Adipocyte-Derived Soluble Factor(s) Inhibits Early Stages of B Lymphopoiesis

Fareena A. Bilwani and Katherine L. Knight

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B lymphopoiesis declines with age, and in rabbits this occurs by 8 wk of age. We found that CFU fibroblasts (CFU-Fs) in the bone marrow (BM) decrease 10-fold by a few weeks of age and that the CFU-Fs preferentially differentiate into adipocytes instead of osteoblasts. BM becomes filled with fat spaces during this time, making rabbit a unique model to study the effects of accelerated fat accumulation on B lymphopoiesis. We show that adipocytes of both rabbit and human secrete a soluble factor(s) that inhibits B lymphopoiesis, and we tested if this inhibition was due to effects on the BM stroma or hematopoietic progenitors. Pretreatment of BM mononuclear cells with adipocyte conditioned medium dramatically inhibited their differentiation into pro-B cells in cocultures with OP9 stromal cells. In contrast, pretreatment of OP9 stromal cells with adipocyte conditioned medium had no effect on B lymphopoiesis. Using human hematopoietic stem cells, we show that inhibition by the adipocyte-derived factor occurred at the common lymphoid progenitor to preproB cell stage. We propose that the age-related decline in B lymphopoiesis is due to a decrease in CFU-Fs, an increase in adipocytes, and an adipocyte-derived factor that blocks B lymphopoiesis at the common lymphoid progenitor to preproB cell stage.

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Human spongy bone samples were taken during knee and hip replacement surgery. Samples were collected in accordance with the principles of the Declaration of Helsinki. Single-cell suspensions of BM were obtained, and RBCs were lysed by 0.85% NH₄Cl.

**Flow cytometry**

Abs used were mouse monoclonal with specificity for human target Ags (Table II). Flow cytometric analysis was performed on a BD Canto II flow cytometer (BD Biosciences) and with FlowJo software (Tree Star, Ashland, OR). All cells analyzed were in the lymphocyte gate; quadrants for positive and negative population were based on cells stained with isotype control Ab.

**CFU-F assay**

Heads of rabbit bones were removed, and BM was flushed from the shaft of femurs. Single-cell suspensions were passed through a 100-μm cell strainer, and RBCs were lysed with 0.85% NH₄Cl. BM mononuclear cells (MNCs) (50–40,000 per well from 1- to 6-wk-old rabbits; 1,000–500,000 per well from 7-wk- to 11-mo-old rabbits) were plated in 96-well plates for 48–72 h. After removal of unattached cells, αMEM medium (Invitrogen, Grand Island, NY) with 20% FCS (Atlanta Biologicals, Lawrenceville, GA) was added, and the medium was changed every 2–3 d. The number of wells with or without fibroblast colonies was counted at day 21. CFU-F was defined as a colony of 50 or more cells with fibroblast-like morphology. Data were analyzed by Mann–Whitney test. Horizontal line represents the average of the ratio of osteoblastic and adipocytic colonies in all age groups.

**Differentiation of CFU-Fs into adipocytes and osteoblasts**

Rabbit CFU-Fs obtained as described earlier were cultured in osteoblast differentiation-inducing (ODI) medium containing 10 mM dexamethasone, 20 mM β-glycerol phosphate, and 50 μM ascorbic acid or in adipocyte differentiation-inducing (ADI) medium containing 0.5 μM isobutylmethylxanthine, 50 μM indomethacin, and 0.5 μM dexamethasone (21). All chemicals were obtained from Sigma Aldrich (St. Louis, MO). Colonies grown in ODI medium were stained for alkaline phosphatase and with alizarin red S, whereas colonies grown in ADI medium were stained with oil red O.

