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Role of the Intracellular Domain of IL-7 Receptor in T Cell Development[1]

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Signals from the IL-7R are uniquely required for T cell development and maintenance, despite the resemblance of IL-7R to other cytokine receptors and the apparent sharing of common signaling pathways. This unique requirement could either reflect unique expression of IL-7R or IL-7, or it could indicate that the IL-7R delivers unique signals. To determine whether the IL-7R provided unique signals, we exchanged its intracellular domain with that of other cytokine receptors: IL-4R, IL-9R, and prolactin receptor (PRLR). Chimeric receptors were used to reconstitute development of IL-7R$^{-/-}$ hemopoietic progenitors by transducing the receptors in retroviral vectors. Whereas IL-7R$^{-/-}$ thymocytes are arrested at the double-negative stage, IL-4R, IL-9R, or PRLR all imparted some progression to the double-positive stage. IL-4R and PRLR gave only small numbers of thymocytes, whereas IL-9R gave robust $\alpha\beta$ T cell development and reconstitution of peripheral CD4 and CD8 cells, indicating that it can duplicate many of the functions of IL-7R. However, IL-9R failed to reconstitute rearrangement of the TCRγ locus or development of $\gamma\delta$ T cells. Thus, the IL-7R signals required in the $\alpha\beta$ T cell lineage (such as survival and proliferation) are not unique to this receptor, whereas rearrangement of the TCRγ locus may require a signal that is not shared by other receptors. ***The Journal of Immunology,*** **2007, 178: 228–234.**

Deletion of IL-7 (1) or its receptor components (2) result in one of the most dramatic phenotypes of any cytokine deficiency and accounts for severe combined immunodeficiency in humans (3). IL-7 is required for early T cell development in the thymus, mainly to protect cells at the double-negative (DN[3]) 2 and DN3 stages from apoptotic cell death (4). This antiapoptotic effect of IL-7 is attributed to expression of the survival proteins Bcl-2 (4) and Mcl-1 (5) and posttranslational suppression of the death proteins Bax, Bad, and Bim. Thus, the IL-7R function at these thymocyte stages can be partially replaced by overexpression of the survival protein Bcl-2 (6, 7) or deletion of the death proteins Bax (8) or Bim (9). In addition to these trophic effects in DN thymocytes, IL-7 is required for rearrangement of the TCRγ locus (10) and promotes selection of CD8 cells (11).

IL-7 is also required for persistence of most of the major subsets of peripheral T cells, including naive CD4 and CD8 cells, memory CD8 cells, and for selection of memory CD4 cells (12–14). Whereas the thymic effect of IL-7 is mainly antiapoptotic, the homeostatic effects on peripheral T cells are also proliferative. IL-7 stimulates cell division through regulation of cyclin-dependent kinases via an activator, Cdc25a (15), and an inhibitor, p27^{Kip1} (16).

IL-7 cross-links the IL-7Rα-chain with common γ chain (γ_c), bringing together their intracellular domains bearing Jak1 and Jak3, respectively. These kinases phosphorylate Y449 on the IL-7Rα intracellular domain, creating a docking site for Stat5 (17, 18) and the p85 subunit of PI3K (19). Although other signaling intermediates are also activated (20), the most compelling evidence published so far implicates AKT and Stat5, because dominant negatives for AKT and for Stat5 inhibited human T cell development in mouse thymic organ culture (21) and the complete deletion of Stat5a and -b blocks T cell development (22).

Although deletion of IL-7 or its receptor components results in a unique phenotype, no unique intracellular mediators have yet been found. Thus, several cytokines activate the Jak1/3-Stat5 pathway, and many more activate the PI3K pathway and control the Bcl-2 family that regulates survival and the cyclin-dependent kinases that regulate cell division. For example, Stat5 is also activated by receptors for IL-2, -4, -9, -15, -21, prolactin receptor (PRLR), and erythropoietin (Epo). There are several possible explanations for the unique requirement for the IL-7 pathway in T cells. First, there could be a unique signal from the IL-7R that has yet to be discovered. Second, there could be strict control of cytokine receptor expression such that only IL-7R is expressed on DN2, DN3, and resting peripheral T cells. Third, there could be strict control of ligand expression such that only IL-7 is available in the niche occupied by these cells. To test the first possibility, that the intracellular domain of IL-7Rα chain provided unique signals to T cells, we exchanged this domain with that from other related receptors. If another intracellular domain could replace the function of IL-7Rα, it would suggest that the unique requirement for the IL-7 pathway was due to strict regulation of receptor and/or ligand expression rather than a unique intracellular signal.

