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Stromal Cell-Independent Maturation of IL-7-Responsive Pro-B Cells

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The proliferation, survival, and differentiation of B cell progenitors in primary hematopoietic tissues depends on extracellular signals produced by stromal cells within the microenvironment. IL-7 is a stromal-derived growth factor that plays a crucial role in B lineage development. We have shown that in the presence of IL-7, pro-B cells proliferate and differentiate to a stage in which they are responsive to stromal cells and LPS, leading to terminally differentiated IgM-secreting plasma cells. In this report, we examine in detail the role of stromal cells in the transition from the IL-7-responsive pro-B cell stage to the mature LPS-responsive B cell stage. We demonstrate that this transition fails to occur, even in the presence of stromal cells and LPS, if constant exposure to IL-7 is maintained. The transition from the large pro-B cell stage to the small cμm1 pre-B cell stage occurs independent of stromal cells. Moreover, the “stromal cell-dependent” maturation that occurs subsequent to the expression of surface IgM leading to responsiveness to B cell mitogens can also be accomplished in the absence of stromal cells if pre-B cells are cultured in proximity to each other or at high cell concentrations. Together these results suggest that stromal cells mediate B cell differentiation by providing the necessary growth requirements (i.e., IL-7) to sustain the development of pre-B cells. The progeny of these pre-B cells can then differentiate through as yet unidentified homotypic interactions, leading to the production of LPS-responsive B cells.


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ells of the hematopoietic system are dependent upon their microenvironment to both commit and progress through a specific cell lineage. B lymphocytes develop in the liver during fetal life and in the bone marrow of adult animals (1). In vivo, B cell progenitors are found in close association with several heterogeneous populations of stromal cells, suggesting that stromal cells support the differentiation of these progenitors (2, 3). It is currently unclear whether stromal cells merely provide the necessary requirements for the growth and survival of B cell progenitors or play an inductive role in regulating lymphopoiesis. Bone marrow stroma is composed of a number of diverse cell types, including adventitial reticular cells, fibroblasts or endothelial-like cells, macrophages, and adipocytes (4, 5). Due to the heterogeneity of the bone marrow, the nature of the interaction between lymphocytes and their environment has been difficult to study.

In vitro systems involving long-term bone marrow cultures have been used effectively to study the microenvironmental interactions of hematopoietic precursors (6–9). Much of our understanding of the biology underlying stromal support of B lymphopoiesis is derived from the study of murine stromal cell lines (10). From these studies, it has become apparent that stromal cells provide a variety of signals to lymphocyte progenitors via both cell-cell interactions and the secretion of soluble mediators (10–12). However, stromal cell lines vary in their ability to promote the proliferation and differentiation of B cell progenitors (13–18). In vitro culture assays using S17 stromal cells have permitted us to study several discrete stages of B cell development (19). Uncommitted progenitors from both day 10 yolk sac and day 12 fetal liver have been shown to be dependent on S17 stromal cells for their growth and differentiation into the B lineage in vitro (20–22). In these cultures, growth factors have been identified (IL-11, MGF, FL, IL-7) that can replace the function of S17 stromal cells in mediating the commitment and maturation of IL-7-responsive pro-B cells from uncommitted progenitors (23, 24). Pro-B cells are predominantly large, cycling cells that are in the process of recombining Ig heavy chain genes and express B220, CD43, BP-1, and L5 (25–28). These IL-7-responsive cells subsequently give rise to small, noncycling pre-B cells that are CD43+, BP-1+, and cytoplasmic μm1 (cμm+)3 cells that undergo rearrangement at the light chain locus (29, 26–28). The events that regulate the transition to an immature surface IgM+ (slgM+) cell and subsequent stages are currently unknown, although stromal cells appear to play a key role in regulating these events (15, 30–33).

In vivo, the development of mature B lymphocytes has been shown to be dependent on IL-7 signaling (34–37). IL-7 was originally identified as a soluble growth factor with lymphopoietic activity (38), and was the first cytokine to be identified and cloned from a stromal cell line (39). IL-7 has been shown to be required for both the proliferation and differentiation of committed B cell progenitors in vitro (32, 40, 41). However, IL-7-responsive cells require stromal cells to mediate their differentiation to mature B cells, which are responsive to the B cell mitogen, LPS (40). The requirements for the transition from an IL-7-responsive pro-B cell to a functionally mature slgM+ B cell remain largely unknown.

3 Abbreviations used in this paper: cμm, cytoplasmic μm1, slgM+, surface IgM+; cμ, surface μm1; BCR, B cell receptor; prodBm1, B220+ day 15 fetal liver cells cultured in IL-7 for 4 days.

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Previous studies have shown that the removal of IL-7 from cultures containing IL-7 and stromal cell-dependent B cell clones results in the induction of V_{H} to D_{H} and V_{L} to I_{L} rearrangements (42, 43). Although some cells become slgM^{+} and phenotypically mature, very few (<1:3000) of these cells respond to LPS, further suggesting that stromal cells are required to mediate the transition to a functionally mature stage of B cell development (44).

The precise role of stromal cells in mediating the maturation of B cell progenitors is currently unclear. We therefore set out to determine the specific stages in which stromal cells interact with B cell progenitors to mediate their differentiation to the LPS-responsive stage. We show that the maturation of pro-B cells cocultured with S17 + LPS is influenced by the presence or absence of IL-7. Upon removal of IL-7, pro-B cells differentiate into small spl pre-B cells that contain a greater number of rearrangements at the \( \kappa \) locus. This transition is independent of stromal cells. Moreover, in the continued presence of IL-7, pro-B cells fail to differentiate into small pre-B cells and fail to undergo stromal cell-dependent maturation. Cells that become surface \( \mu^{+} \) (sIgM^{+}) in our culture system can mature to the LPS-responsive stage if they interact with stromal cells in a contact-dependent manner. The nature of the stromal cell-mediated signal(s) that influence differentiation to the mitogen-responsive stage remain to be elucidated. Interestingly, we have found that the “stromal cell-dependent” maturation event can also be accomplished by culturing IL-7-responsive pre-B cells in proximity to each other or at a high cell density. These results raise the possibility that stromal cells mediate lymphopoiesis by providing the necessary growth factors (such as IL-7) that regulate the growth and differentiation of B cells that, at the appropriate stage, mature further through homotypic interactions.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the animal colony of the Wellesley Hospital Research Institute. Timed pregnancies were established by mating mice overnight and observing vaginal plugs the following morning on day 0. Pregnant females were killed by cervical dislocation on day 15 of gestation.

