B-lineage cell deficits in bone marrow of lpr/lpr mice

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Abstract

Analyses of bone marrow (BM) lymphocytes in C57BL/6 mice homozygous for the lpr mutation (B6.lpr) disclosed low numbers of pre-B and B cells, as compared with age-matched control B6 mice. BM depletion in B6.lpr mice was selective for B-lineage cells, appeared in young adults, and developed markedly with age and disease progression, contrasting with the peripheral lymphocyte hypercellularity. Normalization of pre-B and B cellularity in BM of B6.lpr mice was observed after administration of polyclonal Ig, that also markedly improved the clinical condition. Isolated pre-B (B220+ lgM−) cells from B6 or B6.lpr mice, however, showed essentially the same rates of IL-7-dependent proliferation and differentiation to B (lgM+) cells in culture, indicating that the BM B-lineage deficit is not the result of an intrinsic defect in B cell generation.

Introduction

Autoimmune syndromes are often associated with deviations of the lymphocyte repertoires characteristic of physiologic autoreactivity. To understand pathogenic processes, it is important to investigate whether such alterations are primary or secondary, and which mechanisms bring them about. It is now established that B and T cell repertoires are continuously selected during life, both along lymphocyte differentiation in bone marrow (BM) and thymus, and at the periphery, by differential persistence and/or expansion of T or B cell clones (1–12). Disease-associated repertoire alterations could thus result from either central or peripheral perturbations in their selection. Recent observations would suggest, however, that central and peripheral B lymphocyte repertoires are intimately related, partly because of specific antibody-dependent ‘feedback’ regulation of B cell production (13–15).

The pathogenesis of the genetically determined ‘lupus-like’ syndromes in mice is not established. Introduction of the lpr mutation in homozygous form onto different genetic backgrounds in mice results in the development of a generalized autoimmune syndrome, bearing clinical similarities with systemic lupus erythematosus in humans (16–18). Common B cell features include polyclonal activation, increased serum autoantibody titers and a skewed B cell repertoire (18–21). Alterations in the T cell compartment, involving major distortions in populations of thymocytes and peripheral T lymphocytes, on the other hand, are unique to the murine lymphoproliferative (lpr) syndrome (16,17,22). The lpr mutation has been localized to the gene encoding Fas (23), a receptor involved in cell death (24,25). Available evidence, however, indicates that lpr mutant mice appropriately delete differentiating thymocytes (26–30). In addition, the pathogenic role of the characteristic double-negative T cells in the lpr syndrome is unclear, while there is evidence for the relevance of B cells and autoantibodies in its pathogenesis (31–34). Furthermore, two recent reports analysing the impact of deficient expression of either class I or class II MHC molecules on the lpr phenotype have described the segregation of increased autoantibody production and nephritis from the characteristic high numbers of CD4+CD8− T cells (35–36).

The murine lupoid syndromes do not seem to be associated with a particular haplotype of Ig or TCR gene segments (16,17,27). This would imply that the development of abnormal B cell repertoires is related to alterations in the selection of the expressed repertoires, possibly already in differentiating BM lymphocytes. In this paper, we have studied BM B-lineage cells in mice carrying the lpr defect and report marked deficits in this cellular compartment that seem to be secondary to the autoimmune environment.

Methods

Animals

C57BL/6 (B6) mice were obtained from Pasteur Institute or Ifa Credo (Orléans, France) and C57BL/6.lpr/lpr (B6.lpr)
B-lineage defect in Ipr/lpr bone marrow mice were provided by Dr F. Loor (Université Louis Pasteur, Strasbourg, France), maintained in our colony, and used between 4 weeks and 15 months of age.

**Flow cytometry**

Cell suspensions and flow cytometric analyses were performed as described (13). Briefly, splenic and bone marrow cell suspensions were incubated in BSS supplemented with 1% FCS and 0.1% sodium azide on ice, with the following mAb, either coupled with biotin (and revealed with streptavidin-phycocerythrin; Becton Dickinson, Mountain View, CA) or directly labeled with FITC (37): anti-IgM [331.12 (38)], anti-B220 [RA3.3A1 (39)], anti-Mac-1 [M1/70 (40)] and anti-CD3 [145-2C11 (41)]. A minimum of 10,000 cells/sample were analysed in a FACSscan analyzer (Becton Dickinson) after exclusion of dead cells by propidium iodide staining (42).

