Definition of a Novel Cellular Constituent of the Bone Marrow That Regulates the Response of Immature B Cells to B Cell Antigen Receptor Engagement

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Definition of a Novel Cellular Constituent of the Bone Marrow That Regulates the Response of Immature B Cells to B Cell Antigen Receptor Engagement

Peter C. Sandel,* Mariya Gendelman, † Garnett Kelsoe, † and John G. Monroe*

Previously we defined a Thy1 dull bone marrow-derived cell population that regulated fate decisions by immature B cells after Ag receptor signaling. The microenvironmental signals provided by this cell population were shown to redirect the B cell Ag receptor induced apoptotic response of immature B cells toward continued recombination-activating gene (RAG) expression and secondary light chain recombination (receptor editing). Neither the identity of the cell responsible for this activity nor its role in immature B cell development in vivo were addressed by these previous studies. Here we show that this protective microenvironmental niche is defined by the presence of a novel Thy1 dull, DX5 dim cell that can be found in close association with immature B cells in vivo. Depletion of this cell eliminates the anti-apoptotic effect of bone marrow in vitro and leads to a significant decrease in the number and frequency of bone marrow immature B cells in vivo. We propose that, just as the bone marrow environment is essential for the survival and progression of pro-B and pre-B cells through their respective developmental checkpoints, this cellular niche regulates the progression of immature stage B cells through negative selection. The Journal of Immunology, 2001, 166: 5935–5944.

Early B lymphocyte development is characterized by a series of checkpoints that are governed by the expression and specificity of the B cell Ag receptor (BCR). The early steps in B cell development proceed in an ordered manner and involve the sequential expression and assembly of the BCR. During the pro-B stage, recombination-activating gene (RAG)-1,2-mediated D→J then V→DJ recombination at the Ig heavy chain locus is necessary for expression of the heavy chain component of the BCR. An analogous process at the Ig light chain locus occurs as cells transit through the pre-B stage (1–7). The relative contribution of B cell intrinsic processes and extrinsic signals from other cells in either initiating recombination or in regulating the subsequent positive selection of cells in which recombination has occurred properly is not fully defined. With regard to extrinsic signals, it is known that stromal cell-dependent contact and microenvironmentally derived cytokines influence the survival of both pro- and pre-B cells. For example, pro-B cells require both physical contact with bone marrow stromal cells and IL-7 for survival and Ig heavy chain rearrangement (8–14), whereas pre-B cells require IL-7 and CXC chemokine receptor 4-stromal-derived factor 1-mediated interactions for development in the bone marrow (15–19). Pre-B cells that receive the proper signals indicating successful Ig light chain recombination and expression proceed to the immature stage of B cell development.

A hallmark of the transition from the pre-B to the immature B cell stage is the gradual down-regulation of RAG-1 and RAG-2 expression, cessation of light chain gene recombination, and assembly and surface expression of conventional BCR complexes. Immature stage B cells represent the first stage where there is expression of the fully assembled BCR and, therefore, marks the first stage where recognition and responses to conventional Ag are possible. Ag encounter at the immature B cell stage can result in multiple outcomes, including cell death, developmental arrest, and secondary light chain gene recombination (receptor editing) (20–26). Our previous studies have indicated that the choice between these different fates can be determined by intrinsic signaling processes initiated upon BCR engagement in immature stage B cells as well as by extrinsic signals determined by the microenvironmental context of these signals (27). In isolation, both immature and the later stage transitional immature stage B cells respond to strong BCR engagement by undergoing apoptosis. This response occurs within 12–16 h after signal initiation and requires only brief (10–20 min) BCR stimulation (28). Furthermore, the immature B cell response to BCR engagement is associated with distinct signal transduction processes that distinguish immature and transitional immature BCR signaling from the mature-stage B cell in which similar stimulation results in activation and proliferation (25, 29, 30). These studies indicate that the intrinsic or programmed response of immature and transitional immature B cells is apoptotic and support the conclusion that the default mechanism of negative selection of these B cells to high avidity Ag encounter is deletion.

However, the apparent intrinsic apoptotic response of the immature B cell observed in vitro contrasts with the ability of immature B cells to undergo receptor editing under some circumstances in vivo (21). The mechanisms that regulate the fate decision between deletion and continued light chain recombination and replacement are not known, although models involving developmental stage and microenvironmental influences have been proposed (31, 32). With regard to the latter, we have documented that...

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Abbreviations used in this paper: BCR, B cell Ag receptor; WBM, whole bone marrow; BMPC, bone marrow protective cell; NRigG, normal rabbit IgG; RAG, recombination-activating gene;
the intrinsic apoptotic response of immature B cells to BCR engagement can be blocked and redirected toward continued Ig light chain gene recombination and receptor editing if these B cells were cultured in direct contact with dissociated bone marrow, but not spleen (27). These findings have suggested that the mechanism of B cell negative selection is regulated by the microenvironmental context in which the immature B cell encounters Ag. We propose that the existence of a cellular niche defined by a bone marrow protective cell (BMPC) population plays a role in determining whether the outcome of BCR engagement results in deletion or permits a continuation of light chain recombination.