Materials and Methods

Cell lines

The phoenix-Eco retrovirus packaging cell line was maintained in DMEM (Mediatech) supplemented with 10% FBS. The Baf/3 cell line is an IL-3-dependent murine hemopoietic line. BaF/3 cells were routinely maintained in RPMI 1640 containing 10% FBS with 1 ng/ml IL-3.

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[3] Abbreviations used in this paper: DN, double negative; γ_c, common γ-chain; PRLR, prolactin receptor; Epo, erythropoietin; WT, wild type; 5FU, 5-fluorouracil; PI, propidium iodide; Q-PCR, quantitative PCR; SOCS, suppressor of cytokine signaling; m, murine.

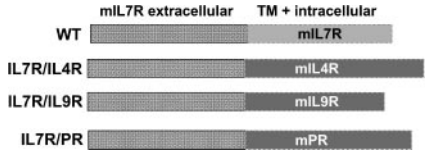

FIGURE 1. Chimeric mIL-7R constructs. Constructs contained the murine IL-7R extracellular domain (1–239 aa) coupled to the transmembrane domain (TM) and intracellular regions from different receptors (murine IL-7R (240–459 aa), murine IL-4R (249–825 aa), murine IL-9R (271–478 aa), or murine PRLR (230–608 aa)).

Retroviral chimeric receptor plasmid construction

Full-length murine IL-7Rα (mIL-7α; wild-type; WT) was cloned from the D1 cell line by RT-PCR. The transmembrane domain and intracellular domain of mouse IL-4Rα, mouse IL-9Rα, or mouse PRLR was amplified, respectively, from pGEM3-mIL4Rα (provided by Dr. A. Keegan, Red Cross, Baltimore, MD) or p9RC4 (provided by Dr. J.-C. Renauld, Universite Catholique de Louvain, Louvain, Belgium) or pECE-PRLR (provided by Dr. W. Doppler, Universität Innsbruck, Innsbruck, Austria). The extracellular domain of mIL-7R was fused to the transmembrane and intracellular domain of mouse IL-4R, mouse IL-9R, or mouse PRLR by PCR strategies, and cloned into pMIG vector (a gift from Dr. J. Keller, National Cancer Institute, Frederick, MD), designated IL-7R/IL4R, IL-7R/IL9R, or IL-7R/PR. All constructs were verified by DNA sequencing.

In vitro response to IL-7 of IL-7Ra$^{-/-}$ bone marrow progenitors transduced with chimeric IL-7Rs: cell cycle and cytology

Chimeric receptor constructs were transfected into the phoenix-Eco packaging cell line using FuGene-6 Transfection Reagent (Roche Diagnostics), and supernatants were collected. Cycling bone marrow stem cells were enriched by injecting IL-7R$\alpha^{-/-}$ mice with 5-fluorouracil (5FU) (150 mg/kg) (Schein Pharmaceuticals) in Dulbecco's PBS 4 days before harvest of bone marrow from tibia and femur. Bone marrow cells were cultured (1×10^6 cells/ml) in X-vivo 10 medium (BioWhittaker) supplemented with 5% FCS, murine stem cell factor (100 ng/ml), murine IL-6 (50 ng/ml), and Flt-3 ligand (100 ng/ml). After 48 h, bone marrow cells were infected overnight with different retroviral supernatants from the packaging line and then the infection was repeated after 72 h. Expression of GFP was analyzed by FACScan flow cytometry (BD Biosciences). Three days later, GFP-positive cells from the infected bone marrow cells were sorted by FACScan cytometry. Sorted bone marrow cells were checked for mouse IL-7R expression with Tri-color-conjugated anti-mIL-7R Ab (eBioscience).