**Coculture of OP9 stromal cells and total BM MNCs with human and rabbit adipocyte CM**

Human and rabbit BM MNCs (10⁶/cm²) were plated in 12-well plates, and adipocytes were generated as described earlier. The adipocytes (as indicated by their morphology upon phase-contrast microscopy) were cultured in αMEM medium without FCS, and CM was collected after 72 h. Adipocytes showed healthy morphology (as evidenced by phase-contrast microscopy) in culture without FCS for 72 h (data not shown). Human or rabbit adipocyte CM (or αMEM without FCS as a negative control) was added (1:2) to cocultures of OP9 stromal cells (5000 cells/well) and rabbit BM MNCs (40,000 cells/well) with αMEM (20%) containing a final concentration of 10 ng/ml each of recombinant human IL-7, stem cell factor (SCF), and FLT-3 ligand (FLT-3L) (Peprotech, Rocky Hill, NJ) in 48-well plates. OP9 cells were plated 24 h prior to the addition of total BM MNCs. Cocultures were fed with fresh medium containing cytokines and adipocyte CM (or αMEM without FCS for the negative control) at day 4. Cells were harvested on day 7 or 8, and live cell counts from three wells were obtained using trypan blue exclusion dye. Cells were stained with anti-CD14, permeabilized with Cytoperm/Cytotox (BD Biosciences), stained with anti-CD79a (IgM), and analyzed by flow cytometry. The dose dependency of CM was tested by adding decreasing amounts of CM to the cultures (1:2 to 1:32). αMEM without FCS was diluted 1:2 in αMEM with 20% FCS and used as a negative control for these experiments. For some experiments, rabbit and human adipocyte CM were centrifuged (2000 rpm) through a filter device with 10-kDa cutoff membrane (Cen tricon; Millipore, Billerica, MA) at 4°C. The <10-kDa fraction of human CM was tested for the presence of adiponectin by ELISA (kit no. ab99968; Abcam, Cambridge, MA), and no detectable adiponectin was found (<25 pg/ml).

**Table I. Number and ratio of osteoblastic and adipocytic colonies/10⁶ BM MNCs generated from CFU-Fs in 14 rabbits of various ages**

<table>
<thead>
<tr>
<th>Age</th>
<th>Osteoblastic Colonies ± SD</th>
<th>Adipocytic Colonies ± SD</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk</td>
<td>38.3 ± 4.1</td>
<td>33.3 ± 4.1</td>
<td>1.15</td>
</tr>
<tr>
<td>3 wk</td>
<td>32.3 ± 5.3</td>
<td>20.7 ± 5</td>
<td>1.56</td>
</tr>
<tr>
<td>4 wk</td>
<td>22.3 ± 7</td>
<td>10 ± 2</td>
<td>2.23</td>
</tr>
<tr>
<td>5 wk</td>
<td>31.3 ± 2.5</td>
<td>38.7 ± 5.7</td>
<td>0.81</td>
</tr>
<tr>
<td>6 wk</td>
<td>5 ± 2.3</td>
<td>1.7 ± 1.7</td>
<td>3.01</td>
</tr>
<tr>
<td>6 wk</td>
<td>3 ± 1</td>
<td>16 ± 1.8</td>
<td>0.18</td>
</tr>
<tr>
<td>7 wk</td>
<td>19.2 ± 6.3</td>
<td>24 ± 7</td>
<td>0.79</td>
</tr>
<tr>
<td>7 wk</td>
<td>22 ± 17</td>
<td>11.6 ± 7.2</td>
<td>1.89</td>
</tr>
<tr>
<td>8 wk</td>
<td>8.2 ± 3.8</td>
<td>10.3 ± 2.1</td>
<td>0.82</td>
</tr>
<tr>
<td>9 wk</td>
<td>3.6 ± 2.6</td>
<td>20.7 ± 7</td>
<td>0.17</td>
</tr>
<tr>
<td>4 mo</td>
<td>4.7 ± 4.6</td>
<td>3 ± 2.6</td>
<td>1.55</td>
</tr>
<tr>
<td>5 mo</td>
<td>6.8 ± 4.2</td>
<td>8.5 ± 7.5</td>
<td>0.8</td>
</tr>
<tr>
<td>6 mo</td>
<td>3.7 ± 3.2</td>
<td>30.8 ± 7.1</td>
<td>0.11</td>
</tr>
<tr>
<td>6 mo</td>
<td>0.3 ± 0.5</td>
<td>2.7 ± 3</td>
<td>0.11</td>
</tr>
<tr>
<td>7 mo</td>
<td>0.3 ± 0.5</td>
<td>3.7 ± 1.5</td>
<td>0.08</td>
</tr>
<tr>
<td>7 mo</td>
<td>0.5 ± 0.8</td>
<td>3 ± 2.2</td>
<td>0.16</td>
</tr>
<tr>
<td>11 mo</td>
<td>0.3 ± 0.5</td>
<td>3 ± 3</td>
<td>0.11</td>
</tr>
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with alizarin red S, whereas colonies grown in ADI medium were stained with oil red O.

