Lymphopenia in Interleukin (IL)-7 Gene–deleted Mice Identifies IL-7 as a Nonredundant Cytokine

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Summary

Interleukin (IL)-7 is a potent stimulus for immature T and B cells and, to a lesser extent, mature T cells. We have inactivated the IL-7 gene in the mouse germline by using gene-targeting techniques to further understand the biology of IL-7. Mutant mice were highly lymphopenic in the peripheral blood and lymphoid organs. Bone marrow B lymphopoiesis was blocked at the transition from pro-B to pre-B cells. Thymic cellularity was reduced 20-fold, but retained normal distribution of CD4 and CD8. Splenic T cellularity was reduced 10-fold. Splenic B cells, also reduced in number, showed an abnormal population of immature B cells in adult animals. The remaining splenic populations of lymphocytes showed normal responsiveness to mitogenic stimuli. These data show that proper T and B cell development is dependent on IL-7. The IL-7–deficient mice are the first example of single cytokine–deficient mice that exhibit severe lymphoid abnormalities.

The biological activities of cytokines, as measured by in vitro assays, had long suggested that these molecules would be important for the development and function of lymphocytes in vivo. However, gene inactivation studies of many of these molecules showed that they were dispensable for the development and normal expansion of lymphocytes. Normal numbers of T and B cells were found in mice that contained induced null mutations of IL-2 (1), IL-4 (2), IL-6 (3) (Dalrymple, S. A., L. A. Lucian, R. Slattery, T. McNeil, D. M. Aud, S. Fuchino, F. Lee, and R. Murray, manuscript submitted for publication), IL-10 (4), IFN receptors (5), and the common receptor for GM-CSF and IL-5 (5a). While all of these animals showed varying phenotypes related to the immune system, the absence of severe lymphoid developmental defects was potentially explained by the redundancy of regulatory cytokine signals. Redundancy implies that removal of a single cytokine signal would not be sufficient to inhibit or severely alter lymphoid development. Alternatively, the idea of redundant function may have arisen simply because the cytokine(s) most crucial for lymphoid development and expansion has not yet been examined by gene-targeting criteria.

To investigate this issue in more detail, we have disrupted the IL-7 gene in mice by standard techniques. Numerous in vitro biological activities of IL-7 have suggested some unique features of this molecule. For example, the stimulation of lymphocytes by IL-7 appears to be more restricted to immature populations (6, 7) compared with many other cytokines, and IL-7 does not always require additional costimulatory agents (8). Proliferation of early B cells in bone marrow stromal cultures seemed to be dependent on the presence of IL-7 (9). IL-7 was also shown to be a maintenance and survival factor for thymocytes (10), an activity unique to this cytokine. Additionally, antibody neutralization experiments predicted that IL-7 plays an important role in B and T cell development in vivo (11, 12).

The receptors for IL-7 include, at minimum, two subunits, the IL-7Rα chain and the IL-2Rγ chain (also referred to as the common γ chain) (13–16). As is the case with most cytokine–receptor interactions, these two receptor subunits also bind several other ligands. The IL-2Rγ chain is necessary for IL-7 binding as well as for IL-2 (17), IL-4 (18, 19), IL-9 (20), and IL-15 binding (21). Another subunit, IL-7Rα, binds IL-7 and at least one additional cytokine, thymic stromal derived lymphopoietin (TSLP)[1] (22). The IL-2Rγ chain has been shown to be deficient in human X-linked severe combined immunodeficiency (XSCID), which results in profound effects on human lymphoid development and function (14).

Gene inactivation studies of common receptor subunits are likely to result in cumulative phenotypes. Therefore, in these types of experiments, it is impossible to discern which aspects

[1] *Abbreviations used in this paper:* ES, embryonic stem; HSA, human serum albumin; HSVtk, herpes simplex virus thymidine kinase gene; TSLP, thymic stromal-derived lymphopoietin; WBC, white blood cell; XSCID, X-linked severe combined immunodeficiency.

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of the overall phenotype relate to the function of a specific cytokine. Cytokine-deficient mice are necessary for understanding the basic role of ligand molecules, for understanding the potential redundancies in the cytokine network, and for providing the framework on which to interpret potentially more complex phenotypes associated with mice lacking common receptor subunits.

We reasoned that the targeted disruption of IL-7 would allow us to address whether this single cytokine, due to its potentially unique activities, would approximate an XSCID-like phenotype, or whether the collective elimination of numerous cytokines that interact with the common IL-2Rγ chain, or other receptor systems, would be necessary to produce severe effects on the development and expansion of lymphocytes.

