Stromal Cell-Independent Maturation of IL-7-Responsive Pro-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 recombinating 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 μ + (cμ+)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.

Stromal Cell-Independent Maturation of IL-7-Responsive Pro-B Cells1

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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 cμ+ 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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3 Abbreviations used in this paper: cμ, cytoplasmic μ; slgM+, surface IgM+; sμ, surface μ; BCR, B cell receptor; prodBmuc−, R220− 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 DJ_{H} and V_{L} to J_{L} rearrangements (42, 43). Although some cells become sIgM^{+} 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 at 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 cIgM^{+} 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 \( \mu^{+} \) (sIgMu^{+}) 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 pro-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 over-night 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. Typically, 2 to 3 \( \mu \)l 5% FCS/balanced salt solution. The recovered cells were centrifuged at 1,000 x g (30 min) to remove debris by gravity sedimentation.

Adherent cells were recovered by scraping with a plastic scraper (no. 3010; Costar, Cambridge, MA) after carefully washing the plates eight times in 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 cIgM 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. Live cells were gated according to forward- and side-scatter characteristics and propidium iodide staining. Cells were stained using Abs (PharMingen) to

Flow cytometric analysis

ProB_{44,8,7} cells (5 x 10^{6} 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 x 10^{5} cells/well). Harvested cells were stained for the expression of surface markers by incubating approximately 10^{6} 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 FACSscan (Becton Dickinson, Mountain View, CA) followed by analysis with Cell Quest version 3.1 software. Cells were stained for cIgM 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. Live 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 Vκ rearrangements
DNA was isolated by a modified direct PCR lysis method (47). A total of
10³ cells/µl were resuspended in PCR lysis buffer (10 mM Tris, pH 8.3, 1.8
mM MgCl₂, 50 mM KCl, 0.45% Nonidet P-40, 0.45% Tween-20, 60 µg/mg
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. Vκ
rearrangements were amplified using primers hybridizing to the framework
3 region of Vκ elements (Vκcon-5’: 5’-GGCTGGCAAG/CTTACTGTG
GCAGTTG(A/G)/(A/G)(AC-3’) (48) and to 3’ of the Jκ5 element
(Jκ 5–3’: 5’-TGGCACAGCTCAGAATAAGGCCTCTC-3’) (49). PCR
products were resolved on 10% Tris, pH 8.3, 50 mM KCl, 1.8 mM MgCl₂,
0.5% Triton X-100, 100 µg/ml BSA (Boehringer Mannheim, Laval, Que-
bec, Canada), 200 mM each dNTP (Boehringer Mannheim), 0.5 µM each
primer (Life Technologies), and 2.5 U of Taq polymerase (Boehringer Mannheim).
PCR amplifications were performed for 10 min at 94°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
CATGAGAGATCTGGCACCACAC-3’ and α-actin 3’: 5’-CGCA
CAATCCTCAGTTCAG-3’) (26) were added during a 10-min pause at
85°C. PCR amplification was permitted to proceed for another 25 cycles of
94°C, 1 min; 60°C, 1 min; 72°C; 2 min; plus 5 s/°C. A 10 min 72°C
cycler primer extension period followed. PCR products were separated on 1.5%
agarose gels and transferred onto nylon membranes (N-Hybond; Amer-
sham, Chalfont, U.K.) by overnight Southern transfer. Membranes were probed with [32P]-labeled Jκ-5 30-mer oligonucleotide
(5’-CTCTTAA CATGAAACCTGTGTCTTACACA-3’) using T4 polynucleotide poly-
merase (NEBlabs, Mississauga, Ontario, Canada) according to the manu-
facturer’s instructions. The Jκ-5 30-mer hybridizes to 20 bases 3’ of the Jκ5 element in the Jκ-C intervening sequence (49). Hybridization was con-
ducted at 42°C for 4 h in 5X SSPE, 5X Denhardt’s solution, and 0.5%
SDS. Blots were washed twice in 2X SSC, 0.1% SDS at 42°C for 5 min,
followed by two washes in 2X SSC, 0.1% SDS for 20 min at 50°C. Blots
were exposed in a PhosphorImager cassettes (Molecular Dynamics, Sunny-
vale, CA). Imaging and quantification was done using a Molecular Dy-
namics PhosphorImager and ImageQuant 4.1 software. Blots were stripped
by boiling in 0.1% SSC for 10 min and reprobed using an α-actin-IN
oligonucleotide (5’-GACATGGAGAAGATCTGGCACCACAC-3’) (26). Vκ5
rearrangements were calculated using the following equation: (Vκ5
sample intensity/relative αctin sample intensity) and are expressed relative
to samples containing 100% S107 cells, which contain both Vκ5
and/or the removal of IL-7. To investigate the influence of S17
stomal cells in mediating the differentiation of B cell progenitors
in the continued presence of IL-7, we transferred proBd4-IL7 cells
into secondary cultures containing irradiated S17 cells in the pres-
ence or absence of IL-7 and LPS for an additional 4 days (Fig. 1). ProBd4-IL7 cells showed a slight increase (4.9-fold) in the incor-
poration of [3H]Tdr after 4 days of culture with S17 cells com-
pared with control cultures containing medium alone (Fig. 1A). The incorporation of [3H]Tdr was used as a convenient assay to
measure the proliferation of proBd4-IL7 cells in these cultures since it
also correlates with an increase in cell number. The proliferation
of proBd4-IL7 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 proBd4-IL7 cells
stimulated with IL-7 had considerable proliferative responses. Pro-
genitors 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 com-
pared with cultures containing only S17 cells. The proliferation of
proBd4-IL7 cells in cultures containing S17 + LPS + IL-7 was
likely due to their response to IL-7 because the [3H]Tdr incorpo-
ration observed in S17 + LPS or LPS-containing cultures was identical to that observed for cultures containing S17 or medium,
respectively.