Cell purification

Cell suspensions were prepared from pooled day 15 fetal livers by passage through a 26-gauge needle; debris was removed by gravity sedimentation on ice for 5 min. The cell suspension was collected and cell viability was determined by trypan blue exclusion. B cell progenitors from day 15 fetal liver cell suspensions were isolated by enriching for B220^{+} cells using 14.8 coated panning plates (2 \times 10^{4} cells/ml/plate). Panning was performed using Optiplex 100-mm plastic petri dishes (Falcon no. 1001, Oxford, CA). Petri dishes were first coated with mouse anti-rat IgG (10 \( \mu \)g/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) at 0.05 M Tris-Cl, pH 9.5, 0.15 M NaCl at room temperature for a minimum of 1 h. The plates were washed three times in 5% FCS/balanced salt solution, followed by the addition of 4 ml of 1.2 diluted 14.8 hybridoma supernatant. Plates were then incubated overnight at 4°C. The plates were washed three times in 5% FCS/balanced salt solution followed by the addition of cell suspensions at 2 \times 10^{4} cells/plate. Cells were incubated at 4°C for 1 h. Adherent cells were recovered by scraping with a plastic scraper (no. 3010; MatTek, Ashland, MA) after carefully washing the plates eight times in 5% FCS/balanced salt solution. The recovered cells were centrifuged at 1250 rpm for 5 min and resuspended in 2 to 3 ml of cold 10% FCS/ OptiMEM (Life Technologies, Grand Island, NY). Cell viability was again determined by trypan blue exclusion. Typically, 2 to 3 \times 10^{3} cells were recovered per day 15 C57BL/6 fetal liver, of which approximately 1.4% were B220^{+}.

Cell culture conditions

Cells were maintained in OptiMEM supplemented with 10% FCS, 50 \( \mu \)M 2-ME, 2.4 g/l NaHCO_{3}, 100 \( \mu \)g/ml penicillin, 100 \( \mu \)g/ml streptomycin, and the appropriate growth factors at saturating concentrations in a humidified atmosphere of 5% CO_{2}, at 37°C. B220^{+} day 15 fetal liver cells were cultured (2 – 5 \times 10^{4} cells/well) in a 24-well plate for 4 days in the presence of recombinant human IL-7 (100 U/ml) (Immunex, Seattle, WA) to further enrich for IL-7-responsive pro-B cells (proB_{A4-IL7} cells). There was a 6- to 10-fold increase in the number of viable cells cultured under these conditions. IL-7 was removed by washing cells twice with 10 ml of 10% FCS/OptiMEM. The differentiation of proB_{A4-IL7} cells to the mitogen-responsive stage was quantitated in a maturation assay by placing 10^{3} cells in a secondary culture (96-well microtiter plate) containing 10^{3} irradiated (2000 rad) S17 stromal cells and 15 \( \mu \)g/ml LPS (Salmonella typhosa W0901; Difco, Detroit, MI). IgM secretion was measured 7 days later in an ELISA assay. A total of 10^{3} S17 stromal cells were determined to be the minimal number of stromal cells required to mediate the maturation of 10^{3} proB/A4-IL7 cells in liquid cultures.

To determine whether the stromal cell-dependent maturation was contact dependent, S17 stromal cells were separated from proB/A4-IL7 cells in secondary cultures using Nunc TC inserts (Nunc, Dusseldorf, Germany) for flat-bottom 96-well microtiter plates. mAbs and reagents used to target stromal cell-mediated maturation were as follows: anti-CD44 (IM7 or KM114), anti-CD40 (9C10 and R1-2), 0.1 \( \mu \)g/ml anti-CD (33.60) (45), 1 \( \mu \)g/ml anti- \( \kappa \) (1050-01) (Southern Biotechnology, Birmingham, AL), anti-lambda (FS1) (46), anti-CD19 (1D3), anti-CD81 (2F7), anti-CD22 (Cy341), and anti-CD40 (3/23). mAbs were purchased from PharMingen (San Diego, CA) without sodium azide and used at 10 \( \mu \)g/ml, unless otherwise stated. FMA (Sigma, St. Louis, MO) and 2-mercaptoethanol (Calbiochem, La Jolla, CA) were used at 1 ng/ml.

ELISA

ELISAs were performed by coating enzyme immunosorbant assay (ELISA) plates (Costar, no. 3950) with 5 \( \mu \)g/ml of affinity-purified goat anti-mouse \( \mu \)-chain Ab (Jackson ImmunoResearch Laboratories) for 30 min at 37°C. Plates were washed twice with cold tap water and blocked for 30 min at 37°C with 5% FCS/PBS followed by an additional eight washes with cold tap water. Ten-fold serial dilutions of culture supernatants in 5% FCS/PBS were added to the plates and incubated for 30 min at 37°C. Plates were washed eight times in cold tap water and a 1:2000 dilution of goat anti-mouse \( \mu \)-chain conjugated to horseshad peroxidase (Sigma) was added for 30 min at 37°C. Plates were again washed eight times in cold tap water followed by the addition of 50 \( \mu \)l of the substrate containing of 0.5 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.05 M phosphate-citrate buffer, and 0.03% sodium perborate (Sigma). Plates were further incubated for 30 min at 37°C and the absorbance was read at 405/630 nm. Control experiments revealed that the rat anti-mouse \( \mu \)-chain mAb, 33.60 (Ox), did not interfere with the detection of secreted IgM in the ELISA assay.

Proliferation assays

B220^{+} cells from day 15 fetal livers were cultured for 4 days (proB/A4-IL7 cells) in the presence of recombinant human IL-7 (100 U/ml). ProB/A4-IL7 cells were subsequently transferred to 96-well microtiter plates (10^{3} cells/well) containing medium, 10^{3} irradiated S17 stromal cells, IL-7 (100 U/ml), LPS (15 \( \mu \)g/ml), S17 + IL-7 or S17 + LPS + IL-7, and cultured for an additional 4 days. Six hours before the end of culture, wells were pulsed with 1 \( \mu \)Ci of \[^{3}H\]TdR (DuPont, Wilmington, DE). Lysed cells were harvested onto microplate filters, and radioactivity was measured in a scintillation counter (Topcount; Canberra Packard, Downers Grove, IL).

Flow cytometric analysis

ProB/A4-IL7 cells (5 \times 10^{3} cells/well) were cultured in 24-well plates in the indicated conditions for 24, 48, or 72 h (irradiated S17 stromal cells were plated at 2 \times 10^{3} cells/well). Harvested cells were stained for the expression of surface markers by incubating approximately 10^{5} cells in 200 \( \mu \)l of PBS and 5% FCS with Abs for 20 min at 4°C. Biotin-conjugated Abs were detected by a subsequent incubation with streptavidin phycoerythrin. Cells were washed three times with 5% FCS/PBS and the fluorescence intensity was measured on a FACScan (Becton Dickinson, Mountain View, CA) followed by analysis with Cell Quest version 3.1 software. Cells were stained for \( \kappa \) expression by fixing the cells in 1% paraformaldehyde for 15 min, followed by two washes in PBS. Cells were incubated with 0.2% Tween-20 in PBS for 15 min at room temperature. Cells were then labeled with goat anti-mouse \( \mu \)-chain Ab conjugated to FITC (Sigma) for 20 min followed by three washes in 5% FCS + 0.2% Tween-20 in PBS. Cells were gated according to forward- and side-scatter characteristics and propidium iodide staining. Cells were stained using Abs (PharMingen) to...
B220 (6B2-PE), BP-1 (6C3-FTC), μ (33.60-FTC) (45), κ (R8-140-FTC), CD22 (Cy34.1-PE), and A5 (FS1-biotin) (46). Iso-type-matched controls were used to determine the background level of staining (<1%).