Previous studies characterizing the activity of this population with regard to the response of the immature B cell to BCR engagement have used coculture models in which the normal compartmentalization of the marrow is lost. Our current studies were designed to explore the anatomical colocalization and the in vivo influence of the BMPC on the development of the immature stage B cell. Furthermore, we demonstrate through in vitro functional studies and population dynamics in vivo that the bone marrow microenvironmental niche that influences the fate decision made by the immature stage B cell subsequent to BCR engagement is marked by the presence of a previously unidentified cell type. Therefore, our current studies implicate that immature stage B cells, like pro-B and pre-B stage cells, are regulated by extrinsic signals from the bone marrow during their development.

Materials and Methods

Reagents

F(ab’)_2 of polyclonal rabbit anti-mouse IgM were generated in our laboratory and have been previously described (33). All Abs for flow cytometry were purchased from Wako Laboratory and have been previously described (33). All Abs for flow cytometry were employed.

Mice

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our colony. BALB/c Rag-2Δ/Δ mice were the kind gift of Jan Erikson (Wistar Institute, Philadelphia, PA) and the Rag-2Δ/Δ x IL-2RγΔ/Δ mice were provided by Taconic Farms (San Diego, CA). Rabbit anti-asialo-GM1 and normal rabbit IgG (NR IgG) were purchased from Wako (Richmond, VA).

Isolation and purification of transitional immature B cells

Transitional immature B cells were isolated from the spleens of mice that had been subjected to 500 rad of whole body irradiation and allowed to reconstitute the bone marrow and peripheral lymphoid compartments for 13–14 days as described in detail elsewhere (26, 34, 35). Splenocytes were depleted of T cells and macrophages as previously described (29, 36) and were >95% IgM<sup>high</sup>, IgD<sup>low</sup>, HSA<sup>high</sup>, and expressed on a wide variety of cells including peripheral T cells, thyocytes, epithelial cells, fibroblasts, neurons, hemopoietic stem cells, NK cells, and NKT cells (38–42).

B cell apoptosis assay

B lymphocytes were cultured alone, with unfractonated bone marrow, or sorted bone marrow populations and harvested 14–16 h after the addition of 20 μg/ml rabbit anti-mouse IgM F(ab’)2. Cells were washed in FACS buffer (1X PBS, 2% FCS, 0.02% NaN3) and fixed in ice-cold 70% ETOH at −20°C overnight. Cells were washed as above and stained with 10 μg/ml propidium iodide, 50 μg/ml RNase for 8 h at room temperature. Cells were analyzed by flow cytometry performed on Becton Dickinson FACScan at the University of Pennsylvania Flow Cytometry Facility, and the number of subdiploid cells was determined by cell cycle analysis using CellQuest software.

Isolation of Thy-1<sup>high</sup> bone marrow subsets

WBM was prepared as above and stained with anti-Thy-1.2-APC and DX5-PE (PharMingen) or the Abs listed for Fig. 1. Stained cells were live gated and sorted based on Thy-1.2<sup>high</sup>, DX5<sup>low</sup> and Thy-1.2<sup>high</sup>, DX5<sup>high</sup> populations using a Becton Dickinson FACSVantage at the University of Pennsylvania Cancer Center Cytometry core. Sorting was performed at 23 psi using a 60-μM sort tip. Postsort purity was always >95% and was usually greater than 98%. Isolated bone marrow populations were rested in culture overnight and then cocultured with CFSE-labeled transitional immature B cells as above except that the B cell-to-bone marrow ratio was increased to 10:1 (B cells-sorted bone marrow cells).

Rag-2 RT-PCR

Total RNA was prepared from transitional immature B cells cultured with sorted Thy-1.2<sup>high</sup>, DX5<sup>low</sup> or Thy-1.2<sup>low</sup>, DX5<sup>high</sup> bone marrow after 22 h using RNA STAT-60 (Tel-Test, Friendswood, TX). cDNA was prepared using 6 μg of total RNA, 500 ng oligo 15-dT primer (Promega, Madison, WI), and 400 U Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD). Rag-2 and β-actin mRNA expression was determined by PCR using previously published primers and amplification conditions (37). Amplified products were resolved on 1.5% agarose gels, transferred to Genescreen Plus (NEN, Boston MA) and detected by Southern analysis using a 32P-labeled oligonucleotides specific to bases 260–284 of the Rag-2 cDNA or 414–434 of β-actin. Quantitation was performed on a Molecular Dynamics Storm 860 Phospholmager.

Histology

BALB/c femurs from normal and sublethally irradiated adult mice were frozen in OCT, sectioned, and acetone fixed. Before staining, bone marrow sections were blocked in 2% BSA, 0.2% cold water fish skin gelatin in PBS. Sections were stained with DX5-FITC and anti-IgM-Cy3 (Jackson ImmunoResearch, West Grove, PA) and mounted in Prolong (Molecular Probes, Eugene, OR). Confocal microscopy was performed at the University of Pennsylvania Cancer Center morphology core facility using a Nikon E600 and a Bio-Rad confocal array.