**Cells and cultures**

BM pre-B cells were enriched by panning (43), using plastic Petri dishes (Optilux, Falcon, CA) coated with 5 mg/ml of anti-B220 mAb [14.8 (44)] or anti-IgM (M-8644; Sigma, St Louis, MO) in 0.05 M KH2PO4/K2HPO4, pH 8. After saturation with RPMI 1640 medium/10% FCS, BM cell suspensions at 5x10⁶ cell/ml were incubated on anti-B220 antibody-coated plates for 1 h at 4°C. After discarding non-adherent cells by repeated washes with RPMI/FCS 10%, adherent cells were harvested and passed onto anti-IgM coated plates, from where non-adherent cells were recovered. This procedure resulted in enriched pre-B cell populations containing 60–80% of B220⁺ IgM⁻ cells and <1% slgM⁺ cells. B220⁺ cells in these populations decayed rapidly in culture (see below) such that up to 98% of all cultured cells were pre-B cells after 4–5 days (see Fig. 6).

Enriched pre-B cells were cultured on cloned stromal cells (S17) and IL-7, supporting pre-B cell growth and differentiation as described (45–47). Briefly, S17 cells were cultured at 5x10⁴ cells/ml in Opti-MEM (Gibco, Gaithersburg, MD) supplemented with 5% FCS, penicillin, streptomycin (Flow, Irvine, UK) and 5x10⁻⁵ M 2-mercaptoethanol (Merck, Darmstadt, Germany), in 24-well culture plates to confluence. After irradiation (2000 rad), 10⁴ to 10⁵ B220⁺ IgM⁻ cells/ml were added to the cultures. Opti-MEM was additionally supplied with recombinant IL-7, obtained from culture supernatants of IL-7-producing transfectants (48), at optimal concentration for pre-B cell growth, as determined in titration experiments. Limiting dilution analyses for determination of the frequency of IL-7 responding pre-B cells were performed in the same culture conditions, using 96-well microtiter plates. Input cell numbers of B220⁺ IgM⁻ ranged from 300 to 1 cell/well in 3-fold dilutions, assessing for growth by microscopy on day 10 of culture (48 cultures at each concentration).

**Results**

Decreased pre-B and B cell numbers in the BM of B6.Ipr mice

While characterizing BM cell populations in B6.Ipr mice, we observed a marked decrease of B-lineage cells in diseased

**Table 1. BM B-lineage cell deficit in B6.Ipr**

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Spleen</th>
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<tbody>
<tr>
<td><strong>B220⁺ IgM⁻</strong></td>
<td><strong>B220⁺ IgM⁻</strong></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>930 ± 181</td>
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<tr>
<td>C57BL/6.Ipr/lpr</td>
<td>248 ± 86</td>
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<tr>
<th>CD3⁺</th>
<th>MAC-1⁺</th>
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<tr>
<td>C57BL/6</td>
<td>122 ± 12</td>
</tr>
<tr>
<td>C57BL/6.Ipr/lpr</td>
<td>297 ± 89</td>
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<tr>
<th></th>
<th>B220⁺ IgM⁺</th>
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<tr>
<td></td>
<td>52 ± 11</td>
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</table>

Mean number of cells (×10⁶) ± SD per femur.

Data were obtained from FACS analyses of bone marrow or spleen cells from five B6 and B6.Ipr mice at 9 months of age. The Ipr mice showed multiple clinical signs of generalized lymphoproliferative syndrome, including lymphadenopathy, proteinuria, skin lesions and splenomegaly.
animals. As shown in Fig. 1, the BM lymphoid compartment of B6. Ipr mice was selectively reduced as compared with age-matched B6 controls. This reduction was due to a selective decrease in the representation of pre-B and B cells, as defined by the surface expression of B220 and IgM, while BM T cells were scored at control levels. As the total numbers of BM cells were comparable in both groups of mice, these results demonstrate a deficit in total numbers of BM B-lineage cells in B6. Ipr mice (Table 1) that is not due to BM infiltration by other lymphoid cells. B cells are more severely depleted than pre-B cells, particularly the subpopulation expressing high levels of B220, corresponding to the most mature B cell population in BM (49).

B-lineage deficiency in B6 Ipr mice was exclusively found in BM. Thus, both spleen (Table 1) and lymph nodes (data not shown) of the same mice contained normal (in young animals) or augmented numbers of B cells.

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In order to evaluate the severity of BM B-lineage deficits with disease progression, we analysed BM compartments in a series of B6. Ipr mice at different ages, together with age-matched controls. As shown in Fig. 2, normal B6 mice show an age-dependent decrease in BM pre-B cell representation, which is much less marked for B cells, as previously described (50). In contrast, B6. Ipr animals, from 2 months onwards, showed B and pre-B cell deficits. By 10 months of age, the representation of B and pre-B cells in the BM of B6. Ipr mice is so low that it comes close to detection limits in flow cytometry.