Materials and Methods

Gene Targeting. An IL-7 gene–targeting vector was constructed using a vector containing a Neomycin resistance gene (neo) flanked by multiple cloning sites. At the 3′ end, a herpes simplex virus thymidine kinase gene (HSVtk) was positioned to permit negative selection against random integration. Both resistance genes are driven by an HSVtk promoter. Cells of the embryonic stem (ES) cell line E14.1 were transfected with linearized vector by electroporation. Culturing was done in DMEM with 15% FCS with 2,500 U/ml recombinant leukemia inhibitory factor (ESGRO™, GIBCO BRL, Gaithersburg, MD) on a layer of Mitomycin C–treated primary embryonic fibroblasts. The cells were selected with G418 (350 μg/ml) and Gancyclovir (2 μM). Surviving clones were screened by Southern blot for homologous recombination. The mutated clones were injected into blastocysts from C57BL/6 mice and implanted into pseudopregnant B6xCBA F1 females. The resulting male chimeras were mated to C57BL/6 females to test for germline transmission. Offspring derived from the ES cells were identified by coat color and tested by Southern blot analysis for the presence of the mutation. Heterozygous animals were crossed to obtain homozygous mutant animals.

Molecular Analysis of IL-7 Deletion. Total RNA was extracted from thymic stromal cells in RNAzol. Reverse-transcriptase PCR reactions were performed (Perkin-Elmer Corp., Norwalk, CT; Boehringer Mannheim Corp., Indianapolis, IN) (primers: sense 5′-GGAATTCCTCCACTGATCCT-3′, Antisense 5′-CTCTCAGTAGTCTCTTTAGG-3′), and the specificity of the PCR bands was confirmed by transfer to Nytran (Schleicher & Schuell, Inc., Keene, NH) and hybridizing the filter with an IL-7 cDNA probe.

Gross Characterization and Blood Counts. All experiments were done on F2 animals, derived from heterozygous parents, aged 5–8 wk unless otherwise noted. The white blood cell (WBC) counts were done using an automated cell counter (Sereno-Baker Diagnostics Inc., Allentown, PA). For differential counts, blood smears were stained with Wright-Giemsa stain and counted under the light microscope. Results are expressed as the mean of at least seven samples ± SD.

FACS® Analysis. Cell populations were isolated from bone marrow, thymus, and spleen. 10^6 cells were stained in 96-well plates for 30 min on ice. The PE-conjugated mAbs were directed against CD43 (S7), CD8, and TCR-$\alpha\beta$. FITC-conjugated mAbs were directed against CD3 and IgM^b. Biotin-conjugated mAb directed against CD24 (human serum albumin, HSA) was obtained from PharMingen (San Diego, CA) as well. The mAb against mouse IgD was obtained from Southern Biotechnology Associates (Birmingham, AL). Tricolor conjugated mAbs directed against CD4 and B220 were obtained from CALTAG Labs (South San Francisco, CA). The avidin-Texas red conjugate was obtained from Leinco Technologies Inc. (Ballwin, MO). Cell surface immunofluorescence analysis was performed by use of a cell sorter (Vantage; Becton Dickinson Immunocytometry Systems, San Jose, CA).

Proliferation Assay. Splenocytes and thymocytes were isolated aseptically and depleted of erythrocytes by osmotic shock. Cells were cultured at 10^5 cells/well in 96-well plates in RPMI with 10% FCS and supplements. The splenocytes were stimulated with 50 μg/ml LPS or 50 μg/ml Con A for 2 d. Thymocytes were stimulated with 2.5 μg/ml Con A with 20 ng/ml IL-2 for 3 d. Cells were pulsed with 0.5 μCi [^{3}H]thymidine for 12 h before harvesting, and [^{3}H]thymidine incorporation was measured.

Results

Generation of IL-7−/− Mice. The IL-7 targeting vector was constructed by use of genomic DNA isolated from a genomic DNA phage library made from 129/SV mice (Stratagene Inc., La Jolla, CA) in order to maximize homology between the targeting plasmid and the IL-7 locus. Two 2.3-kb fragments were cloned 5′ and 3′ of the neo gene (Fig. 1 *A*). Homologous recombination would create a complete deletion of exon 4 (28.3% of coding sequence) and 300 bp of flanking sequence. DNA from resistant ES colonies was digested with BamHI and XbaI and Southern blots were probed with a 3.1-kb external probe located 3′ of the targeting sequences (Fig. 1 *B*). Hybridization with a neo gene probe was performed to ensure a single integration site (data not shown). The frequency of homologous recombination was 1 out of 1,166 doubly resistant clones. Appropriately targeted ES cells were injected into C57BL/6 blastocysts. IL-7−/− mice were generated from matings between IL-7+/− mice at the expected Mendelian frequency.