BMPC depletion

Neonatal BALB/c mice were treated with rabbit anti-asialo-GM1 or NR IgG for 8 wk to deplete Thy<sup>1low</sup>, DX5<sup>low</sup> cells. Mice were injected i.p. with 800 μg of Ab for 3 wk a week beginning on neonatal day 1. For week 4–8, the dose was increased to 1.6 mg injected i.v. Experimental and rabbit serum Ig controls were given two injections of 1.6 mg of IgG, 1 wk apart.

Results

The BMPC is present within a heterogeneous population of Thy-1<sup>high</sup> cells in the bone marrow

We previously identified the BMPC as a Thy<sup>1high</sup> cell population found in the bone marrow of wild-type and Rag<sup>-2<sup>−/−</sup></sup> mice (27). Thy1 (CD90) is a GPI-anchored membrane glycoprotein that is expressed on a wide variety of cells including peripheral T cells, thyocytes, epithelial cells, fibroblasts, neurons, hemopoietic stem cells, NK cells, and NKT cells (38–42). The heterogeneity of the Thy<sup>1high</sup> fraction of the bone marrow is evident by the presence of subsets of cells expressing B220, CD5, CD11c, CD11b (data not shown), and DX5 (Fig. 1A). We also detected MHC class II-expressing cells in this population (data not shown). CD4- and CD8-expressing cells were mostly undetectable in the Thy<sup>1high</sup> population, although they were a significant component of the Thy<sup>1low</sup> population (data not shown).

To determine which population(s) within the Thy<sup>1high</sup> component of the bone marrow was responsible for the BMPC activity, Thy<sup>1high</sup> cells coexpressing B220, MHC class II, CD11c, or DX5 were sorted from normal BALB/c bone marrow. After sorting,
each subpopulation was placed in coculture with transitional immature B cells isolated and purified from sublethally irradiated and autoreconstituted BALB/c mouse spleens. Immature B cells from this source were chosen because they can be isolated as a homogeneous population of immature stage B cells, lacking pre-B and mature B cells (25, 27). Before placement in the cocultures, the immature cells were labeled with CFSE so that they could be subsequently identified and analyzed after stimulation with anti-BCR Abs. As with our previous studies (27), CFSE, in conjunction with propidium iodide staining to measure DNA content, allows us to determine the frequency of apoptotic transitional immature B cells in mixed cell cultures following BCR engagement.

The anti-apoptotic effect of the BMPC within the unfractionated bone marrow is demonstrated in each of the experiments depicted in Fig. 1, B–D. In each case, addition of WBM to cultures of CFSE-labeled transitional immature stage B cells at a ratio of 2:1 (WBM cells:transitional immature B cells) resulted in a decreased number of apoptotic CFSE-tagged cells in both unstimulated and anti-BCR-stimulated cultures. As with our previous studies (27), the Thy1dull subset was as effective as the WBM in this protective effect. For comparison, it should be noted that the Thy1dull population comprises only ~5% of the total bone marrow. Approximately 20–40% of these are DX5pos (1–2% of the bone marrow). In contrast to the other cellular elements of the bone marrow tested above, a purified population of DX5pos of Thy-1 dull cells was observed to be as effective as the unfractionated Thy1dull population for protecting the immature B cell from anti-BCR-induced apoptosis (Fig. 2B). In contrast, the DX5neg subset of the Thy1dull bone marrow was ineffective in blocking the BCR-induced apoptotic response. In this case, the background and BCR-induced apoptotic

The BMPC is a DX5-expressing cell within the Thy1dull subpopulation of the bone marrow

In addition to the lineage markers tested in the above studies, we also observed a significant population of Thy1dull bone marrow cells to be positive for expression of the DX5 marker (Fig. 1A). DX5 is a marker expressed on NK cells and NK T cells (43, 44). Thy-1 dull DX5pos and Thy-1 dull DX5neg bone marrow cells (Fig. 2A) were isolated by FACS to >95% purity and cultured with CFSE-labeled transitional immature stage B cells. The Thy1dull population comprises only ~5% of the total bone marrow. Approximately 20–40% of these are DX5pos (1–2% of the bone marrow). In contrast to the other cellular elements of the bone marrow tested above, a purified population of DX5pos of Thy-1 dull cells was observed to be as effective as the unfractionated Thy1dull population for protecting the immature B cell from anti-BCR-induced apoptosis (Fig. 2B). In contrast, the DX5neg subset of the Thy1dull bone marrow was ineffective in blocking the BCR-induced apoptotic response. In this case, the background and BCR-induced apoptotic