**Table II. Monoclonal Abs used in this study**

<table>
<thead>
<tr>
<th>Ab</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Source</th>
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<tbody>
<tr>
<td>Anti-CD14a</td>
<td>TUK4</td>
<td>Pacific blue</td>
<td>Serotec</td>
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<tr>
<td>Anti-CD79a</td>
<td>HM47</td>
<td>PE</td>
<td>BD PharMingen</td>
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<td>Anti-CD34</td>
<td>563</td>
<td>PE</td>
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<td>Anti-CD38</td>
<td>HIT2</td>
<td>Allophycocyanin</td>
<td>BD PharMingen</td>
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<td>OKT3</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-CD14</td>
<td>61D3</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-CD15</td>
<td>H198</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-CD19</td>
<td>HIB19</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
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<td>SJ25-C1</td>
<td>Allophycocyanin-Cy7</td>
<td>Invitrogen</td>
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<td>Anti-CD56</td>
<td>MEM188</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
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<td>Anti-CD7</td>
<td>EBio124-D1</td>
<td>PE-Cy5</td>
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<td>Anti-CD10</td>
<td>HI10a</td>
<td>PE-Cy7</td>
<td>BioLegend</td>
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<tr>
<td>Anti-IgM</td>
<td>G20-127</td>
<td>Allophycocyanin</td>
<td>BD PharMingen</td>
</tr>
</tbody>
</table>

*aCross-reactive with rabbit.*
and CD14+ myeloid-lineage (without adipocyte CM). After 7-d cocultures of total BM MNCs with OP9 stromal cells with or for the presence of cytoplasmic CD79a+ B- and CD14+ myeloid-lineage cells example of lymphocyte gate. Cocultures of OP9 stromal cells and rabbit BM MNCs. Effect of adipocytes on the generation of B lineage cells in rabbits and human BM stroma were generated as described earlier. Adipocytes were generated in 12-well plates (as described earlier) in the bottom of Transwells. Transwells of 1.0-μm pore size (Millipore, Billerica, MA) were plated with 5 × 10^6 OP9 stromal cells 24 h prior to addition of 20 × 10^6 total BM MNCs/well. Cocultures containing oMEM (20% FCS) and IL-7, SCF, and Flt-3L (10 ng/ml each) were fed with fresh medium at day 4; cells were harvested at day 10. Live cell counts and analysis by flow cytometry were performed as described earlier.

**Pretreatment of OP9 stromal cells and rabbit BM MNCs with adipocyte CM**

OP9 stromal cells (10^5) or rabbit BM MNCs (4 × 10^6) were treated 24 h with oMEM (20% FCS) and adipocyte CM (1:2) (or with oMEM with no FCS as negative control) for 5 d. OP9 stromal cells were then detached using 0.25% trypsin (Sigma). Treated OP9 stromal cells (detached with 0.25% trypsin) and BM MNCs were washed with oMEM (20% FCS) and used in coculture experiments.

**Coculture of human HSCs with rabbit adipocyte CM**

CD34+ cells were enriched from human CB using EasySep CD34+ positive selection mixture (Stem Cell Technologies, Vancouver, Canada). CD34+ CD38− Lineage− (Lineage markers: CD3, CD14, CD15, CD19, CD56) HSCs (5000 cells/well) were then sorted directly onto OP9 stromal cells (5000 cells/well) using the FACSaria flow cytometer (BD Biosciences) in 48-well plates containing oMEM 20% FCS and adipocyte CM (1:2) (or oMEM with no FCS as negative control) with IL-7, SCF, and Flt-3L (10 ng/ml each). Cocultures were fed with fresh medium and adipocyte CM (1:2) (or oMEM with no FCS as a negative control) at days 4 and 10; cells were harvested at days 10 and 14. Live cell counts from three wells were obtained using trypan blue exclusion dye. Analysis for the presence of CLPs and preproB cells was performed by surface anti-CD7, anti-CD10, anti-CD34, and anti-CD19, and cytoplasmic anti-CD79a staining. ProB cells were identified by surface anti-CD7 and anti-CD19 and cytoplasmic anti-CD79a and anti-μ staining. All samples were analyzed by flow cytometry. In our coculture system, both CLPs and preproB cells were CD34+ but CD10−. In agreement with Ichii et al. (22), committed B lineage cells expressed CD10 in addition to CD19 and CD79a.

**Statistical analysis**

Mann–Whitney U test and Student t test were performed using Prism Statistical Software. A p value of <0.05 was considered statistically significant.

**Results**

Changes in the frequency and differentiation potential of CFU-Fs

We hypothesized that the decline in B lymphopoiesis in rabbits was due to changes in the BM environment. We tested if there is...
a decrease in the number of CFU-Fs, which differentiate into osteoblasts and adipocytes, and/or a change in the capacity of CFU-Fs to differentiate into osteoblasts with age. CFU-Fs were quantified (Supplemental Fig. 1A) (23) by limiting dilution analysis of BM MNCs from rabbits of various ages. We used the CFU-F assay because the resulting colonies derived from BM were shown previously to be multipotent and clonal in origin (19). By phase-contrast microscopy, the CFU-Fs had fibroblast-like morphology, and no macrophages were seen (Supplemental Fig. 1A), as reported previously (19). The frequency of CFU-Fs in 1- to 4-wk-old rabbits was generally greater than 100 in 10^6 BM MNCs and declined dramatically by 9 wk of age to less than 10 in 10^6 cells (Fig. 1A, 1B). The low number of CFU-Fs was maintained for at least a year, as evidenced by the frequency of only nine CFU-Fs per 10^6 total BM MNCs in an 11-mo-old rabbit, the latest age we tested (Fig. 1B). We conclude that the number of CFU-Fs decreased nearly 10-fold by 9 wk of age.