We next determined whether IL-7-stimulated proBd4-IL7 cells
were able to differentiate as well as proliferate when cocultured
with S17 stromal cells in the continued presence of IL-7. Matura-
tion 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). ProBd4-IL7 cells
were harvested and transferred to secondary cultures (10³ cells/ well) containing the reagents noted in Figure 1B for an additional
7 days. IgM secretion was not detected in cultures in which
proBd4-IL7 cells were transferred to either IL-7, S17, S17 + IL-7,
or medium alone. However, proBd4-IL7 cells cultured with S17 + LPS
underwent significant differentiation resulting in IgM-secret-
ing 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 IL-7 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. 8A). 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 for 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. 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]ThdR (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. 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.

Cultures of proB d4-IL7 cells stimulated with S17 + LPS + IL-7 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 + IL-7 and S17 + LPS. The data suggest that the additional pro-B cells found in cultures containing S17 + LPS + IL-7 did not differentiate into
It has been reported previously that B lineage clones, which are containing either proB d4-IL7 (10^3 cells/well) or proB d8-IL7 cells (10^3 cells/well) was comparable to that generated in the presence of IL-7 for 4 days. Cells were stained using FITC or phycoerythrin-conjugated Abs as described in Materials and Methods. Cultures containing IL-7 resulted in a more than threefold expansion of viable cells (Table I). The absolute number of A5^+ cells recovered from cultures at the end of 48 h was as follows: IL-7 (3.2 x 10^5), media (1.1 x 10^3), S17 (4.1 x 10^3), S17 + LPS (4.4 x 10^3), and S17 + IL-7 (8.2 x 10^3). Isotype controls were >99% negative.

Isotype controls were placed in secondary cultures containing S17 (4.1 x 10^3), S17 + LPS (4.4 x 10^3), LPS (4.4 x 10^3), and cultured in the indicated conditions for an additional 48 h. Cells were stained using FITC or phycoerythrin-conjugated Abs as described in Materials and Methods. Cultures containing IL-7 resulted in a more than threefold expansion of viable cells (Table I). The absolute number of A5^+ cells recovered from cultures at the end of 48 h was as follows: IL-7 (3.2 x 10^5), media (1.1 x 10^3), S17 (4.1 x 10^3), S17 + LPS (4.4 x 10^3), and S17 + IL-7 (8.2 x 10^3). Isotype controls were >99% negative.

It was possible that many of the proB d4-IL7 cells in the IL-7 + S17 + LPS-stimulated cultures were unable to differentiate into LPS-responsive B cells as a result of the culture conditions. The extended time that proB d4-IL7 cells were cultured in the presence of IL-7 may have resulted in nonfunctional rearrangements. To assess the differentiative capacity of B cell progenitors cultured in IL-7 for either 4 (proB d4-IL7) or 7 days (proB d7-IL7), we measured the frequency of progenitors that gave rise to LPS-responsive B cells when placed in secondary cultures containing S17 + LPS. The frequency of proB d4-IL7 cells (1:127 x/\sqrt{1.7}) was comparable to the frequency of proB d7-IL7 cells (1:63 x/\sqrt{1.7}) that generated IgM-secreting cells in secondary cultures. Furthermore, the amount of IgM secretion observed in S17 + LPS cultures containing either proB d4-IL7 (10^5 cells/well) or proB d8-IL7 cells (10^5 cells/well) was similar when measured 7 days later (5.5 μg/ml vs 7.1 μg/ml, respectively). Therefore, short-term cultures (7 days) containing pro-B cells stimulated with IL-7 maintain progenitors with the potential to differentiate in the presence of S17 + LPS as efficiently as cultures stimulated with IL-7 for 4 days. Together, these results indicate that stromal cells significantly enhance the generation of IgM-secreting plasma cells from proB d4-IL7 cells in the absence of IL-7. However, in the presence of IL-7, the stromal cell-mediated differentiation of IL-7-responsive pro-B cells does not occur.