FIGURE 1. FACS analysis and sorting of Thy1 dull bone marrow populations. A, BALB/c bone marrow was analyzed for coexpression of Thy1.2 (Thy1 expression) and other hematopoietic lineage and stromal cell markers. FACS analysis was performed on the Thy1.2 dull bone marrow population coupled with simultaneous analysis of the indicated surface proteins. Solid lines represent isotype controls for the appropriate primary Abs. B–D, Thy1 dull, Thy1 dull B220pos, Thy1 dull B220neg, Thy1 dull I-A^dpos, Thy1 dull I-A^dneg, Thy1 dull CD11cpos, and Thy1 dull CD11cneg populations were sorted from BALB/c WBM by high speed FACS sorting, rested overnight, and placed in culture with CFSE-labeled transitional immature B cells. Cocultures were at ratios of 2:1 (WBM-transitional immature B cells) for unsorted bone marrow and 1:10 (sorted populations-transitional immature B cells) for the sorted populations. Cultures were stimulated with 20 μg/ml of rabbit anti-mouse IgM F(ab)2 fragments (anti-BCR) for 16 h, fixed, and stained with propidium iodide for identification of apoptotic cells. Responses by the CFSE-labeled cells in the cocultures were compared with those in cultures of CFSE-labeled transitional immature B cells in medium alone (none). Bars represent the mean percent hypodiploid (apoptotic) CFSE-labeled cells in duplicate cultures ± SD.
responses were not significantly different from cultures of transitional immature B cells in the absence of any cocultured cells.

Another documented characteristic of the response of immature B cells to BCR signaling in the presence of BMPC-containing bone marrow is continued or induced Rag-1 and Rag-2 mRNA expression (27). To determine whether this response is also mediated by the Thy1 dull/DX5 pos component we measured Rag-2 mRNA levels in cultures containing transitional immature B cells in the presence of Thy1 dull/DX5 pos cells sorted from bone marrow of Rag-2/−/− mice after anti-BCR stimulation (Fig. 2C). Normalized expression levels of Rag-2 were nearly 4-fold higher in anti-BCR-stimulated cultures containing Thy1 dull/DX5 pos cells as compared with similarly cultured B cells in the absence of anti-BCR stimulation. This level of induction or maintenance is comparable to that observed previously using unfractionated bone marrow (27). Furthermore, induced apoptosis in the absence of any cocultured cells (data not shown), indicating that this response requires both BCR signals as well as the Thy1 dull/DX5 pos component of the bone marrow.

The BMPC is a novel cellular constituent of the bone marrow

The Thy1 pos, DX5 pos phenotype of the BMPC is characteristic of NK cells and NK T cells (42–45). Like conventional T cells, NK T cells depend upon RAG-mediated DNA recombination for their development. However, as is evident from data depicted in Fig. 3A, equivalent anti-apoptotic activity was mediated by bone marrow from wild-type or Rag-2/−/− mice substantiating that, like the Rag-2 expression studies shown in Fig. 2C, the anti-apoptotic activity of the BMPC requires neither NK T nor conventional T cells. Also, bone marrow from sublethally irradiated, d14 autoreconstituted normal BALB/c mice also continued to exhibit BMPC activity (Fig. 3B) indicating that the BMPC activity resides within a relatively radio-resistant compartment of the bone marrow.

To examine the role of NK cells in mediating the protective effect of bone marrow on BCR-mediated apoptosis of immature B cells, we cultured transitional immature B cells with bone marrow from IL-2Rγ−/− mice. Mice lacking the IL-2Rγ protein exhibit diminished numbers of T cells and B cells, and no mature NK cells (46). For our experiments, we used IL-2Rγ−/− mice that had been

![FIGURE 2.](image-url)

**FIGURE 2.** Thy1 dull/DX5 pos bone marrow cells protect transitional immature B cells against BCR-mediated apoptosis and facilitate BCR-induced Rag-2 expression. A, RBC-depleted WBM was stained with anti-Thy1.2-APC (Thy1 expression) and DX5-PE (DX5 expression) and analyzed for the relative expression of these two markers. B, Thy1 dull/DX5 pos and Thy1 dull/DX5 neg cells were sorted from BALB/c bone marrow by FACS at 23 psii, rested overnight in culture and then added to CFSE-labeled transitional immature B cells at either 2:1 (WBM-transitional immature B cells) for the unfractionated WBM or 1:10 for sorted Thy1 dull populations. Cultures were stimulated with (hatched bars) or without (open bars) 20 μg/ml of rabbit anti-mouse IgM F(ab)2 fragments (anti-BCR) and incubated for 18 h. Cultures were harvested, fixed, and stained with propidium iodide for identification of apoptotic cells. Responses by the CFSE-labeled cells in the cocultures were compared with those in cultures of CFSE-labeled transitional immature B cells in the absence of any cocultured cells. Bars represent the mean percent hypodiploid (apoptotic) CFSE-labeled cells in duplicate cultures ± SD.