CFU-Fs give rise to mature BM stromal cells, osteoblasts, adipocytes, and chondrocytes (24). Osteoblasts reportedly support all stages of B lymphopoiesis in BM (12), whereas adipocytes negatively regulate hematopoiesis (13). We hypothesized that the decline in B lymphopoiesis may reflect not only a decrease in the number of CFU-Fs but also increased capacity of the CFU-Fs to differentiate into adipocytes instead of osteoblasts. We tested this possibility by culturing CFU-Fs from rabbits 2 wk to 11 mo of age in ODI or ADI medium. Colonies grown in ODI medium were stained for the presence of osteoblastic colonies using alkaline phosphatase, a marker of osteogenic differentiation of CFU-Fs (Supplemental Fig. 1B, 1C), and for the presence of minerals deposited by osteoblasts using alizarin red S stain (Supplemental Fig. 1D, 1E). Colonies grown in ADI medium were stained for the presence of adipocytic colonies by oil red O, which stains fat globules (Supplemental Fig. 1F, 1G). Because these CFU-Fs can differentiate into osteoblasts and adipocytes, we assume that they are mesenchymal stem cells. We calculated the ratio of osteoblastic to adipocytic colonies and found that CFU-Fs from 2- to 8-wk-old rabbits readily differentiated into osteoblasts as evidenced by an average ratio of osteoblastic to adipocytic colonies of 1.3 (Fig. 1C, Table I). In contrast, for rabbits 9 wk of age and older, the average ratio was 0.4, indicating that the CFU-Fs from older rabbits preferentially differentiated into adipocytes (Fig. 1C, Table I). We conclude that in vitro, CFU-Fs from rabbits >2 mo of age preferentially differentiate into adipocytes rather than into osteoblasts. Although we cannot rule out the possibility that the apparent increase in differentiation to adipocytes is due to preferential survival and/or expansion of other cell populations, the finding correlates with the increase in fat spaces at 2 mo of age, as observed in H&E-stained BM sections (Supplemental Fig. 2) and as described previously (25).

**Adipocyte inhibition of B lymphopoiesis**

The increase in fat spaces in rabbits >8 wk of age, as seen in the H&E stain of BM (Supplemental Fig. 2), led us to ask if adipocytes inhibit the generation of proB cells, thereby contributing to the decline in B lymphopoiesis. We tested this possibility by adding primary rabbit adipocyte CM to cocultures of OP9 stromal cells and BM MNCs from rabbits >8 wk of age [in which BM is devoid of CD79a<sup>+</sup> (Igα) progenitor B lineage cells (8)] and measuring the number of CD79a<sup>+</sup> (Table II) B lineage cells generated. We found that in the presence of CM, almost no CD79a<sup>+</sup> cells were generated (Fig. 2A–C); inhibition of the generation of CD79a<sup>+</sup> B lineage cells by CM was dose dependent (Fig. 2E, 2F). These data indicate that adipocytes secrete a factor(s) that inhibits B lymphopoiesis. This finding was confirmed using a Transwell system in which rabbit BM MNCs were cocultured with OP9 stromal cells above the Transwell with primary rabbit adipocytes below the Transwell. We found a striking decrease in the capacity of OP9 stromal cells to promote the generation of CD79a<sup>+</sup> B lineage cells in the presence of adipocytes (Fig. 2G, 2H). In contrast, the adipocyte CM did not decrease generation of CD14<sup>+</sup> myeloid lineage cells and, in fact, appeared to increase myelopoiesis (Fig. 2B, 2D, 2G). We also generated adipocytes from human CFU-Fs (21) and found that like rabbit, human adipocyte CM impaired generation of CD79a<sup>+</sup> B lineage cells from rabbit BM MNCs (Fig. 3A, 3B). We conclude that adipocytes secrete a soluble factor(s) that selectively inhibits B lymphopoiesis. By filtering the CM through a 10-kDa membrane, we found that inhibitory activity resided in the <10-kDa fraction (Fig. 4A, 4B), indicating that the inhibitor is small in size. Similar results were obtained with the <10-kDa fraction of human adipocyte CM (data not shown).

