Expression and function of the interleukin 7 receptor in murine lymphocytes

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ABSTRACT   A monoclonal antibody, A7R34, that recognizes the high-affinity interleukin 7 receptor (IL-7Ra) and blocks the binding between IL-7 and IL-7Ra has been produced. Cell surface staining with A7R34 demonstrated that IL-7Ra is expressed in both B- and T-cell lineages. In the bone marrow, immature B-lineage cells that do not express surface IgM were IL-7Ra$^+$. In the thymus, IL-7Ra was detected in CD4$^-$8$^-$ T cells and also in CD4 or CD8 single-positive cells but not in CD4$^+$8$^+$ double-positive cells. In the peripheral lymphoid tissues, both CD4 and CD8 single-positive cells were the major cell types that express IL-7Ra. Addition of A7R34 to a long-term B-precursor-cell culture inhibited proliferation of the B-lineage cells, indicating that IL-7 is an absolute requirement for *in vitro* B-cell genesis. Consistent with this *in vitro* result, continuous injection of A7R34 into an adult mouse resulted in a decrease of B-precursor cells and also of thymocytes, whereas a considerable fraction of mature B and T cells in the peripheral tissues persisted over 2 weeks of the experiment. When A7R34 injection is started from day 14 of gestation, it is possible to produce mice that lack B cells. These results indicate that IL-7 is an essential molecule for generation of both B and T cells in murine bone marrow and thymus, respectively. Moreover, IL-7Ra would be the sole receptor system regulating these processes.

B-cell genesis in the adult mouse is regulated by a meshwork of stromal-cell components in the bone marrow (1, 2). Establishment of the stromal-cell lines that can support B-cell genesis from multipotent hematopoietic stem cells facilitated identification of the molecules that are required for this process (3–6). Among a number of molecules that are expressed in the stromal-cell lines, interleukin 7 (IL-7) is the first molecule that has been shown to be able to induce proliferation of B-cell precursors (7, 8). Initial use of recombinant IL-7 demonstrated that IL-7 can induce proliferation of pre-B cells (8–10). Subsequent studies further demonstrated that IL-7, when used in combination with the stromal cell lines or the ligand for c-kit, can act on earlier stages of B precursors (11–15). Besides the proliferative activity on B-precursor cells, it was reported that IL-7 induces proliferation of both mature and immature mouse T cells (16–22). In fact, studies of mice that received continuous IL-7 injections or bore the IL-7 transgene indicate that elevation of IL-7 results in enormous expansion of both T and B cells (23–26).

Despite all such positive evidence suggesting the role of IL-7 in lymphopoiesis, whether IL-7 is a functional requirement for *in vivo* lymphocyte production is yet to be determined. To address this question, we have produced an antagonistic monoclonal antibody (mAb) to the high-affinity IL-7 receptor (IL-7Ra) (27) and have investigated its expression and function. Our study demonstrates that IL-7Ra is

expressed in surface IgM-negative (sIgM$^-$) B precursors and also CD4$^-$8$^-$, CD4$^+$8$^-$, and CD4$^-$8$^+$ T cells. In addition, an *in vivo* injection experiment using this mAb indicates that IL-7 is actually a functional requirement for generation of both T and B cells.

MATERIALS AND METHODS

Preparation of Murine IL-7R–Human IgG1 Chimeric Protein. A cDNA fragment corresponding to the whole IL-7Ra coding region (28) was generated by reverse transcription–PCR amplification from mRNA of the IL-7-dependent pre-B cell line DW34 (29). The primers used were 5′-CTCAGAAT-GATGGCTCTGGG and 5′-AATTCATTTGTTTTGG-TAAAAACTAGACAT as sense and antisense primers, respectively. The fragment was cloned into the *Sma* I site of pUC19. Sequence analysis of this IL-7Ra gene indicates that it encodes only a truncated form of IL-7Ra (N-terminal 281 aa) due to the loss of a nucleotide at position 846. However, this gene encodes the intact extracellular and transmembrane domains of IL-7Ra. *Kpn* I–*Bam*HI fragment of thus cloned IL-7Ra gene was inserted to CD4Rg (a kind gift of B. Seed, Massachusetts General Hospital) (30) from which the *Xho* I–*Bam*HI fragment containing the CD4 gene was removed and transfected to CHO DUKXB11 (DHFR$^-$) cell line (a kind gift of L. A. Chasin, Columbia University) (31) plus pAdD26SV(A)-3 (32), which contains the mouse dihydrofolate reductase coding sequence. A high producing clone, CHOIL-7RIg-1-1-M, was cultured in Dulbecco's modified Eagle's medium/F-12 medium (GIBCO) supplemented with 1% Nutridoma-NS (Boehringer Mannheim). The recombinant protein was purified from the supernatants by Prosep-A (Bioprocessing, Princeton).