**Detection of V\(\kappa\) rearrangements**

DNA was isolated by a modified direct PCR lysis method (47). A total of 10^7 cells/μl were resuspended in PCR lysis buffer (10 mM Tris, pH 8.3, 1.8 mM MgCl\(_2\), 50 mM KCl, 0.45% Nonidet P-40, 0.45% Tween-20, 60 μg/ml proteinase K), and incubated at 56°C for 1 h. Samples were heated to 90°C for 15 min to inactivate the proteinase and used directly for PCR. V\(\kappa\) rearrangements were amplified using primers hybridizing to the framework 3 region of V\(\kappa\) elements (V\(\kappa\)con-5′: 5′-GGCTGCAA(C/G)TGCTGTGGCAGCTTG(A/G)TC(T/G)GC-3′ (48) and to 3′ of the J\(\kappa\)5 element (J\(\kappa\)5-3′: 5′-TGGCCAGCTCACTGGATAATGAGCCCTC-3′ (49)). PCR products were analyzed by agarose gel electrophoresis.

**Detection of J\(\kappa\) rearrangements**

SSP amplification was performed in two cycles after a 10 min hot start at 85°C. To control for the number of cell equivalents used from sample to sample, 0.5 μM α-actin primers (α-actin 5′: 5′-GA CATGAGGAGATCTGGACCCAC3′-3′ and α-actin 3′: 5′-CGCA CAACTCTACGCCATCAG-3′) (26) were added during a 10-min pause at 85°C. PCR amplification was performed to proceed for another 25 cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 2 min; plus 5 s/cycle. A 10 min 72°C primer extension period followed. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. 

**PCR reactions**

PCR amplifications were conducted for five cycles after a primer extension period followed. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The concentration of secreted IgM in the culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA).

**Mature B lineage markers**

We have reported previously that the differentiation of B cell progenitors from early fetal liver is dependent upon the presence of stromal cells (40, 50, 51). To determine the specific developmental stages in which stromal cells influence the differentiation of B cell progenitors, we examined the interactions between IL-7-responsive pro-B cells from B220-ε-enriched day 15 fetal liver and the stromal cell line, S17 (52). Fetal liver is the predominant site of lymphopoiesis in the developing fetus during this gestational period and is a ready source of B cell progenitors that have not undergone significant κ rearrangements (49). Previous studies in our laboratory demonstrated that B cell progenitors require an IL-7-responsive phase to differentiate to a stage in which they can interact with stromal cells to achieve a state of mitogenic responsiveness (40, 53, 54). B220+ (a B lineage isoform of CD45) cells were therefore enriched from day 15 fetal livers and cultured for 4 days in the presence of saturating concentrations of IL-7 to obtain a relatively homogeneous population of IL-7-responsive B cell progenitors (proB44+IL-7 cells). This population expressed surface markers characteristic of the late pro-B cell stage of development (i.e., B220+CD19+CD43+HSA-BP-1+, A5-), but was negative for mature B lineage surface markers such as IgM and class II MHC (data not shown). B220+ fetal liver-derived progenitors cultured in the presence of IL-7 for 4 days increased in cell number ninefold, whereas viable cells were not obtained from cultures containing medium alone.

**Cells responsive to IL-7**

Cells responsive to IL-7 differentiate into IgM-secreting plasma cells when transferred to irradiated stromal cells in the presence of LPS (40). In these studies, maturation to the LPS-responsive stage may have been dependent on signals provided by stromal cells and/or the removal of IL-7. To investigate the influence of S17 stromal cells in mediating the differentiation of B cell progenitors in the continued presence of IL-7, we transferred proB44+IL-7 cells to secondary cultures containing irradiated S17 cells in the presence or absence of IL-7 and LPS for an additional 4 days (Fig. 1). ProB44+IL-7 cells showed a slight increase (4.9-fold) in the incorporation of [3H]Tdr after 4 days of culture with S17 cells compared with control cultures containing medium alone (Fig. 1A). The incorporation of [3H]Tdr was used as a convenient assay to measure the proliferation of proB44+IL-7 cells in these cultures since it also correlates with an increase in cell number. The proliferation of proB44+IL-7 cells cocultured with S17 stromal cells was not due to the presence of IL-7 because S17 stromal cells do not produce IL-7 mRNA (55). In contrast, cultures containing proB44+IL-7 cells stimulated with IL-7 had considerable proliferative responses. Progenitors cultured with S17 + LPS + IL-7 showed a significant increase (>32-fold) in thymidine incorporation compared with cultures containing medium alone and a 6.6-fold increase compared with cultures containing only S17 cells. The proliferation of proB44+IL-7 cells in cultures containing S17 + LPS + IL-7 was likely due to their response to IL-7 because the [3H]Tdr incorporation observed in S17 + LPS or LPS-containing cultures was identical to that observed for cultures containing S17 or medium, respectively.

**Next we determined whether IL-7-stimulated proB44+IL-7 cells were able to differentiate as well as proliferate when cocultured with S17 stromal cells in the continued presence of IL-7. Maturation was assessed by measuring the IgM secreted by the progeny of mature B cells that responded to LPS. We have previously used this assay to accurately measure the number of progenitors that have differentiated in our culture system (40). ProB44+IL-7 cells were harvested and transferred to secondary cultures (10^5 cells/well) containing the reagents noted in Figure 1B for an additional 7 days. IgM secretion was not detected in cultures in which proB44+IL-7 cells were transferred to either IL-7-, S17, S17 + IL-7, or medium alone. However, proB44+IL-7 cells cultured with S17 + LPS underwent significant differentiation resulting in IgM-secreting plasma cells as demonstrated by a 52-fold increase in the amount of secreted IgM. These results show that the removal of...
IL-7 from a 4-day IL-7-responsive culture of pro-B cells does not permit their differentiation into LPS-responsive B cells. Cultures of proB d4-IL7 cells stimulated with S17 LPS contained more cells than cultures stimulated with S17 LPS as indicated by the sixfold increase in [3H]thymidine incorporation (Fig. 1A). We therefore expected to observe a corresponding increase in the amount of secreted IgM in the maturation assay (Fig. 1B). Contrary to expectation, we detected equivalent amounts of secreted IgM in both cultures containing S17 LPS and S17 + LPS. The data suggest that the additional pro-B cells found in cultures containing S17 + LPS + IL-7 did not differentiate into mature B cells even though S17 + LPS were present. A titration of proB d4-IL7 cells revealed that the quantity of secreted IgM increases as a function of the initial number of cells cultured in the presence of S17 LPS, demonstrating that our assay was not limiting (see Fig. 2A). A similar titration was also observed in cultures containing S17 + LPS + IL-7 (data not shown).

To directly test whether there was an equivalent frequency of B cell progenitors that differentiated into IgM-secreting plasma cells in cultures containing S17 LPS or S17 + LPS + IL-7, proB d4-IL7 cells were harvested, sorted into sμ− (circles) and sμ+ (squares) fractions (99 vs 1%, respectively) and subsequently cultured in limiting dilutions with LPS, S17 + LPS (open symbol), or S17 + LPS + IL-7 (closed symbol) for 14 days followed by the detection of secreted IgM in an ELISA. The fraction of wells negative for IgM production was plotted against the number of initial cells cultured per well. B. The frequency of proB d4-IL7 cells that gave rise to functionally mature B cells was determined as the number of initial cells per well where 37% (arrow) of the wells were negative for IgM production.