To evaluate the inactivation of the IL-7 gene we performed reverse-transcriptase PCR analysis of thymic stromal cell RNA from 2-wk-old mice, using primers at the 5′ and 3′ end of the cDNA. Hybridization with the cDNA showed that there was no appropriate specific IL-7 message found in IL-7−/− animals (Fig. 1 *C*). β-actin PCR showed equivalent amounts of RNA in each sample (data not shown). The induced mutation was designed to remove two of four α-helical bundles of the cytokine structure, but also resulted in apparent mRNA instability. Attempts to culture thymic stromal cells to detect IL-7 biological activity were unsuccessful from both IL-7−/− and IL-7+/+ mice.

Macroscopic Analysis of IL-7−/− Mice. The IL-7−/− mice appeared healthy at birth and developed normally. Both sexes were fertile. In the mutant mice, the thymus and the spleen were reduced in size and weight, and lymph nodes or Peyer's patches were not detected. Histologically, the thymus of the IL-7−/− mice showed cortical and medullary areas and the spleen showed a normal structure with red and white pulp (data not shown).

IL-7−/− Mice Exhibited Severe Peripheral Blood and Tissue Lymphopenia. IL-7–mutant animals were tail bled and ex-

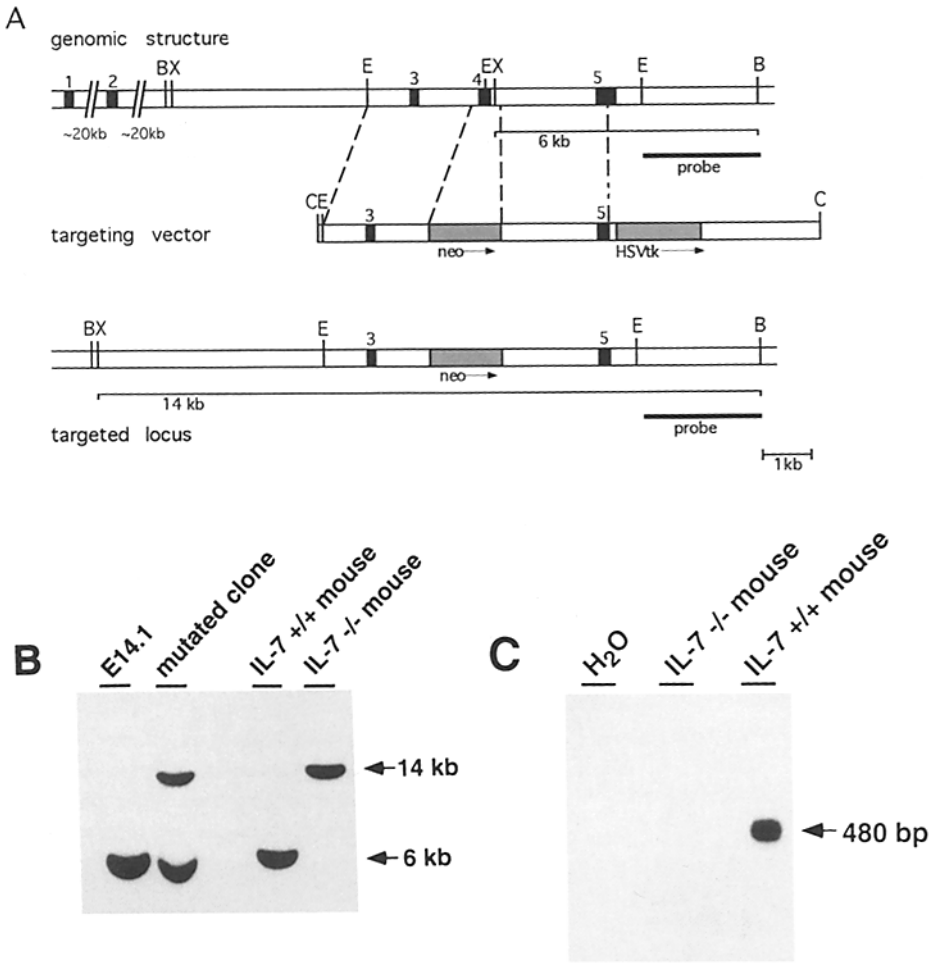

Figure 1. Targeting of the IL-7 gene. (*A*) The IL-7 gene locus is shown with restriction enzyme sites: *B*, BamHI; *E*, EcoRI; *X*, XbaI. Exons are represented as black boxes. The gene-targeting vector contains two 2.3-kb fragments of the IL-7 gene as shown. Exon 4 and 300 bp of the flanking introns were replaced with the neo gene. The vector was linearized with ClaI, *C*, before transfection. The predicted structure of the targeted locus is shown, with the length of diagnostic restriction fragments and location of probe sequences used for Southern blot analysis. (*B*) Southern blot analysis of E14.1 parental cells, an appropriately targeted ES clone, and tail DNA from F2 offspring derived from heterozygous parents is shown. DNA was digested with BamHI and XbaI and hybridized with the probe shown in Fig. 1 *A*. (*C*) Reverse-transcriptase PCR of thymus stromal RNA from 2-wk-old