mAbs, Cell Staining, Immunoprecipitation, and IL-7 Binding Assay. Production of anti-IL-7R mAb is described in the *Results*. Biotin-labeled A7R34, fluorescein isothiocyanate (FITC)-labeled RA3-6B2 (anti-B220) (33), and FITC-labeled anti-Mac1 (34) were prepared as described (35). FITC-labeled S7 (36) was a kind gift of W. Müller (Cologne University). Apophycocyanin-conjugated A7R34 was kindly prepared by R. H. Hardy (Fox Chase Cancer Center). FITC-labeled anti-Thy1.2, FITC-labeled anti-Lyt2, and phycoerythrin (PE)-labeled L3T4 were purchased from BRL. PE-labeled streptoavidin was purchased from Becton Dickinson.

Biotinylation of cell surface protein was carried out by the method of Ingalls *et al.* (37). Briefly, 70Z/3 cells were surface labeled with sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce) at 0.5 mg/ml for 30 min in Hepes-buffered saline (0.1 M Hepes, pH 8.0/0.15 M NaCl) at room temperature. The labeled cells were lysed with the precipitation buffer [50 mM

Abbreviations: IL, interleukin; IL-7R, IL-7 receptor; IL-7Ra, the high-affinity IL-7R; LTBM-W, Whitlock–Witte type long-term bone marrow culture; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PE, phycoerythrin; sIgM, surface IgM.

Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/leupeptin (1 μg/ml)/5 mM EDTA/0.1% NaN₃] for 45 min on ice. The lysates were precleared overnight with 100 μl of goat anti-rat immunoglobulin-conjugated Dynabeads (Dynal, Oslo), precipitated with A7R34- or anti-rat-immunoglobulin-conjugated Dynabeads, and subjected to SDS/PAGE under reducing conditions in 7.5% gels. After transferring to nitrocellulose membrane (Hybond-C, Amersham), the membrane was blocked with phosphate-buffered saline containing 5% (vol/vol) skim milk and 1% bovine serum albumin, incubated with horseradish peroxidase-conjugated avidin (Bio-Rad), and developed by the ECL detection reagent (Amersham).

Murine recombinant IL-7 (Intergen, Purchase, NY) was radiolabeled using a solid-phase iodination technique with Iodo-Beads (Pierce), by following the manufacturer's recommendation. The receptor binding assay was performed by the method of Park *et al.* (38).

RESULTS

The spleen cells from a Wister rat that was immunized with the IL-7Ra–IgG1 fusion protein was fused with X63.Ag8 cells as described (35). The hybridoma supernatants were screened by a growth inhibition assay of the IL-7-dependent cell line DW34. Among several hybridoma clones that were selected by the initial screening, only A7R34 remained after the second recloning.

A series of specificity tests for A7R34 indicated that it recognizes an epitope near the ligand binding site of IL-7Ra. (*i*) A7R34 stained the CHO cells that were transfected with the pSRα vector containing the gene encoding N-terminal 281 aa of IL-7Ra but not those transfected with the control vector without the inserts (Fig. 1*A*). (*ii*) Immunoprecipitation of the biotinylated surface protein of 70Z/3 cells by A7R34-coated magnetic beads gave a 75- to 80-kDa major band and two smaller bands (Fig. 1*B*). The size of the major band precipitated by A7R is consistent with a previous report that 65- to 75-kDa and 150- to 160-kDa (the dimer form) bands were cross-linked to labeled IL-7 (27, 28). However, the nature of smaller bands, whether they are IL-7Ra-associated molecules or degradated products of IL-7Ra, is yet to be determined. (*iii*) A7R34 inhibited the binding of ¹²⁵I-labeled IL-7 to DW34 as efficiently as unlabeled IL-7 (Fig. 1*C*).

