The effects of microenvironment and internal programming on plasma cell survival

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Keywords: antibody, bone marrow, CXCL12, plasma cell, stromal cell

Abstract

Two populations of plasma cells (PCs) are formed after immunization. A short-lived population in the spleen and lymph nodes provides rapid protection. A long-lived population, mainly in the bone marrow, provides lasting immunity. The mechanisms responsible for the differences in PCs life span remain largely unknown. The goal of the current study was to compare the intrinsic survival capacity of isolated short-lived (spleen) versus long-lived (bone marrow) PCs. We approached this question by using a previously established in vitro model that measures PC survival in a supportive stromal environment. Regardless of the tissue source or isolation time point after immunization, the two PC populations showed similar intrinsic ability to survive in vitro. To test differences in the stromal microenvironments, stromal cells from marrow, spleen or lymph nodes were evaluated for ability to support PCs survival. Survival of isolated PC was always greater when co-cultured with marrow stromal cells compared with those from spleen (or lymph node) despite the finding that IL-6, necessary for PC survival in culture, was secreted by all three stromal cell sources. Additionally, low expression of B-cell-activating factor belonging to the tumor necrosis factor-family was detected in all three stromal isolates. In contrast, marrow stromal cells were distinguished by cell-surface phenotype and CXC chemokine ligand (CXCL)12, IL-7 and stem cell factor expression. Although CXCL12 has been suggested as a possible survival factor for PC, addition or neutralization of CXCL12 had minimal effect on PC survival. We conclude the mechanisms regulating PC longevity appear extrinsically driven and marrow favored, but the factors that give marrow stromal cells a unique advantage remain unknown.

Introduction

Two seemingly distinct plasma cell (PC) populations develop during the course of an immune response. One population of PCs is formed early (within 4 days) following antigen encounter in secondary immune tissue, such as the spleen. These early PCs generally have a low affinity for antigen and possess a short life span (1–3). Later in the immune response (at 1–2 weeks), a second population of PCs develops that supplies the body with lasting immunity against viruses and protein antigens (4–7). These late PCs have been selected in germinal centers to survive based on high affinity for antigen and possess an extended life span. Long-lived PCs are predominantly found in the bone marrow and, to a much lesser degree, in the spleen (5–7). Both short-lived and long-lived PC populations are integral components of an efficient immune response, providing immediate and long-term protection. Despite the important roles the two PC populations play in maintaining humoral immunity, the mechanisms responsible for the apparent differences in the PC life span are poorly defined. It is possible that the life span of a PC may be intrinsically pre-programmed. A study by Shapiro-Shelef et al. (8) demonstrated the importance of B lymphocyte-induced maturation protein (Blimp)-1 not only in the formation of PCs but also in the maintenance of long-lived PCs in the bone marrow. PCs may also differ in life span based on their ability to handle increased antibody production via either the unfolded protein response and X-box-binding protein-1 expression (9) or in proteosome activity (10). However, data also support an extrinsic control of PC survival, particularly in the bone marrow. Several microenvironmental factors have been implicated and various approaches have been used—intact animals, culture of unfractionated bone marrow,
culture of purified mature PCs with exogenous factors and/or stromal cells and immunofluorescence localization in intact bone marrow tissue (11–16). Many studies, using isolated PCs and myeloma cells, have revealed the importance of IL-6 in maintaining PCs survival in vitro (11, 13, 17–21). Recent compelling data show that reduction in tumor necrosis factor (TNF) family members B-cell-activating factor belonging to the TNF-family (BAFF) and/or APRIL in intact animals causes rapid decline of a majority of long-lived PCs (12, 14, 15). Finally, an exquisite study identified the specific niche for PCs in the bone marrow, using immunofluorescence of marrow frozen sections. PCs were shown to associate only with stromal cells expressing CXC chemokine ligand (CXCL12) in vivo (16), a finding that complements the suggestion that CXCL12 can extend PC life span in vitro (11).

