Differential Roles of Stromal Cells, Interleukin-7, and kit-Ligand in the Regulation of B Lymphopoiesis

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Newly formed B lymphocytes are a population of rapidly renewed cells in the bone marrow of mammals and their steady state production presumably depends on a cascade of regulatory cells and cytokines. Although considerable information had been forthcoming about the role of interleukin-7 (IL-7) in potentiating pre-B-cell proliferation, few studies have addressed the possibility that multiple cytokines are involved in the progression of early events in cellular differentiation and proliferation in this hematopoietic lineage. Our laboratory previously described pre-B-cell differentiation mediated by the bone marrow stromal cell line S17. In this study, we further delineate the role of stromal cells in differentiation and proliferation of pre-B cells. These experiments show that the stromal cell line S17 potentiates the proliferative effect of IL-7 on B-lineage cells and that this S17-derived potentiator can be replaced with recombinant kit-ligand (KL). Our results further show that pre-B-cell formation from B220+, Ig- progenitor cells and expression of μ heavy chain of immunoglobulin is uniquely dependent on the presence of S17 stromal cells and cannot be reproduced with IL-7, KL, or costimulation with both IL-7 and KL. These data contribute to a rapidly evolving model of stromal cell regulation of B-cell production in the marrow and suggest unique roles for IL-7, KL, and as yet uncharacterized stromal cell-derived lymphokines in this process.

STUDIES OF THE regulation of B-lymphocyte differentiation have only recently become feasible, owing primarily to the development of lymphoid long-term bone marrow cultures (LBMC) that support both early and late events in this lineage.13 These cultures have been essential in defining regulatory cells and cytokines that affect the differentiation and proliferation of early B-lineage cells. Although a number of cells appear to play a role in providing positive and negative signals that maintain steady-state production of B cells, data from BMC have established that the essential regulatory component for B-cell development in vitro appears to be a population of adherent fibroblastic stromal cells.4 The cells are presumed to be representative of the population of reticular cells present in intersinusoidal spaces of the marrow medullary cavity.6

Several laboratories have recently cloned representative stromal cell lines from BMC,7,14 and these cell lines have proven invaluable in studies of cytokines that regulate the proliferation and differentiation of B-lineage cells. Interleukin-7 (IL-7) was cloned from an SV40-transformed stromal cell line and was originally characterized as a proliferative factor for B-lineage cells.14,15 In addition, more recent studies have shown a role for IL-7 in the proliferation of thymocytes, mature peripheral T cells,18,19 and lymphokine-activated killer (LAK) cells.20 Several groups recently identified the ligand for the protein product of c-kit, a membrane-bound receptor with tyrosine kinase activity,21,22 and in one case the ligand for this receptor was cloned from a murine BM stromal cell line.23-25 Kit-ligand (KL) was recently shown to interact synergistically with IL-7 and to stimulate proliferation of B-lineage cells.26 In addition to these proliferative stimuli, stromal cells secrete an activity(s) that allows B220+ precursors to mature to Ig expression. Stromal cell line S17 was previously shown to secrete cytokines that potentiated this differentiative step, and that process appeared to involve little or no proliferation.10

During the course of studies aimed at characterizing this differentiative activity, S17 cells were observed to secrete an activity that synergized with IL-7 to potentiate the proliferation of LBMC nonadherent cells. Further analysis indicated that activity was due to secreted KL, and this observation raised the question of whether that factor was the S17-derived differentiative activity that allowed matura-

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ent cell lines cells were plated in 96-well disposable culture dishes (Corning Glassworks, Corning, NY) and used after a confluent cell layer was established. For short-term BM coculture experiments, cells were transferred to 24-well cell culture plates and used as confluent adherent layers of supporting cells that were separated from target cells by a cellulose 0.45 μm microporous membrane (Millipore-HA; Millipore, Bedford, MA).

In some experiments, conditioned medium (CM) was prepared by growing adherent cell lines to confluence in α-Modification of Eagle’s Medium (Mediatech Celgro, Washington, DC) containing 5% FCS (JRH Scientific, Woodland, CA), 5 × 10⁻⁵ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 10⁻⁵ U/mL penicillin G, and 100 μg/mL streptomycin. CM was collected at 96 hours of culture and stored as sterile unconcentrated CM in small aliquots at −85°C.

