Inhibition of Murine B and T Lymphopoiesis In Vivo by an Anti-Interleukin 7 Monoclonal Antibody

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Summary

The effects of interleukin 7 (IL-7) on the growth and differentiation of murine B cell progenitors has been well characterized using in vitro culture methods. We have investigated the role of IL-7 in vivo using a monoclonal antibody that neutralizes IL-7. We find that treatment of mice with this antibody completely inhibits the development of B cell progenitors from the pro-B cell stage forward. We also provide evidence that all peripheral B cells, including those of the B-1 and conventional lineages, are derived from IL-7-dependent precursors. The results are consistent with the rapid turnover of B cell progenitors in the marrow, but a slow turnover of mature B cells in the periphery. In addition to effects on B cell development, anti-IL-7 treatment substantially reduced thymus cellularity, affecting all major thymic subpopulations.

In the adult mouse, B lymphocytes develop from progenitor cells in the bone marrow. This development proceeds in an ordered fashion and can be characterized by the sequential acquisition of Ig gene rearrangements and cell surface markers (1, 2). The principal marker of the B lineage in murine bone marrow is the CD45R isoform identified by the 6B2 mAb and is designated B220 (3). The earliest cells committed to the B lineage, however, are B220− (4). A number of other cell surface antigens have been described whose expression is characteristic of particular stages in the sequence of B cell development (1) which culminates in the expression of functional surface IgM.

The early development of B cells in the marrow is dependent on stromal cells and is mediated by cell contact and secreted cytokines (5). The role of cytokines in the development of B lymphocytes has been characterized primarily using in vitro methods in which particular cytokines such as IL-7 (6), mast cell growth factor (kit ligand) (7) or its antagonist (8), or insulin-like growth factor 1 (9) can be shown to regulate the proliferation of B cell progenitors. In vitro data suggest that as Ig genes rearrange, B cell progenitors progress from a stage in which they are stromal cell dependent and IL-7 independent to a stage in which they require IL-7 (10, 11). Very little information has been available on the actual role of these molecules in vivo, although treatment of normal mice with recombinant IL-7 has been shown to greatly augment B lymphopoiesis (12).

The extent to which the B cells in the peripheral lymphoid organs are derived from IL-7-dependent precursors remains unclear. Whereas pre-B cells in the marrow turn over very rapidly, the rate at which cells turn over in the periphery appears to be much slower. This view is based on studies using bromodeoxyuridine administration (13) which indicate that the majority of peripheral B cells are long-lived. A small proportion of peripheral B cells turns over more rapidly and these are presumably replaced by newly developed marrow-derived immature cells. B cells of the B-1 lineage, found primarily in the peritoneum, self-renew in the adult and are derived from immature progenitors only during fetal development (14–17). The role of IL-7 in the development of this B cell lineage is completely unknown.

IL-7 also has been shown to stimulate the proliferation of murine (18, 19) and human mature T lymphocytes (20), and thymocytes (21). IL-7 is produced by thymic stromal cells (6) as well as bone marrow–derived stromal cells, and so it might be expected that T cell precursors would require IL-7 at some stage of development.

We have generated an mAb which neutralizes murine IL-7. In the current study, mice were treated with this antibody in order to determine to what extent B cell development was dependent on IL-7 and at what stage early progenitors began to require IL-7. In addition, thymocytes and peripheral lymphocytes were studied for the effects of in vivo treatment with anti-IL-7. We found that this treatment manifested profound inhibitory effects on B and T lymphopoiesis.
Materials and Methods

Mice. All experiments used BALB/cByJ female mice between 8 and 10 wk of age (the Jackson Laboratory, Bar Harbor, ME). Timed pregnant BALB/cByJ mice were also obtained from the Jackson Laboratory.

mAbs for In Vivo Treatment. A murine IgG2b mAb was generated against human and mouse IL-7 by subcutaneous immunization of BALB/c mice with 10 μg of Escherichia coli-derived recombinant human IL-7 in CFA. Immunized mice were boosted intravenously with antigen in saline 3 d before fusion of the immune spleen cells with P3X63Ag8.653 myeloma cells (American Type Culture Collection, Rockville, MD). Hybridomas were cloned in HAT medium and selected for binding to 125I-IL-7 (murine and human). The M25 mAb was found to bind and neutralize both human and murine IL-7 in vitro. The activity of the antibody against IL-7 was measured using the 2B murine pre-B cell clone (22) which is IL-7 dependent. Inclusion of 10 μg/ml of M25 in cultures of 2B cells resulted in ~50% inhibition of cellular proliferation induced by 10 ng/ml of murine IL-7. The M25 mAb was approximately fourfold more inhibitory for the activity of human IL-7 than murine IL-7. In all experiments, control mice were treated with an isotype-matched (IgG2b) mAb. Large scale preparations of both the M25 and control antibodies were generated as culture supernatants and purified on protein A affinity columns. All antibody preparations were tested and found to contain <10 pg of endotoxin per mg of antibody.