mice. The thymuses from IL-7−/− mice were much smaller than those from control animals. Equivalent amounts of RNA from mutant mice are likely to represent an enrichment of the stromal cell type known to produce IL-7. The PCR was performed with primers located at the 5′ and 3′ end of the cDNA. The blot was hybridized with IL-7 cDNA.

amined for peripheral WBC counts. Animals were grouped from 5 wk to 14 wk. Fig. 2 *A* shows a significant decrease in WBC counts in the mutant animals irrespective of their age. All other blood parameters such as red blood cells, platelets, hemoglobin, and hematocrit were in normal ranges. These same samples were used to produce blood smears, and were evaluated for cell types. The reduced WBC in the mutant animals was due to a severe reduction of lymphocytes from an average of 68.2% to 9.5% (Fig. 2 *B*). The nonlymphoid cells in these smears gave normal ratios and absolute numbers of monocytes and granulocytes.

The bone marrow, thymus, and spleen were then evalu-

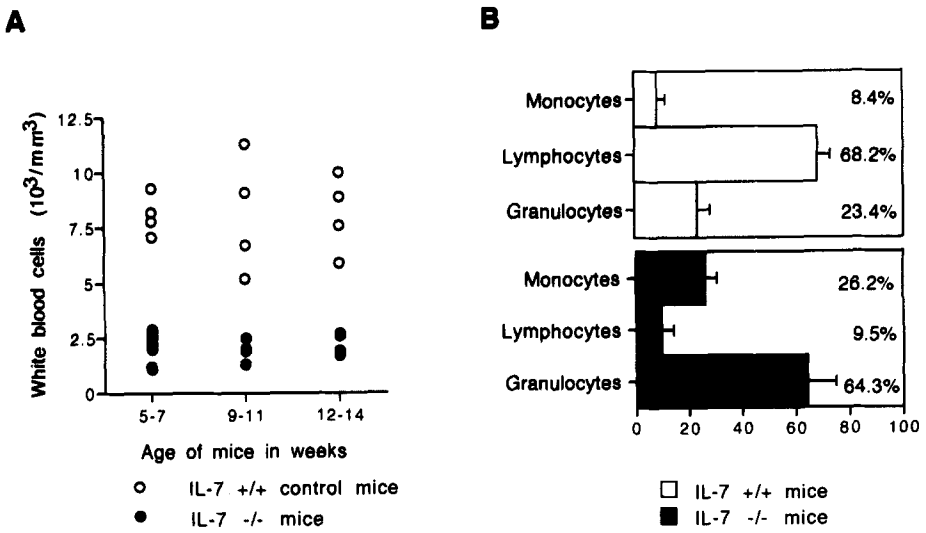

Figure 2. WBC analysis in IL-7−/− and IL-7+/+ control mice. (*A*) WBC counts in 5–14-wk-old IL-7−/− and IL-7+/+ control mice. (*B*) Differential blood count of the same blood samples of Fig. 2 *A*. The data represent mean ± SD values of experiments of 17 IL-7−/− mice and 12 control mice.

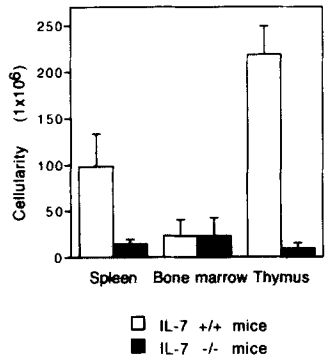

Figure 3. Analysis of lymphoid cellularity in IL-7−/− and IL-7+/+ control mice. Total cell numbers of spleen, thymus, and bone marrow (collected from two femurs) from mice aged 5–7 wk. The data represent mean ± SD values of experiments with nine IL-7−/− mice and seven control mice.

ated for cellularity. While IL-7 mutants had normal numbers of bone marrow cells, both thymus and spleen cellularities were reduced to <5 and 15% of controls, respectively (Fig. 3).

Collectively, these data show a strong dependence on IL-7 for proper expansion of lymphoid lineages. The lymphopenic state persisted in mice up to 4 mo of age, and to date, has shown no increase of peripheral lymphocytes. Additionally, this stable phenotype has shown little animal-to-animal variation.