We next investigated the cell types expressing IL-7Ra. In the thymus, IL-7Ra expression was observed in CD4⁻8⁻ double-negative T cells and also CD4⁺8⁻ and CD4⁻8⁺ single-positive cells, whereas no IL-7Ra⁺ cells could be detected in CD4⁺8⁺ double-positive cells (Fig. 2*A*). In the spleen and lymph node, IL-7R⁺ cells were present mostly in CD4⁺8⁻ and CD4⁻8⁺ single-positive T cells (Fig. 2*B* and unpublished observation). In contrast to these peripheral organs, most IL-7Ra⁺ cells in the bone marrow were B220⁺ cells and the cells that express a high level of IL-7Ra were basically B220^dull cells (Fig. 2*C*). Upon maturation to sμ⁺ B cells, IL-7Ra expression is downregulated.

Because A7R34 blocks IL-7 binding to IL-7Ra, it is expected that all IL-7Ra-dependent processes are suppressed by this mAb. To test whether B-cell genesis in a Whitlock–Witte type long-term culture (LTBM-W) (39) is dependent on IL-7, we added A7R34 (20 μg/ml) to a LTBM-W 4 weeks after the initiation of culture. The proliferation of B220⁺ cells was blocked and they disappeared from the culture (data not shown). This indicates that B-cell genesis in a LTBM-W is absolutely dependent on IL-7Ra.

To investigate the *in vivo* role of IL-7Ra in lymphocyte production, 2 mg of A7R34 was injected into 8-week-old mice every other day. Fourteen days after the initial injection, the cells in various lymphoid organs were counted and analyzed for the expression of surface markers (Fig. 3). C2-7(γ2aκ), which does not react to normal lymphoid tissues, was injected as a class-matched control mAb. A7R34 injection resulted in a striking reduction of the B220⁺ cells in the bone marrow and also of the thymocytes, whereas a decrease of the lymphocytes in the spleen and lymph nodes was rather slight. B220^dull sμ⁻ and B220^dull sμ⁺ populations in the bone marrow and CD4⁺8⁺ cells in the thymus were affected most profoundly, though all cell fractions decreased in the absolute number. Interestingly, despite such a big deficit of lymphocyte recruitment from the bone marrow and thymus, a considerable number of both T and B lymphocytes in the peripheral organs persisted.

While the results in Fig. 3 clearly indicate that IL-7Ra is functioning in *in vivo* B-cell genesis, the presence of persistent sμ⁺ cells in the bone marrow of A7R34-injected animals
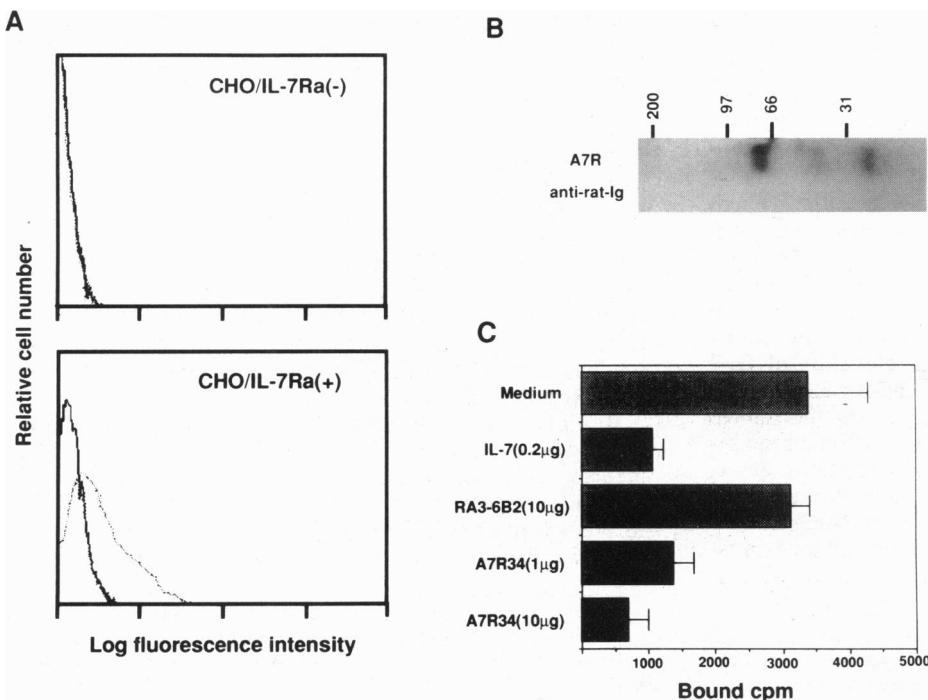
A