![FIGURE 3.](image-url)

**FIGURE 3.** Bone marrow from lymphocyte-deficient, Rag-2−/−, or IL-2Rγ−/− mice protects transitional immature B cells against BCR-induced apoptosis. CFSE-labeled transitional immature B cells were cocultured with normal WBM, and the BCR-induced apoptotic responses were measured as in Figs. 1 and 2 and compared with cultures of CFSE-labeled transitional immature B cells in medium alone (none) as well as to responses in cocultures of WBM from BALB/c × Rag-2−/− mice (A), sublethally irradiated BALB/c mice (B), or C57Bl6 Rag-2−/− × IL-2Rγ−/− mice (C). All cocultures were at a ratio of 2:1 WBM-transitional immature B cells. Bars represent the mean percent hypodiploid CFSE-labeled cells in triplicate cultures ± SD.
bred onto a \textit{Rag-2}^{−/−} background so as to eliminate T, B, NK T, as well as NK cells. As shown in Fig. 3C, BCR-induced apoptotic responses by transitional immature B cells were almost completely eliminated when these cells were cultured in the presence of bone marrow from \textit{IL-2Rγ}^{−/−} mice. Although because of the tight error bars in this comparison one might argue that there is some loss in protection that is associated with this mutation, in fact across three experiments, we never observed an inability of the \textit{IL-2Rγ}^{−/−} bone marrow to mediate BMPC activity. Therefore, based on all of our analyses, we conclude that the Thy1\textsuperscript{dull} DX5\textsuperscript{pos} BMPC is not a conventional T, NK T, or NK cell.

\textit{Immature B cells and DX5\textsuperscript{pos} cells are present in the same bone marrow compartment in vivo}

Bone marrow is a highly organized structure containing hematopoietic stem cells, stromal cells, dendritic cells, monocytes, T cell precursors, NK cells, developing and recirculating B cells, as well as other less well-defined cells. Stromal reticular cells provide developmental signals and allow developing B cells to migrate through the bone marrow (47–50). Lymphopoiesis begins near the surrounding bone endosteum, and as B cells develop, they move centripetally along stromal reticular cells toward the bone marrow center (49). Finally, they transverse the sinusoidal endothelium and enter the circulation via the central sinus and its tributaries (49).

Our studies up to this point have relied on disassociated bone marrow and peripheral transitional immature B cells to reveal BMPC activity. However, in vivo the BMPC might not be present in the same bone marrow microenvironment as immature stage B cells. To determine whether BMPC and IgM-expressing cells colocalized in situ, we made serial sections from whole mouse femurs and identified IgM- and DX5-expressing cells by immunofluorescence. Fig. 4A diagrammatically depicts our interpretation of the data in Fig. 4B. Based on the migration and maturation of B cell progenitors described previously by Osmond and colleagues (49, 50), we observed a gradual increase in the green fluorescence intensity of IgM-expressing cells as they proceeded from the bone endosteum to the area of the central sinus. We believe that this increase in IgM expression reflects the stepwise maturation from the pre−immature−transitional immature B cell stages. Importantly, it is clear from the analysis in Fig. 4B that the microenvironmental distribution of the IgM-expressing (green fluorescent) and DX5-expressing (red fluorescent) cells overlap. Therefore, these results provide evidence for the opportunity for contact between the two populations of cells.

Although the majority of IgM-expressing cells in the bone marrow of adult mice are immature B cells, \textasciitilde10–20\% of the IgM-expressing B cells are mature recirculating B cells (51, 52). Our histological identification in Fig. 4B does not distinguish between mature and immature IgM-expressing B cells. To determine

\textbf{FIGURE 4.} Localization of BMPC and immature B cells within the bone marrow. A, Diagrammatic depiction of data from \textit{B} highlighting the orientation of the bone endosteum, the sequential development from the IgM\textsuperscript{neg} pre-B through IgM\textsuperscript{bright} late immature B cells as described by Osmond and colleagues (49, 50). DX5\textsuperscript{pos} cells are depicted as in \textit{B} in red. \textit{B}, Low magnification epifluorescent analysis of bone section from an adult BALB/c femur. Section is stained for IgM (green fluorescence) and DX5 (red fluorescence) expression. \textit{C}, Confocal microscopy of bone marrow section from a femur of a BALB/c mouse 13 days post irradiation with 500 rad. The section is stained for DX5 (green fluorescence) and IgM (red fluorescence) expression. \textit{D}, High magnification confocal microscopy of a femur from a sublethally irradiated BALB/c mouse 13 days post irradiation stained as in \textit{C} to identify DX5\textsuperscript{pos} and IgM\textsuperscript{pos} cells.
whether immature B cells and DX5-expressing cells are found in direct contact in the bone marrow, we analyzed bone marrow sections prepared from sublethally irradiated mice that had been allowed to reconstitute their lymphoid compartments for 13 days. These mice lack mature splenic B cells in their periphery and bone marrow (36–38). Bone marrow sections from these irradiated mice were analyzed by confocal imaging to identify IgM-expressing B cells and DX5-expressing cells (this time using red and green fluorescence tags, respectively). As in the epifluorescence analyses described above, we also observed colocalization of IgM- and DX5-expressing cells (Fig. 4C). Furthermore, ×20 magnification of a region of this section (Fig. 4D) indicates that some of these cells are in close association as evidenced by the presence of red and green fluorescent cells with yellow junctions indicating overlapping fluorescence emissions.