**FIGURE 1.** The response of IL-7-responsive B cell progenitors under various conditions. A, B220 + B cell progenitors from day 15 C57BL/6 fetal livers were cultured in the presence of IL-7 for 4 days (proB d4-IL7 cells). ProB d4-IL7 cells were harvested and cultured (10^5 cells/well) in the indicated conditions for an additional 4 days. DNA synthesis was measured by pulsing cultures with [3H]TdR (1 μCi) 6 h before harvesting. Results are expressed as the mean of five replicates ± SD and are representative of three independent experiments. B, Parallel proB d4-IL7 cell cultures were incubated for 7 days followed by the detection of secreted IgM in an ELISA. Results are expressed as the mean ± SEM from 12 pooled experiments.

**FIGURE 2.** sμ− cells arising in IL-7-stimulated cultures require stromal cells to respond to LPS. A, B220 + day 15 fetal liver cells were cultured in the presence of IL-7 for 4 days. Cells were sorted into sμ− (circles) and sμ+ (squares) fractions (99 vs <1%, respectively) and subsequently cultured in limiting dilutions with LPS, S17 + LPS (open symbol), or S17 + LPS + IL-7 (closed symbol) for 14 days followed by the detection of secreted IgM in an ELISA. The fraction of wells negative for IgM production was plotted against the number of initial cells cultured per well. B, The frequency of proB d4-IL7 cells that gave rise to functionally mature B cells was determined as the number of initial cells per well where 37% (arrow) of the wells were negative for IgM production.
It has been reported previously that B lineage clones, which are dependent on stromal cells + IL-7, differentiate upon the removal of IL-7 or stromal cells (56–58). Consequently, cells lose surface membrane proteins characteristic of pro-B cells including c-Kit, A5, CD43, and BP-1 and gain the expression of CD25 and sIgM. However, only a small fraction of the sIgM+ cells (<1:3000) become reactive to LPS, suggesting that these cells require additional maturational events that enable them to become mitogen responsive (42, 44, 58). To determine the precise stage of B cell development that stromal cells are required to mediate differentiation events, we examined the maturation of proBd4-IL7 cells to the small pre-B cell stage in the presence or absence of stromal cells by flow cytometry (Fig. 3). ProBd4-IL7 cells kept for an additional 48 h in the presence of IL-7 alone expressed B220, A5, and BP-1, but did not express sIgM or κ. The majority of these cells were also large in size as measured by their forward light-scattering characteristics. In contrast, when IL-7 was removed from culture and replaced by medium, S17, or S17 + LPS, proBd4-IL7 cells rapidly (within 24 h, data not shown) decreased in size and lost the surface expression of A5 and BP-1 (Fig. 3). Moreover, we observed an increase in the proportion of cells expressing CD2 and κμ compared with cultures containing IL-7. This surface phenotype is characteristic of small, resting pre-B cells (26, 28, 59). However, proBd4-IL7 cells cultured with S17 + IL-7 maintained a pro-B cell phenotype. These results suggest that differentiation to the small pre-B cell stage is less likely to occur in the presence of stromal cells and IL-7. Apparently, it is the removal of IL-7, rather than the presence of S17 stromal cells, that drives the pro-B cell to pre-B cell transition.

By 48 h, 5 to 10% of the pre-B cells cultured in the absence of IL-7 were sμ+κ−, whereas cultures that contained IL-7 consisted of <1% sμ+κ− cells. However, the absolute numbers of sμ+κ− cells were roughly equivalent (~5 × 10^4) in all cultures (Table I). The difference between the relative and absolute numbers of sμ+κ− cells was likely due to the proliferation of IL-7-responsive pro-B cells, which resulted in a marked reduction of viable cells. The loss of the large, A5+ pro-B cell population in cultures lacking IL-7 could not be attributed to the selective death of this population because the absolute number of cells with a small pre-B cell phenotype in cultures without IL-7 is much greater than the absolute number of large, A5+ pro-B cells.

Stromal cells are not necessary for the differentiation of pro-B cells to the small pre-B cell stage

It has been reported previously that B lineage clones, which are dependent on stromal cells + IL-7, differentiate upon the removal of IL-7 or stromal cells (56–58). Consequently, cells lose surface membrane proteins characteristic of pro-B cells including c-Kit, A5, CD43, and BP-1 and gain the expression of CD25 and sIgM. However, only a small fraction of the sIgM+ cells (<1:3000) become reactive to LPS, suggesting that these cells require additional maturational events that enable them to become mitogen responsive (42, 44, 58). To determine the precise stage of B cell development that stromal cells are required to mediate differentiation events, we examined the maturation of proBd4-IL7 cells to the small pre-B cell stage in the presence or absence of stromal cells by flow cytometry (Fig. 3). ProBd4-IL7 cells kept for an additional 48 h in the presence of IL-7 alone expressed B220, A5, and BP-1, but did not express sIgM or κ. The majority of these cells were also large in size as measured by their forward light-scattering characteristics. In contrast, when IL-7 was removed from culture and replaced by medium, S17, or S17 + LPS, proBd4-IL7 cells rapidly (within 24 h, data not shown) decreased in size and lost the surface expression of A5 and BP-1 (Fig. 3). Moreover, we observed an increase in the proportion of cells expressing CD2 and κμ compared with cultures containing IL-7. This surface phenotype is characteristic of small, resting pre-B cells (26, 28, 59). However, proBd4-IL7 cells cultured with S17 + IL-7 maintained a pro-B cell phenotype. These results suggest that differentiation to the small pre-B cell stage is less likely to occur in the presence of stromal cells and IL-7. Apparently, it is the removal of IL-7, rather than the presence of S17 stromal cells, that drives the pro-B cell to pre-B cell transition.

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Stromal cells are not necessary for the differentiation of pro-B cells to the small pre-B cell stage

It has been reported previously that B lineage clones, which are dependent on stromal cells + IL-7, differentiate upon the removal of IL-7 or stromal cells (56–58). Consequently, cells lose surface membrane proteins characteristic of pro-B cells including c-Kit, A5, CD43, and BP-1 and gain the expression of CD25 and sIgM. However, only a small fraction of the sIgM+ cells (<1:3000) become reactive to LPS, suggesting that these cells require additional maturational events that enable them to become mitogen responsive (42, 44, 58). To determine the precise stage of B cell development that stromal cells are required to mediate differentiation events, we examined the maturation of proBd4-IL7 cells to the small pre-B cell stage in the presence or absence of stromal cells by flow cytometry (Fig. 3). ProBd4-IL7 cells kept for an additional 48 h in the presence of IL-7 alone expressed B220, A5, and BP-1, but did not express sIgM or κ. The majority of these cells were also large in size as measured by their forward light-scattering characteristics. In contrast, when IL-7 was removed from culture and replaced by medium, S17, or S17 + LPS, proBd4-IL7 cells rapidly (within 24 h, data not shown) decreased in size and lost the surface expression of A5 and BP-1 (Fig. 3). Moreover, we observed an increase in the proportion of cells expressing CD2 and κμ compared with cultures containing IL-7. This surface phenotype is characteristic of small, resting pre-B cells (26, 28, 59). However, proBd4-IL7 cells cultured with S17 + IL-7 maintained a pro-B cell phenotype. These results suggest that differentiation to the small pre-B cell stage is less likely to occur in the presence of stromal cells and IL-7. Apparently, it is the removal of IL-7, rather than the presence of S17 stromal cells, that drives the pro-B cell to pre-B cell transition.