Antibodies. Hybridoma clone 14.8 was obtained from the ATCC (TIB 164) and grown as previously described in RPMI 1640 medium (GIBCO) containing 2% FCS and 1% Nutridoma-SP (Boehringer-Mannheim, Indianapolis, IN). 14.8 CM was concentrated 10-fold on Amicon XM-50 ultrafiltration membranes (Amicon, Danvers, MA) for use in these experiments. Affinity-purified goat-antirat Ig, fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat-antimouse λ and goat-antimouse κ light chains of Ig, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated affinity-purified goat-antimouse μ-heavy chain of IgM antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). All antibodies were used at saturating concentrations in antibody binding studies.

Recombinant growth factors. Recombinant human IL-1α (specific activity, 2.7 × 10⁸ U/mL) was obtained from Hoffman LaRoche (Nutley, NJ). Recombinant murine IL-7 (10¹⁰ U/mg) was obtained from Biosource International (Westlake Village, CA). The cDNA for secreted form of mouse KL (sKL) was cloned from the cDNA of a generous gift from Dr S.I. Nishikawa. The 1.5-kb PstI and Xho I cDNA fragment was a gift from JTT (TIB 164) and grown as previously described in RPMI 1640 medium (GIBCO) containing 2% FCS and 1% Nutridoma-SP (Boehringer-Mannheim, Indianapolis, IN). 14.8 CM was concentrated 10-fold on Amicon XM-50 ultrafiltration membranes (Amicon, Danvers, MA) for use in these experiments. Affinity-purified goat-antirat Ig, fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat-antimouse λ and goat-antimouse κ light chains of Ig, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated affinity-purified goat-antimouse μ-heavy chain of IgM antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). All antibodies were used at saturating concentrations in antibody binding studies.

Preparation of cell suspensions. Mice were killed by cervical dislocation. Femurs and tibias were removed and the marrow plug flushed from bones with RPMI 1640 (GIBCO) containing 5% heat-inactivated FCS (HIHFS; Hyclone Laboratories, Logan, UT) using a syringe fitted with a 23-gauge needle. Bone spicules and debris were allowed to settle into an underlayer of HIHFS and cells were transferred to a second tube. Cell viability and cell counts were determined in a hemocytometer.

Cell depletion. Adherent cells were depleted from BM cell suspensions or LBMC using G10 Sephadex. Sterile G10 columns were prepared exactly as previously described. Briefly, sterile 10-mL syringe barrels were plugged with glass wool, autoclaved, and filled with 8 mL of sterile preswollen G10 Sephadex. Columns were washed with sterile phosphate-buffered saline (PBS) and equilibrated with 20 mL warm (37°C) RPMI 1640 containing 5% HIHFS. Two milliliters of medium containing no more than 10⁷ BM cells was applied to each column. Columns were incubated for 30 minutes at 37°C and nonadherent cells washed from columns with 30 to 35 mL of warm (37°C) medium.

Monoclonal antibody 14.8 binds to a determinant on the 220 Kd molecular weight form of CD45 (B220) cell surface molecule of lymphoid cells. Depletion of cells with this cell surface antigen results in the removal of B-lineage cells that express either sIg (B cells) or cytoplasmic μ-heavy chains of IgM (pre-B cells). In our studies, 14.8” cells were depleted from cell suspensions on antibody-coated polystyrene Petri dishes (Falcon 1001; Becton Dickinson, Oxnard, CA) exactly as previously described. Petri dishes were first coated with affinity-purified goat antirat Ig (50 μg in 5 mL Tris buffer, pH 9.5) at room temperature for 70 minutes and then washed three times with PBS, pH 7.2. Plates were then incubated with 100 μg 14.8 antibody in 3 mL PBS at 4°C for 30 minutes. 14.8-coated plates were washed three times with ice-cold PBS, filled with 5 mL PBS containing 5% HIHFS, and held at 4°C until use. All plates were used the same day that they were prepared.