As negative controls, we tested CM from rabbit BM undifferentiated CFU-Fs and osteoblasts, as well as CM from human BM

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**FIGURE 3.** Effect of human adipocyte CM on generation of B lineage cells. (A) Rabbit BM MNCs were cocultured with human adipocyte CM on OP9 stromal cells for 7 d and then tested for CD79a<sup>+</sup> B lineage cells by flow cytometry. (B) Number of CD79a<sup>+</sup> B lineage cells generated in the absence (–) and presence (+) of human adipocyte CM. Error bars show average ± SD of triplicate wells. Data are representative of three independent experiments.

**FIGURE 4.** Effect of <10-kDa fraction of adipocyte CM on generation of B lineage cells. (A) OP9 stromal cells and BM MNCs were cocultured for 7 d in the presence of <10-kDa fraction of adipocyte CM. Cells were analyzed by flow cytometry for the presence of CD79a<sup>+</sup> B lineage and CD14<sup>+</sup> myeloid lineage cells in the lymphocyte gate (as shown in Fig. 2A). Cocultures without (w/o) (negative control) or with adipocyte CM (positive control) are included. (B) Number of CD79a<sup>+</sup> B lineage cells. Error bars show average ± SD of triplicate wells. Data are representative of three independent experiments.
With age, mouse BM stroma loses the capacity to support development of early B cell precursors (26), and we asked if the adipocyte-derived soluble factor(s) could inhibit B lymphopoiesis by altering the lymphopoietic capacity of BM stroma or if it could directly alter the capacity of HSCs and/or early lymphoid progenitors to differentiate into B lineage cells. We first tested these possibilities by treating the OP9 stromal cells, which are known to support B lymphopoiesis (27), with rabbit adipocyte CM and then coculturing these pretreated cells with rabbit BM MNCs in the absence of adipocyte CM. Such treatment did not alter the capacity of OP9 stromal cells to support the generation of CD79a^+ B lineage cells (Fig. 5A, 5B). These data suggest that short-term exposure of the stromal cells to the adipocyte-derived factor does not alter their B lymphopoietic capacity, although we cannot rule out the possibility that continuous presence of inhibitory factor(s) with the stromal cells may have an effect on them.

In contrast, after treatment of BM MNCs from rabbits >2 months of age—which have HSCs and early B cell progenitors such as CLPs in their BM but no CD79a^+ proB or preB cells (8)—with adipocyte CM for 24 h followed by culturing them with OP9 stromal cells in the absence of adipocyte CM, we found greatly impaired capacity to differentiate into CD79a^+ B lineage cells (Fig. 6A, 6B) even after the removal of adipocyte CM (Fig. 6A). We conclude that the adipocyte-derived inhibitory molecule does not appear to change the capacity of BM stromal cells to support B lymphopoiesis but instead to directly alter the capacity of HSCs and/or early lymphoid progenitors such as CLPs to differentiate into B lineage cells. Although we cannot rule out the possibility that the inhibitory molecule acts by interfering with access to growth factors, especially IL-7, we do not think this is likely because IL-7, SCF, and FLT3L were added to the cultures after removal of CM.

### FIGURE 5

Effect of pretreatment of OP9 stromal cells with adipocyte CM on the capacity to support B lymphopoiesis. (A) OP9 stromal cells treated with adipocyte CM for 5 d, washed, and cocultured with BM MNCs in the absence of adipocyte CM for 8 d. Cells were analyzed by flow cytometry for CD79a^+ B lineage and CD14^+ myeloid lineage cells. Untreated OP9 stromal cells were cocultured with BM MNCs without (negative control) or with adipocyte CM (positive control). (B) Number of CD79a^+ B lineage cells. Error bars show average ± SD of triplicate wells, and data were analyzed using Student t test. Data are representative of two independent experiments.

### FIGURE 6

Effect of pretreatment of BM MNCs with adipocyte CM on differentiation into B lineage cells. (A) BM MNCs treated with adipocyte CM for 24 h were washed and cocultured with OP9 stromal cells and then analyzed at day 7 by flow cytometry for CD79a^+ B-lymphoid and CD14^+ myeloid lineage cells. Untreated BM MNCs were cocultured with OP9 stromal cells in the absence (negative control) or presence of adipocyte CM (positive control). (B) Number of CD79a^+ B lineage cells. Error bars show average ± SD of triplicate wells, and data were analyzed using Student t test. Data are representative of three independent experiments.

Undifferentiated CFU-Fs and osteoblasts. Although we found some inhibitory activity in the generation of CD79a^+ B lineage cells (Supplemental Fig. 3A–C), the adipocyte-enriched cultures had greater inhibitory activity.