B

C

FIG. 1. Specificity of A7R34. (*A*) CHO cells were transfected with the pSRα mammalian expression vector encoding the N-terminal 281 aa residue of IL-7Ra or the vector alone and stained with A7R34 plus FITC-conjugated anti-rat IgG. Solid line, FITC-conjugated anti-rat IgG alone; dotted line, A7R34 plus FITC-conjugated anti-rat IgG. (*B*) Immunoprecipitation of biotinylated surface proteins of 70Z/3 by A7R34-conjugated magnetic beads. Molecular masses in kDa are shown. (*C*) Inhibition of ¹²⁵I-labeled IL-7 binding to DW34 by A7R34. Each bar represents arithmetic mean (+SD) of triplicate assays.
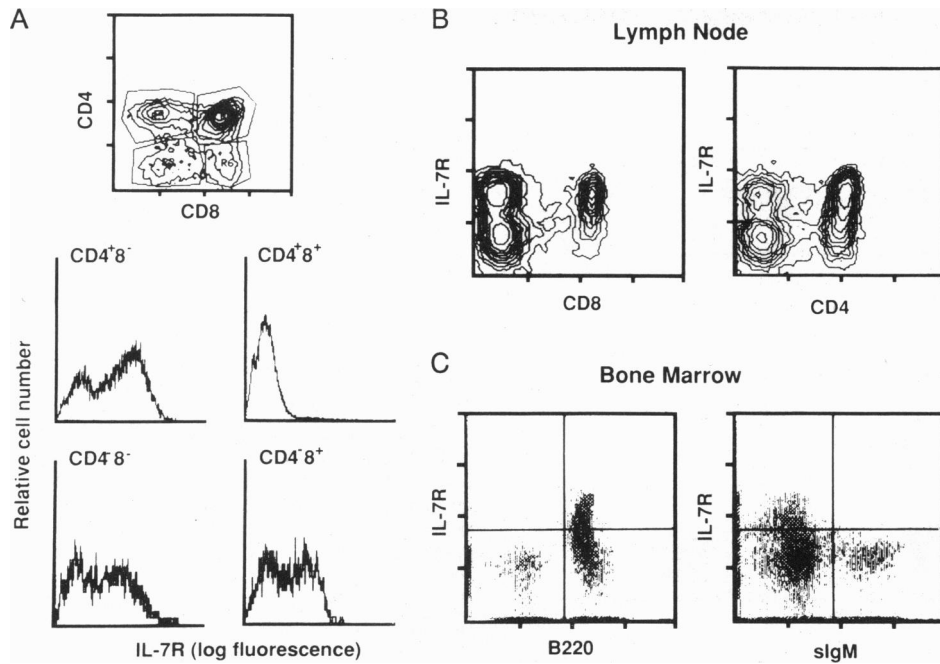
FIG. 2. Expression of IL-7Ra in various lymphoid organs of C57BL/6 (B6) mice. (A) Thymocytes from a 4-week-old mouse were stained with FITC-conjugated anti-Lyt2, PE-conjugated anti-L3T4, and apophycocyanin-conjugated A7R34. IL-7Ra expression of thymocytes in the CD4$^+$CD8$^-$, CD4$^+$CD8$^+$, CD4$^-$CD8$^-$, and CD4$^-$CD8$^+$ populations is presented separately. (B) Inguinal lymph node cells from a 4-week-old mouse were three-color stained by the same combination of antibodies used for thymocyte staining. (C) Bone marrow cells from an 8-week-old mouse were stained with biotin-conjugated A7R34 in combination with FITC-conjugated anti-B220 or FITC-conjugated anti-mouse IgM. The stained cells were incubated with PE-conjugated avidin and analyzed.