Using isolated mature PCs, the main goal of the present work was to evaluate the intrinsic survival capacities of ‘short-lived’ versus ‘long-lived’ PCs. Previously, we established a model system to evaluate PC survival in vitro, whereby, PCs are FACS sorted based on CD138⁺ (Syndecan-1) surface expression and co-cultured with primary bone marrow stromal cells. In this system, PC survival is concomitant with antibody secretion (13). In the present study, we found that PCs from spleen or bone marrow, whether harvested early or late after immunization, were surprisingly similar in their intrinsic survival capacity in vitro. Because most long-lived PCs reside in the marrow, we subsequently compared the ability of stromal cells from spleen or lymph node with those from bone marrow to support the survival of long-lived PCs. Marrow stromal cells were superior in this regard, and further characterization showed that marrow stromal cells were distinguished in high expression of CXCL12 compared with those from spleen or lymph node. However, experiments using neutralizing antibody or addition of CXCL12 indicated a minor role for CXCL12 in survival of long-lived PCs isolated from bone marrow in vitro.

Methods

Animals

Female BALB/c mice (5 weeks or 22 months of age) were purchased from Harlan Laboratories (Indianapolis, IN, USA) through contract with the National Institute of Aging (Bethesda, MD, USA). Tg(Cxcr4-EGFP)73Gsat mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) (24), 10.588 and 10.938 (the latter two mAbs were prepared in our laboratory against unknown antigens on stromal cells from Whitlock-type long-term bone marrow cultures). Cells were then stained with biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and strepavidin APC (BD PharMingen) and analyzed using FACSComp (Becton Dickinson, Mountain View, CA, USA). In addition, bone marrow, splenic and lymph node stromal cells were cultured in Permanox chamber slides (Nalge Nunc International, Rochester, NY, USA), fixed in 4% PFA and stained with Cy3-conjugated antibody to mouse α-actin (clone I4, Sigma-Aldrich). Alpha-actin is found in smooth muscle (25, 26), myofibroblasts (27) and bone marrow stromal cells (24, 26, 28). Fluorescence was visualized with a Leica DM IRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Uptake of Dil-acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA, USA) was analyzed using flow cytometry (15).

Culture of adherent layers and stromal cell isolation from unimmunized mice

Bone marrow: to obtain reticular-like stromal cells, Whitlock-type long-term bone marrow cultures that support B lymphopoiesis (LTBMC-B) were initiated and maintained by established methods (22, 23). Bone marrow stromal cells were obtained from LTBMC-B by FACS sorting directly into flat-bottomed 96-well plates as previously described (22) or, in some cases, by column depletion of macrophages and B-cell precursors (described below). Isolated stromal cells were plated at a density of 1 × 10⁶ cells per well. Spleen and lymph nodes: spleens were perfused with 0.125% Collagenase D (Roche Diagnostics, Indianapolis, IN, USA) in HBSS with Ca²⁺, Mg²⁺ and then washed several times by vigorous pipetting. The spleens were then cut into 1–3 mm pieces and further incubated in the Collagenase D solution for 3 h at 37°C. Lymph nodes (axial, inguinal, and cervical) were pooled and treated with Collagenase identically to spleen fragments except that perfusion was omitted. The fragments from either tissue were dispersed by vigorous pipetting, filtered through 100 μm Nitex membrane (Sefar America, Depew, NY, USA), washed and centrifuged. Splenic and lymph node cultures were set up at a density of 30 × 10⁶ cells per 100 mm plate or 15–30 × 10⁶ cells per 100 mm plate, respectively, based on prior empirical conclusions of satisfactory outgrowth of the adherent layer. The cultures were used 1.5–4 weeks later and were maintained in the same medium as were LTBMC-B (22). Reticular-like stromal cells were enriched from the adherent cell populations and from developing lymphocytes by column depletion using biotinylated antibodies against CD11b (eBioscience, San Diego, CA, USA) and CD43 (BD PharMingen, San Diego, CA, USA), respectively, followed by anti-biotin magnetic beads (Miltenyi Biotec, Auburn, CA, USA). The resulting negative fractions were plated onto 96-well plates at a density of 0.75–1.0 × 10⁵ cells per well.

