Autoantibodies Inhibit Interleukin-7–Mediated Proliferation and Are Associated With the Age-Dependent Loss of Pre-B Cells in Autoimmune New Zealand Black Mice

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Surface IgM⁺B220⁺ B cell precursors can be categorized as either leukosialin (CD43/CD37) negative (late stage pre-B cells) or positive (pre-B/early pre-B cells). In autoimmune New Zealand Black (NZB) mice, bone marrow small pre-B cells (IgM⁺CD43⁺B220⁻) and pre-B/early pre-B cells (IgM⁺CD43⁺B220⁻) declined significantly with age. In particular, subpopulations of pre-B/early pre-B cells expressing the heat stable antigen (HSA) were found in lower proportions with age. Significant decreases in interleukin-7 (IL-7) cytokine forming units (CFU) were also seen in NZB mice by 6 to 8 months of age and accompanied alterations in the numbers of pre-B and pro-B cells in bone marrow. Concomitant with reduced numbers of B lineage precursor cells and IL-7 CFU in vivo, NZB mice produced serum IgM antibodies that strongly inhibited IL-7 CFU responses in vitro. Two monoclonal IgM antibodies (5G9, 2F5) derived from LPS stimulated 10-month-old NZB splenocytes recognized pre-B cell surface antigens on both pre-B cell lines and on IL-7 stimulated bone marrow pro-B/pre-B cells. However, these monoclonal antibodies (MoAb) failed to significantly stain ex vivo bone marrow cells. The 5G9 and 2F5 MoAbs also partially inhibited IL-7 CFU in vitro. These results suggest that NZB bone marrow becomes increasingly deficient in B cell precursors and especially in IL-7 responsive pre-B cells with age. IgM serum antibodies and monoclonal IgM antibodies derived from older NZB mice inhibit pre-B cell growth to IL-7. The production of such autoantibodies may interfere with B cell development in aging NZB mice by preventing IL-7–mediated proliferation.

IN ADULT BONE MARROW, B lineage cell development occurs in a highly regulated manner characterized by initial rearrangement and expression of μ heavy chain gene segments at the early pre-B cell stage followed by light chain gene rearrangement in late-stage pre-B cells.1,3 Proliferation of B lineage precursors, particularly after Vh to D-Jh gene rearrangement and cytoplasmic μ chain expression during the early pre-B cell phases,4,7 is required for adequate production of small pre-B cells and B cells during the lifetime of the individual. Proliferation of B lineage precursor cells in the mouse occurs within an environmental milieu of both supportive and inhibitory soluble mediators and cellular elements.9,12

In previous studies, we and others have shown that the development of B cell precursors is abnormal in adult mice of the autoimmune New Zealand Black (NZB) and (NZB × NZW) F1 strains.13-15 In these mice, the number of detectable bone marrow small pre-B cells declines in an age-dependent manner. More recently, we have reported that the dysfunction in B lineage cell development in NZB mice is progressive with age and eventually affects not only pre-B and pro-B cell populations, but also early B lineage progenitor cells that have negligible B220 antigen expression.16 Importantly, our adoptive transfer studies indicate that the decreased production of pre-B cells in NZB mice is not the result of intrinsic defects in the B lineage precursors themselves. Therefore, in aging NZB mice, abnormalities in the microenvironment may be of particular importance in reduced B lineage cell formation.

NZB mice are known to produce increasing titers of antibodies to self antigens with age, including autoantibodies reactive with lymphoid cell associated antigens.17,19 In this report, we have assessed the loss of phenotypically and functionally defined subpopulations of B lineage precursors in aging NZB mice. Furthermore, we demonstrate that NZB mice produce antibodies capable of inhibiting interleukin-7 (IL-7)–mediated proliferation by pre-B cells. Such antibodies may be important in the diminished production of pre-B cells seen in aging NZB mice.
were routinely acquired using a FACSscan (Becton Dickinson, Mountain View, CA) and analyzed using Lysis II software.

**IL-7 CFU assay and inhibition by antibodies.** Bone marrow cells were cultured in semi-solid media containing 0.9% methylcellulose as previously described. Briefly, mixtures of bone marrow cells, murine recombinant (r)IL-7 (Biosource International, Camarillo, CA) and methylcellulose in α-MEM with 30% FCS and 0.1 mol/L 2-ME were vortexed and aliquoted into triplicate wells of 12 well plates. Each well contained $2 \times 10^5$ cells and 50 U of rIL-7 in 1 mL of medium. In co-culture experiments bone marrow cells from 11- to 20-month-old NZB mice were mixed with equal numbers ($2 \times 10^5$) of bone marrow cells from 1- to 2-month-old NZB mice. Cells were cultured for 7 days under 5% CO₂ at 37°C and colonies of 25 or more cells scored using an inverted microscope. Cells were removed from cultures by treating wells with 1 U/well cellulase (Sigma, St Louis, MO) for 30 minutes. Triplicate wells were pooled and cells fluorescently labeled as above for subsequent flow cytometric analysis using the FACSscan. In inhibition experiments, cultures were established as above, but graded concentrations of either anti-mouse IgM, affinity purified, or MoAbs as culture supernatants were included as indicated.