questions whether IL-7Ra is really an absolute requirement. To solve this problem, we started A7R34 injection before mature B lymphocytes appear, namely, from day 14 of gestation. A7R34 at 4 mg (2 mg intravenously and 2 mg subcutaneously) was injected every other day into a pregnant mother and then 100 μg of A7R34 was directly injected into the peritoneal cavity of pups. At 12 days after birth, the bone marrow cells were harvested and analyzed. Virtually no sμ$^+$
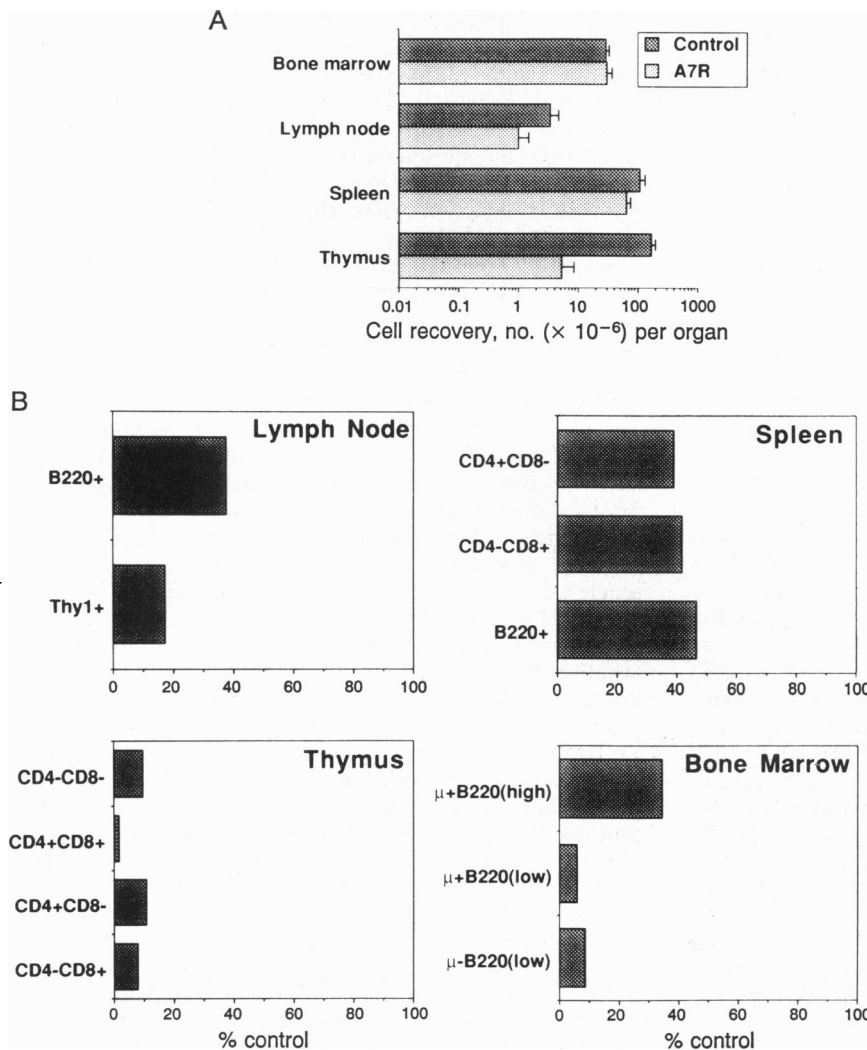