Comparison of the phenotype of stromal cells

Stromal cells isolated from the bone marrow, spleen and lymph node were stained with hamster mAbs that bind to primary bone marrow stromal cells; anti-CD28 (Caltag, Burlingame, CA, USA) (24), 10.588 and 10.938 (the latter two mAbs were prepared in our laboratory against unknown antigens on stromal cells from Whitlock-type long-term bone marrow cultures). Cells were then stained with biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and strepavidin APC (BD PharMingen) and analyzed using FACSComp (Becton Dickinson, Mountain View, CA, USA). In addition, bone marrow, splenic and lymph node stromal cells were cultured in Permanox chamber slides (Nalge Nunc International, Rochester, NY, USA), fixed in 4% PFA and stained with Cy3-conjugated antibody to mouse α-actin (clone I4, Sigma-Aldrich). Alpha-actin is found in smooth muscle (25, 26), myofibroblasts (27) and bone marrow stromal cells (24, 26, 28). Fluorescence was visualized with a Leica DM IRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Uptake of Dil-acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA, USA) was analyzed using flow cytometry (15).
Isolation of PCs and co-culture with stromal cells

PCs were isolated as described in our previous paper with modifications (13). PCs were purified from spleen and bone marrow at early (day 4) or late (day 8–10) time points following secondary immunization (29, 30). To purify splenic PCs, T cells and macrophages were removed by staining with biotinylated anti-CD3 (145-2C11, eBioscience) and anti-CD11b followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec) and retention on a magnetic column. The PCs from spleen or bone marrow were then enriched by positive selection based on CD138/Syndecan-1 expression using PE-conjugated anti-CD138/Syndecan-1 antibodies (281-2, BD PharmMingen) (31), followed by incubation with anti-PE magnetic beads (Miltenyi Biotec). PCs were further purified by FACS™ sorting for CD138+ cells from this enriched fraction. The latter step was necessary to ensure high purity of PC isolates. Purity was confirmed by light microscope examination of hematoxylin/eosin-stained cytocrifuged cells after FACS sorting. Greater than 95% of the isolated cells had the distinctive morphology of differentiated PCs (13, 32).

Five thousand freshly isolated PCs were added to each well containing 7.5–10 × 10^3 bone marrow, splenic or lymph node stromal cells in a total volume of 150 ml. Supernatants (120 ml) were collected twice weekly to determine the quantity of secreted Ig (frozen at −20°C) and fresh media was added after supernatant collection.

Recombinant CXCL12 and neutralizing anti-CXCL12 antibody

Bone marrow PCs were isolated as described above and cultured in round-bottom 96-well plates (2500 PCs per well; four wells per treatment group) in the presence of varying concentrations of recombinant mouse CXCL12 (R&D Systems, Minneapolis, MN, USA) for 7 days. In addition, neutralizing anti-human/mouse CXCL12 antibody (clone 79014, R&D Systems) or mouse IgG1 isotype control antibody (eBioscience) was added to PC/stromal co-cultures initiated as described above and harvested at day 7.

ELISAs

Antigen-specific IgG1, IgM and IL-6 ELISAs were modified from previous descriptions (13, 33). For antigen-specific ELISA, plates were coated with 10 µg ml⁻¹ TNP–KLH; for IgM and IL-6 ELISA, plates were coated with 2 µg ml⁻¹ rat anti-mouse IgM or rat anti-mouse IL-6, respectively. Biotin-conjugated rat anti-mouse IgG1, IgM or IL-6 antibodies were used at 1 µg ml⁻¹ for detection and were developed with strepavidin-conjugated alkaline phosphatase (Southern Biotechnology Associates, Inc.) and p-nitrophenyl phosphate (Sigma-Aldrich). Two-fold serial dilutions of isotype-specific standards were included on every test plate at a range of 1000–1.92 ng ml⁻¹. Recombinant IL-6 (Biosource, Camarillo, CA) was used for the standard curve in IL-6 ELISA at a range of 200–0.4 ng ml⁻¹. Samples were analyzed by quantitating values within the linear range of the standard curve. The following ELISA antibodies were purchased from BD Pharmingen: unlabeled rat anti-mouse IgM (II/41), purified mouse IgG1 (A112-2), purified mouse IgM (G155-228), biotin-conjugated rat anti-mouse IgG1 (A85-1) and biotin-conjugated rat anti-mouse IgM (R6-60.2). Unlabeled rat anti-mouse IL-6 (MP5-20F3) and biotin-conjugated rat anti-mouse IL-6 (MP5-32C11) were purchased from eBioscience.