B Lymphopoiesis in the Bone Marrow Was Blocked at the Transition to Pre-B Cells. Hardy et al. (23) have characterized murine B lymphopoiesis by a series of developmentally regulated cell surface molecules. B cells are classified from most immature (fraction A) to increasingly more mature populations (fractions B–F) using cell surface markers such as B220, IgM, S7, and HSA. We used these general criteria to evaluate the bone marrow development of B cells in the IL-7–mutant mice. Fig. 4 *A* shows that the mutant animals contained almost no $B220^+/IgM^+$ bone marrow cells, in contrast to control animals. As these cells represent later stages of B lymphopoiesis in the bone marrow, we used four-color flow cytometry to evaluate the more immature populations contained within the $B220^+/IgM^-$ fraction. Fig. 4 *B* shows that, when gated on $B220^+/IgM^-$ cells, the population of $B220^+/IgM^-/S7^-/HSA^+$ cells was not present, indicating that the transition of fractions B and C to fraction D in mutant animals was blocked. Thus, the transition to the pre-B cell population (fraction D) was severely impaired.

Reduced Thymocyte Numbers Exhibited Normal Composition of Thymic Subsets. The remaining 5% of thymocytes in the mutant animals were examined for CD4, CD8, and CD3 expression. Fig. 5 shows that the thymic subpopulations were present in normal ratios, and suggests that proliferation and expansion rather than differentiation were affected by the lack of IL-7. A minor observation, which was consistent in all mice tested, was a slight increase in the percentage of $CD4^+/CD8^+$ cells in the IL-7−/− animals.

Analysis of Splenic Abnormalities in IL-7−/− Mice. The spleens of mutant animals exhibited a reduction in cellularity (Fig. 3). As splenocytes represent a collection of cell types, we evaluated the absolute numbers of lymphocytes in this tissue. Based on FACS® staining, we found a severe reduc-

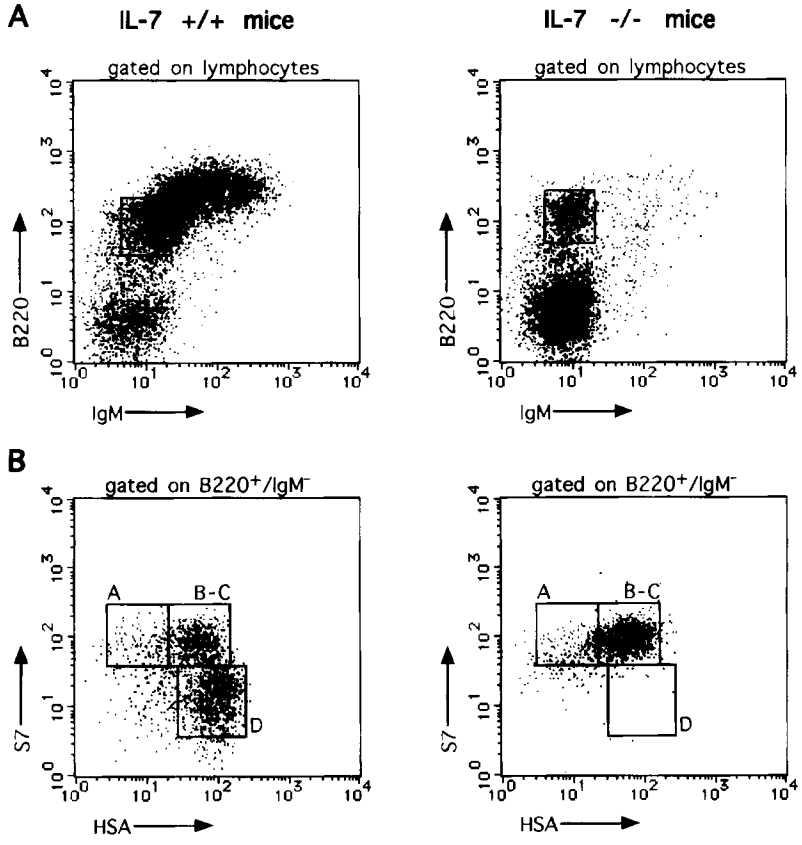

Figure 4. Immunofluorescent analysis of bone marrow from IL-7−/− and IL-7+/+ control mice. (*A*) Bone marrow stained with FITC-anti-IgM and tricolor-anti-B220 gated on lymphocytes. (*B*) Bone marrow stained with PE-anti-S7 and biotinylated anti-HSA followed by streptavidin-Texas red gated on $IgM^-/B220^+$ cells (*square* in Fig. 4 *A*). The boxes indicate B cell fractions A–D. The data are results of a representative experiment.

