSF, and PBSF in 6 functionally different stromal cell clones. Only CBR-BM,L#21 seemed to express lower amounts of PBSF, which might account for its universal lack of pre-B cell support. Although this protocol allows only for semiquantitative determination of mRNA levels, we could rule out the complete lack of expression of any of the cytokines in any of the studied stromal cell clones.

**DISCUSSION**

The aim of this study was to establish a series of stromal cell clones not only from BM but also from FL and to dissect
functions of the stromal cell compartments of these organs at the cell clone level. We took advantage of an inducible, temperature sensitive allele of the SV40 TAg used as a transgene in mice. It allowed us to clone immortalized stromal cells directly from the organs but perform functional studies in the absence of the large TAg. This is especially important, because it has been shown that large TAg expression in human stromal cells alters cytokine expression.37 Because the transgene is under the control of an MHC class-I promoter, it is presumably expressed by all stromal cell types. We used parallel culture conditions with either L-valine or D-valine containing culture medium to inhibit overgrowth, and thereby overrepresentation of fibroblasts in the stromal cell clone collection. Therefore, this set of established stromal cell clones might represent a high proportion of the in vivo stromal cell compartment. Conditional immortalization and block in differentiation was shown for all stromal cell clones (Fig 1).

We analyzed the obtained stromal cell clones for expression of hematopoietic cell lineage markers and for surface molecules found to be relevant for interactions between leukocytes and nonhematopoietic cells (Table 2). Because of the lack of Mac-1, CD3, CD4, CD8, GR-1, and B220 we excluded the possibility of having cloned transformed hematopoietic lineage cells. Removal of IFNγ and addition of BM hematopoietic cells did not affect the phenotype of any stromal cell clone (data not shown). Most of our cell clones express VCAM-1 (CD54), which has been shown to mediate the binding of cobblestone forming area cells (CAFC) and B-cell progenitors to stromal cells in vivo. None of our stromal cell clones express CD44, which has been shown to be involved in hematopoiesis by MoAb blocking experiments in LTMC. Expression of 6C3/BP-1 on stromal cells has been correlated with their capacity to support B lymphopoiesis. However, BP-1+ stromal cells supporting B-cell proliferation have also been reported. Some of our stromal cell clones support B-lineage cell proliferation but do not express BP-1, confirming the latter. ICAM-1 (CD54) is implicated in the binding of leukocytes to endothelium. Because more than half of our stromal cell clones from BM and FL express ICAM-1, it might play a role in the interactions between hematopoietic cells and stromal cells during hematopoiesis. Null mutations of the ICAM-1 gene, however, did not reveal its essential requirement for the generation of blood cells in vivo. The functions of the glycosphatidylinositol (GPI) anchored molecules HSA (CD24), Sca-1, and Thy-1 (CD90) are not fully understood. We found Sca-1 and Thy-1 expression restricted to BM and FL stromal cells, respectively (Table 2 and Fig 4). Possibly because of redundancy among different adhesion mechanisms between stromal cells and hematopoietic progenitors we were not able to find correlations between surface molecule expression and hematopoiesis supportive functions.

We defined three types of morphology by phase contrast light microscopy and TEM (Fig 2 and Fig 3). FL derived cells display a more epithelial-like morphology, whereas BM derived clones appear more fibroblastic. Morphological changes and stromal cell differentiation have been reported depending on the culture conditions.

The morphology differences within our set of stromal cell clones are prominent and stable over an extended time period, and the cell culture conditions are the same for all of them, these morphological differences might well be because of different cell types or to different stages of cell differentiation. Strong correlation was found between the capacity of stromal cell clones to accumulate fat in the presence of hydrocortisone.
and their support of CFU-S in vitro, confirming a correlation found in Dexter type LTBMC.\(^9\) Interestingly, the stromal clone morphology correlates well with the origin of the cell clones, i.e., type A and B were only found among BM, type C only among FL derived clones.

Functional assays included the long-term maintenance of early myeloid progenitors (CFU-S\(_{12}\)) (Table 3) and the support of final differentiation to mature granulocytes from 5-FU BM progenitors (Fig 5A). The actual number of CFU-S\(_{12}\) progenitors in the cocultures most likely depends on (1) differentiation from more primitive cells, (2) self-renewal of CFU-S\(_{12}\), and (3) differentiation to more mature cells or cell death. Therefore, whenever a coculture was assayed, the CFU-S\(_{12}\) progenitor cell content resulting from all three factors was measured. Because some of our clones support the presence of CFU-S\(_{12}\) for a period of 8 to 10 weeks, but do not support the long-term generation of differentiated hematopoietic cells, we conclude that progression to or self-renewal of the CFU-S\(_{12}\) progenitor compartment is supported under the given culture conditions, but the differentiation and proliferation to mature cells is not. In LTBMCs one doubling of CFU-S\(_{12}\) per week has been originally found by Dexter.\(^9\) Our data range between 0.5 and 2 doublings per week for CFU-S\(_{12}\) progenitors, depending on the stromal cell clone used as supportive stromal layer.

While 5 out of 6 BM derived stromal cell clones support the generation of high numbers of 8C5'/Mac-1' cells from 5-FU progenitors, FL derived stromal cell clones are not able to induce this maturation/proliferation (Fig 5A). The support of granulocyte expansion by BM derived stromal cell clones is dominant over FL derived non-supporters, as shown by the coculture of hematopoietic progenitors with a mixture of all stromal cell clones.

We also analyzed the effect of the different stromal cell clones on the IL-7 dependent proliferation of early (B220'/c-kit') and late (B220'/c-kit') B-cell progenitors from FL and BM. Short-term analysis (8 days) revealed that the stromal cell clones least supportive of granulocyte expansion best supported the differentiation and proliferation of pre-B cells from 5-FU progenitors (Fig 5A). Myeloid and lymphoid
HEMATOPOIESIS described in Material and Methods. One third of each reaction was panel. The amount of template RNA from each stromal cell clone gene p-actin.

The first one occurred, no matter what hematopoietic cells on the cell clone level. Two different 1L-7 dependent types of differentiation support thus seem to be mutually exclusive of pre-B cell proliferation support were revealed, an early (9 weeks) and a late (3 weeks) support of mainly c-kit' cells (Fig 5C). (1) The cocultures were started with, but only on CBR-BM,L#19.

The second type was typical for FL derived hematopoietic cells and occurred on 10 out of 12 stromal cell clones. C-kit" cells were not able to differentiate to c-kit" cells on CBR-BM,L#19, ie, after 3 weeks this coculture was highly enriched for c-kit" pre-B cells (Fig 5C). Based on these functional assays we were able to dissect our stromal cell specific supporters of BM-derived pre-B cells were not found. We considered this stromal cell clone collection a novel valuable tool useful for studies in hematopoiesis or gene therapy, now also available to other investigators.

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REFERENCES


29. Harrison DE, Lerner CP: Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-FU. Blood 78:1237, 1991
35. Whitlock CA, Tidmarsh GF, Mueller-Sieburg C, Weissman IL: Bone Marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. Cell 48:1009, 1987
44. Dustin ML, Springer TA: Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J Cell Biol 107:321, 1988
48. van de Rijn M, Heimfeld S, Spangrude GI, Weissman IL: Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci USA 86:4634, 1989
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