ELISPOts

The protocol to enumerate TNP–KLH-specific PCs was modified from previous descriptions (13). First, 96-well Multi-screen hemagglutinin 0.45 µm plates (Millipore, Bedford, MA, USA) were coated with 10 µg ml⁻¹ TNP–KLH overnight at 4°C. Total cells harvested from bone marrow and spleens were added to the plates at 1 × 10^5 cells, serially diluted 1:4 and incubated for 4 h at 37°C. Bound antigen-specific antibody was detected with goat-anti-mouse Ig (H + L-chain) (Southern Biotechnologies) and plates were developed as described previously (34). Spots were counted by image analysis using the AlphaImager System (Alpha Innotech, San Leandro, CA, USA).

Reverse transcription-PCR analyses of Blimp-1 and Pax5 mRNA

RNA was isolated from purified splenic and bone marrow PCs using the Qiagen RNeasy kit (Valencia, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized using the Amersham First Strand cDNA synthesis kit (Buckinghamshire, UK). PCR analysis was performed using a GeneAmp 2400 thermal cycler (PerkinElmer, Wellesley, MA, USA) under conditions published by the Calame laboratory (35). Primer oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA): Blimp-1 5’ primer GCGAACAAGGAATCTCTGTGT and Blimp-1 3’ primer AGGATAAACCCCGGATGGT as reported by Shaffer et al. (35); Pax5 5’ GCCGTGTGAGAGAGACGACT and Pax5 3’ AAGAATCTGAGGGTGCTAT (35); β-actin 5’ primer GTGGGGCAGAGGACACA and β-actin 3’ primer CTCCTATAATGCACGCAGTTTC. PCR products were resolved by electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide and compared against a 100-bp DNA ladder (Promega, Madison, WI, USA). RNA isolated from CH12 cells stimulated with 25 µg ml⁻¹ LPS (24 h) and splenocytes stimulated with Con A (48 h) were used as positive controls for Blimp-1 and Pax5 amplification, respectively. Negative controls included samples without reverse transcriptase or RNA.

Real-time PCR analysis

RNA isolation and subsequent cDNA synthesis from purified bone marrow, splenic and lymph node stromal cells were performed as described above. Real-time PCR analysis was performed as described previously (13). Real-time oligonucleotides were designed using Primer Express Software (Applied Biosystems) and synthesized by GibcoBRL-Invitrogen: CXCL12 5’ primer CAACCCACATGCTCATCATT; CXCL12 3’ primer GGTTAGAGAAGTGGAGCAAGA; IL-7 5’ primer TCTGTGGCTGTCACCATC; IL-7 3’ primer GGACATTGATTCCTTCACTGTATC; Stem Cell Factor (SCF) 5’ primer GAATCTCGAAAGAGCAGAAG; SCF 3’ primer AGGGACTTTCTGCTGCAACA; B cell-activating factor belonging to the TNF-family (BAFF) 5’ primer TGGTGAAGAAGCAAGCATT; BAFF 3’ primer CTGTGCACTTCCGAGGAT;
GAPDH 5′ primer GTGAGGCCGGTGCTGAGTAT; and GAPDH 3′ primer TCATGAGCCCTTCCACAATG. The number of copies of transcript per milliliter were determined by generating a GAPDH standard curve with each PCR run, using 10-fold serial dilutions ranging from \(1 \times 10^2\) copies per milliliter to \(1 \times 10^5\) copies per milliliter of plasmids. The ratios of copies per microliter of the transcript of interest to copies per microliter of GAPDH in individual samples were calculated. Data are presented as the fold difference of expression for each sample as compared with lymph node stromal cells.

Statistical analysis
Two-way analysis of variance or student’s t tests were used to determine statistical differences between experimental groups.