FIG. 3. Effect of A7R34 injection on lymphopoiesis of adult mice. B6 mice were given an intravenous injection of A7R34 or a class-matched control mAb, C2-7, every other day. Fourteen days after the initiation of the injection, the cells were harvested from the inguinal lymph nodes, spleen, thymus, and one femur of each mouse. After measuring the cell numbers in each organ of three mice individually, the cells were pooled and analyzed for the expression of surface antigens. (A) The total number of cells recovered from each organ is expressed as the arithmetic mean (+SD) from three mice. (B) The absolute number of various lymphocyte subpopulations in each organ was calculated after flow-cytometric analysis and is expressed as the percentages of values from the control mice.
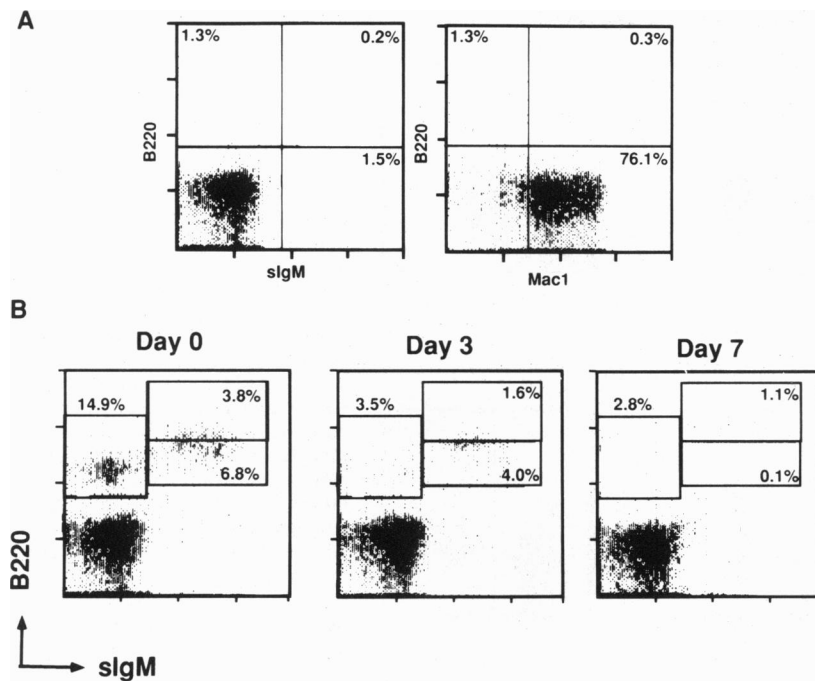
FIG. 4. Nearly complete suppression of B-cell genesis by A7R34 injection into young mice. (*A*) Two pregnant B6 mice were injected every other day with 4 mg of A7R34 from day 14 of gestation until the day of delivery. Thereafter, 100 μg of A7R34 was injected every other day into the pups, and 12 days after birth, two pups from different mothers were sacrificed, and the bone marrow cells were harvested, pooled, and analyzed for the expression of B220, sIgM, and Mac1 antigens. (*B*) Four-week-old B6 mice were injected every other day with 2 mg of A7R34. Zero, 3, and 7 days after the initiation of injection, the bone marrow cells from two mice were harvested, pooled, and analyzed for the expression of B220 and sIgM antigens.

cells were present in the bone marrow, though a small number of B220$^+$ cells were present (Fig. 4A). Interestingly, all the remaining B220$^+$ cells were also S7/CD43$^+$ (data not shown). According to Hardy *et al.* (36), IL-7-reactive B progenitors are included in the B220$^+$S7$^+$ population. Thus, these results indicate that B220$^+$S7$^+$ progenitors are constantly generated but cannot further proliferate to give rise to more mature B-lineage cells in the absence of IL-7. We next determined the time course of the disappearance of B-lineage cells by A7R34 injection into 4-week-old mice. Fig. 4B demonstrates that disappearance of B220$^+$ cells by A7R34 treatment is very rapid. Particularly, B220^{dull}sμ$^+$ cells had almost all disappeared as early as 7 days after the initiation of the injection. Again, all B220$^+$sμ$^-$ cells remaining in the bone marrow were S7/CD43$^+$.

DISCUSSION

The present study demonstrates that IL-7Ra is expressed and functions during early stages of B-lineage differentiation. In agreement with previous studies that indicate the absence of IL-7 reactivity in sIgM$^+$ mature B cells (9, 10, 36), IL-7Ra expression of mature B cells is below the detectable level. Thus, like the recombination activating genes and the surrogate light chain (for review, see refs. 40 and 41), IL-7Ra belongs to a group of the molecules whose expression is downregulated upon the pre-B to sIgM$^+$ B transition, though the order of each event is yet to be determined.