The effect of adipocytes on stromal cells and hematopoietic progenitors

Because reagents are readily available to identify HSCs and CLPs. We first showed that the rabbit adipocyte CM inhibited differentiation of human CD34^+ HSC-enriched cells into CD79a^+ B lineage cells in cocultures with OP9 cells (data not shown). We then cocultured human HSCs and OP9 cells with rabbit adipocyte CM and analyzed the cells for CLPs, preproB cells, and proB cells (28–30). At day 10, we found that the percentages and absolute numbers of CLPs (CD7^+ CD34^+) were similar in cultures with or without adipocyte CM (Fig. 7A, 7B). However, we found a significant decrease in the percentage and absolute numbers of preproB cells (CD79a^+ CD34^+) in the cocultures that contained the inhibitory factor (Fig. 7A, 7C). In addition, when we analyzed CD7^+ cells, of which ~70% are CD34^+, we also found that the percentage and absolute numbers of CD7^+ CD79a^- early lymphoid progenitors (CLPs) were similar in cultures with or without CM (23.9 versus 22.7%; 4,834 versus 5,417 cells) (Supplemental Fig. 4). Similarly, when we analyzed CD79a^+ cells (~60% of which are CD34^+), we found a significant decrease in the percentage and absolute numbers of early B lineage cells (preproB) in
cultures that contained CM (13.6 versus 5.6%; 3,194 versus 1,143 cells) (Supplemental Fig. 4). By day 14, almost no proB cells were found in the presence of the inhibitory factor (Fig. 7D, 7E). We determined the total number of live cells at days 7, 10, and 14 using trypan blue exclusion dye and did not find a significant difference at any time point in the number of live cells in the presence or absence of adipocyte CM. In addition, we searched for preproB cells at day 7, and similar to days 10 and 14, we found almost no preproB cells in the presence of adipocyte CM (data not shown). These data demonstrate that rabbit adipocytes do not affect generation of CLPs from HSCs but do inhibit differentiation of these cells to preproB cells (Fig. 8).

**Discussion**

B lymphopoiesis declines with age in various species including humans, rabbits, and mice. In mice, this decline is attributed to defects in both HSCs and the BM microenvironment (4–6), whereas in rabbits the defect appears to be with BM stroma (9). The BM stroma is composed of various cell types including osteoblasts and adipocytes, which are derived from CFU-Fs. Osteoblasts support early stages of B cell development, whereas adipocytes have been shown to negatively regulate hematopoiesis (12, 13). Although the frequency of CFU-Fs does not change appreciably in aged mice or humans (14, 15), we found a 10-fold decline in CFU-Fs in rabbit BM within a few weeks of age. In addition, these CFU-Fs, like those in aged humans and mice, appear more likely to differentiate into adipocytes than into osteoblasts (15–17). This observation is based on use of CFU-Fs that were enriched by their adherence property, and we assume that this was similar in all age groups of rabbits. The rapid decline in the frequency of CFU-Fs in rabbit BM along with the decreased potential to differentiate into osteoblasts coincides
with the time at which B lymphopoiesis in rabbit BM wanes (Fig. 8A). We suggest that these changes result in fewer osteoblasts and that the increase in fat leads to secretion of large amounts of inhibitor and a concomitant decline in B lymphopoiesis (Fig. 8B).

Age-related increases in fat are also found in the thymus and are associated with reduced numbers of thymic progenitors and thymic epithelial cells (31, 32). In 1974, Tavassoli et al. (33) found that by removing adipocytes from rabbit BM, hematopoiesis was increased, and more recently, Naveiras et al. (13) removed adipocytes from mouse BM and showed that hematopoiesis, including B lymphopoiesis, was increased. In this study, we showed that rabbit BM-derived adipocytes secrete a soluble factor(s) that inhibits the generation of B cells at the CLP to preproB cell stage. Because human BM shows an increase in fat with age (34), we suggest that adipocytes negatively affect early stages of B lymphopoiesis in BM of the elderly. Adipocytes secrete numerous molecules, including leptin, resistin, and adiponectin, all of which play a role in immune responses (35). Of these, adiponectin was shown to inhibit B lymphopoiesis (11, 36); however, using a <10-kDa adiponectin-free fraction from CM, we showed that the inhibitory activity in our study is independent of adiponectin.

Adipocytes could inhibit B lymphopoiesis by altering the BM stroma and/or by direct action on hematopoietic progenitors. Treatment of the stroma with the adipocyte inhibitor did not alter the capacity of stroma to support differentiation of HSCs to B lineage cells. However, we could only selectively treat the stroma with CM prior to the addition of hematopoietic progenitors, and we cannot rule out the possibility that CM affects the stroma but that the changes are transient and not observed in our cultures. In contrast, 24-h pretreatment of the inhibitor with hematopoietic progenitors profoundly affected differentiation of the progenitors into B lineage cells, indicating that the inhibitor induced a stable change in the progenitors, making them unable to differentiate into B lineage cells even in the presence of BM stroma and B lymphopoietic cytokines.