Results
Characterization of purified splenic and bone marrow PCs.
The humoral immune response is characterized by a dynamic expansion, contraction and accumulation of PCs in different anatomical locations. The current paradigm holds that numbers of PCs peak in the spleen at day 4 following antigen boost and then quickly decline (6, 30). These PCs are believed to be short lived. Concurrent with the decline of short-lived PCs in the spleen, PCs begin to accumulate in the bone marrow (5, 29, 30). In mice, these long-lived bone marrow PCs may survive in vivo for a year or more (6, 7). In order to assess whether PCs differ in the ability to survive, we used an experimental model where a workable yield of PCs could be isolated from spleen and bone marrow. TNP–KLH was used as an immunogen and CD138+ PCs were isolated from the spleen and bone marrow 4 or 8–10 days following antigen boost. In accordance with previous data (6), the spleen had greater numbers of antigen-specific PCs (Fig. 1A) and greater levels of antigen-specific antibody (Fig. 1B) as compared with the bone marrow at the early time point after antigen boost. Between 4 and 9–10 days, the PCs in the spleen showed an extraordinary drop in number, while antigen-specific PCs increased in the bone marrow (Fig. 1A). At the later time point, antibody secretion had clearly shifted to the bone marrow (Fig. 1B). These data suggest that the splenic and bone marrow PCs isolated here are representative of the populations described as short-lived and long-lived populations of PCs, respectively (5, 6, 30).

PCs isolated from bone marrow and spleen were similar in morphology (Fig. 1C), which was comparable to previous descriptions of freshly isolated PCs (13, 36, 37). PCs isolated from both spleen and bone marrow expressed Blimp-1.
mRNA message (Fig. 1D), a transcription factor increased in PCs and important for PC differentiation (38, 39). In contrast, message for the transcription factor Pax5, repressed by Blimp-1 (35, 40), was detectable in splenocytes but absent in the isolated PC populations (Fig. 1D), reflecting the high purity of the isolates.

Comparison of intrinsic survival qualities of splenic and bone marrow PCs.

Bone marrow PCs are reported to be longer lived than most splenic PCs (6, 7). We previously tested the ability of bone marrow stromal cells to provide a survival environment for PCs in vitro (13). We found that antibody secretion is prolonged in PC/stromal cell co-cultures for 2–3 weeks beyond that detected in PCs cultured in media alone (13), which has been confirmed by others (14). In the co-culture system, relative levels of antibody correlate with the numbers of surviving PCs (13). The PC/marrow stromal cell co-culture system was used here to determine whether intrinsic differences exist in the longevity of short-lived and long-lived PCs, when placed in a supportive environment. Since the quantity of antibody secreted differs from experiment to experiment in PC isolates, relative antibody levels were normalized by calculating the amount of antigen-specific antibody at each time point relative to the value at day 1. If bone marrow PCs possess a survival advantage over splenic PCs, then antibody levels in cultures of splenic PCs should decline more rapidly than in cultures of bone marrow PCs. However, relative decline in antibody levels was similar between splenic and bone marrow PCs, independent of the time after immunization (Fig. 2A and B). Similar to previous observations using the stromal cell co-culture assay (13), antibody levels from both PC populations declined rapidly within the first week of co-culture followed by an attenuated reduction for the remaining 2–3 weeks of culture period. These data suggest that whether isolated at early (4 days) or late (8–10 days) times following antigen boost, splenic and bone marrow PCs are similar in ability to survive in vitro during the initial co-culture period.

The influence of the microenvironment on PC survival.

The above results suggested that most bone marrow and splenic PCs have similar intrinsic survival profiles. Several in vitro studies in mice and humans indicate that PC survival may be impacted by the microenvironment (13, 16, 19, 21, 29, 41). Consistent with the idea of extrinsic control of PC longevity, PCs associate in niches where reticular cells are found: in the bone marrow (13, 42, 43), in the red pulp of the spleen (2, 3, 44, 45) and in the medullary cords of the lymph nodes (4, 46). Since PCs tend to preferentially accumulate over time in the bone marrow rather than in the spleen or lymph nodes, we tested the possibility that reticular-like stromal cells from bone marrow provide a more favorable environment for PC survival than reticular-like cells from the spleen and lymph node. Primary adherent cell cultures were established from each organ and stromal cells were purified. The lymph node and splenic stromal cells were similar to bone marrow stromal cells in that they were reticular-like in morphology and all expressed α-actin, a cytoskeletal fiber characteristic of bone marrow stromal cells (Fig. 3A).