The number of Gr-1pos granulocytes in the bone marrow is over 10 times that of immature B cells. Therefore, if the close association of the immature B cells with the DX5pos population was random, we should detect a greater frequency of these associations with the Gr-1pos cells. However, quantitation of the number of close associations (determined by overlapping fluorescence emissions) between the IgM-expressing and Gr-1pos cells in the bone sections from the day 13 postirradiated mice (Table I), we determined that 34% of the IgM-expressing cells are found in close association with DX5-expressing cells as compared with 2% of Gr-1pos cells. Therefore, we believe that the association between the DX5-expressing cells and IgM-expressing immature B cells is selective and not the result of random interactions.

**In vitro and in vivo depletion of asialo-GM1pos cells eliminates BMPC activity**

For previously characterized DX5-expressing cells, expression is generally associated with coexpression of the surface marker asialo-GM1 (53–56). FACs analysis revealed that nearly one-hundred percent of the Thy1dull, DX5pos bone marrow cells also expressed asialo-GM1 (Fig. 5A). For this analysis, WBM from BALB/c mice was gated on the Thy1dull, DX5pos bone marrow cells and simultaneously analyzed for asialo-GM1 expression. Expression of asialo-GM1 was relatively homogenous for all of the cells within the Thy1dull, DX5pos gate.

To directly establish that the asialo-GM1-expressing cells also contained the BMPC activity, we treated WBM in vitro with rabbit anti-asialo-GM1 and rabbit complement and then assessed it for BMPC activity. Anti-asialo-GM1 and complement treatment reduced the frequency of Thy1dull, DX5pos cells in the bone ~10-fold below that present in samples depleted with NRIgG and complement controls (Fig. 5, B and C). As we would predict, this depletion resulted in a marked reduction in the ability of the bone marrow to protect transitional immature B cells from anti-BCR-induced apoptosis (Fig. 5D). This diminished activity was not observed in the normal rabbit serum-treated bone marrow. Because our studies have indicated that only the Thy1dull, DX5pos population contains BMPC activity and that virtually all of these cells are asialo-GM1pos, our results indicate that BMPC activity is localized to the Thy1dull, DX5pos, asialo-GM1pos cells in the bone marrow.

Rabbit anti-asialo-GM1 was also found effective for the depletion of the BMPC in vivo (Fig. 6). BALB/c mice were given two i.v. injections of rabbit anti-asialo-GM1 or NRlG at the same concentration 1 wk apart. BMPC activity of bone marrow isolated from treated mice was assessed in vitro 3 days after the second injection. Treatment of adult mice with anti-asialo-GM1 resulted in a 70% decrease in the frequency of Thy1dull, DX5pos cells as compared with mice injected with NRlG (Fig. 6, A and B). This depletion resulted in a marked reduction in the BMPC activity that was not observed with bone marrow isolated from NRlG-treated mice. Although there was a clear and consistent difference in the activity of the bone marrow from the anti-asialo-GM1- and control-treated mice, unlike the in vitro depletion studies described previously, abrogation of BMPC activity was not complete. We believe that this disparity is due to the fact that depletion of the Thy1dull, DX5pos cells by in vivo Ab administration was not as efficient as treatment in vitro. Nevertheless, these results demonstrate that in vivo treatment simultaneously decreased the frequency of Thy1dull, DX5pos cells and markedly reduced the BMPC activity of the bone marrow.

**Depletion of the BMPC selectively disrupts the pre- and immature B cell compartments in the bone marrow**

To directly establish that the Thy1dull, DX5pos cells and corresponding BMPC activity by in vivo administration of anti-asialo-GM1 Ab provided us with a means to assess the relevance of the BMPC for the development and/or selection of immature B cells in vivo. BALB/c mice were treated with rabbit anti-asialo-GM1 Ab or NRlG over an 8-wk period, beginning at neonatal day 1. This long-term treatment was chosen to eliminate the BMPC early during the establishment of the B cell repertoire. After this treatment, experimental and NRlG-treated control mice were sacrificed at 9 wk of age, and their bone marrow and spleens were harvested and analyzed by FACs to determine the frequency and number of B cells within the pro-, pre-, and immature B cell compartments.