BM or LBMC cells were first depleted of adherent cells on G10 Sephadex. The remaining cells were then transferred to antibody-coated plates (2.5 × 10⁷ cells/plate) and incubated at 4°C for 70 minutes. In all experiments, cells were depleted on three sequential plates to stringently remove appropriate cells. Nonadherent cells from this series of depletions were collected as 14.8” (B cell and pre-B cell depleted) BM cells for use in experiments. Antibody depletions were checked by staining nonadherent cells with fluorescent labeled 14.8 antibody. Depletions were further monitored for efficiency of pre-B-cell removal by staining depleted cells for expression of Ig light chain and μ-heavy chain expression as described below.

Short-term BMC. BM cells were depleted of adherent cells on G10-1 Sephadex and pre-B cells by adherence to 14.8-coated Petri dishes as described above. Nonadherent and 14.8” cells were cultured at 10⁶ cells/mL in RPMI 1640 containing 5% FCS, 5 × 10⁻⁵ mol/L 2-mercaptoethanol, essential and nonessential amino acids (GIBCO), 2 mmol/L L-glutamine, MEM vitamins (GIBCO), 10⁻⁵ U/mL penicillin G, and 100 μg/mL streptomycin. In experiments that required an underlayer of stromal cells, BM cells were cultured in diffusion chamber culture inserts (Millipore-HA; Millipore, Bedford, MA) to prevent cell contact between nonadherent BM cells and stromal cells as previously described.

Lymphoid BMC. Lymphoid permissive BMC were set up exactly as originally described by Whitlock and Witte. Briefly, suspended BM cells were cultured at an initial density of 1 × 10⁷ cells/mL in 10 mL RPMI 1640 containing 5% FCS in 100-cm² polystyrene tissue culture grade petri dishes (Corning #1005) at 37°C and 6% CO₂ in ambient air. Cultures were initially fed an additional 5 mL of the same medium on day 3 of culture. For the remainder of culture, 10 mL of medium was removed once weekly and replaced with 10 mL of identical fresh cell culture medium. After 3 to 4 weeks, cultures were observed for the appearance of foci of lymphoid cells and nonadherent cells were collected for assays.

Antibody labeling of cells and fluorescence microscopy. B and pre-B cells were identified by immunofluorescent staining of Ig light and heavy chains on fixed cytocentrifuged preparations as previously described. Briefly, 10⁷ cells were cytocentrifuged onto glass slides, air dried, and fixed in absolute ethanol containing 5% glacial acetic acid at −20°C for 15 minutes. Fixed preparations were rehydrated in 3 vol of PBS for 15 minutes each, and incubated with FITC-goat antimouse κ light chain (50 μg/mL) and FITC-goat antimouse λ light chain (50 μg/mL) containing 0.1% NaN₃ in a humidified chamber for 30 minutes at room temperature. Slides
were then rinsed with PBS and incubated with TRITC-goat antimouse μ-heavy chain (50 μg/mL) under the same conditions. Slides were rinsed in three changes of PBS with the last wash left overnight at 4°C. Washed slides were mounted in Elvanol (Fluoromount-G; Southern Biotechnology) and examined under epifluorescence illumination on a Zeiss Universal microscope. Cells with detectable green fluorescence (light chain\(^+\)) were considered B cells. Only cells with detectable μ-heavy chain of Ig but no detectable light chain were considered pre-B cells in these studies.

**Proliferation assays.** Cells were incubated at a final concentration of 2.5 \(\times\) 10\(^4\) cells/mL in 200 μL in 96-well multi-well dishes (Corning) and pulsed with 1 μCi \(^{3}H\)-Tdr/well at 24 to 60 hours of incubation. Cells were harvested onto glass wool fiber strips with an automated cell harvester (Cambridge Institute, Boston, MA) at 18 hours after pulse with radiolabeled nucleotide and incorporated radioactivity determined by liquid scintillation counting (LKB/Wallack Model 1410, Gaithersburg, MD) in an aqueous fluor (Biosafe-II; Research Products International, Mt Prospect, IL).