To test the ability of splenic and lymph node stromal cells to support PC survival, bone marrow PCs were co-cultured with the various stromal cells and relative antibody levels were assessed over time. Antibody levels were always greater when PCs were co-cultured with bone marrow stromal cells in comparison with stromal cells from spleen or lymph node (3/3 experiments) (Fig. 3B). Bone marrow stromal cells were more supportive at the outset, since antibody production declined more rapidly in lymph node and splenic stromal cell co-cultures. By day 4 through the remainder of
the culture period, antibody secretion from splenic and lymph node co-cultures was 25–50% of that observed from bone marrow co-cultures. These data suggest that the bone marrow microenvironment is more favorable to PC survival than either the spleen or lymph node. Notably, antibody was detected in all co-cultures for at least 14 days (Fig. 3B), implying that stromal cells from bone marrow, spleen or lymph node are all capable of supporting PC survival to some degree.

Bone marrow stromal cells produce IL-6, which is well documented as a stromal cell-derived factor that influences survival of both normal mouse (11, 13, 21) and malignant (17–20, 42) human PCs. We next determined whether IL-6 protein levels differed among stromal cells enriched from bone marrow, spleen and lymph node, by measuring IL-6 in the supernatants of the various stromal cells by ELISA. Although bone marrow stromal cells promoted greater PC survival, the constitutive levels of IL-6 were appreciably greater in supernatants from splenic and lymph node stromal cells (Fig. 3C). Splenic and lymph node stromal cells secreted four to five times more IL-6 than the bone marrow stromal cells (21–25 ng ml⁻¹ IL-6 from splenic and lymph node stromal cells versus 0–4 ng ml⁻¹ IL-6 from bone marrow stromal cells). Thus, IL-6 alone did not account for the favorable survival of PCs observed in bone marrow stromal co-cultures.

Because the bone marrow, splenic and lymph node stromal cells differed functionally, we further characterized the stromal cell isolated for cell-surface phenotype. Using antibodies specific for cell-surface molecules previously found on bone marrow stromal cells (CD28, 10.938 and 10.588), lymph node and splenic stromal cells virtually lacked expression of all three molecules tested (Table 1). Antibody secretion from PCs co-cultured with stroma initiated from CD28⁻/⁻ mice was similar to secretion from those co-cultured with stroma from wild-type counterparts (data not shown), suggesting it is not necessary for PC survival. Additional characterization of stromal cells isolated from bone marrow, spleen and lymph node was done using quantitative real-time PCR to detect CXCL12 and BAFF, which have been proposed to maintain PC survival (11, 14), as well as the well-known stromal-derived factors IL-7 and SCF (22, 47). Bone marrow stromal cells were clearly distinguished from splenic and lymph node stromal cells in CXCL12, IL-7 and SCF mRNA expression (Table 2). CXCL12 mRNA levels from bone marrow stromal cells were at least 94-fold greater than that detected in either splenic or lymph node stromal cells (Table 2). Thus, the high expression of CXCL12 may be one reason bone marrow stromal cells provide better support for PC survival and will be explored further below. Bone marrow stromal cells also had greater numbers of mRNA transcripts for both SCF and IL-7 (12- to 74-fold and 33-fold greater, respectively, Table 2). While both of these cytokines are essential for B-cell development, little or no role has been found for these cytokines in terms of maintaining PC survival. The addition of exogenous rIL-7 to PC cultures in the absence of stromal cells did not enhance antibody secretion or resultant PC survival and no differences were seen in antibody secretion from PCs co-cultured with stromal cells isolated from IL-7-deficient mice (data not shown). Finally, BAFF has recently
The number of copies of target to the number of copies of GAPDH. A standard curve. Target mRNA levels were calculated by normalizing with levels detected in lymph node stromal cells. RNA isolated from reticular stromal cells.

Table 1. Comparison of stromal cells cultured from bone marrow, spleen and lymph node

<table>
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<th>Bone marrow</th>
<th>Spleen</th>
<th>Lymph node</th>
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<tr>
<td>CD28</td>
<td>250^b</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>10.588^c</td>
<td>725</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>10.938^c</td>
<td>2695</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td>ac-LDL^d</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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ac-LDL, acetylated low-density lipoprotein.

aBone marrow, spleen and lymph node stromal cells were stained with the following hamster antibodies: anti-CD28, 10.588 and 10.938 and analyzed by flow cytometry. In addition, stromal cells were incubated in the presence of Dil-ac-LDL and analyzed using flow cytometry.

bPositive staining was determined based on the geometric mean fluorescence intensity compared with isotype control staining.

cThe 10.588 and 10.938 antibodies are hamster antibodies raised specifically against cell-surface molecules of mouse bone marrow reticular stromal cells.

dUptake of ac-LDL.