Some groups of mice studied for BM B-lineage composition were also examined for clinical parameters of disease development, i.e. lymphadenopathy, proteinuria, skin lesions, body weight, total serum Ig concentrations, anti-DNA and rheumatoid factor antibodies, as well as for the sub-set composition and functional activities of peripheral lymphocytes. In this series, the BM B-lineage deficit was detected before any other disease parameter in B6 Ipr mice and its severity correlated with disease progression, as defined by the clinical and immunological assays. It would appear, therefore, that the BM lymphoid composition is a valuable parameter to assess disease activity in B6. Ipr mice.

This notion was reinforced by the recovery of a normal pattern of BM lymphoid composition in B6. Ipr mice treated with pooled normal isologous Ig (IVIg) (15) according to administration schedules currently used in the therapy of certain human autoimmune diseases (51, 52). As can be seen in Fig. 3, the BM B-lineage cell representation in 10-month-old B6. Ipr mice that had received IVIg 6 months before was comparable to normal age-matched controls, while untreated animals showed a profound deficiency in pre-B and B cells. Interestingly, the normalization of BM B-lineage content in IVIg-treated B6. Ipr mice correlated, in all mice examined, with a markedly improved clinical status, as compared with untreated B6 Ipr (see 15 for an extended description of the clinical effects of IVIg in B6. Ipr mice).

BM B-lineage cell defects in B6. Ipr mice are not intrinsic to the cell populations affected

The B6. Ipr defect in BM B-lineage cells could represent primary abnormalities in these cell populations, or else be secondary to local or systemic environmental alterations. In order to directly address this question, the proliferative and differentiative capacities of BM pre-B cells from B6. Ipr and control B6 mice were compared. Isolated B220+ slgM+ (pre-B) cells were cultured on feeder layers of cloned stromal cells (45–47) in medium supplemented with recombinant IL-7 (48). In these conditions, pre-B cell growth could be quantitatively assessed both by cell numbers and by limiting dilution analyses of the frequency of cells initiating IL-7-dependent growth, while the rate of differentiation to IgM+ B cells could be compared after IL-7 withdrawal (53).

As shown in Fig 4, isolated pre-B cells from B6 or B6. Ipr
Fig. 3. Flow cytometric analyses (see Fig. 1) of total BM cells of 10-month-old B6.Ipr treated at 4 months of age (left panels) with murine polyclonal IgG (see text). Sham-treated control B6.Ipr (right panels) at this age showed a pronounced lymphoid depletion (upper right panel), due to the absence of B-lineage cells (lower right panel) in BM, as compared with IgG-treated animals (left panels) and to control B6 mice (shown in Fig 2).

Fig. 4. IL-7-dependent proliferation of B220+ slgM− cells isolated from B6 (white circles) or B6.Ipr (black circles) BM and cultured on S17 stromal cells. Data are representative of two independent experiments, and each point indicates the mean and SD of quadruplicate cultures.

mice proliferate with similar kinetics in response to IL-7 in vitro. Furthermore, very similar frequencies of IL-7 responsive cells, 1/11 versus 1/13 for B6 and B6.Ipr respectively (Fig. 5), could be scored in the two types of BM cells by limiting dilution analysis. Upon IL-7 withdrawal from the cultures, pre-B cells decrease in size, as assessed by light scatter parameters in flow cytometry (Fig. 6), and decay logarithmically as described (53), while a population of small surface IgM− B cells increases in frequency (Fig. 6). The representation of B cells in these cultures upon IL-7 withdrawal was 3-fold lower in B6.Ipr cultures, as compared with B6 (Fig. 6; day 4), a consistent finding in three independent experiments, at the various days of assay after IL-7 withdrawal. Figure 7 shows, however, that the actual numbers of newly formed B cells were comparable in both strains, since more cells were recovered in cultures of B6.Ipr pre-B cells and the increased B cell representation after IL-7 withdrawal in these cultures is primarily due to pre-B cell decay. In summary, total numbers of newly formed B cells differentiating from B6 or B6.Ipr pre-B cells at IL-7 withdrawal were comparable, while death of pre-B cells in the absence of IL-7 seemed to be reduced in Ipr BM cultures (Fig. 7).

These observations indicate that there is no detectable intrinsic B6.Ipr B-lineage cell defect, in terms of IL-7-dependent growth or of B cell differentiation from pre-B cells in vitro.