**Northern analysis.** Total RNA was extracted by RNazol (Cinna Biotec, Friendsville, TX), fractionated on agarose/formaldehyde gels, stained with ethidium bromide, and transferred to Nitroplus 2000 (Separation Science Inc, Waltham, MA). Hybridization and washing procedures were performed as previously described in detail.\(^{26}\) After the initial hybridization with the Xho I-IL-7 cDNA fragment, blots were stripped by boiling in H\(_2\)O, and filters were rehybridized with the 1.5-kb Pst I cDNA fragment of GAPDH\(^{10}\) to control for quantitative loading of lanes.

**Presentation of data.** In all experiments that address the formation of pre-B cells in culture, the absolute number of pre-B cells generated is presented. This calculation of actual pre-B cells lost or gained in cultures prevents potential misinterpretation of data due to secondary frequency changes caused by net losses or gains in another cell population from the BM inoculum. In these experiments, cell viability for treated and control cultures did not vary more than 5%.

For proliferation assays, data presented are as mean counts per minute of at least three independent samples and include the standard error of the mean for each treatment group.

**RESULTS**

**S17 stromal cells and IL-7 synergize in regulating the proliferation of B-cell precursors.** During the course of experiments aimed at characterizing cytokines produced by S17 stromal cells, the line was found to produce an activity that synergized with IL-7 in potentiating the proliferation of B-lineage cells. As shown in Fig 1, IL-7 alone supported proliferation of nonadherent lymphoid cells from LBMC, and this effect was saturable with maximal stimulation of proliferation achieved at 50 U/mL recombinant murine IL-7 (rmuIL-7). However, the magnitude of this proliferative response was significantly elevated when cultures were stimulated with IL-7 in the presence of S17 stromal cells. We also noted low but significant proliferation of LBMC nonadherent cells on S17 stroma alone, suggesting that S17 and IL-7 synergized in this assay. This synergistic proliferation response was not observed when S10 stromal cells or NIH-3T3 cells were used in place of S17 cells in this assay (data not presented).

In initial experiments, we noted considerable variation of proliferative stimulation indices for LBMC cells and identified several characteristics of nonadherent LBMC cells that affected the magnitude of the proliferative response. A direct relationship was observed between the age of LBMC used and proliferative response to IL-7 addition alone (data not shown). This elevation of IL-7-mediated proliferation in older cultures tended to obscure the synergistic response described above and, for that reason, all of the experiments presented used nonadherent cells from LBMC during the first 6 weeks after they began producing significant numbers of nonadherent cells. We also separated LBMC nonadherent cells into B220\(^+\) and B220\(^-\) cells in an attempt to determine whether both populations responded to S17 and IL-7 with a synergistic proliferative response. Both populations responded to this proliferative stimulus (data not presented); however, this result was not particularly revealing because both populations contained essentially identical numbers of Cp\(^+\), Ig-light chain\(^-\) cells. This finding suggests that, although B220 and Ig expression are coordinately expressed in normal BM cells, this is apparently not true for lymphoid cells from LBMC.

Finally, a number of experiments were set up in reduced oxygen tension (5% O\(_2\) in N\(_2\)) as previously described.\(^{14,15}\) No differences in IL-7 or S17 potentiated cell proliferation were found in companion cultures incubated under low or ambient oxygen tension. Ambient oxygen tension incubators were used for all of the cultures presented.

**S17 stromal cells do not produce detectable IL-7 messenger RNA (mRNA).** Although the synergistic costimulation of S17 stromal cells and IL-7 on lymphoid cell proliferation did not appear to be due to increases in IL-7 concentration (Fig 1), it remained possible that S17 cells expressed IL-7 in a manner that resulted in the observed increase in proliferation. To determine whether IL-7 mRNA transcripts could be detected in the S17 stromal cells used in this study,
confluent adherent layers were harvested at 72 hours after feeding. RNA was prepared as described, and Northern blot analysis performed. IL-7 message was detected in RNA prepared from S10 but not from S17 stromal cells (Fig 2). To assure that equivalent amounts of RNA had been loaded in each lane, blots were stripped and rehybridized with a 1.5-kb Pst I cDNA fragment of GAPDH.31