**Preparation of NZB serum IgM.** Sera from at least three NZB and BALB/c mice of each age group were pooled and passed over an antienzyme IgM (μ heavy chain specific) agarose column (Sigma Immunochemicals, St Louis, MO). IgM immunoglobulins were eluted from the column with 0.1 mol/L glycine buffer, pH 2.4. Eluates were neutralized, dialyzed against phosphate-buffered saline (PBS), and, where required, concentrated using a Centriprep-10 concentrator. IgM concentrations were determined via ELISA assay and inhibition analysis using the FACScan. In inhibition experiments, cultures were established as above, but graded concentrations of either anti-mouse IgM, affinity purified, or MoAbs as culture supernatants were included as indicated.

**Derivation of NZB pre-B cell reactive MoAbs.** Splenocytes from a 10-month-old NZB mouse were cultured with 10 μg/mL LPS (S. typhimurium) for 3 days. Cultured cells were removed, washed, and fused with SP20 cells via polyethylene glycol. Hybridomas were selected with HAT media. After appearance of growing hybridomas in the culture wells, supernatants were screened for IgM secretion by ELISA and for binding to the pre-B cell line, A5C6 (cytoplasmic and surface μ,κ; mRNA present for VpreB), glutaraldehyde fixed to microtiter plates in ELISA assays. IgM hybridoma supernatants reactive with A5C6 cells were further tested for positive surface staining by fluorocentriflow cyrometry. Positive hybridomas were cloned by limiting dilution; MoAbs were prepared as culture supernatants, 50% ammonium sulfate precipitated fractions; and/or enriched from culture supernatants via EZSep™ reagent (Pharmacia Biotech, Uppsala, Sweden) as per manufacturer's instructions with no differences in the results obtained.

**Cell lines and surface staining.** The following murine and human cell lines were examined for surface staining by NZB-derived MoAbs: A5C6 (surface μ,κ,λ); murine pre-B cell line; WEHI 231 (surface μ,κ) murine B cell line; A20 (surface γ,κ) murine B cell line; ex vivo murine thymocytes and the murine T cell line, EL4; P388D1 murine macrophage line; and KG-1a, human promyelocytic cell line. Staining of κ cell lines was with 5 μg MoAb followed by PE-anti-κ antibody; staining of κ' cell lines was with biotinylated anti-IgM MoAb (reactive with NZB IgM) and PE-streptavidin or with FITC-anti-IgM (for A20).

**Statistical analyses.** Values were determined to be statistically significant by paired Student's t-test.

**RESULTS**

**NZB mice exhibit loss of CD43+ pre-B cells and CD43+ pro-B cells with age.** Murine bone marrow cells that lack surface IgM and express the B220 and CD43 (S7) surface antigens have been shown to be pro-B/early pre-B cells in contrast to B220+CD43− late-stage small pre-B cells. We have previously shown that alterations in the pro-B/early pre-B cell (IgM+CD43+B220+) versus late pre-B cell (IgM+CD43+B220−) compartments occur in adult NZB mice with age; these initial observations were confirmed and extended in this study. As shown in Fig 1, NZB mice exhibited declining numbers of bone marrow IgM+CD43+B220+ pre-B cells with age. Indeed, by 6 to 9 months of age, CD43+ pre-B cells were reduced by ~80% in NZB bone marrow and remained depressed through 21 months of age. Significant decline in bone marrow pre-B cells in normal BALB/c mice

![Fig 1. NZB mice exhibit decreased pre-B and pro-B cells with age.](attachment-url)
NZB ANTI-BODIES INHIBIT PRE-B CELL GROWTH

Fig 2. HSA' pro-B cells decrease in NZB mice with age. (A-D) Bone marrow cells were labeled with anti-IgM-biotin/streptavidin Cyochrome, anti-CD43-PE, and anti-B220-FITC for flow cytometric analysis. (E-H) Bone marrow cells were labeled with anti-HSA-biotin/streptavidin Cyochrome, anti-CD43-PE, and anti-B220-FITC. CD43'B220' cells were gated and HSA staining analyzed. (A, E) Two-month-old BALB/c; (B, F) 3-month-old NZB; (C, G) 9-month-old NZB; (D, H) 20-month-old NZB. Data are expressed as percentage of bone marrow cells (A-D) and percentage of CD43'B220' cells (populations ABC) (E-H). Data are representative of six to nine individuals of comparable age.

has not been observed until 15 to 17 months of age, therefore, the decline in NZB pre-B cells occurs in an accelerated manner relative to normal mice.