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present in cultures containing IL-7 (Fig. 3). This is readily observed in IL-7-stimulated cultures, as the $3.2 \times 10^4 \Delta S^5$ cells present at the end of 48 h cannot account for the absolute number of $\Delta S^5$ cells that are present in cultures containing media ($1.1 \times 10^7$), S17 ($4.1 \times 10^7$), or S17 + LPS ($4.4 \times 10^7$).

Surprisingly, only 1 to 2% of the cells cultured in any condition were $\kappa^+$, even though 5% of the cells in cultures that did not contain IL-7 were $\mu^+$ (Fig. 3). Surface staining for A light chain did not account for this discrepancy (data not shown). Moreover, the majority of the $\kappa^+$ cells were also $\Delta S^5$, suggesting that these are transitional cells that have recently rearranged their light chain locus. The surface expression of $\kappa$ may be regulated independently of rearrangements at the $\kappa$ locus (60). Thus, it was possible that proB$\Delta S^4$,IL-7 cells cultured in the absence of IL-7 contain rearrangements at the $\kappa$ locus, and additional maturation events are required for an increase in the surface expression of $\kappa$ protein. Previous studies have shown that in the absence of IL-7, transcription of both the RAG-1 and RAG-2 genes increase (57, 61). Moreover, the removal of IL-7 leads to an increase of sterile $\kappa$ transcripts, which normally precedes rearrangement at the $\kappa$ light chain locus (48, 61). The data presented in Fig. 4A show the relative level of VJ$\kappa$ rearrangements per cell equivalents in proB$\Delta S^4$,IL-7 cell cultures tested under various conditions for an additional 48 h. We consistently observed a twofold increase in the number of $\kappa$ rearrangements per cell in cultures lacking IL-7. That the increase in $\kappa$ rearrangements was independent of stromal cells is evident from the observation that pro-B cells cultured in medium alone gave identical results. These findings are consistent with the suggestion that stromal cells do not directly influence either the surface phenotype or $\kappa$ rearrangements of differentiating pre-B cells. Instead, it is the absence of IL-7 that induces the transition to the small pre-B cell stage (Fig. 3) and the concomitant rearrangement at the $\kappa$ light chain locus (Fig. 4).

**Immature $\mu^+$ B cells are dependent upon stromal cells to mature to the LPS-responsive stage**

IL-7 plays a significant role in maintaining pro-B cells in a stromal cell-independent state. However, even in the absence of stromal cells, $\mu^+$ cells consistently arise at a low frequency (~1%) in IL-7-stimulated pro-B cell cultures (see Fig. 3). To determine whether these $\mu^+$ cells were competent to make a

mitogen response, we cultured sorted $\mu^+$ and $\mu^-$ proB$\Delta S^4$,IL-7 cells in limiting dilutions with LPS (Fig. 2B). We found that the frequency of progenitors generating IgM-secreting progeny in the presence of LPS was only 3-fold greater in the $\mu^+$ population than in the $\mu^-$ population. However, the number of LPS-responsive cells was still 16-fold less than the frequency of progenitors ($\mu^+$ or $\mu^-$) that will mature when cocultured with S17 + LPS (Fig. 2B). These results suggest that the majority of $\mu^+$ that arise in IL-7-stimulated cultures remain refractory to LPS until they encounter differentiation signals provided by S17 stromal cells.

To determine whether this stromal cell-mediated maturation was contact dependent, we separated S17 cells from proB$\Delta S^4$,IL-7 cells using a transwell insert (Fig. 5). This culture system permits the diffusion of soluble factors through the Anopore membrane but

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>IL-7</th>
<th>Media</th>
<th>S17</th>
<th>S17 + LPS</th>
<th>S17 + IL-7</th>
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</thead>
<tbody>
<tr>
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<td>48</td>
<td>11</td>
<td>9.9</td>
<td>7.5</td>
<td>34</td>
</tr>
<tr>
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<td>1.2</td>
<td>22</td>
</tr>
<tr>
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<td>23</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>47</td>
<td>8.4</td>
<td>3.7</td>
<td>2.7</td>
<td>32</td>
</tr>
<tr>
<td>$\kappa A$</td>
<td>12</td>
<td>6.2</td>
<td>7.3</td>
<td>4.7</td>
<td>12</td>
</tr>
<tr>
<td>CD2</td>
<td>21</td>
<td>8.3</td>
<td>12</td>
<td>8.6</td>
<td>25</td>
</tr>
<tr>
<td>$\mu A$</td>
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<td>0.54</td>
<td>0.53</td>
<td>0.67</td>
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<tr>
<td>kappa</td>
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<td>0.17</td>
<td>0.22</td>
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<tr>
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<td>5.8</td>
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</tr>
<tr>
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<td>6.6</td>
<td>6.0</td>
<td>4.5</td>
<td>26</td>
</tr>
</tbody>
</table>

No. of cells recovered

(\(\times 10^3\))

<table>
<thead>
<tr>
<th>Alive</th>
<th>48</th>
<th>11</th>
<th>9.9</th>
<th>7.5</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>

$^d$ Absolute number of B cell progenitors \(\times 10^3\) after 48 h of culture.

$^c$ Large cells were arbitrarily defined as those with FSC > 460 channels.

$^b$ Initial number of pro-B cells cultured was \(1.5 \times 10^6\).

$^a$ Live cells were determined by trypan blue exclusion.