Failure to detect constitutive IL-7 mRNA production by S17 stroma did not exclude the possibility that its production could be induced. This is a relevant point in view of a report by Sudo et al that a stromal cell line that did not constitutively express IL-7 message could be induced to do so after 72 hours of coculture with IL-1.26 As illustrated in Fig 2, the addition of 1 to 100 U/mL rIL-1α to confluent layers of S17 for 72 hours before RNA extraction did not result in induction of IL-7 mRNA production (only data from 10 U/mL IL-1α treatment shown). IL-7 message remained undetectable in these preparations after 30 rounds of PCR amplification (data not presented). On the other hand, S10 cells did have detectable constitutive levels of IL-7 mRNA and this steady state level of IL-7 gene transcription was not obviously elevated by treatment with rIL-1α (Fig 2).

CM from S17 cells can synergize with IL-7 and stimulate proliferation of B-cell precursors. From the above studies it was not clear whether the IL-7 synergistic activity associated with S17 cells was cell bound or released into the medium. CM was collected from confluent S17 stromal cells as described and used 1× at 50% of culture volume in proliferation assays. As shown in Fig 3A, S17 CM synergized with 50 U/mL rIL-7 in stimulating the proliferation of nonadherent cells from LBMCI in the absence of an adherent cell layer. As before, this synergistic effect was achieved with 50 U/mL rIL-7 and further increases were not observed with higher concentrations of IL-7 (data not shown).

The contribution of S17 stromal cells in the proliferation of B-cell precursors can be replaced by KL. One recent report suggested that the ligand for the c-kit-encoded receptor may play a role in the proliferation of lymphoid cells.28 Because kit-ligand mRNA transcripts can be easily detected in S17 cells (unpublished results), we questioned whether the synergistic effect observed here was due to KL. As shown in Fig 3B, supernatants from COS-1 cells transfected with a cDNA clone encoding KL synergized with IL-7 in this assay and completely reproduced the effect of S17 cells or supernatants. This effect was not observed with control COS-1 supernatants (Fig 3B).

Neither IL-7 nor KL support pre-B-cell formation. Although the above data indicate that IL-7 and KL together stimulate the proliferation of B-lineage cells, it was also important to determine whether these cytokines potentiate differentiation of early Ig- B-cell progenitors (pro-B cells) and initiation of Cμ-heavy chain expression. IL-7 is not produced by the S17 cell line (Fig 2), and for that reason is almost certainly not the factor responsible for the differentiative activity produced by those stromal cells. However, these data do not rule out the possibility that IL-7 also has the ability to potentiate maturation of immature B-cell precursors. The data in Fig 4 clearly show that the addition of rmu IL-7 to B220-depleted BM cells at concentrations of 25 to 500 U/mL did not stimulate pre-B-cell formation. However, the addition of IL-7 to cultures that also contained S17 CM consistently resulted in greater numbers of pre-B cells formed as compared with cultures that contained only S17 CM (Fig 4). This difference was increased by extending cultures to 48 hours (Fig 4).

Because KL could replace S17 in the IL-7 costimulation assay for cell proliferation described above, we questioned whether the differentiation activity previously ascribed to S17 might reside with KL. As shown in Fig 5, neither COS-1 supernatants containing rKL (Fig 5A) nor the combination of rKL and IL-7 (Fig 5B) potentiated differentiation of Cμ+ Ig light chain+ cells from B220+ BM at any concentration tested. In addition, these data show that, whereas the presence of IL-7 in these cultures again resulted in greater numbers of pre-B cells in cultures containing S17 CM, the addition of KL did not affect the number of pre-B cells found in cultures that were potentiated with either S17 alone or those containing both S17 and IL-7.