In contrast to the loss of CD43' pre-B cells in adult NZB mice with age, populations of IgM CD43'B220' pro-B/early pre-B cells were generally reduced to a lesser extent. As shown in Fig 1, NZB mice at 6 to 9 months of age had IgM CD43'B220' cell proportions, which were approximately 30% to 45% of that seen in young NZB mice.

Expression of HSA on NZB B cell precursors with age. In normal adult BALB/c mice, approximately one third of CD43'B220' B cell precursors express dull staining with antibodies to the HSA (HSA'dull; population A of Hardy et al21). Those CD43'B220' cells with higher HSA expression (populations B and C of Hardy et al21) are regarded as more mature than are those with lower HSA surface densities. In young NZB mice, approximately 70% of CD43'B220' cells were HSA' (Fig 2) and the representation of such cells within the CD43'B220' population decreases to approximately 50% by 6 months of age. Taken together with the numbers of pro-B/early pre-B cells seen in NZB mice with age (Fig 1A), this suggests preferential loss of HSA'CD43'B220' precursor cells with age.

IL-7 responsive B lineage cells (IL-7 CFU) are decreased in older NZB mice. As previously shown, a subset of pre-B cells proliferates in vitro in response to rIL-7 when bone marrow cells are plated in semi-solid media.5-10 To determine the effects of age on the incidence and activity of these B lineage precursors in NZB bone marrow, IL-7 CFU were enumerated at day 7 after culture initiation. As shown in Fig 3, NZB bone marrow exhibited significantly lower IL-7 CFU at 6 to 9 months of age and by 11 to 20 months of age were extremely low. As previously reported, comparable declines in IL-7 CFU were not seen in BALB/c bone marrow cells until ~2 years of age.20 As shown in Fig 3, equal mixtures of bone marrow cells from young and old NZB mice did not lead to reduction in the number of expected IL-7 CFU. Therefore, active suppression of IL-7 CFU by old NZB bone marrow cells was not demonstrable in this culture system.

NZB serum IgM specifically inhibits IL-7 CFU. IgM was isolated from pooled sera of NZB and BALB/c mice at 1 to 2 months, 6 to 9 months, and 10 to 16 months of age. The capacity of these IgM antibodies to affect BALB/c bone marrow IL-7 CFU were measured. As shown in Fig 4, IgM from BALB/c mice of all age groups tested had little effect
IL-7 CFU from older NZB mice are resistant to inhibition by NZB IgM antibodies. As shown above, IgM antibodies from older NZB mice inhibited the majority of bone marrow IL-7 CFU. However, a population of B lineage progenitor cells remained that formed IL-7 CFU and were resistant to NZB IgM antibodies in vitro. If, in vivo, autoantibodies are responsible for the decrease in NZB B lineage precursors capable of producing IL-7 CFU, then we would predict that the remaining low numbers of IL-7 CFU detected in bone marrow from older NZB mice would be resistant to NZB IgM antibody in vitro. Consistent with this prediction, bone marrow cells from young NZB mice yielded IL-7 CFU that were mainly (~60%) inhibited by NZB IgM antibodies; in contrast, IL-7 CFU from bone marrow cells of older NZB mice were resistant to inhibition by NZB IgM antibodies. Notably, the remaining IL-7 CFU in older NZB bone marrow were lymphoid, based on forward and side angle light scatter, and were B lineage as shown by their positive B220 and 6C3/BP-1 staining (data not shown). NZB MoAbs react with pre-B cells and prevent IL-7 dependent growth. Splenocytes from a 10-month-old NZB mouse were stimulated with LPS in vitro, fused with SP2/O cells, and the resultant IgM secreting hybridomas screened for reactivity with the A5C6 pre-B cell line by ELISA and by fluorescence flow cytometry. Three pre-B cell reactive IgM MoAbs were obtained: 3D1, 5G9, and 2F5. The reactivity of these MoAbs with the A5C6 pre-B cell line are shown in Fig 6; reactivity with a panel of lymphoid and nonlymphoid cell lines is summarized in Table 1. As shown, these MoAbs reacted with pre-B cell lines. The 2B4 and 2A9 MoAbs, derived from the same NZB fusion, serve as non-pre-B cell reactive negative controls.