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**FIGURE 4.** Withdrawal of IL-7 leads to a greater proportion of $\kappa$ rearrangements in pre-B cells, independent of stromal cells. A, B220$^+$ B cell progenitors were isolated from day 15 fetal liver and incubated with IL-7 for 0, 2, or 4 days. On day 4 of culture, cells were placed in secondary cultures for an additional 48 h. DNA was prepared and subjected to PCR amplification for the analysis of VJ$\kappa$ rearrangements. The PCR products from \(10^3\) cell equivalents were analyzed by Southern blotting and probed using an oligonucleotide sequence hybridizing 3’ of the J$\kappa$5 element in the J$\kappa$C intervening sequence. Band intensities were quantified using a PhosphorImager. Amplification of actin was used to control for the number of cell equivalents. VJ$\kappa$5 rearrangements corrected for actin are expressed relative to \(10^3\) cell equivalents of S107, which is VJ$\kappa$5 rearranged on one allele. B, The relative VJ$\kappa$5 rearrangements of S107 cells titrated into MC9 cells (germ line for $\kappa$ rearrangements).
prevents contact between the stromal cells and proB<sub>d4-IL7</sub> cells. When contact with S17 in the presence of LPS was permitted, there was significant IgM secretion in the culture supernatant compared with cultures containing LPS alone. However, in cultures containing the transwell, the amount of secreted IgM was significantly reduced. This result demonstrates that small pre-B cells require contact with stromal cells to differentiate to a stage of LPS responsiveness. It was possible that stromal cell contact was necessary for the induction of secreted growth factors that mediate the pre-B to mature B cell transition. To assess this possibility, we cultured irradiated 18.81 pro-B cells or 70Z/3 pre-B cells with either S17 or LPS alone in the lower chamber of cultures containing primary proB<sub>d4-IL7</sub> cells in the upper chamber. The incubation of B lineage cell lines with S17 cells did not overcome the requirement for the pre-B stromal cell contact (data not shown). In addition to mediating the maturation of B cell progenitors, S17 cells enhance the cloning efficiency of mature splenic B cells in response to LPS (40). It was possible that LPS indirectly influenced the maturation of B cell progenitors by inducing growth factors or surface receptors on S17 cells. Stromal cells are known to secrete cytokines and regulate the expression of adhesion molecules in response to LPS and other cytokines on S17 cells. Stromal cells are known to secrete cytokines and regulate the expression of adhesion molecules in response to LPS and other cytokines (62, 63). To determine whether S17 cells could mediate the maturation of pre-B cells in the absence of LPS, we cultured proB<sub>d4-IL7</sub> cells in the presence of S17 for various lengths of time and placed the harvested cells in secondary cultures containing 15 μg/ml of LPS (Fig. 6). IgM secretion was measured 7 days after the addition of LPS in the secondary cultures to assess the degree of maturation that had occurred. Compared with the proB<sub>d4-IL7</sub> cells that were cultured with S17 + LPS for the entire 7-day period, we found that proB<sub>d4-IL7</sub> cells cultured with S17 for 48 h and subsequent culture with LPS in the absence of S17 generated cells that secreted equivalent amounts of IgM (Fig. 6). Control cultures containing proB<sub>d4-IL7</sub> cells incubated with LPS alone consistently contained only low levels of secreted IgM. Together, these results suggest that within 48 h, stromal cells alone are sufficient to directly mediate the maturation of pre-B cells into LPS-responsive B cells.

Abs recognizing CD19 or the B cell receptor (BCR) inhibit the generation of IgM-secreting cells

Previous studies have demonstrated that progression through the B cell lineage requires signaling through both the pre-BCR and BCR (64, 65). Signaling through the pre-BCR promotes the differentiation of pro-B cells to the small, c<sup>+</sup> pre-B cell stage (64, 65). We have attempted to block the interaction between proB<sub>d4-IL7</sub> cells and S17 cells in our culture system using mAbs directed against surface Ags associated with BCR signaling (Fig. 7). Anti-CD44 (IM7) (66, 67) and anti-VLA-4 mAbs (68, 69) have been shown to block B cell progenitor-stromal cell interactions in Whitlock-Witte cultures, preventing the subsequent maturation of these progenitors. However, we found that these Abs had no effect on B cell maturation in our culture system. These results suggest that the critical interaction mediated by these integrins occurs before the small pre-B cell stage. Strikingly, we did not observe secreted IgM in cultures containing mAbs recognizing μ heavy chains or κ light chains (Fig. 7). Similar results were obtained with PMA + ionomycin, which mimics the downstream effects of BCR signaling (70). Moreover, the amount of IgM secretion detected in S17 + LPS-stimulated proB<sub>d4-IL7</sub> cell cultures containing anti-μ, anti-κ Abs or those containing PMA + ionomycin was substantially less than that observed in cultures containing LPS alone.

Abs recognizing the pre-BCR (FS1, anti-μ) (46) as well as anti-CD81, anti-CD22, and anti-CD40 had no effect on blocking the pre-B cell-stromal cell interaction. In addition, we found that these Abs were unable to mediate the maturation of proB<sub>d4-IL7</sub> cells by replacing the activity of stromal cells in our culture system (data not shown). In contrast, mAbs recognizing...
CD19 (1D3) significantly reduced the number of cells that generated LPS-responsive cells in the presence of S17 LPS (Fig. 7). Murine CD19 has recently been shown to associate with CD21, CD35, and CD81 (71). Moreover, anti-CD19 Abs enhance signaling through both the m/surrogate light chain complex of primary pre-B cells and in mature B cells (71). The inhibition of IgM secretion in proBd4-IL7 cell cultures containing anti-CD19 Abs may have been due to the modulation of a negative signal similar to that observed with the anti-μ and anti-κ Abs. Alternatively, the presence of anti-CD19 Abs in these cultures may have interfered with CD19-dependent cell-cell interactions.

ProBd4-IL7 cells cultured in proximity to each other differentiate into LPS-responsive B cells in the absence of stromal cells

We found that proBd4-IL7 cells cultured at a high cell density (10⁴ cells) generate IgM-secreting cells in response to LPS in the absence of stromal cells (Fig. 8A), whereas cultures containing 5- to 10-fold fewer proBd4-IL7 cells produced 200-fold less IgM. This observation suggests that at high proBd4-IL7 cell concentrations, stromal cells are not required to mediate the proBd4-IL7 cell to mature B cell transition. Although >99% of the proBd4-IL7 cells express surface markers characteristic of pro-B cells, we considered the possibility that rare stromal cells may be present in sufficient numbers at the high cell concentrations to mediate this interaction. Alternatively, B cell progenitors themselves at high cell concentrations may mediate B-B interactions that replace the requirement for stromal cells. To address this issue, proBd4-IL7 cells were cocultured in flat-bottom, U-bottom, and V-bottom plates with LPS or S17 + LPS (Fig. 8B). ProBd4-IL7 cells cultured in U-bottom or V-bottom plates (10³ cells/well) in the presence of LPS generated significantly greater amounts of IgM (100-fold) compared with proBd4-IL7 cells incubated in flat-bottom plates with LPS. Moreover, the supernatants from U- or V-bottom plate cultures containing as few as 500 proBd4-IL7 cells stimulated with LPS alone (data not shown). A titration of S17 cells in flat-bottom plates containing 10³ pro-B cells stimulated with LPS revealed that there was substantially less IgM secreted in cultures containing fewer than 10³ S17 cells, and essentially no IgM secretion was detected in cultures containing 100 S17 cells (data not shown). Therefore, the maturation of proBd4-IL7 cells observed in U- or V-bottom plate cultures containing LPS in the absence of S17 cells was unlikely to be due to contaminating stromal cells. These results suggest that proBd4-IL7 cells cultured in proximity to each other do not require stromal cells to mediate the transition from a pre-B cell to a mature B cell generating secreted IgM in response to LPS stimulation.