The bone marrow IgMpos, IgDneg immature B cell compartment in the anti-asialo-GM1-treated mice was severely affected as compared with the rabbit-IgG-treated mice (Fig. 7). The number and frequency of IgMpos, IgDneg B cells was reduced by 75 and 62%, respectively, in the bone marrow of treated mice as compared with control mice treated with NRlG (Fig. 7, A and B). In addition, the

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**Table I. Selective association of the BMPC with bone marrow immature B cells**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Number of Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Cells in Direct Contact with DX5pos Cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percentage of Cells in Direct Contact with DX5pos Cells</th>
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<tr>
<td>IgMpos cells</td>
<td>1 46 15 32.6</td>
<td>2 46 14 30.4</td>
<td>3 20 8 40.0</td>
</tr>
<tr>
<td>3 547 15 32.6</td>
<td>34.3 ± 5.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.9</td>
<td>2.2 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR-1pos cells</td>
<td>1 540 10 1.9</td>
<td>2 521 11 2.1</td>
<td>3 547 15 2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> From femurs of BALB/c mice 13 days after sublethal irradiation with 500 rad.

<sup>b</sup> Number of cells per 0.26 mm² of sectioned femur.

<sup>†</sup> Mean ± SD.
frequency and number of pre-B cells was also decreased by treatment with anti-asialo-GM1, although not to the same extent as that observed for the immature B cell compartment. From these results, the BMPC appears to be important for the development and/or maintenance of the immature and pre-B cell compartments. In contrast, the depletion of BMPC did not result in a significant alteration in the number or the frequency of pro-B cells in the bone marrow, defined as B220pos, CD43 pos, IgM neg cells. Despite the decreased number and frequency of IgM pos, IgD neg cells in the bone marrow of mice treated with anti-asialo-GM1, the number of IgM pos, IgD pos, and IgD neg B cells in the spleen was not significantly altered (Fig. 7C). These latter results argue that the Thy1 dull, DX5 pos BMPC plays a necessary role in the generation or maintenance of the immature B cell pool in the bone marrow but not in the periphery.

Discussion

B cell development is an ordered process that involves the stepwise expression and assembly of the BCR complex. It is the immature stage B cell that first expresses the Ag-binding form of the BCR and can, therefore, be subjected to negative selection processes to eliminate B cells with potential reactivity to self-Ags. This selection begins in the bone marrow and continues in the splenic transitional immature B cell compartment. Previously, we identified a Thy1 dull population in the bone marrow of adult mice that alters the response of immature B cells to BCR engagement (27). In vitro coculture of immature stage B cells with Thy1 dull bone marrow cells abrogated the intrinsic apoptotic response of immature stage B cells to BCR cross-linking (receptor editing). In this study, we further characterized this bone marrow compartment and determined its relevance to the immature B cell compartment in vivo. In this study, we have demonstrated that this BMPC activity within the Thy1 dull bone marrow population is confined to DX5 pos cells. Consistent with our previous studies, the Thy1 dull, DX5 pos subset also facilitates Rag-2 expression in immature stage B cells following BCR-cross-linking. Although the BMPC constitutes only 0.5–1% of the total bone marrow, cocultures of Thy-1 dull, DX5 pos BMPC and immature stage B cells at ratios as high as 1:20 abrogate the intrinsic apoptotic response of the immature stage B cells to BCR engagement. In contrast, similar effects on the response of immature B cells by

FIGURE 5. Treatment with anti-asialo-GM1 in vitro eliminates the BMPC and its activity. A, FACS analysis of WBM gated on Thy1.2 dull (Thy1 dull ), DX5 pos cells and simultaneously analyzed for asialo-GM1 expression. Unfilled histograms represent nonspecific binding by the isotype-matched negative control. Filled histograms represent relative asialo-GM1 expression. B and C, FACS analysis of bone marrow treated with either anti-asialo-GM1 and rabbit complement or NR IgG and rabbit complement in vivo. D, Frequency of apoptotic transitional immature B cells in the presence of WBM treated with anti-asialo-GM1 or NR IgG in vitro with (□) or without (□) induction by anti-BCR Abs. CFSE-labeled transitional immature B cells were cultured with unlabeled bone marrow at 2:1 (WBM-transitional immature B cells) with 20 μg/ml anti-IgM F(ab) fragments (anti-BCR) for 16 h, and the frequency of apoptotic CFSE-labeled cells was determined by propidium iodide staining. Bars represent the mean of triplicate cultures ± SD.