B-lineage progenitors that precede B220 and Cμ expression are not expanded in the presence of IL-7 and KL. To determine whether IL-7, KL, or the combination of IL-7 and KL would stimulate expansion of cells that differentiate to Cμ expression in the presence of S17, B220-depleted BM cells were cultured for up to 48 hours in the presence of 100 U/mL rIL-7 or a combination of 100 U/mL IL-7 and 10%...
Fig 3. Effect of IL-7, S17 CM, and rKL on the proliferation of nonadherent cells from lymphoid LBMC. (A) LBMC cells were cultured in the presence of 50 U/mL rIL-7, 50% S17 CM, or 50% S17 CM and 50 U/mL rIL-7; or (B) 1% to 25% CM from COS-1 cells transfected with pMKL (○---○); 50 U/mL rIL-7 and 1% to 25% CM from COS-1 cells transfected with pMKL (●-●); CM from untransfected COS-1 cells (Δ---Δ); or 50 U/mL rIL-7 and 1% to 25% CM from untransfected COS-1 cells (Δ---Δ). Cultures were pulsed at 48 hours with 1 μCi [3H]-Tdr and harvested 18 hours later for liquid scintillation counting. Data are means and standard errors of three independent samples and are representative of five independent experiments.

Fig 4. Effect of cell-free supernatants from BM stromal cell lines S17 and rmu IL-7 on the differentiation of Cp⁺, Ig-light chain cells from B220⁺ progenitors from normal mouse BM. Adherent cells were removed by G10 Sephadex chromatography and B220⁺ cells were depleted on 14.8 monoclonal antibody-coated petri dishes. Depleted BM cells were cultured for 24 or 48 hours in the presence of CM from stromal cell line S17 (○-○) or medium only (○---○) and 0 to 500 U/mL rmu II-7. At the termination of culture, cells were counted and evaluated for Ig heavy and light chain expression. Only cells with detectable μ-heavy chain but no light chain were recorded. All data were converted to absolute numbers of cells per culture and are representative of four independent experiments.

Fig 5. Effect of S17 cells, rKL, and rmu IL-7 on the differentiation of Cp⁺, Ig-light chain cells from B220⁺ progenitors from normal mouse BM. BM cells were depleted of adherent cells and B220⁺ cells as described in Materials and Methods. Depleted BM cells were cultured in the presence of an S17 stromal layer (●) or in medium alone (○). Supernatants from COS-1 cells transfected with pMKL (solid symbols) or control COS-1 supernatants (open symbols) were added to cultures at 0% to 10% volume. Cultures were evaluated in the absence (A) or presence (B) of 100 U/mL rIL-7. Ig-heavy and light chain expression was evaluated at 24 hours. Only cells with detectable μ-heavy chain but no light chain were recorded. All data are presented as absolute numbers of cells per culture and are representative of three independent experiments.
COS-sKL and then exposed to S17 CM in secondary culture for 24 hours. As before, pre-B cells were not generated in primary cultures containing either IL-7 or sKL + IL-7 (Table 1). However, when S17 CM was added to these cultures at either 24 or 48 hours and cultures extended for and additional 24 hours (a total culture period of 48 and 72 hours, respectively) Cμ+ cells were generated (Table 2). Importantly, the number of pro-B cells (precursors of Cμ+ pre-B cells) in cultures maintained for up to 72 hours in the presence of IL-7 or sKL and IL-7 was not different from that found from primary cultures enumerated at 24 hours (Table 1). Taken together, these data strongly suggest that the addition of IL-7 and sKL to these in vitro cultures does not result in either expansion of B220+ B-cell progenitors or differentiation of these cells to Cμ expression.

**DISCUSSION**

The aim of this study was to determine if IL-7 and an IL-7 synergistic activity secreted by the S17 stromal cell line, which appears to be KL, could potentiate the differentiation of B220+ B-cell precursors into pre-B cells. The data clearly show that neither IL-7 nor KL alone or in combination mediated that event. Instead, the combination of IL-7 and KL apparently costimulate proliferation of pre-B cells after they have differentiated in the presence of as yet uncloned molecules produced by stromal cells. These results corroborate previous observations that KL and IL-7 act synergistically to stimulate proliferation of B-lineage cells and significantly extend these findings to show that additional cytokines are responsible for a separable developmental step in which the maturation of immature B-cell precursors to an IL-7/KL responsive state occurs.