Discussion

Stromal cells are known to promote the proliferation and differentiation of B lymphocytes. However, many stromal cell lines mediate only the early proliferative stages of B lymphopoiesis, and fail to support differentiation beyond the pre-B cell stage (10, 13, 72, 73).
Similar to primary stroma (30), S17 stromal cells promote the differentiation of IL-7-responsive B cell progenitors to a sIgM + stage in which these mature B cells are responsive to LPS in both liquid and soft agar cultures (40, 19). In this report, we have shown that IL-7-responsive pro-B cells will proliferate in response to saturating concentrations of IL-7, even if provided with an environment suitable for supporting differentiation to the mature LPS-responsive stage. IL-7-responsive B cells found in cultures containing S17 + LPS + IL-7 are similar to the proB54-IL7 cells present at the initiation of the maturation assay. Therefore, there is a greater number of IL-7-responsive B cell progenitors in cultures containing S17 + LPS + IL-7 that have the potential to further differentiate in the presence of stromal cells compared with cultures that are not stimulated with IL-7. However, these additional IL-7-responsive pro-B cells fail to undergo stromal cell-mediated maturational events leading to the stage of LPS responsiveness. These results suggest that IL-7 regulates the development of B cell progenitors in vivo by selectively increasing the pool of IL-7-responsive pro-B cells. Pro-B cells that become unresponsive to IL-7 stimulation proceed to the small pre-B cell stage and become capable of the further differentiation in the presence of stromal cells. Previous studies have described the differentiation of stromal cell + IL-7-dependent pro-B cell clones to the pre-B cell stage when IL-7 is removed from these cultures (42, 44). We have found that this pro-B to pre-B cell transition is independent of whether or not stromal cells are present (Fig. 3). Within 48 h of removing IL-7 from these cultures, cells become smaller and lose the expression of several surface markers characteristic of pro-B cells such as A5 and BP-1. Moreover, we also observe an increase in the proportion of CD2 + and mµ - cells upon the removal of IL-7, indicating that an increase in pre-B cell differentiation has occurred. Therefore, IL-7 negatively regulates B cell differentiation by maintaining cells in a proliferative, pre-B cell state, independent of stromal cells.

The possibility that IL-7 regulates the expression of A5 and BP-1 is suggested by a significant decrease in surface expression of these markers, already obvious 24 h after IL-7 withdrawal, even though total cell numbers remain approximately the same (Fig. 3, data not shown). Essentially all pro-B cells (>95%) stimulated with IL-7 express A5 as determined by surface staining with F41 mAbs. However, mµ - cells are not detected in this population despite the fact that approximately 18 to 27% of these A5 + cells express mµ . Previous studies have shown that surrogate light chain (A5/N5µpreB) is expressed on the surface of pro-B cells before the formation of mµ in a complex with a surrogate heavy chain (gp130) (46, 74). However, it remains unclear whether the gp130 protein is expressed past the large pro-B cell stage. In vivo, B cell progenitors that produce a functional pre-BCR proceed to the small pre-B cell stage as a result of the pre-BCR. Our failure to observe µ at the cell surface in IL-7-stimulated cultures may be due to the fact that the majority of µ-chains found in fetal liver-derived mµ pro-B cells cultured fail to associate with the surrogate light chain. Recent studies have described an early c-Kit + mµ - pro-B cell population in the bone marrow of normal mice in which only half of the mµ-chains expressed have the capacity to form a pre-BCR (75). Moreover, fetal-associated µ heavy chains have been identified that permit pre-B cell proliferation in fetal liver, despite their failure to efficiently associate with a surrogate light chain (76). It is therefore possible that IL-7 promotes the proliferation of a small population of pro-B cells that have rearranged their heavy chain locus but fail to generate a functional pre-BCR. Upon the removal of IL-7, a greater proportion of proB34-IL7 cells express mµ (Fig. 3). Presumably, those that express a functional pre-BCR at the cell surface exit the cell cycle and differentiate into small pre-B cells.

Several groups have shown that transgenic complementation of RAG-deficient mice with a µ heavy chain permits the progression of developmentally arrested pro-B cells to the small pre-B cell stage (64, 65, 77). Transition to the small pre-B cell stage is associated with decreased expression of A5 transcripts (64, 77, 78). Cells at the small pre-B cell stage also exhibit an increase in the expression of germ-line κ transcripts concomitant with increased recombination at the κ locus (61). Pre-B cells from µ/Rag-2 - / - mice were also shown to have substantially decreased proliferative responses to IL-7 (65). These observations suggest that a functionally rearranged heavy chain promotes the differentiation of IL-7-responsive pro-B cells to an IL-7-unresponsive pre-B cell stage. The removal of IL-7 in pro-B cell cultures similarly mediates the transition to the small pre-B cell stage in which κ rearrangements occur. Although it is possible that stromal cells have a functional role in regulating the availability of IL-7 in vivo, we have shown that the pro-B cell to pre-B cell transition occurs independent of stromal cells in our culture system. A stromal cell-dependent stage of maturation does occur subsequent to the expression of sIgM. Thus, mµ + cells still require stromal cells to generate IgM-secreting cells in response to LPS stimulation.

Studies in which IL-7Rα - / - B cell progenitors from Whitlock-Witte cultures were reconstituted with mutant IL-7Rα-chains via retroviruses-mediated gene transfer have demonstrated that the proliferative signals generated by IL-7 signaling are distinct from those mediating differentiation (41). Mutant IL-7Rα receptors that abrogated the ability of the IL-7R to induce the proliferation of B cell progenitors were able to promote the differentiation of these cells to the mµ + stage. In contrast, other chimeric IL-7Rα-chains proficient in stimulating proliferation could not mediate this transition. These studies show that the differentiative function of IL-7 signaling is independent of proliferation but does not rule out the possibility that the proliferative response to IL-7 inhibits further differentiation. It is likely that IL-7-responsive pro-B cells are not at a stage responsive to stromal cell-mediated maturation and only become so following the absence of IL-7 signaling. Alternatively, it is possible that the signals generated through the IL-7 receptor are dominant to stromal cell-mediated signals and thereby prevent the cell from progressing to the next stage of differentiation.

Contrary to previous reports (42, 57, 58), we do not observe an increase in the percentage of κ + cells upon the removal of IL-7. Clearly, there is an increase in the proportion of pre-B cells expressing CD2, mµ - , and sµ - chains, as well as an increase in the number of κ rearrangements when IL-7 is removed from culture. However, there is little difference in terms of the absolute number of sµ - or κ + mature B cells compared with cultures containing IL-7, even after 72 h. Cultures stimulated with IL-7 contain a three- to fivefold greater number of viable pro-B cells and consequently contain relatively fewer sµ - cells. Interestingly, the proportion of κ rearrangements increased in the absence of IL-7, although the proportion of cells expressing κ at the cell surface did not. It is possible that κ-rearranged pre-B cells require additional maturation signals that enable the surface expression of κ. This possibility is in agreement with a previous report that showed a high proportion of stromal cell-dependent pre-B cell colonies with rearranged light chain genes but undetectable mature κ transcripts (60). Contact between the S17 cells and the B cell progenitors was prevented by culture in semisolid medium. The lack of cell contact may have eliminated the signals required to promote the expression of mature κ transcripts and subsequent expression of IgM on the cell surface. Alternatively, many of the κ-rearranged cells we observed in Figure 4 may have been nonproductively rearranged and were therefore unable to express κ on the cell surface.