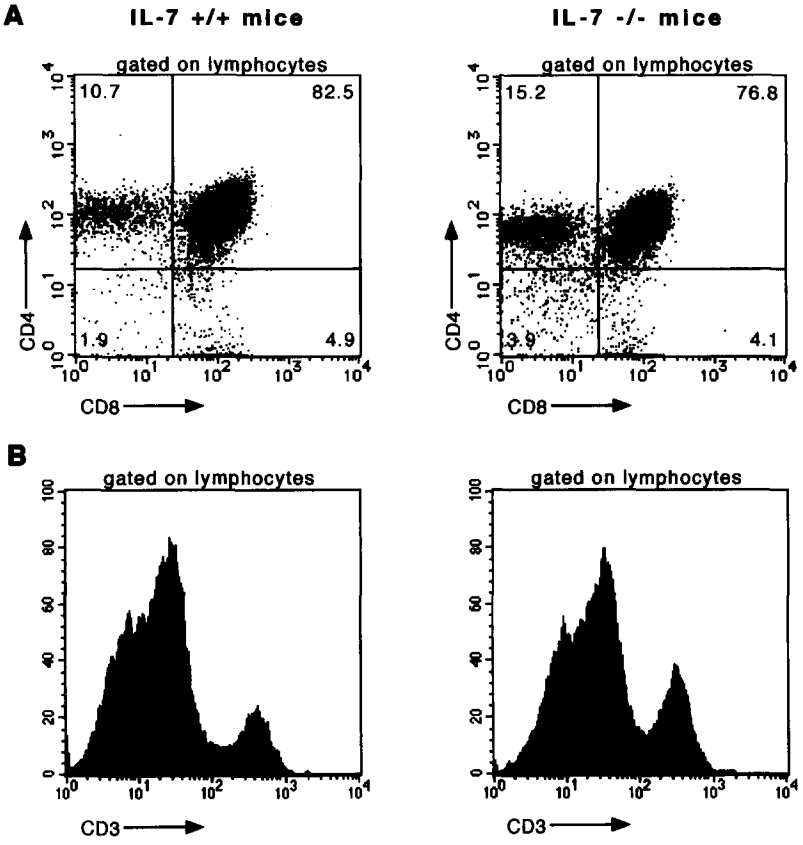

Figure 5. Immunofluorescent analysis of thymocytes from IL-7−/− and IL-7+/+ control mice. (*A*) Thymus cells stained with PE-anti-CD8 and tricolor-anti-CD4 gated on lymphocytes. The percentage of cells in each quadrant is indicated. (*B*) Thymus cells stained with FITC-anti-CD3 gated on lymphocytes. The data are results of a representative experiment.

tion in absolute numbers of mature splenic B and T cells (Fig. 6 *A*). In addition, the relative proportion of B cells was reduced within the spleens of IL-7-deficient mice (Fig. 6 *B*). Three-color FACS® analysis of spleen cells was performed to determine the phenotype of the remaining lymphocytes. A population of $B220^+/IgM^+$ mature B cells were present, and at least some of these cells were IgD^+, although at a lower percentage than in controls (Fig. 7, *A* and *B*). In addition, a population of $B220^+/IgM^-$ cells existed in the mutant animals, which was not found in the control animals, suggesting a significant number of immature B cells in the adult spleen. Analysis of the remaining splenic T cell populations showed normal staining patterns of CD4, CD8, and CD3 (data not shown).

Thymocyte and Splenocyte Response to Mitogenic Stimuli. As the IL-7-mutant animals displayed reduced but stable numbers of splenic lymphocytes and thymocytes, we evaluated the response of these cells to LPS, Con A, or Con A plus rIL-2. The B cell response to LPS was reduced if total splenocytes were used. However, as the spleen showed reduced numbers of B cells as well as an additional reduction in the ratio of B ($B220^+/IgM^+$) to non-B cells, we normalized the per-