None of these MoAbs significantly stained freshly isolated bone marrow cells as determined by fluorescence flow cytometry (data not shown). However, the activated and proliferating pro-B/pre-B cells resulting from IL-7 stimulation of bone marrow cells in vitro were weakly stained with all three MoAbs (Fig 6). These results suggest that IL-7 stimulation may increase the density of surface antigens recognized by these MoAbs.

Similar to results obtained with NZB serum IgM antibody, the NZB MoAbs 2F5 and 5G9 also inhibited bone marrow...
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Fig 5. Older NZB mice have IL-7 CFU that are resistant to NZB serum antibody. NZB bone marrow cells (2 × 10⁶), from young (1-2 months) (A) and old (7 months) (B) NZB mice, were cultured in IL-7 CFU assays in the presence/absence of 1 μg/mL IgM affinity purified from sera of 2-, 6-, and 16-month-old NZB or BALB/c mice. Colonies were counted 7 days later. *Significantly different from cultures in the absence of added IgM at P < .05. All cultures were performed in triplicate. The data are representative of three experiments.

Fig 6. NZB MoAbs recognize pre-B cell antigens. Staining of the A5C6 pre-B cell line with MoAbs is shown in A-D. Staining of pro-B/pre-B cells expanded from BALB/c bone marrow after 7 days culture with rIL-7 is shown in E-H. Staining was performed using 5 μg MoAb and biotinylated anti-μ-light chain antibody followed by PE-streptavidin and analyzed by flow cytometry. MoAbs used are: 3D1, (A, E); 5G9, (B, F); 2F5, (C, G); and 2B4, (D, H). Staining by secondary reagents alone is indicated by arrows.
Thymocytes that greater than 3294 MERCHANT, GARW, AND RILEY

cates that less than 25% of cells stained positive with the MoAb indicated when compared with secondary reagent staining alone. A minus sign indicates inhibition of CFU-GM or IL-3-induced myeloid colony seen was specific for B lineage responses, since significant reductions of B lineage precursors responsive to growth factors (eg, IL-7) could have dramatic effects on the steady-state proportions and numbers of pre-B cells. In this regard, we have previously shown that decreased mitotic activity occurs among large B cell precursors in NZB mice with age. In contrast to HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} cells, HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} cells have been shown to yield IL-7 responses in vitro.\textsuperscript{2} The reduced presence in particular of HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} pro-B/early pre-B cells in older NZB bone marrow, with few IL-7 responsive precursors, strongly suggests that a defect occurs in the generation and/or function of HSA\textsuperscript{-} and IL-7 responsive precursors within the pro-B/early pre-B cell compartment in the aging NZB mouse.

The stromal microenvironment required for pre-B cell proliferation appears functional in aging NZ mice. We have previously shown that primary bone marrow stromal cell cultures from older adult autoimmune (NZB × NZW) F1 mice were able to support the growth of B lineage precursor cells from either normal mice or autoimmune NZ mice under Whitlock-Witte conditions.\textsuperscript{15} Similarly, Yoshida et al,\textsuperscript{16} have shown that 6-month-old NZB bone marrow can yield B lineage precursor cells in Whitlock-Witte culture. Furthermore, IL-7 mRNA is detected in bone marrow from NZB mice by PCR methods (B. Garvy and R. Riley, data not shown). These experiments suggest that nonlymphoid cells required to support B lineage cell production and differentiation can be recovered from older NZ mice and function under in vitro conditions.

In contrast to the above, we have also shown that older NZB bone marrow permits B lineage cell development on adoptive transfer into SCID recipients, consistent with a lack of intrinsic maturational defects in NZB B lineage cells.\textsuperscript{16} These experiments implicate the microenvironment in the

IL-7 CFU by approximately 40% to 60% (Fig 7). The inhibition seen was specific for B lineage responses, since significant inhibition of CFU-GM or IL-3-induced myeloid colonies was not observed (data not shown). Unlike MoAbs 2F5 and 5G9, the 3D1 MoAb, although reactive with pre-B cell lines and IL-7–stimulated bone marrow pro-B/pre-B cells, failed to inhibit IL-7 CFU. Similarly, the non–pre-B cell reactive IgM MoAbs 2B4 and 2A9 also failed to inhibit IL-7 CFU (Fig 7).