We consistently observe approximately 1% sµ + cells continuously arising in cultures containing fetal liver-derived IL-7-responsive pro-B (Fig. 3). Although the frequency of LPS-responsive
cells found in the sIgM− population is three times that found in the sIgM+ population, the sIgM+ B cells still require stromal cells to differentiate to an LPS-responsive stage (Fig. 2). Therefore, it is likely that the stage of maturation mediated by stromal cells is subsequent to μ being expressed on the cell surface. These observations are consistent with reports from other in vitro culture systems in which the surface deposition of IgM on immature B cells is insufficient to render these cells mitogen responsive (44).

We have previously tried to replace the stromal cell-mediated maturation signal with several known cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-11, LIF, steel factor, M-CSF, IGF-1, and TSLP), but have been unsuccessful in overcoming the requirement for stromal cells at this stage of development (19, 40, 54). In this report, we have attempted to identify the molecules involved in this interaction by targeting several adhesion and signaling molecules known to be on the surface of pre-B/immature B cells (Fig. 7). Abs recognizing the cell adhesion molecules CD44 (66, 67) and VLA-4 (68, 69) were shown to completely inhibit the production of lymphoid and myeloid cells in long-term bone marrow culture (8, 9). We propose that the critical CD44 and/or VLA-4 interactions between B cell progenitors and stromal cells occur before the pre-B cell stage because we have failed to detect any inhibition in the generation of IgM-secreting cells in response to LPS in our culture systems (Fig. 7). This is consistent with the observation that the inhibition of lymphopoiesis only occurred when Abs were present during the first week of culture. At this stage, cultures primarily consist of early progenitors (12). Moreover, we could not induce the differentiation of pre-B cells in the presence of hyaluronate (data not shown), which is one of the stromal cell ligands that associate with CD44 (67, 79, 80). Recently, several chimeric mice containing progenitors lacking either the β1 or α4 integrins of VLA-4 have revealed that B lymphopoiesis from fetal liver progenitors can occur in their absence (81, 82). However, α4−/− bone marrow progenitors fail to reconstitute the B cell compartment of irradiated recipients. It is possible that VLA-4 is required for the development of B cell progenitors in the bone marrow but not for fetal liver progenitors. However, stromal cells derived from VCAM-1 knockout mice supported the normal development of B cell progenitors in vivo and in vitro, suggesting that the VLA-4/NCAM interaction is not essential for the development of B cells (83).

Abs recognizing the BCR (μ and κ) may have prevented the maturation of pre-B cells to the LPS-responsive stage in our culture system by blocking a critical interaction between stromal cells and pre-B cells. Alternatively, these Abs may have induced apoptosis in immature B cells or may have prevented maturation to the plasma cell stage. We favor these latter hypotheses because the level of IgM secretion in cultures containing anti-μ (or anti-κ) Abs, or in cultures containing PMA and ionomycin, was less than the background levels of IgM observed in cultures containing LPS alone. We also observe that proB44,IL-7 cells cultured alone in U-bottom plates for 24 h and then transferred to secondary cultures containing LPS in flat-bottom plates mature to the LPS-responsive stage as measured by IgM secretion (data not shown). The same number of proB44,IL-7 cells cultured in flat-bottom plates with LPS do not mature in the absence of stromal cells and therefore do respond to LPS and secrete IgM. The U-bottom plate enables pre-B cell interactions, and this stage of differentiation occurs within 24 h of cell contact. When the addition of anti-μ and anti-κ Abs was delayed for 24 h, we still failed to detect significant IgM secretion (data not shown). We propose that these reagents block maturation subsequent to the mature LPS-responsive B cell stage because proB44,IL-7 cells cultured alone for 24 h in U-bottom plates are sufficient to mediate their maturation into LPS-responsive B cells. Interestingly, B cell maturation could not be rescued in cultures containing IL-4, anti-μ, and anti-CD40 Abs, or combinations of the Abs described in Figure 7 (data not shown).

We have observed partial inhibition of maturation leading to IgM secretion with mAbs recognizing CD19 but not with anti-murine A5, CD81, CD22, or CD40 Abs. CD19 associates with CD21, CD81, and Leu-13 to form a complex involved in regulating the activation threshold of the BCR (71, 84–86). Recently, it has been shown that the anti-CD19 mAb used in this report (ID3) can stimulate signaling in pre-B cells and synergize with complexes containing μ heavy chain (71, 87, 88). This suggests that CD19 modulates signaling through the pre-BCR. However, we did not observe a decrease in the maturation of pre-B cells cocultured with stromal cells in the presence of Abs recognizing A5 (46) or CD81 (89). This is in contrast to T cell differentiation, in which the 2F7, anti-CD81 mAb blocked the development of CD4+CD8+ TCRαβ thymocytes in fetal thymus organ cultures (89). Moreover, fibroblasts transfected with CD81 could support the differentiation of CD4−CD8− into CD4+CD8+ T cells. Our observations therefore suggest that immature B cells differentiating in vitro are sensitive to both BCR and CD19 receptor engagement similar to previous studies, whereas Abs recognizing the pre-BCR fail to have this effect on differentiation (88, 90, 91).

Although stromal cells are required to mediate the transition from an immature B cell stage to the LPS-responsive stage leading to the secretion of IgM, we have also demonstrated that pro-B cells, incubated at high cell density or in proximity (i.e., U-bottom/V-bottom plates) to each other, mature and become mitogen responsive in the absence of stromal cells. It is unlikely that these results can be explained in terms of contaminating stromal cells for several reasons. First, proB44,IL-7 cells are approximately 99% homogeneous with respect to B220 expression, a B lineage isofrom of CD45 that is not expressed on stromal cells (10). Furthermore, experiments in which decreasing numbers of S17 stromal cells were titrated into cultures of 10^3 pro-B cells revealed that a minimum of 10^5 irradiated S17 were required to mediate the maturation of proB44,IL-7 cells. However, fewer than 500 pro-B cells cultured in a V-bottom plate differentiated to a mature B cell stage and secreted IgM in response to LPS. The data presented in the U-bottom experiments raise the possibility that pre-B cells can associate with each other to mediate further maturation. It is possible that pre-B cells contain analogous surface proteins to those on stromal cells, which are responsible for mediating the pre-B to mature B cell transition. Alternatively, stromal cells may provide a supportive framework for immature B cells to develop. This scaffold may permit the association of immature B cells that differentiate through, as of yet, unidentified homotypic interactions. The S17 stromal cell-mediated maturation of proB44,IL-7 cells in our culture system was dependent on cell contact, but this interaction may have only been required to bring sufficient numbers of immature B cells together. In vivo, there is a clustering of small, B220+ slgM+ cells within the lumen of sinusoids (2). It has been proposed that these immature B cells accumulate in closed sinusoidal segments for a period of maturation before being released into the bloodstream. It is therefore possible that the final maturation of immature B cells occurs within these sinusoid compartments through homotypic interactions. The proteins involved in mediating the late maturational stages remain to be identified. The finding that pre-B cells can functionally mature in the absence of stromal cells in vitro provides a novel way to effectively identify the molecules involved in mediating the final stages of B cell maturation in primary lymphoid organs.
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References


24. Bone marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. Cell 48:1009.


78. Arakawa, H., and S. Takeda. 1996. Early expression of Ig µ chain from a transgene significantly reduces the duration of the pre-B cell stage but does not affect the small pre-B stage. *Int. Immunol.* 8:1339.