GFP-positive cells from different groups were plated in a 6-well plate and incubated with or without mIL-7 for 48 h, and cell cycle was determined by propidium iodide (PI) staining as described previously (23). In brief, cells were placed in detergent buffer containing 50 μg/ml RNase A (Roche) at a concentration of $1–2 \times 10^6$ cells/ml, and then mixed with an equal volume of PI (50 μg/ml; Sigma-Aldrich) and incubated at room temperature in the dark for 1 h. DNA contents were assayed by flow cytometry. For cytological analysis, sorted cells were cultured with or without mIL-7 for 72 h, prepared by cytospin onto slides and stained with Hama 3 staining set (Biochemical Sciences).

Reconstitution of IL-7R$\alpha^{-/-}$ bone marrow stem cells using retroviruses

IL-7R$^{-/-}$ mice (B6.129S7-IL7r^{tm1Im}) and Rag2$^{-/-}$ mice were purchased from The Jackson Laboratory and maintained by homozygous breeding at the National Cancer Institute-Frederick Cancer Research and Development Center animal facility. Cycling bone marrow stem cells were enriched, cultured, and infected as described above. On the fourth day of in vitro culture, 2×10^6 infected bone marrow cells were injected i.v. into Rag2$^{-/-}$ recipients previously given three Gy whole body irradiation. As a control for PCR and quantitative PCR (Q-PCR), 2×10^6 normal bone marrow cells from C57 mice were used to reconstitute Rag2$^{-/-}$ mice.

Four weeks after bone marrow reconstitution, mice were sacrificed and thymus and spleen cell suspensions were stained with PE-conjugated anti-CD4, CyChrome-conjugated anti-CD8, PE-conjugated anti-TCR-$\gamma\delta$, or CyChrome-conjugated anti-TCR-β Abs (BD Pharmingen). Stained cells were analyzed on a FACScan flow cytometry.

PCR analysis of rearrangement of TCRγ locus

Genomic DNA from thymus was extracted using DNeasy Tissue kit (Qiagen). The primers for detection of gene rearrangement were derived from

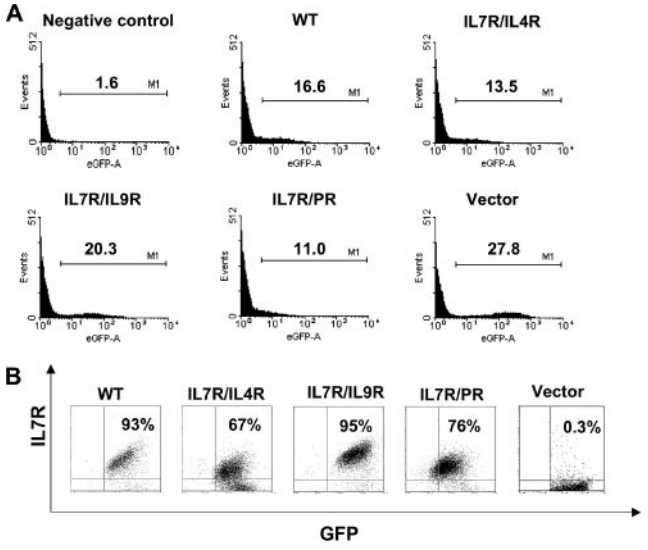

FIGURE 2. Retroviral transduction of chimeric IL-7Rs into bone marrow cells from IL-7R$\alpha^{-/-}$ mice. IL-7R$\alpha^{-/-}$ mice were treated with 5FU, then bone marrow cells were prepared and infected with retroviruses expressing different IL-7R chimeric constructs. *A*, Infection efficiency was monitored by GFP expression. Numbers indicate the percentage of GFP-positive cells. *B*, FACS analysis of mIL-7R expression on the cell surface of bone marrow cells transduced with different chimeric receptor and sorted for GFP expression.

sequences as published previously (24). The following oligonucleotides were used (the bp number in the parentheses indicates the precise position in the locus as reported in EMBL/GenBank/DDBJ under accession no. AF037352): No. 1, Vγ2 leader peptide sense (8280 bp): 5′-CTGGGAATTCAACCTGGCAGATG-3′; No. 2, Vγ2 sense (8460 bp): 5′-CATGGGAAGTTGGAGCAACCTGAAATATC-3′; No. 3, Vγ2 antisense (8734 bp): 5′-GCTTCGTCTTCTTCCTCCAAGGAATA-3′; and No. 9, Jγ1 antisense (33661 bp): 5′-CGGGATCCCAGAGGGAATTACTATGAGC-3′.