**DISCUSSION**

Both NZB and (NZB × NZW) F1 (BWF1) autoimmune mice have been shown to undergo age-dependent reduction in bone marrow pre-B cells.\textsuperscript{13-15} Previous studies have shown that cμ\textsuperscript{+} pre-B cells were essentially absent in bone marrow of 4-month-old NZB mice.\textsuperscript{13} Although extensively analyzed, the mechanisms responsible for this dysregulation remain obscure. In this report, we demonstrate that 1) late stage pre-B cells (IgM \textsuperscript{+} CD43 \textsuperscript{-} B220 \textsuperscript{-}) are preferentially decreased in adult NZB bone marrow; 2) loss of both HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} pro-B/early pre-B cells and IL-7 responsive B cell precursors (IL-7 CFU) was observed with age in NZB mice; and 3) both IgM serum antibodies from older NZB mice and NZB-derived monoclonal antibodies could inhibit IL-7–mediated pre-B cell proliferation in vitro.

In normal mice, HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} cells represent pro-B cells, before Ig gene rearrangement, whereas HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} cells represent pre-B/early pre-B cells that are in the process of rearranging V\textsubscript{H} to Dh-Jh and expressing cytoplasmic \(\mu\) heavy chains.\textsuperscript{3} Relative proportions of HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} cells decreased in favor of the HSA\textsuperscript{hi} CD43\textsuperscript{+} B220\textsuperscript{+} subpopulation in aging NZB bone marrow. Therefore, it is likely that preferential loss of the more differentiated HSA\textsuperscript{+} subpopulations occurs with age in NZB mice.

Because proliferation of pro-B/early pre-B cells undergoing \(\mu\) chain rearrangement is required for population of the resting late-stage pre-B cell compartment,\textsuperscript{17} decreased incidences of B lineage precursors responsive to growth factors...
aging NZB mouse as important to the loss of pre-B cell generation. We have noted that the temporal reduction in small pre-B cells and IL-7 responsive B lineage cells in NZB bone marrow approximates the age dependence of autoantibody titers, polyclonal B cell activation, and hypergammaglobulinemia seen in this strain. 17,18 Therefore, we tested whether NZB mice produce antibodies that may affect their development.

With age, NZB serum IgM gains the capacity to inhibit the formation of IL-7-dependent pre-B cell colonies in IL-7 CFU assays. The presence within the NZB splenic B cell repertoire of autoantibodies capable of abrogating pre-B cell proliferation to IL-7 was confirmed with NZB MoAbs. That NZB mice can produce antibodies capable of recognizing surface antigens on B lineage precursor cells is consistent with previous reports wherein older NZB mice produced high titers of autoantibodies reactive with the Fcγ receptor and human patients with systemic lupus erythematosus produced autoantibodies reactive with isofoms of CD45. 22,23 Further experiments to characterize the antigens recognized by both IL-7 CFU inhibitory NZB serum antibody and MoAbs are in progress. Importantly, these studies indicate that the antibody repertoires expressed in older NZB mice can encompass specificities reactive with pre-B cells and these autoantibodies have profound effects on pre-B cell proliferation. Of interest, the capacity of the NZB MoAbs to bind to IL-7 stimulated, but apparently not fresh ex vivo pre-B cells, suggests that the antigens recognized are activation dependent. Therefore, the target cells for this autoimmune regulation may be pre-B cells at the time of IL-7-mediated activation and proliferation rather than resting pre-B cells.

The mechanisms by which these NZB antibodies affect pre-B cell responses to IL-7 are not understood. Although these antibodies may act directly on IL-7 responsive pre-B cells, it is also possible that they act in an indirect manner via accessory cells or in tandem with accessory cell derived inhibitory factors. It is also of interest that pre-B cells responsive to IL-7 demonstrate heterogeneity with regard to inhibition by NZB serum IgM antibody. The occurrence of such antibody-resistant pre-B cells in older NZB mice probably accounts for a substantially diminished, but continued, development of new B cells even in the presence of these autoimmune regulatory mechanisms.

In summary, our results suggest that autoantibodies, by inhibiting IL-7-dependent pre-B cell proliferation, could contribute to the decreased production of pre-B cells in aging NZB mice. Normal mice of several strains also show increased autoreactivity, hypergammaglobulinemia, and diminished pre-B cell numbers concomitant with advanced age. Whether similar autoantibody mediated mechanisms underlie the reduction in pre-B cells seen in normal mice during senescence and, temporally accelerated, in autoimmune mice awaits further studies.

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REFERENCES


Autoantibodies inhibit interleukin-7-mediated proliferation and are associated with the age-dependent loss of pre-B cells in autoimmune New Zealand Black Mice

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