Stromal cell line S17 has been previously shown to potentiate differentiation of early B-lymphoid cells from the marrow, but proliferative activity produced by this cell line has thus far been limited to effects on B220+ BM cells. In this study we demonstrated that, although S17 stroma was not efficient in stimulating proliferation of isolated LBMC nonadherent cells, its presence potentiated the proliferative effect of IL-7. Two observations suggested that the synergistic stimulation of proliferation by IL-7 and S17 cells was not due to the contribution of additional IL-7 to the assay by S17 cells. First, the level of IL-7-induced proliferation in these assays was saturable, and did not increase significantly between 50 and 1,000 U/mL (Fig 1). We also could not demonstrate IL-7 mRNA in S17 cells by Northern analysis (Fig 2) or after 30 cycles of PCR amplification (unpublished observation). These data suggested the presence of a unique S17 stromal cell-derived cytokine that synergized with IL-7 in lymphoid cell proliferation.

One recent report suggested that BM adherent cells produce KL and that KL costimulated IL-7-mediated growth of B-lineage cells from normal BM. However, the adherent cells used in that study were an uncloned primary cell line and likely contained several cell types. We found that the cloned S17 stromal cell line produced several species of KL mRNA by Northern analysis (unpublished data) and tested whether this molecule could account for the S17-derived synergistic activity described herein. Recombinant KL reproduced the IL-7-potentiating effect of S17 supernatants in the absence of a stromal cell underlayer (Fig 3A). While this observation does not rule out the possibility that S17 produces multiple cytokines that potentiate IL-7 effects, it strongly suggests that KL is a primary mediator of this effect.

Our previous studies showed that S17 stromal cells potentiated differentiation of pre-B cells (Cμ+, Ig light chain-negative cells) from B220+ progenitor cells resident in the marrow. Previous reports from this laboratory have shown that this pre-B–differentiation activity is released from BM stromal cells into the growth medium and that it resides with a relatively small (<10 Kd molecular weight) molecule in supernatants from the S17 cell line. The results presented here strongly suggest that this previously described activity of S17 cells does not appear to be due to KL, IL-7, or the combination of these two cytokines. Differentiation and proliferation of B-cell progenitors, then, appear to be regulated by different stromal cell-derived cytokines. S17 cells do not have detectable IL-7 mRNA or directly stimulate significant proliferation of B-lineage cells, but are efficient in stimulating differentiation of pre-B cells and potentiating IL-7-mediated expansion of differentiated cells.

**Table 1. Effect of IL-7 and sKL on the Expansion of Pro-B Cells In Vitro: Pre-B Cell Generation in Secondary Culture After Extended Culture Addition**

<table>
<thead>
<tr>
<th>Primary Culture Supplement</th>
<th>Length of Primary Culture (h)</th>
<th>At Termination of Primary Culture</th>
<th>24 h After Addition of S17 CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24</td>
<td>&lt;0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>IL-7</td>
<td>48</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>IL-7 + sKL</td>
<td>24</td>
<td>&lt;0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>IL-7 + sKL</td>
<td>48</td>
<td>0.2</td>
<td>2.1</td>
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BM cells were depleted of B220+ cells on 14.8 antibody-coated plates and initially cultured for 24 or 48 hours in the presence of IL-7 or IL-7 and sKL before addition of S17 CM and IL-7 for an additional 24 hours. At termination of cultures, absolute numbers of Cμ+, Ig- cells were determined by immunofluorescence. Data are representative of four virtually identical experiments.
McNiece et al.\textsuperscript{26} reported that the combined addition of IL-7 and KL stimulated differentiation of B220-depleted BM cells to detectable B220 expression. This conclusion is not supported by our studies and there are several possible explanations for this apparent difference. While our data are reported as absolute cell numbers from short-term cultures, McNiece et al. used 14-day cultures and analyzed only the frequency of B220-expressing cells at the termination of culture. This difference in analysis is further complicated by the possibility that B220\textsuperscript{+} cells remained in the cell population used for cultures. B220 depletion in our hands requires several rounds of depletion for efficient removal of detectable B220\textsuperscript{+} and Cp\textsuperscript{+} cells from BM cell suspensions. B220 expression on these cells varies quantitatively and pre-B cells express lower density of this antigen than that found for B cells (unpublished observations). McNiece et al.\textsuperscript{26} report using a single depletion step and the resulting cell population used for culture may have included substantial numbers of cells that express B220 at low density. Certainly any remaining B220\textsuperscript{+} pre-B cells would respond dramatically to the combination of IL-7 and KL used and predominate in cultures by 14 days. It should also be noted that the studies reported herein use Cp\textsuperscript{+} expression as an independent marker of efficiency of B220 depletion to circumvent detection problems associated with possible B220 antigen modulation during the depletion procedure. Finally, because the two reports have used different endpoints (B220 and Cp\textsuperscript{+} expression), it remains possible that IL-7 and KL stimulate either differentiation to B220 expression or proliferation of B220\textsuperscript{+}, Cp\textsuperscript{+} cells, but other stromal cell-derived cytokines are necessary for Cp\textsuperscript{+} expression. Experiments are currently in progress to further resolve these differences.