PCR were performed with a hot start: (94°C for 6 min) 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, for 30 cycles (extension, 7 min at 72°C). PCR products were separated on a 1.4% agarose gel.

Q-PCR analysis of rearrangement of TCRγ locus

Q-PCR analysis was performed using SYBR Green Chemistry (Qiagen) according to the manufacturer's instructions in 10-μl final volumes in 384-well microtiter plates. Thermocycling conditions using an Applied Biosystems ABI-7900 SDS were as follows: 95°C for 15 min and 40 cycles of 95°C for 15 s and 55°C for 1 min.

Quantification (percentage of expression) was achieved by normalizing a particular sample to a reference sample (normal bone marrow reconstitution thymus) and endogenous control (primer 2 and 3).

Western blot analysis of phospho-Stats

Chimeric receptor constructs were transfected into the phonenix-Eco packaging cell line, and supernatants were collected. BaF/3 cells were infected with different retroviral supernatants. One week later, GFP-positive cells were sorted by FACScan cytometry and cultured in the presence of mIL-3. BaF/3 cells were washed twice with PBS and put into fresh RPMI 1640 containing 10% FBS without IL-3 overnight. Cells were stimulated with mIL-7 for 20 min and were lysed for 15 min on ice in a 1% Triton X-100 buffer containing 62.5 mM Tris-HCl, 10% glycerol, and 50 mM DTT supplemented with protease inhibitor mixtures (Calbiochem). Extracts were cleared by centrifugation at $14,000 \times g$ for 10 min at 4°C. Triton X-100 lysates were resolved on a 12% PAGE gel (Invitrogen Life Technologies) by blotting with Abs against phospho-Stat5, phospho-Stat6, or β-actin (Cell Signaling Technology), followed by the appropriate secondary Abs conjugated to HRP (Cell Signaling Technology), and then visualized by a BM Chemiluminescence blotting system (Roche).

Intracellular staining of phospho-Stat5

Spleen cells were prepared from Rag2$^{-/-}$ mice that had been reconstituted 60 days earlier with IL-7R$^{-/-}$ bone marrow progenitors transfected with