Mice were also treated in some experiments with a mixture of two murine monoclonal alloantibodies to murine IgD of the α allotype. These were H6/1, an IgG2b, and FF1-4D5, an IgG2a (23). Mice were injected intravenously in the retroorbital sinus with 100 μg each of the anti-IgD antibodies, once at the initiation of the experiment. This antibody treatment activates the immune system similarly to the injection of heterologous anti-IgD polyclonal antibodies (24, 25), and results in the depletion of most peripheral IgM+ B cells (data not shown) as previously reported for anti-IgD polyclonal antibodies (26, 27).

mAb Conjugates for Flow Cytometry. The following mAbs were used for the dissection of murine B and T cell subsets: 6B2, rat IgG2a anti-B220; b-7-6, rat IgG1 anti-CDM; BP-1, mouse IgG2a anti-metallopeptidase (kindly supplied by Dr. Max Cooper, University of Alabama, Birmingham, AL); M1/69, rat IgG2b anti-heat stable antigen; S7, rat IgG2a anti-CD43; GK1.5, rat IgG2b anti-CD4; and 53-6.72, rat IgG2a anti-CD8. Polyclonal goat anti-IgD (δ chain specific) was prepared as previously described (27). For four-color analysis, a Texas red goat anti-IgM (μ chain specific) preparation was used (Southern Biotechnology Associates, Birmingham, AL). Biotinylated mAbs were developed with PE-Streptavidin (Becton Dickinson & Co., Mountain View, CA) as a second step. The rat mAbs were semi-purified from serum-free supernatants by ammonium sulfate precipitation. The BP-1 antibody was purified from ascites using affinity chromatography on a protein A column. The various antibodies were biotin and fluorescein conjugated using standard protocols. Antibodies were conjugated with Cyanine 5.18 dye, (generously provided by Dr. Alan Waggoner, Carnegie-Mellon University, Pittsburgh, PA) as previously described (28).

Flow Cytometry. All tissues were stained with multiple mAbs simultaneously in the presence of 5% normal rat serum and 50% of a supernatant of the 2.4G2 anti-FcγRII hybridoma. Stained cells were analyzed using a FACStar®PLUS (Becton Dickinson & Co., San Jose, CA) equipped with filters for four-color immunofluorescence. In all experiments, the data were gated on small cells by forward and 90° light scatter. The data were analyzed using Repproman (True Facts Software, Seattle, WA).

Colony Assays. Bone marrow cells from treated mice were cultured at 5 × 10^6 cells/0.5 ml in 16-mm tissue culture wells in Super McCoy's medium containing FCS, 0.13% agarose, and either 25 ng/ml of IL-7 (29) or 20 ng/ml of GM-CSF. Colonies were enumerated on day 7.

Results

The M25 anti-IL-7 antibody was tested for its effects on B lymphopoiesis in vivo. Adult BALB/c mice were injected intraperitoneally with 3 mg of M25 or isotype control mAb every third day for 10 d. Bone marrow cells were removed from femurs and tibias and assayed by flow cytometry for the presence of B lineage cells. In several experiments, the recovery of total bone marrow cells per mouse did not differ significantly between control and M25 treated groups and ranged between 3 and 5 × 10^7. A striking reduction was observed (Fig. 1) in the number of IgM^+ /CD45R^dim (immature) B cells (quadrant 1) as well as IgM^+ /CD45R^ bright pre-B and pro-B cells (quadrant 3) as a result of anti-IL-7 treatment.

The IgM^+ /CD45R^dim B cells (Fig. 1, quadrant 2) were not reduced by in vivo treatment with M25 mAb. To test whether the population of IgM^+ B cells that remains after M25 treatment is made up of mature B cells, bone marrow...
cells were tested for the expression of IgD as shown in Fig. 2. As expected, the IgM⁺/IgD⁻ immature B cells were substantially reduced in number after M25 treatment whereas the IgD⁺ mature B cells were unaffected.