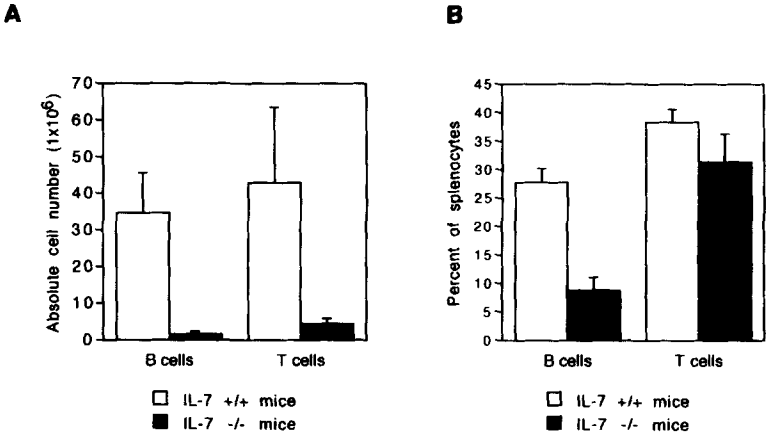

Figure 6. Analysis of lymphoid cellularity in the spleen. (*A*) Absolute cell number of B cells and T cells in the spleen. The numbers are based on FACS® staining with B220 and IgM or TCR-$\alpha\beta$. The data represent mean ± SD values of experiments with four IL-7−/− mice and four control mice. (*B*) Relative percentage of B cells and T cells in total splenocytes.

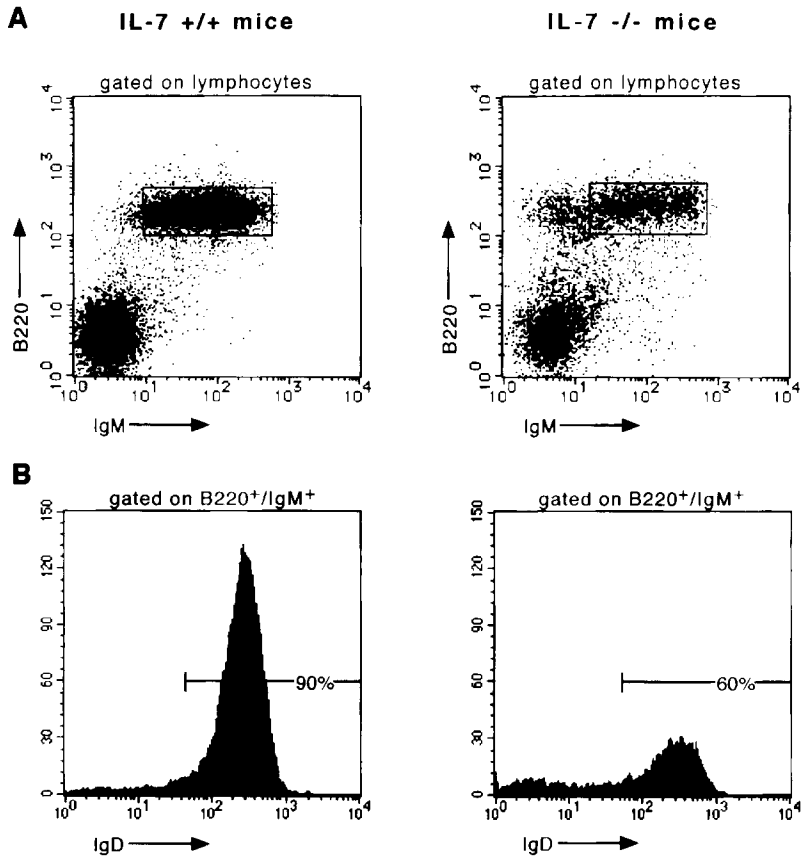

Figure 7. Immunofluorescent analysis of splenocytes from IL7−/− and IL7+/+ control mice. (*A*) Spleen cells stained with FITC-anti-IgM and tricolor-anti-B220 gated on lymphocytes. (*B*) Spleen cells stained with PE-anti-IgD gated on IgM+/B220+ cell (*rectangle* in Fig. 7 *A*). The percentage of IgD+ cells is indicated. The data are results of a representative experiment.

Table 1. *Thymocyte and Splenocyte Response to Mitogenic Stimuli*

A. Proliferation of splenic B cells	Uncorrected cell numbers		Corrected cell numbers	
	Unstimulated	LPS	Unstimulated	LPS
IL7 +/+	120 ± 13	143,804 ± 12,321	120	143,804
IL7 −/−	115 ± 26	56,766 ± 8,386	367	181,083
B. Proliferation of splenic T cells				
	Unstimulated	Con A		
IL7 +/+	330 ± 61	8,975 ± 1,639		
IL7 −/−	367 ± 22	8,296 ± 2,043		
C. Proliferation of thymocytes				
	Unstimulated	Con A + IL2		
IL7 +/+	122 ± 32	116,766 ± 21,008		
IL7 −/−	96 ± 9	161,396 ± 11,655		

Splenic B or T cells and thymocytes were examined for proliferative responses to the indicated stimuli. Results of a representative experiment are shown with average cpm ± SD of triplicate wells.

centage shown in Fig. 6 *B*. Table 1 *A* shows that the proliferative response of B cells was similar to controls when the cell population was normalized. T cells in the spleen also gave reduced overall numbers of cells, but a normal ratio of T to non-T cells, so no correction factor was necessary, and Table 1 *B* shows that the Con A response of splenic T cells was normal. Similarly, the response of the remaining thymocytes to Con A plus rIL-2 was similar for both groups of animals (Table 1 *C*).