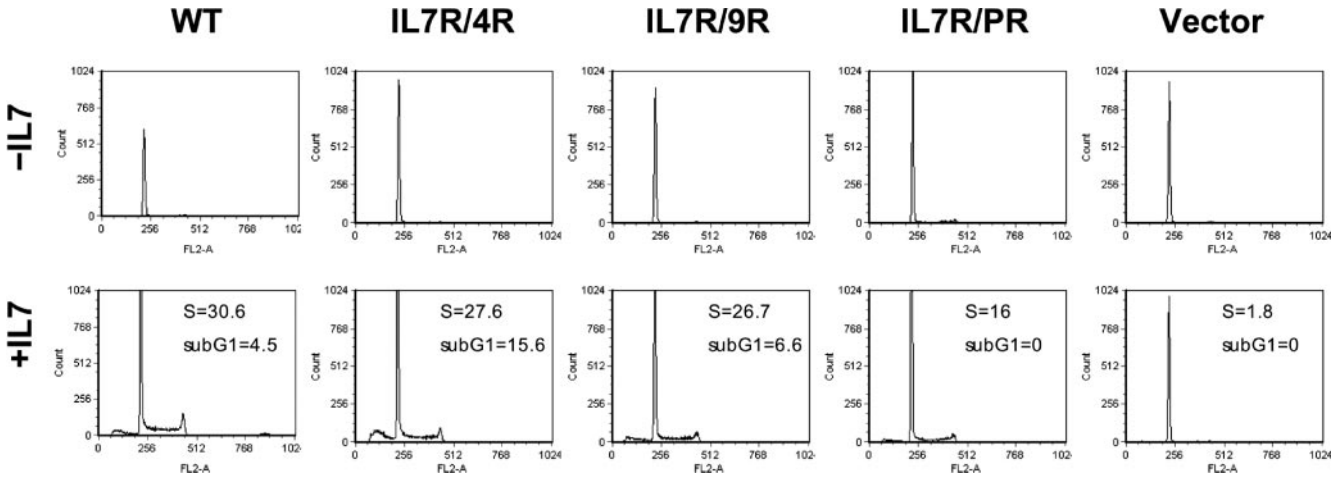

FIGURE 3. Chimeric IL-7Rs signal a response to mIL-7. IL-7R$\alpha^{-/-}$ mice were treated with 5FU, then bone marrow cells were prepared and infected with retroviruses expressing different IL-7R constructs. Infected cells were sorted for GFP expression, cultured with or without IL-7 for 48 h and analyzed by PI staining. When cultured without mIL-7, there were no cells in S phase or sub-G_1. Stimulation of cells with any of the chimeric receptors induced entry into S phase and some cells underwent apoptosis. This analysis was repeated for six mice per group in two separate experiments.

WT or IL-7R/IL9R. Red cells were lysed with a buffer of NH_4Cl (8.29 g/L), $KHCO_3$ (1 g/L), and EDTA (.04 g/L), and cells were stimulated 20 min with mIL-7 (25 ng/ml) then stained with anti-CD4 conjugated with Alexa 405 (Invitrogen Life Technologies) and washed twice. Cells were prepared for intracellular staining of phospho-Stat5 (25) by resuspending in 90 μl of permeabilization buffer (Caltag Laboratories; Reagent B), adding 10 μl of PE-conjugated anti-phospho-Stat5 (BD Pharmingen) or isotype control. Cells were incubated for 1 h in the dark, washed twice in PBS plus 5% FCS, and analyzed on a FACScan, gating on CD4 cells.

Results

To compare signaling from IL-7Rα to that of other related cytokine receptors, chimeric receptors were constructed. The extracellular regions of the IL-7Rα chain were coupled to the transmembrane and intracellular domains of other receptors as shown in Fig. 1. The IL-7Rα-chain docks and activates Stat5 as do IL-9Rα, PRLR, and IL-4Rα, and the latter also activates Stat6. In contrast, three of these receptors heterodimerize with γ_c (IL-4, -7, and -9), whereas the PRLR is a homodimer. All the receptors incorporate Jaks 1 and 3. Thus, the IL-9R complex resembles the IL-7 complex most closely in that both complexes incorporate γ_c and activate Stat5.

Following transduction of IL-7R$\alpha^{-/-}$ bone marrow cells, all the retroviral constructs expressed GFP, although to a varying extent (Fig. 2*A*). GFP level, in declining order, was as follows: vector, IL-9R, WT IL-7R, IL-4R, and PRLR. The vector may express the most GFP because of an advantage in ribosomal initiation compared with the other bicistronic messages. The surface expression of the receptors on transduced bone marrow cells (Fig. 2*B*) showed significant variation between chimeric constructs, with IL-9R and WT IL-7R being the highest, and IL-4R and PRLR being lower. Although the surface expression of IL-4R and PRLR was lower on average, the constructs conferred a functional response to IL-7. As shown in Fig. 3, the survival and proliferation of IL-7R$^{-/-}$ bone marrow cells was stimulated by IL-7 in all the transduced cell types, although PRLR was the least vigorous. This suggests that the intracellular domains and signaling pathways, diverse as they are, may be roughly equivalent in bone marrow cells in terms of inducing survival and proliferation. From these bone marrow progenitors, we have previously characterized the cell types generated by ectopic expression of WT IL-7R to be predominately of the myeloid lineage (26). The cell types that arose in culture from progenitors harboring chimeric receptors are shown in Table I. All the constructs responded to IL-7 stimulation by generating immature myeloid cells. The most striking distinction was the capacity of the IL-4R intracellular domain to generate mast cells. The IL-9R intracellular domain generated occasional megakaryocytes. The early signaling triggered by chimeric receptors in bone marrow progenitors was analyzed by observing the phosphorylation of Stats. As shown in Fig. 4, Stat5 phosphorylation was induced by the WT and IL-7R/IL-9R chimeric receptors, whereas the IL-7R/