To determine the precise stage at which B cell development was arrested by the anti-IL-7 mAb, bone marrow cells from mice treated with M25 anti-IL-7 or control antibody were tested for the expression of a number of markers previously used to characterize different stages of B cell development (1). BP-1 is a marker specific for pre-B cells (30). In mice treated with M25, BP-1⁺ cells were reduced from 17% to 1% of the bone marrow cells as shown in Fig. 3. The BP-1⁻/CD45R⁻ cells (quadrant 2) are earlier B lineage cells. This population was also greatly reduced, from 15% in the control bone marrow to 5% in the M25 treated bone marrow (Fig. 3). These data indicate that the M25 mAb is arresting B cell development at a stage before the expression of the BP-1 marker.

On CD45R⁺/IgM⁻ bone marrow cells, S7 (CD43) and heat stable antigen (HSA) have been used as markers to characterize the earliest B lineage cells (1). S7⁺/HSA⁻ cells represent the earliest B lineage cells in this category which are followed in maturation by the S7⁻/HSA⁻ cells and then the S7⁻/HSA⁺ cells. For this analysis, bone marrow cells were stained with four antibodies specific for IgM, CD45R,
S7, and HSA. The data were gated to include all CD45R+IgM- cells and then analyzed for the expression of S7 and HSA as shown in Fig. 4. Many fewer cells fell within these gates from M25 treated mice compared with control mice, being 24% of the total (small scatter gated) bone marrow in the controls and 4% in the M25 treated mice. However, the total number of cells in the S7+/HSA/ B lineage population (quadrant 1) was essentially unaffected by treatment with M25 anti-IL7. The S7+/HSA as well as the S7-/HSA bright B lineage cells (quadrants 2 and 4) were greatly reduced (>90%) by treatment of mice with M25 anti-IL-7 mAb. The S7-/HSA bright cells represent the pre-B cells and late pro-B cells and include the BP-1+ population (1).

In contrast to the effects of M25 on the B lineage in the marrow, little or no effect was seen on peripheral B cell populations under the conditions of treatment used in this study. To directly determine if peripheral B cell populations are derived from IL-7-dependent precursors, mice were treated with anti-IgD mAbs. Treatment of mice with goat anti-IgD induces a T cell-dependent immune response in which IgD+

B cells are stimulated to differentiate into IgG1 secreting cells. By 2 wk after anti-IgD antibody injection however, the activated B cells die (26, 27). Thus, injection of anti-IgD antibody, would provide a simple way to deplete IgM+/IgD+ B cells and could be used to determine if IL-7 were required for regrowth of this population. For this reason, mice were treated with anti-IgD and treated subsequently for 3 wk with M25 or control antibody. Analysis of the B lineage in the bone marrow (Fig. 5) indicates that at day 23 after anti-IgD treatment, IgM+ cells are present in normal numbers with the exception of the CD45Rbright subset. Treatment with anti-IgD (day 0) followed by M25 anti-IL-7 antibody (1 mg intraperitoneally, every third day) resulted in the complete absence of IgM+ bone marrow cells. By day 23 after injection of anti-IgD antibody, B cells have begun to repopulate the LNs of the anti-IgD treated mice (Fig. 6). However, treatment with anti-IL-7 mAb prevents the reappearance of IgM+ B cells in the LNs of anti-IgD treated animals. Small numbers of IgM- B cells persist in the LNs of anti-IgD treated mice. These cells are IgG+ (data not shown), and therefore represent the B cells that are switched as a result of anti-IgD treatment. Similarly, a substantial population of

Figure 5. Inhibition of B lymphopoiesis by M25 in anti-IgD treated mice. Adult BALB/c mice were treated on day 0 with anti-IgD. Mice were also left untreated or were treated intraperitoneally with either M25 or control antibodies, days 0-21 every third day, 1 mg per injection. Mice were killed on day 23 and bone marrow cells analyzed by flow cytometry for the expression of CD45R and IgM.

Figure 6. Inhibition of peripheral B cell repopulation by M25. LN cells from anti-IgD and M25 (for Control and Untreated) treated adult BALB/c mice shown in Fig. 5 were analyzed by flow cytometry for the expression of CD45R and IgM.
IgM+ B cells in the spleen and peritoneal cavity persist in anti-IgD treated mice (data not shown). These populations are CD23− and probably resist anti-IgD by virtue of low levels of IgD on their surface. These B cells probably represent the marginal zone B cells of the spleen and B-1 cells of the peritoneum.