Table 2. Comparison of various target transcripts expressed in purified primary stromal cells

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<tr>
<th></th>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Lymph node</th>
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<tr>
<td>CXCL12</td>
<td>95.6^a</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>SCF</td>
<td>74.2</td>
<td>6.5</td>
<td>1.0</td>
</tr>
<tr>
<td>IL-7</td>
<td>33.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>BAFF</td>
<td>0.6</td>
<td>0.6</td>
<td>1.0</td>
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^aData are shown as the fold difference in copy number as compared with levels detected in lymph node stromal cells. RNA isolated from stromal cells was analyzed for transcripts indicated using real-time PCR. Copy number per λ was determined for each sample based on a standard curve. Target mRNA levels were calculated by normalizing the number of copies of target to the number of copies of GAPDH.

The presence of CXCL12 was notably less than in cultures with IL-6 (Fig. 4B) and (ii) addition of both factors showed neither an additive nor a synergistic effect (Fig. 4C). However, the importance of CXCL12 may be transient and/or dependent on the presence of factors other than IL-6 in the milieu as antibody concentration is much greater in PC cultures following the addition of rIL-6. Instead, when IL-6 is present, CXCL12 appeared dispensable.

To determine if CXCL12 played a role in the ability of stromal cells to support PC survival, neutralizing anti-CXCL12 antibody was added to co-cultures containing bone marrow stromal cells and isolated PCs. Antibody levels following 7 days of co-culture with or without neutralizing antibody were similar (Fig. 5A), suggesting that in the presence of stromal cells, PCs do not require CXCL12 for survival. Further, the addition of rCXCL12 had little enhancing effect on antibody secretion in co-cultures containing stromal cells from bone marrow, spleen or lymph node (Fig. 5B). Taken together, the results suggest that the role of CXCL12 in maintaining PC survival is minimal.

Discussion

It is remarkable that most PCs appear to be long lived in the bone marrow, while only a minor fraction of splenic PCs are long lived. If long-term survival is intrinsic to marrow PCs, then antigen-activated B cells must choose between alternate gene expression pathways as they become PCs. If the contrary hypothesis is correct—that is, cues from the microenvironment control PC life span—it must be presumed that the marrow is enriched in factors that are deficient in peripheral lymphoid tissues. Identifying these specific elements is essential to a complete understanding of humoral immunity. In fact, the weight of evidence currently supports the microenvironment hypothesis; however, the survival potential of isolated short-lived and long-lived PCs had not been directly compared. While splenic PCs (short lived) and bone marrow PCs (long lived) are thought to have differing life spans in vivo, their tissue of origin appears irrelevant in vitro when presented with supportive marrow. Our studies conclude that PC survival does not appear to be pre-programmed, but rather survival is strongly manipulated by the stromal environment.

Identifying the external signals responsible for maintaining PC longevity remains a current challenge despite numerous reports on multiple candidate factors. Different factors appear to be relevant in different experimental situations. For example, the role of IL-6 has been delineated in vitro (11, 13, 21) and evidence for BAFF and APRIL as mediators of non-malignant PC longevity has been shown mostly in vivo (12, 14, 15). In contrast, anatomical studies have revealed associations of PCs with CXCL12-expressing reticular stromal cells in bone marrow sections (16).

Several studies have shown that IL-6 is critical for PC survival in vitro (11, 13, 17–21, 42), and co-culture of PCs yields up-regulation of IL-6 expression in bone marrow stromal cells (13). However, the appearance of PCs in the marrow of IL-6-deficient mice suggests that IL-6 is sufficient, but not necessary, in vivo or that it is compensated for by another factor.
Data presented here indicate that the level of CXCL12 mRNA message is many-fold greater in stromal cells isolated from the bone marrow as compared with the spleen and lymph node. A strong case can be made for the importance of CXCL12 to developing marrow PCs. Notably, plasmablasts emigrate to the marrow by chemotaxis toward CXCL12 (50). Once the plasmablasts find appropriate survival niches in the marrow and complete terminal differentiation to PCs, the migratory response to CXCL12 wanes. For example, ovalbumin peptide-specific bone marrow antibody-secreting cells isolated 6 days following secondary immunization exhibit a strong migration toward CXCL12 but lose the migratory capacity if isolated from the marrow 12 days following antigen boost (48). Despite the loss of chemotactic responsiveness to CXCL12, PCs retain surface expression of the receptor to CXCL12 (48–51), and lymph node PCs exhibit extracellular signal-related kinase-1 and 2 phosphorylation in response to CXCL12 (51). Moreover, PCs appear to co-localize with CXCL12+ stromal cells (16).