FIGURE 6. Treatment with anti-asialo-GM1 Ab in vivo eliminates the Thy1 dull, DX5 pos BMPC. A and B, FACS analysis of Thy1.2 dull (Thy1 dull ) and DX5 coexpressing cells within the WBM from mice treated with either anti-asialo-GM1 or rabbit IgG in vivo (see Materials and Methods). C, CFSE-labeled transitional immature B cells were cultured with bone marrow from BALB/c mice treated with anti-asialo-GM1 or NR IgG in vivo at 2:1 (WBM-transitional immature B cells) with (□) or without (□) 20 μg/ml rabbit anti-mouse IgM F(ab)2 fragments (anti-BCR) for 16 h. Percent apoptotic cells was determined by determining the frequency of hypodiploid cells by propidium iodide DNA staining. Bars represent the mean of triplicate cultures.
depicted in Fig. 4, we can discern that the bone marrow contains lyzing confocal images from studies similar to and including those are found interspersed with the immature B cell population. Anation appeared to be specific for immature stage B cells as only 2% of GR-1-expressing cells were observed to be in close association with DX5pos cells despite the fact that this latter population is much more prevalent in the bone marrow. Analyzed a different way, ~40% of the DX5pos cells were found to be in close contact with IgMpos immature B cells (as determined by overlapping fluorescence) as compared with <1% with GR-1pos cells. These observations are important because our previous in vitro studies have demonstrated that direct contact with a BMPC is required to protect immature B cells against BCR-induced apoptosis and to facilitate Rag gene expression (27). Based on these analyses, we conclude that at any given time, only about one-third of the immature stage B cells in the bone marrow are in close physical contact with a BMPC. The explanation for this limited interaction, we believe, is that only those autoreactive immature stage B cells that bind Ag in the bone marrow require the BMPC for their continued development or survival. Therefore, we suggest that the IgMpos immature B cells that are not found in direct contact with the BMPC represent those cells that are not self-reactive and are directed toward emigration from the bone marrow without continued light chain recombination.

We have observed that depletion of Thy1dull, DX5pos cells results in a significant reduction in the sizes of the pre- and immature B cell compartments in the bone marrow. Mice treated with anti-asialo-GM1 Ab for 8 wk after birth exhibit significant decreases in both the frequency and number of IgMpos, IgDneg immature B cells in the bone marrow. In contrast to the 60% decrease in the frequency of immature bone marrow B cells and a 40% decrease in the frequency of pre-B cells, the pro-B cell population in mice injected with anti-asialo-GM1 Ab was not affected. These data suggest that the Thy1dull, DX5pos asialo-GM1-expressing BMPC is necessary for maintenance of the immature B cell compartment and that the effects of the BMPC are selective and limited to the pre-B and immature B cell stages in the bone marrow. In this regard, following the long-term depletion studies reported here we saw no significant effect on the peripheral IgMpos pool, again supporting the conclusion that the effect of BPCM depletion is selective for the bone marrow immature and pre-B compartments. The fact that the number of peripheral B cells was not reduced despite a reduction in the immature precursor pool likely reflects homeostatic expansion of the emigrated B cells that are not affected by depletion of the BMPC (i.e., nonself-reactive B cells). In studies not shown, short term (4 day) depletion studies revealed that the acute loss of bone marrow pre-B and immature B cells is associated with a transient increase in the number of these cells in the spleen. Our interpretation of these results is that the BMPC may influence the retention of immature B cells in the bone marrow in addition to upregulating survival signals for B cells engaging Ag. As with the long-term depletion studies, the pro-B compartment was not affected by acute depletion of asialo-GM1pos, DX5pos cells.

![Figure 7](http://www.jimmunol.org/Downloaded-from-April-15,-2017) (PDF: [5942 MICROENVIRONMENTAL CONTROL OF IMMATURE B CELLS](http://www.jimmunol.org/Downloaded-from-April-15,-2017))
The observed effects on the pre-B cell compartment could indicate a role for the BMPC in the survival or development of pre-B cells. Alternatively, the affected B220<sup>+</sup>, IgM<sup>-</sup> B cells that would appear by these analyses to be pre-B cells may in fact be self-reactive immature B cells that have modulated their BCR expression. In so doing, we postulate that they have functionally continued light chain recombination in an attempt to edit their self-reactive receptors. However, in the absence of BMPCs, these BCR nonexpressing immature B cells would now be targeted for deletion.

Finally, if the diminished populations of immature stage B cells in the bone marrow of BMPC-deficient mice are due to the inability of immature stage B cells to undergo receptor editing we must infer that the majority of normal immature stage B cells attempt receptor editing. As shown above, mice treated with anti-asialoGM1 to deplete the bone marrow of Thy-1<sup>-</sup> DX5<sup>+</sup> BMPC exhibited a 62% decrease in the frequency of IgM<sup>+</sup> B cells in the bone marrow. These data agree favorably with previous studies in which the frequency of receptor editing among immature B cells was estimated to be as high as 47% (57).

In conclusion, our studies suggest that, like pro- and pre-B cells, the development of immature B cells is also influenced by the cellular microenvironment of the bone marrow. However, whereas pro- and pre-B cells require bone marrow stromal cells for differentiation, the bone marrow microenvironment may provide survival signals to immature stage B cells that encounter Ag. Based upon these and earlier studies (27), the influence of BMPC on the development of immature B cell pool is to block or delay the intrinsic apoptotic response of autoreactive immature stage B cells and sustain continued light chain recombination. By supporting light chain rearrangement as long as signals from the BCR and the BMPC are present, the likelihood of generating nonreactive BCR will be increased. Therefore, fate decisions during immature B cell development are regulated not only by the intrinsic signals of the BCR but also by extrinsic cues provided by the microenvironment. The balance of these signals may significantly influence the frequency of B cells emerging from the immature B cell pool and the repertoire of those cells that enter the mature B cell pool.

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References