Developing embryos were treated in utero with M25 anti-IL-7 which effectively crosses the murine placenta. Pregnant female BALB/c mice were treated from day 10 of gestation with daily intraperitoneal injections of M25 or control antibody. The spleen and liver of the newborn mice were tested for the presence of B lineage cells as shown in Fig. 7. The B lineage comprised about 20% of the spleen, including 6.4% IgM+ B cells, in newborns delivered from control antibody treated mothers. Treatment in utero with M25 anti-IL-7 profoundly inhibited the development of the B lineage leaving only 0.5% IgM+ cells and 2.6% IgM− B cells (pre-pro-B). These data provide further evidence that the precursors that give rise to peripheral B cells, including cells of the B-1 lineage, are IL-7 dependent. Very similar results were obtained with the newborn liver cells (data not shown).

Fig. 7. Inhibition of B lymphopoiesis in utero. Pregnant mice were treated with daily intraperitoneal injections, 2 mg per injection, with either M25 or control antibody from day 10 of gestation. Spleen cells from the newborn animals were analyzed by flow cytometry for the expression of CD45R and IgM. The percentages of the total spleen by quadrant were: (for Control) 1, 6.4%; 2, 12.7%; and (for Anti-IL7) 1, 0.6%; 2, 2.6%.

In spite of evidence discussed above that the peripheral B cells are derived from IL-7-dependent precursors, adult mice treated for up to 3 wk with M25 anti-IL-7 show very little reduction in the numbers of peripheral B cells (data not shown). The same is true of peripheral T cells. There is, however, a significant and reproducible effect of M25 antibody treatment on the thymus. After 10 d of treatment with M25 (3 mg intraperitoneally, every third day) thymic cellularity

is substantially reduced relative to control-treated mice (Fig. 8). Absolute numbers of cells within each subset are decreased (Fig. 9), although the double negative cells were the least affected being reduced by only 43% compared with a reduction of 80% of the double positives, 60% of the CD8+ subset, and 83% of the CD4+ subset. The total number of double positive thymocytes (quadrant 2) in the control group averaged 7.1 × 10^7 cells per thymus as compared with 1.4 × 10^7 cells per thymus in the M25-treated group.

The effects of M25 in vivo appear to be lymphoid specific as bone marrow cells from the treated animals generated normal numbers of CFUs in response to GM-CSF as shown in Fig. 10. The number of CFUs in response to IL-7 was greatly reduced in M25 treated animals, suggesting that most


of the progenitor cells of the CFU–IL-7 were themselves IL-7 dependent.

**Discussion**

In this study, we have demonstrated that treatment of adult mice with M25 mAb against IL-7 arrests the development of B cells in the marrow as measured by the complete inhibition of the appearance of IgM+IgD– cells. Development of IgM–CD45R+ pre-B cells in the bone marrow is also substantially, although not completely, inhibited.

The persistence of a minor subpopulation of IgM–/CD45R+ cells in the marrow of M25 anti-IL-7 treated mice allowed the analysis of the precise stage at which the B lineage was inhibited. Hardy et al. (1) were able to stage murine B cell development in the adult marrow using flow cytometry, including antibodies against CD43 (S7), HSA, and BP-1, as well as CD45R and surface IgM. We have used these markers and found that the earliest B lineage cells detectable with these markers, which are S7+/HSAhi, are the latest B lineage cells that survive in anti-IL-7 treated mice. All subsequent stages of developing B cells, as identified by the expression of the HSA and BP-1 antigens, were profoundly reduced in these animals. These results indicate that the earliest B cell progenitors may not require IL-7 for survival. Such cells have been reported to require contact with stromal cells for survival (1). The possibility that their close association with stromal cells makes them inaccessible to antibodies cannot be completely ruled out in this study, although other studies have demonstrated the ability of mAbs to enter this intimate compartment (31).

The stage at which B cell development is blocked in anti-IL-7 treated animals demonstrates an interesting contrast to the B cell deficiencies generated in mice with targeted mutations that interrupt early B cell development, including those that interfere with Ig gene rearrangement, RAG-1 (32), RAG-2 (33), and JαT (34), as well as those that interfere with the expression of the pre-B cell receptor, λ5T (35) and µMT (36). In fact, all of these knockout mice appear to arrest B cell development at the same stage (32–34), which corresponds to the expression of the µm-λ5-Vpre-B complex.