Discussion

The present results demonstrate that (i) early in life (2 months of age), while the BM pre-B cell content of B6.Ipr mice appears quite normal, BM B cell numbers are already significantly diminished, as compared with normal, age-matched controls; (ii) the numbers of BM pre-B and B cells in B6.Ipr mice decrease in parallel with the age-dependent development of the autoimmune syndrome in the periphery; (iii) the BM B-lineage deficit in B6.Ipr animals was reversed by administration of high doses of normal Ig, that also improved the clinical condition of the mice; (iv) isolated B cell precursors from
normal B6 or B6 Ipr mice contain similar numbers of IL-7-reactive precursors and proliferate equally well in response to IL-7 in vitro; (v) upon IL-7 withdrawal in culture, pre-B cells from B6.Ipr mice survive somewhat better than pre-B cells from control B6 mice, but the total numbers of newly formed B cells are comparable in both strains.

Interestingly, a similar deficit in BM B-lineage cells was also scored in MRL.Ipr/lpr mice (data not shown). Furthermore, a similar recovery of B-lineage BM composition was detected in MRL.Ipr/lpr mice whose clinical conditions had improved following treatment with linomide (54 and data not shown). These observations would suggest that a BM B-lineage cell deficit is generally associated with the murine lpr syndrome.

The finding of a disease-associated B-lineage cell deficit in the BM of B6.Ipr autoimmune mice is in striking contrast to the peripheral B hypercellularity and increased functional activity. The BM B-lineage depletion could in principle result from decreased rates of production and/or differentiation of pro-B cells or from increased rates of loss/elimination of pre-B cells and B cells. As shown here, the defect does not seem to result from intrinsic growth defects of pre-B cells, as assayed in vitro. This conclusion is in line with previous observations (55) reporting full BM reconstitution, after irradiation of B6 mice and transfer of B6.Ipr BM. Furthermore, and although B-lineage cells were not analysed, the degree of bone marrow chimerism in tetraparental embryo aggregation chimeras between MRL.Ipr and DBA/2 mice has been found to correlate with the degree of chimerism in the skin (34).
findings of others (32-34) using different types of chimeric
an intrinsic defect of IprB cells. Rather, this could suggest that,
secondary to the peripheral autoimmune syndrome It should
be pointed out that this conclusion is not in opposition to the
observations, BM B-lineage cell elimination has been shown
established (58,59), some of which could be due to ligand-
animals could be B6.Ipr
In this case, BM B-lineage deficits in
Ipr
in the periphery of B6.
BM deficit is not due to decreased rates of B-lineage cell
BM cells (55). In summary, it would appear that the B6./pr
pre-B cells if starved of IL-7 is in line with a general
defect in apoptotic mechanisms, imposed by the loss of Fas protein in this strain (23–25,56). On the other hand, there is little evidence for Fas expression in BM B-lineage cells (57).
Increased rates of pre-B/B cell export from BM could be
compatible both with the BM deficit and the peripheral hypercellularity, but no pre-B lymphocytes have been found in the periphery of B6./pr mice (data not shown). We are thus left with the possibility that the BM B-lineage deficit in B6./pr mice results from increased (local) elimination A considerable loss of pre-B and B cells in BM of normal mice has been well established (58,59), some of which could be due to ligand-
dependent deletion, as suggested both in transgenic models
and in normal mice (7,8,13,14,60–61). Most related to our
observations, BM B-lineage cell elimination has been shown to result from increasing the concentration of circulating Ig (13–15), suggesting that peripheral Ig production could
physiologically ‘feed back’ upon central B lymphopoiesis (62).
In this case, BM B-lineage deficits in B6./pr animals could be
secondary to the peripheral autoimmune syndrome. It should
be pointed out that this conclusion is not in opposition to the
findings of others (32–34) using different types of chimeric
animals, showing that autoantibody production results from
an intrinsic defect of IprB cells. Rather, this could suggest that,
in autoimmune syndromes, peripheral lymphocyte activities
mediate central events in B lymphopoiesis, such as the
inhibition of central B cell production by peripheral hyper-
activity of B cells, contributing to maintain a pathological
‘disease-specific circuit’
Whatever the origin of the BM B-lineage cell deficit in these
autoimmune syndromes, it may contribute to the establishment
and development of the disease, as a limited BM output will
necessarily restrict the range of diversity from which peripheral
repertoires are selected. This could lead to continuous selec-
tion of peripheral clones and their amplification to pathogenic
levels, as demonstrated by Weigert et al. in Ipr mice (19,20).
Furthermore, if the BM deficit in lupus-like autoimmune syn-
dromes selectively impinges on some V regions, it could
restrict the availability of critical clonal reactivities that are
necessary in physiology (e.g. ‘regulatory’ clones) and thus
be a fundamental component of pathogenesis.

References
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