Discussion

Gene targeting analysis of cytokine molecules has led to a better understanding of the role of these molecules during immune responses and normal regulation of the immune system. However, a consistent finding in the cytokine gene disruption literature has been the absence of severe lymphoid defects due to inactivation of a single cytokine gene. One collective interpretation of these experiments would suggest that the normal appearance and expansion of lymphocytes is not critically dependent on any individual cytokine. Another possibility is that cytokines playing a more crucial role in the appearance and expansion of lymphocytes may be as yet uncharacterized, or not yet analyzed by gene-targeting experiments.

The data we have presented in this report would favor the latter of these two possibilities. This report identifies the first single-cytokine gene-deficient mouse that exhibits severe lymphoid abnormalities. Inactivation of the IL-7 gene has shown a stable phenotype of severe peripheral blood lymphopenia. This defect can be traced back to abnormal B lymphopoiesis in the bone marrow. The transition between pro-B, fractions B/C ($B220^+/IgM^-/S7^+/HSA^+$) and pre-B, fraction D ($B220^+/IgM^-/S7^-/HSA^+$) was blocked. According to the B cell development scheme suggested by Hardy et al. (23), fractions B and C show a strong proliferative response to IL-7, whereas fraction D is unresponsive to IL-7. Our data indicate that the proliferative response of cells in fractions B and C to IL-7 is not due to IL-7 exclusively. However, the differentiation of fraction B/C to fraction D showed an absolute dependence on IL-7. The peripheral lymphopenia can also be traced to inefficient thymic development where only 5% of normal thymocyte numbers exist. However, the relative ratios of thymic subpopulations were normal, and only overall numbers of cells were reduced. This is consistent with IL-7 being described in vitro as a proliferation and maintenance factor for thymocytes, but not as a differentiation factor (10, 24, 25).

Further analyses revealed an interesting phenotype of the few remaining lymphocytes. In addition to reduced numbers of $B220^+/IgM^+$ cells, there was a population of $B220^+/IgM^-$ cells, which was not present in control mice. These cells may represent premature migration of B cells from bone marrow to spleen, or alternatively, abnormal splenic B lymphopoiesis. The remaining $B220^+/IgM^+$ cells in the mutant animals contained surface IgD^+ cells, but in lower numbers and percentages. T cell populations in the spleen were also reduced in number, but normal population of cells as analyzed by CD4, CD8, and CD3 were present.

It is useful to compare the phenotype of the mice we describe here to the recent report of IL-7Rα −/− mice (22). IL-7Rα −/− mice exhibited a similar overall reduction of B cells, but showed a block in B cell development at the transition of pre-pro-B cells (fraction A) to pro-B cells (fraction B). The IL-7 −/− mice were inhibited in the transition from B/C to D, which represents a later developmental block. This difference is likely to be due to other cytokines that use the IL-7Rα chain, such as TSLP. Paradoxically, treatment of mice with anti-IL-7 antibodies does not fit precisely with this idea (11). Instead of showing the predicted block equivalent to IL-7–deficient mice, antibody-treated mice appeared to be blocked at a stage similar to the IL-7Rα −/− mice. Perhaps the antibody treatment is affecting other cytokines that interact through the IL-7Rα chain, as it is difficult to imagine that IL-7 neutralization by an antibody could result in a phenotype more severe than in the corresponding gene inactivation study presented here.

In contrast to the stable reduction of thymocytes we reported, IL-7Rα −/− mice inexplicably showed two different phenotypes in thymic development. One was similar to the IL-7 −/− mice and the other was more severe. Although the reason for the variability is unclear, the more severe phenotype is again likely due to other molecules interacting through the IL-7Rα chain.

The IL-7 −/− mice exhibited similar lymphocyte abnormalities to IL-2Rγ–deficient mice (26), even though this receptor subunit is shared by at least four other cytokines. Neither the gene inactivation of IL-2 (1) or IL-4 (2), or the double mutation of IL-2 and IL-4 (27) showed any defects in lymphopoiesis. This indicates that most of the lymphocyte developmental abnormalities seen in an IL-2Rγ–deficient model of murine XSCID are due mainly to IL-7, and not other cytokines that bind to IL-2Rγ.

The data shown here indicate that IL-7 is a unique and nonredundant cytokine, in that it is essential for normal lymphocyte development. To date, IL-7–deficient mice have demonstrated the most profound effect on lymphoid development in contrast to gene inactivation of other cytokines previously thought to be important for lymphocyte development.

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