Therefore, it appears that the pre-B cell receptor functions as an important transition in B cell development by providing a positive selection for B cells that have productive IgH rearrangements. Anti-IL-7 treatment arrests B cell development at an earlier stage, when the B lineage becomes IL-7 dependent, which follows the final commitment to the B lineage when IgH rearrangements are just beginning.

The early pre-pro-B cell which is unaffected by anti-IL-7 treatment is still responsive to IL-7 treatment in vitro. The growth of IL-7–responsive CFUs from the bone marrow of anti-IL-7 treated animals is substantially reduced, but there is a significant and highly reproducible residual CFU response by such cells. Addition of MGF with IL-7 to these marrow cultures greatly increases the number of early B lineage CFUs (data not shown).

Peripheral B cells in normal adult animals were relatively unaffected by treatment with the anti-IL-7 antibody under the conditions of this study (10 d of treatment). A kinetic study was done (data not shown) which demonstrated that this was the minimal exposure required to completely deplete pre-B and immature B cells from the marrow. Longer treatment schedules of up to 3 wk were also unable to substantially decrease the number of peripheral B cells. These results are consistent with the results of others which indicate that the majority of peripheral B cells are long-lived (13).

Newly developed B cells however, are probably short-lived in the periphery, as suggested by treatment of normal mice with IL-7. After 6 d of treatment, B lymphopoiesis was dramatically stimulated and the number of mature peripheral B cells doubled, yet within 2 wk after cessation of treatment, B cell numbers returned to normal (12).

Injection of mice with anti-IgD antibody results, within 2 wk, in the death of the IgM+IgD+ B cells that are stimulated by this antibody. As nearly all IgM+ B cells in peripheral LNs also express IgD, this treatment depletes nearly all LN IgM+ B cells and allows repopulation of the LN with freshly generated IgM+ B cells to be observed (26, 27).

Treatment of mice with M25 anti-IL-7 after anti-IgD treatment completely inhibited the repopulation of the peripheral LN with IgM+ B cells. This indicates that these peripheral IgM+ cells are indeed derived from IL-7–dependent bone marrow precursors. These data also argue against the possibility that the persistence of the peripheral B cells in mice treated with anti-IL-7 is due to inefficiency of the antibody.

In anti-IgD treated mice, specific subpopulations of splenic and peritoneal B cells were not depleted, probably as a result of relatively low expression of IgD. These subpopulations represent the splenic marginal zones and the B-1 cells of the peritoneal cavity. B-1 cells in the peritoneum self-renew and are not derived from precursors in the adult marrow (14, 15, 17). The precursors of the B-1 lineage are found in the fetal liver, are distinct from the precursors of conventional B cells, and are the predominant B lineage cells in the developing fetus (16, 17, 37). We found that treatment of fetuses in utero with the M25 anti-IL-7 antibody from day 10 of gestation completely inhibited the appearance of IgM+ cells in the spleen or liver of the neonates. These data would indicate...
that the B-1 lineage is derived from IL-7-dependent precursors in early development. However, resistance of peripheral adult B-1 cells to the effects of anti-IL-7 treatment might result from their self-renewal and thus their autonomy from IL-7.

CD45R* IgM+ IgD+ B cells in the marrow were unaffected by treatment with M25 anti-IL-7 in contrast to the immature IgM+/IgD- B cells in the marrow, which were eliminated by this treatment. IgD is expressed on developing B cells after IgM and has been shown to be a marker of relatively mature virgin B cells (38). Like the IgM+/IgD- B cells in the marrow, mature B cells in the periphery were not eliminated by anti-IL-7 treatment. These data suggest that the IgD+ cells in the marrow are derived from recirculating peripheral B cells.

In addition to its effects on B cell development, anti-IL-7 treatment substantially inhibited thymic cellularity, resulting in a three- to fourfold reduction in cell number. All the major thymocyte subsets were affected by the treatment. This suggests that IL-7 may be important for proliferative expansion, but not differentiation of thymocytes. Similar effects were observed when mice were treated in utero with anti-IL-7.

We conclude that IL-7 is an essential factor for the development of B lineage cells that become IL-7 dependent when they reach the pro-B cell stage and begin to rearrange H chain genes. Our data also indicate that most or all peripheral B cells are derived from IL-7-dependent precursors, although most peripheral B cells are stable and do not represent a high turnover cell population. Finally, our data indicate that IL-7 is important, but possibly not essential in T cell development in that it promotes lymphocyte growth within the thymus.

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