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 slgM. However, only a small fraction of the slgM^+ cells (<1 x 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 proB d4-IL7 cells to the small pre-B cell stage in the presence or absence of stromal cells by flow cytometry (Fig. 3). ProB d4-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 slgM 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, proB d4-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 CD22 and slgM compared with cultures containing IL-7. This surface phenotype is characteristic of small, resting pre-B cells (26, 28, 59). However, proB d4-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 slgM^+, whereas cultures that contained IL-7 consisted of <1% slgM^+ cells. However, the absolute numbers of slgM^+ cells were roughly equivalent (~5 x 10^5) in all cultures (Table I). The difference between the relative and absolute numbers of slgM^+ cells was likely due to the proliferation of IL-7-responsive pre-B cells, which resulted in a 3.2-fold increase in the number of viable cells by 48 h (Table I). ProB d4-IL7 cell cultures that were not stimulated with IL-7 contained a greater proportion of dead cells and 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.
present in cultures containing IL-7 (Fig. 3). This is readily observed in IL-7-stimulated cultures, as the $3.2 \times 10^4 \alpha S^5$ cells present at the end of 48 h cannot account for the absolute number of $\alpha S^5$ cells that are present in cultures containing media ($1.1 \times 10^5$), $S17$ ($4.1 \times 10^5$), or $S17 + LPS$ ($4.4 \times 10^5$).

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 $\kappa^+$ (Fig. 3). Surface staining for $\lambda$ light chain did not account for this discrepancy (data not shown). Moreover, the majority of the $\kappa^-$ cells were also $\alpha S^5$, 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 $proB_{A4-IgL7}$ 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 Figure 4A show the relative level of $V\kappa5$ rearrangements per cell equivalents in $proB_{A4-IgL7}$ 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 pro-B cells. Instead, it is the absence of IL-7 that induces the transition to the small pro-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 $\mu^-$ $proB_{A4-IgL7}$ 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 $proB_{A4-IgL7}$ cells using a transwell insert (Fig. 5). This culture system permits the diffusion of soluble factors through the Anopore membrane but...
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 cell 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 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 (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 LPS. IgM secretion was quantitated 7 days later as the mean ± SEM from three independent experiments. 

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>μ</sup><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-μδ) (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 $\mu$/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-$\mu$ and anti-$k$ 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 ($10^4$ 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 ($10^3$ 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 $10^3$ pro-B cells stimulated with LPS revealed that there was substantially less IgM secreted in cultures containing fewer than $10^3$ 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 slgM+ 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 proB34-I8 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 maturation 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 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 cμ - 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, pro-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 FSI mAbs. However, cμ - cells are not detected in this population despite the fact that approximately 18 to 27% of these A5 + cells express cμ . Previous studies have shown that surrogate light chain (A5/VpreB) is expressed on the surface of pro-B cells before the formation of cμ 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 cμ - pro-B cells cultured fail to associate with the surrogate light chain. Recent studies have described an early c-Kit + cμ - pro-B cell population in the bone marrow of normal mice in which only half of the cμ -chains expressed have the capacity to form a pre-BCR (75). Moreover, fetal-associated cμ - 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-I8 cells express cμ (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 c 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/Rag2–/– 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 pre-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 slgM. Thus, sμ - cells still require stromal cells to generate IgM-secreting cells in response to LPS stimulation.

Studies in which IL-7Ra–/– B cell progenitors from Whitlock-Witte cultures were reconstituted with mutant IL-7Ra-chains via retrovirus-mediated gene transfer have demonstrated that the proliferative signals generated by IL-7 signaling are distinct from those mediating differentiation (41). Mutant IL-7Ra 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 cμ - stage. In contrast, other chimeric IL-7Ra-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, cμ -, 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 slgM 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 $s_{\mu}^{+}$ population is three times that found in the $s_{\mu}^{-}$ population, the $s_{\mu}^{+}$ 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 $\mu$ 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 $\beta_1$ or $\alpha_4$ integrins of VLA-4 have revealed that B lymphopoiesis from fetal liver progenitors can occur in their absence (81, 82). However, $\alpha_4^{-/-}$ 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/VCAM interaction is not essential for the development of B cells (83).

Abs recognizing the BCR ($\mu$ and $\kappa$) 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-$\mu$ (or anti-$\kappa$) Abs, or in cultures containing PMA + ionomycin, was less than the background levels of IgM observed in cultures containing LPS alone. We also observe that proB$^{s_{\mu} IL-7}$ 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 proB$^{s_{\mu} IL-7}$ 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-$\mu$ and anti-$\kappa$ 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 proB$^{s_{\mu} IL-7}$ 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-$\mu$, 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 $\mu$ 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$^{+}$ TCR$\alpha\beta$ 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, proB$^{s_{\mu} IL-7}$ cells are approximately 99% homogeneous with respect to B220 expression, a B lineage isoform 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 $10^3$ irradiated S17 were required to mediate the maturation of proB$^{s_{\mu} IL-7}$ 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 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.
Maturation of IL-7-Responsive Pro-B Cells

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