In agreement with our previous results that no active B lymphopoiesis is supported on a stromal cell clone that does not express IL-7 (11), the proliferation of B-progenitor cells in a LTBM-W is completely suppressed by A7R34, and most B220$^+$ cells eventually die even on the stromal cell layer of a LTBM-W. This indicates that IL-7Ra is an absolute requirement for B lymphopoiesis in a LTBM-W. *In vivo* B-cell genesis was also suppressed by A7R34 injection. This suppression was more conspicuous in the immature B-cell populations (namely, B220^{dull}sμ$^-$ and B220^{dull}sμ$^+$ cells) than in B220^{high}sμ$^+$ cells. As a consequence of this deficit in B-cell recruitment, the absolute number of B cells in the peripheral organs decreased to 50% of the control group. However, even in the bone marrow, a mature B220^{high}sμ$^+$ population persisted over the period of this experiment. This could be

because the IL-7Ra-independent pathway of B-cell genesis is present or because a considerable fraction of mature B cells has a life span longer than 2 weeks. It might be difficult to exclude the first possibility unless we obtain B-cell-less mice by A7R34 injection. In fact, it would be interesting to know whether mature B cells can persist despite the life-long treatment with A7R34, and if not, how long it takes to deplete all mature B cells by blocking B-cell recruitment. Such a long-term A7R34 injection into adult mice would provide insight into the life span of mature B cells. Nevertheless, the results in Fig. 4 indicate that B-cell-less mice can be produced by A7R34 injection when the injection is started at the embryonic stage. Thus, at least in the early life, interaction between IL-7 and IL-7Ra is essential for B-cell genesis. If the same is true for B-cell genesis in the adult bone marrow, our results in Fig. 4 suggest that a half of spleen B cells can survive longer than 2 weeks. It is interesting to note that a small number of B220$^+$S7$^+$ cells are constantly generated even in the B-cell-less mice produced by A7R34 injection. Because IL-7-reactive B-cell progenitors are included in B220$^+$S7$^+$ population (36), this indicates that the earliest part of B-cell differentiation from multipotent stem cells to the IL-7-reactive stage may occur without IL-7Ra.

The major T-cell populations that express IL-7Ra are CD4$^-$8$^-$ immature thymocytes and CD4$^+$8$^-$ and CD4$^-$8$^+$ single-positive T cells in the thymus and the peripheral lymphoid tissues. However, the level of IL-7Ra expression within each differentiation stage is variable (Fig. 2). Thus, whether the level of IL-7Ra expression in each T-cell fraction could be a marker reflecting a differentiation stage or a functional status is an important question. Our present result on IL-7Ra expression in T cells is consistent with a series of previous *in vitro* studies that demonstrated that IL-7 is able to stimulate proliferation of CD4$^-$8$^-$ immature thymocytes and mature peripheral T cells and also to induce cytotoxic activity of CD8$^+$ mature lymphocytes (16–22). The present study demonstrates that IL-7Ra is functioning in T-cell production in the thymus. A nearly 30-fold reduction of the thymus cellularity was induced by A7R34 injection, and probably as a consequence of this, the number of peripheral T cells was also reduced to 20–40% of that in control animals. However, we cannot address the question of whether IL-7Ra plays a role in maintaining IL-7Ra$^+$ cells in peripheral tissues

because of the tremendous effect of A7R34 on T-cell recruitment in the peripheral organs. The fact that both CD4^{+} and CD8^{+} cells remained in the peripheral tissues after the long-term treatment of A7R34 suggests that the role of IL-7Ra in the peripheral tissues might be small.

By taking the IL-7Ra expression pattern in the thymus into consideration, it is clear that IL-7 plays an essential role in the proliferation of CD4[-]8^{-} thymocytes. However, of note is the fact that a significant number of both CD4^{+}8^{+} T cells and mature single-positive cells persist over 2 weeks of treatment. Moreover, 10[7] thymocytes were present in the mice that received the continuous A7R34 injection from day 14 of the gestation, though no B cells were present in the bone marrow (the same mice shown in Fig. 4A). Thus, if all IL-7Ra^{+} cells are accessible to the injected A7R34, it is likely that the role of IL-7 in T-cell genesis might be a redundant process involving other cytokines.

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