These observations suggest additional roles for CXCL12 may exist. Indeed, Cassese et al. (11) reported that 10 ng ml⁻¹ CXCL12 increased PC survival in vitro. However, we found that survival is only slightly enhanced even at much higher concentrations of CXCL12. In addition, neutralizing CXCL12 in PC/stromal cell co-cultures had little effect on PC survival, and the need for CXCL12 appears minimal if IL-6 is added to the cultures. Instead, CXCL12 may have a compensatory role in terms of PC survival: when IL-6 is lacking, CXCL12 can promote survival, although not to the extent of IL-6. This could explain, in part, why PCs are still detected in IL-6−/− mice (52). Thus, IL-6 appears to have a more important role in vitro maintaining PC survival and CXCL12 may be dispensable when stromal cell support and stromal cell-derived factors are provided.

Recent studies, mostly based on in vivo experiments, emphasize a role for BAFF and/or APRIL in maintaining PC and myeloma cell survival (12, 14, 15). O’Connor et al. (14) reported that bone marrow PCs decrease substantially in vivo when transmembrane activator and CAML interactor (TACI), one of the receptors for BAFF and APRIL is blocked and, further, that adding BAFF to in vitro bone marrow cultures enhances PC survival. In addition, two recent reports show convincing evidence that APRIL is important for the survival of PCs in mouse bone marrow (12, 15). Our results show negligible BAFF transcript in stromal cells isolated from bone marrow, spleen and lymph node; however, substantial BAFF and APRIL expression has been identified in human CD14+ bone marrow cells and in human osteoclasts derived ex vivo (53). Osteoclasts and monocytes are relatively rare cells in the marrow but may well provide a source of BAFF and/or APRIL in mouse bone marrow. It is not clear, however, if osteoclasts/monocytes are part of the specific marrow niches for PCs, as has been described in anatomical detail for CXCL12-expressing stromal cells.

While the in vitro results described here may not exactly mimic what occurs in vivo, these experiments do allow us, in part, to disengage intrinsic influences on PC survival from extrinsic influences and support the notion that the two populations do not differ intrinsically in survival capabilities. Taken together, the data presented here provide a strong case for extrinsic regulation of PC longevity. Future studies using this experimental system should allow further dissection of critical components and events necessary for bone survival.
Fig. 5. Effects of neutralizing CXCL12 antibody or rCXCL12 on PC/stromal cell co-cultures. (A) Bone marrow PCs were co-cultured with bone marrow-purified stromal cells in the presence or absence of neutralizing anti-CXCL12 antibody (10 μg ml−1) or isotype control antibody (10 μg ml−1). Day 7 supernatants were collected and antibody concentrations determined by ELISA. Data are expressed as the percentage increase in antibody secretion following addition of rCXCL12 as compared with PCs co-cultured with organ-specific stromal cells alone.

marrow PC survival as well as the further elucidation of the unique role of bone marrow stromal cells in maintaining PC longevity.

Acknowledgements

We thank Joseph Brewer for CH12 RNA, John Dye for assistance with fluorescence microscopy imaging, Pegha Mohseni and Shubin Zhang for technical assistance and Kara Johnson for insightful discussions. We also thank Patricia Simms and the Loyola FACS Core Facility for technical assistance and Kara Johnson for insightful discussions. This work was supported by National Institutes of Health Grants R01AG13874 and K07AG00997 (PLW) and Columbia College Chicago Faculty Development Grant (HAMWW).

Abbreviations

BAFF: B cell-activating factor belonging to the tumor necrosis factor-family
Blimp-1: B lymphocyte-induced maturation protein

References