These data contribute to our working knowledge of differentiation and expansion of this essential immune responsive cell population. In vivo kinetic data suggest that proliferation of Cp\textsuperscript{+} pre-B cells may not account for significant cell expansion in this lineage\textsuperscript{38} and could not account for the necessary expansion of cells that have related antibody specificity after Ig heavy chain rearrangement. Lee et al.\textsuperscript{39} concluded that the majority of cells that proliferate in response to IL-7 are B220\textsuperscript{+} and our results are clearly in agreement with that study. In addition, our studies have not detected significant expansion of pre-B-cell precursors (pro-B cells) in extended culture of B220-depleted BM in the presence of KL and IL-7 (Table 2). Taken together, these data suggest that the B-lineage cell population responsible for clonotype expansion expresses B220 but not Cp. Cells with this phenotype have been previously described\textsuperscript{38} and are notably more frequent in embryonic liver than in postnatal mouse BM.\textsuperscript{39} Candidate target cells for IL-7-mediated proliferation may express B220 and have D-J Ig gene rearrangements but do not express detectable Cp\textsuperscript{+}-heavy chain.\textsuperscript{40} Cells with this phenotype have been shown to proliferate for extended periods in the presence of IL-7 and BM stromal cells\textsuperscript{40} and may represent a discrete proliferating compartment in the B lineage that accounts for the necessary clonotype expansion in the marrow. Although these cells are capable of in vivo reconstitution of B cells in scid mice,\textsuperscript{41} their relevance to expansion of normal B-lineage cells in the marrow, normal population size, and growth requirements of their normal counterparts require further investigation.

Regulation of B-cell development apparently depends on separable cytokines that affect cellular proliferation and differentiation. Differentiation of pro-B cells to initiate Ig-heavy chain gene expression is regulated by secreted factors from S17 stromal cells that do not in and of themselves stimulate significant proliferation of these cells. Pre-B cells that express Cp\textsuperscript{+} heavy chain and develop in the presence of stromal-derived differentiation cytokines are then receptive to IL-7-induced proliferation. The IL-7-dependent proliferative response of at least some of these pre-B cells can be upregulated by costimulation with KL; however, to date we have not detected differentiative potential for pre-B-cell formation by any combination of these two cytokines. Costimulation with KL and IL-7, then, either results in recruitment of additional cells into cell cycle or expression of functional IL-7 receptor complexes on developing B-lineage cells that did not previously express them.

This working model of B-cell differentiation in the marrow contrasts dramatically with the regulation of proliferation and differentiation in myeloid lineages. Colony-stimulating factors potentiate both proliferation and differentiation of target cells and purely differentiative signals have not been described. It will be important now to further characterize and clone differentiative cytokines produced by S17 cells and to determine whether they function as commitment or differentiation stimuli in the expression of differentiated cell phenotype in the lymphopoietic system. The role of KL in lymphopoiesis appears to be primarily a potentiating signal for the expansion of cells that have already undergone heavy chain gene rearrangement and expression of Cp\textsuperscript{+}. However, the proliferation of earlier cells in the lineage will likely involve related molecules.

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Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis

LG Billips, D Petitte, K Dorshkind, R Narayanan, CP Chiu and KS Landreth