Table I. *Cell types recovered after IL-7 stimulation of hemopoietic progenitors transfected with chimeric IL-7Rs*

		Cell Type (percentage)				
Chimericreceptor	IL-7	Primitive Myeloid	Differentiated Neutrophil	Macrophage	Megakaryocyte	Mast Cell
Vector	(−)	6	94	0	0	0
	+	10	90	0	0	0
WT	(−)	9	91	0	0	0
	+	32	68	0	0	0
IL-7/IL-9	(−)	8	92	0	0	0
	+	50	50	0	Occasional	0
IL-7/IL-4	(−)	6	94	0	0	0
	+	42	39	0	0	19
IL-7/PRL	(−)	5	94	1	0	0
	+	23	77	0	0	0

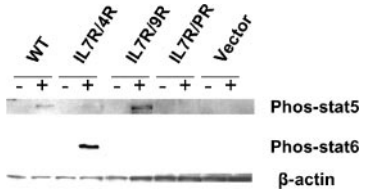

FIGURE 4. Stat5 and -6 phosphorylation induced by chimeric receptors in BaF/3 cells. BaF/3 cells were transduced with different chimeric receptor then deprived of mIL-3 overnight. Cells were stimulated with (+) or without (−) mIL-7 for 20 min. Triton X-100 lysates were analyzed by Western blotting with Abs against phospho-Stat5, phospho-Stat6, or β-actin. WT, IL-7R/4R, and IL-7R/9R activated Stat5 and only IL-7R/IL-4R activated Stat6. This analysis was repeated in two separate experiments for each construct.

IL-4R chimeric receptor induced both Stat5 and Stat6 phosphorylation. The IL-7R/PRLR chimeric receptor did not induce detectable Stat5 phosphorylation, although it triggered a survival and proliferative response in myeloid progenitors as shown in Fig. 3.

We then examined the ability of the intracellular domains from these different receptors to support development of thymocytes, a process that normally requires signals from the IL-7R during the DN stage. As shown in Fig. 5*A*, all the chimeric receptors induced a varying degree of development to the double-positive thymocyte stage compared with the vector control. The magnitude of this reconstitution differed substantially as shown in Fig. 5, *B* and *C*, showing that the IL-9R intracellular domain induced significant thymopoiesis ranging from 10 to 70% of the magnitude of the IL-7R intracellular domain. The IL-4R intracellular domain functioned efficiently in just one of six mice, whereas the PRLR intracellular domain did not support significant levels of development. These results show that the types of signals required to transit the DN stage must not be uniquely inducible by the IL-7R intracellular domain. For example, cell survival may be the primary function of IL-7R at the DN stage, and all of these receptors have been shown to promote survival in cells that normally express them. At the later stage of CD8 development, which is dependent on IL-7 (11), there was no apparent inadequacy of the IL-9Rα signals to support CD8 cells.

Development of the $\gamma\delta$ lineage of T cells requires signals from the IL-7R. However, WT IL-7Rα did not reconstitute robust $\gamma\delta$ development in the thymus as we reported previously (17), perhaps because most $\gamma\delta$ cells develop early in life whereas our studies used adult progenitors and adult recipients. In this study, we also found very few $\gamma\delta$ T cells in the thymus after WT IL-7Rα reconstitution (data not shown). Another measure of the IL-7 response is rearrangement the TCRγ locus, which is required for development of $\gamma\delta$ cells. We previously showed that rearrangement of the γ locus is dependent on signals from IL-7R, and this, at least in part, accounts for the block in $\gamma\delta$ development in the absence of IL-7R signals. Because rearrangement of the TCRγ locus also occurs in at least half of $\alpha\beta$ T cells (27), its detection does not require the development of a large population of $\gamma\delta$ T cells, and this rearrangement is therefore more readily detectable in IL-7R-reconstituted mice than $\gamma\delta$ cells themselves. Using the PCR shown in Fig. 6*A*, rearrangement was restored by WT IL-7R but was undetectable in mice reconstituted with other receptors including IL-9R, which gave robust $\alpha\beta$ T cell development (Fig. 6*B*). Q-PCR data in Fig. 6*C* for TCRγ rearrangement confirmed that IL-9R was much less functional in reconstitution of $\gamma\delta$ cells compared with WT IL-7R. Thus, the IL-7R intracellular domain may be uniquely effective for inducing rearrangement of the TCRγ locus.