Whereas adipocytes were shown to alter the proliferation and differentiation capacity of HSCs (37), no effect on other hematopoietic progenitor cells including multipotent progenitors or CLPs has been reported. In this study, we show that adipocytes block B lymphopoiesis at the CLP to preproB cell stage. We think that the inhibitor affects the differentiation of CLPs into preproB cells rather than compromising the survival of preproB cells because in the presence of the inhibitory molecule, we did not find a significant decrease in the number of live cells, nor did we find a distinct population of preproB cells at earlier time points in the cultures. We suggest that the block in differentiation of CLPs into preproB cells by adipocytes explains the lack of proB cells and the arrest of B lymphopoiesis in rabbit BM. Although a decrease in the differentiation of CLPs to preproB cells in aged mice has been reported, the possibility that this is due to changes in CM stroma was not addressed (38). The transition of CLPs to preproB cells requires transcription factors early B cell factor-1, E2A, and Pax-5, and we suggest that the block in differentiation of CLPs into preproB cells is due to changes in expression of one or more of these transcription factors.

CLPs can differentiate into myeloid cells as well as B lineage cells, and they can express transcription factors required for myeloid cell differentiation (39). In some experiments, we found an increase in myeloid cells in cultures containing adipocyte CM, suggesting that adipocytes may promote myelopoiesis. If confirmed, then adipocyte inhibitor(s) may upregulate myeloid-specific transcription or promote survival of early myeloid progenitors such as common myeloid progenitors and granulocyte-macrophage progenitors. Alternatively, adipocytes may alter the differentiation potential of HSCs. Recent data suggest that subsets of human HSCs are myeloid- or lymphoid-biased (40), and we suggest that adipocytes contribute to regulating the balance between the numbers of myeloid- and lymphoid-biased HSCs with age.

In summary, we conclude that the decline in numbers of CFU-Fs and their decreased capacity to form osteoblasts, combined with an apparent increase in fat that secretes an as-yet-unidentified inhibitory molecule, likely contribute to the decline in B lymphopoiesis in rabbits (Fig. 8B). We show that adipocytes inhibit the differentiation of human CLPs into preproB cells by direct action on hematopoietic progenitors. Our studies include both rabbit and human, showing that adipocyte CM inhibits B lymphopoiesis using either human or rabbit HSCs. We suggest that rabbit is a useful model to elucidate the mechanism by which adipocytes inhibit B lymphopoiesis. Further, we propose that B lymphopoiesis and immune responses can be enhanced by modulating the activity of fat cells.

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**FIGURE 8.** Model for the mechanism(s) by which B lymphopoiesis declines in rabbit BM. (A) Decline in the number of proB and preB cells in rabbits has a remarkable correlation with a decrease in the number of CFU-Fs and an increase in fat in the first 8 wk of life. (B) Decline in the numbers of CFU-Fs and their capacity to differentiate into osteoblasts rather than adipocytes leads to an increase in fat spaces in adult rabbit BM. The adipocytes secrete a soluble factor(s) that inhibits B lymphopoiesis at the CLP to preproB cell stage. Consequently, no proB or preB cells are found in adult rabbit BM.
Disclosures
The authors have no financial conflicts of interest.

References
FIGURE LEGENDS OF SUPPLEMENTAL FIGURES:

Supplemental Figure S1. Generation of adipocytes and osteoblasts from rabbit CFU-Fs.
(A) Morphology of rabbit CFU-F as shown by phase contrast microscopy of BM fibroblast colony (20 X magnification). Rabbit CFU-Fs were grown in osteoblast differentiation-inducing (ODI) medium and adipocyte differentiation-inducing (ADI) medium. Generation of osteoblasts was verified by staining for alkaline phosphatase after fixing cells with ethanol and stained with fast red and 1-naphthyl phosphate disodium salt. Colonies containing more than 50 alkaline phosphatase-positive cells were counted as osteoblastic colonies. Representative example of alkaline phosphatase positive osteoblastic colonies is shown at 2.5X (B) and 10X (C) magnification by phase contrast microscopy. Osteoblastic colonies were also stained for evidence of mineralization by fixing in ethanol and staining with alizarin red S. Colonies containing more than 50 cells positive for alizarin red S were considered as osteoblastic colonies. Phase contrast microscopy of alizarin red S-positive osteoblast colony at 2.5X (D) and 10 X (E) magnification is shown. Differentiation of CFU-Fs into adipocytes was verified by oil red O stain. Adipocyte colonies fixed in buffered formalin were stained with oil red O dissolved in isopropanol. Cells were counter stained with Harris hematoxylin. Example of oil red O positive adipocyte colony is shown at 20X (F) and 40X (G) magnification.

Supplemental Figure S2. H&E stained BM sections of 2-week-old (A) and 2-month-old (B) rabbits. Bones of rabbit were decalcified and the middle part of the diaphysis was cut equidistant from the head of the bone and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin using standard protocols. Note increase in fat spaces in BM sections of 2-month-old compared to 2-week-old rabbits (25X magnification).
Supplemental Figure S3. Effect of CM from undifferentiated CFU-Fs and osteoblasts generated from rabbit and human BM CFU-Fs on production of B-lineage cells. CM from undifferentiated rabbit (A) and human (B) CFU-Fs were added to co-cultures of rabbit BM MNCs and OP9 stromal cells. In addition, CM from rabbit (A) and human (C) osteoblasts was added to co-culture of rabbit BM MNCs and OP9 stromal cells. At day 8, co-cultures were analyzed for the presence of CD79a^+ B- and CD14^+ myeloid-lineage cells by flow cytometry. Co-cultures without (w/o) (negative control) and with adipocyte CM (positive control) were also set up simultaneously. Data from 2 independent experiments are shown in A and B. Data from 2 independent samples (I and II) of human osteoblast CM are shown in C.

Supplemental Figure S4. Effect of rabbit adipocyte CM on generation of CD7^+ CD79a^- early lymphoid progenitors and CD7^- CD79a^+ early B-lineage cells from human HSCs. (A) Human HSCs were co-cultured for 10 days with rabbit adipocyte CM on OP9 stromal cells and analyzed by flow cytometry for the presence of CD7^+CD79a^- (early lymphoid progenitors/CLPs) and CD7^-CD79a^+ (early B-lineage cells/pre-proB). Both of these cell populations were CD19^- and cytoplasmic μ^- (data not shown). (B) Number of early lymphoid progenitors/CLPs. (C) Number of early B-lineage cells/pre-proB.
FIGURE S1.
FIGURE S3

A  EXPERIMENT 1

W/o Adipocyte CM  |  With Adipocyte CM  |  With Undifferentiated CFU-Fs CM  |  With Osteoblast CM

CD14  |  Cyto CD79a

5.7 %  |  81.8 %  |  20.5 %  |  24.4 %

3.1 %  |  57.8 %  |  55.4 %

B  EXPERIMENT 2

W/o Adipocyte CM  |  With Adipocyte CM  |  With Undifferentiated CFU-Fs CM  |  With Osteoblast CM

CD14  |  Cyto CD79a

3.3 %  |  72.1 %  |  8.6 %  |  9.6 %

35.3 %  |  9.3 %  |  51.8 %  |  55.6 %
**FIGURE S3**

**EXPERIMENT 1**

- **W/o Adipocyte CM**
  - CD14: 4.7%
  - Cyto CD79a: 83.7%

- **With Adipocyte CM**
  - CD14: 53%
  - Cyto CD79a: 6.6%

- **With Undifferentiated CFU-Fs CM**
  - CD14: 9.7%
  - Cyto CD79a: 75.1%

**EXPERIMENT 2**

- **W/o Adipocyte CM**
  - CD14: 8.3%
  - Cyto CD79a: 64.1%

- **With Adipocyte CM**
  - CD14: 37%
  - Cyto CD79a: 15.8%

- **With Undifferentiated CFU-Fs CM**
  - CD14: 20.2%
  - Cyto CD79a: 47.4%
FIGURE S3

C

<table>
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<tr>
<th>Condition</th>
<th>CD14</th>
<th>Cyto CD79a</th>
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<tr>
<td>W/o Adipocyte CM</td>
<td>12.1%</td>
<td>57.4%</td>
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<tr>
<td>With Adipocyte CM</td>
<td>23.8%</td>
<td>13.8%</td>
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<tr>
<td>With Osteoblast CM (I)</td>
<td>19.4%</td>
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<td>With Osteoblast CM (II)</td>
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</table>
**FIGURE S4**

A. W/o Adipocyte CM  

- 22.7% Cyto CD79a+  
- 13.6% CD7+  

With Adipocyte CM  

- 23.9% Cyto CD79a+  
- 5.6% CD7+

B. Early lymphoid progenitors (CLPs) (10^3) / well

- Without CM: 5  
- With CM: 4

C. Early B-lineage (pre-proB) cells (10^3) / well

- Without CM: 3  
- With CM: 2

*ns*  

***p < 0.0009***