IL-7 is required for homeostatic survival of T cells after they leave the thymus. The number of splenic T cells is shown in Fig. 7*A*. These data with peripheral T cells, like the thymus, indicate that IL-9R was quite effective and even exceeded the effect of WT IL-7R for $\alpha\beta$ T cells. This result suggests that the homeostatic requirement for IL-7, like the development of $\alpha\beta$ T cells, does not require signals unique to IL-7R. $\gamma\delta$ T cells in contrast were virtually undetectable in the spleens of IL-9R-reconstituted mice, whereas $\gamma\delta$ T cells were apparent in spleens of WT IL-7R-reconstituted mice (Fig. 7*B*). Thus, other receptors could not replace IL-7R to induce rearrangement of the TCRγ locus and repopulation of $\gamma\delta$ cells in thymus and spleen. The signaling function of the

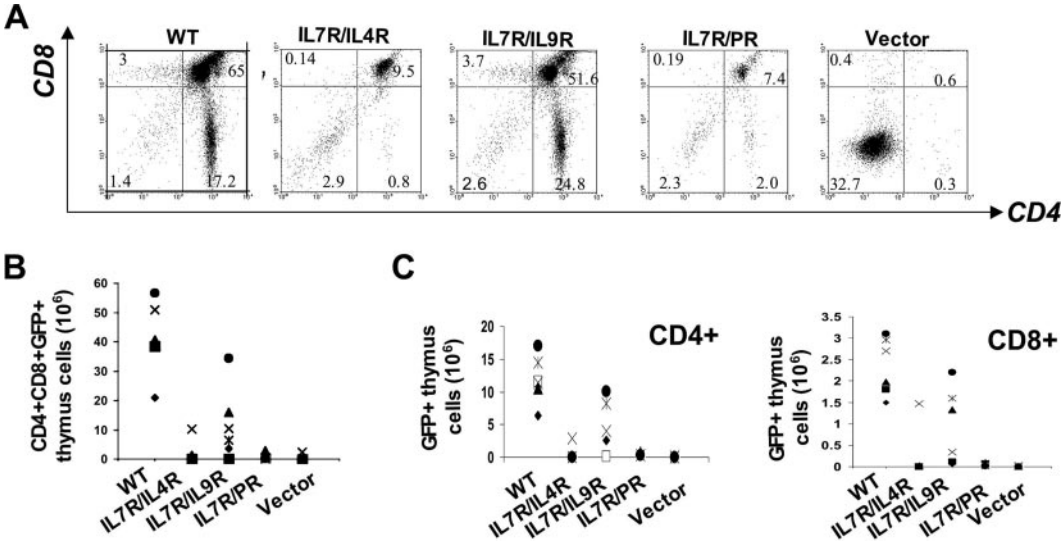

FIGURE 5. Reconstitution of T cell development in the thymus. IL-7R$\alpha^{-/-}$ mice were treated with 5FU, then bone marrow cells were prepared, infected with retroviruses expressing different IL-7R constructs, and injected into Rag2$^{-/-}$ mice. Four weeks later thymocytes were analyzed. *A*, Thymocytes were stained for CD4 and CD8 and gated on GFP-positive cells. Numbers indicated the percentage of total thymocytes. Samples from representative individual mice are shown. *B*, Recovery of CD4$^+$CD8$^+$ cells in thymus. Data are from three experiments of six individual mice. Some dots overlap, especially on the axis. *C*, Recovery of single-positive cells in thymus. Data are from three experiments of six individual mice. Some dots overlap, especially on the axis.

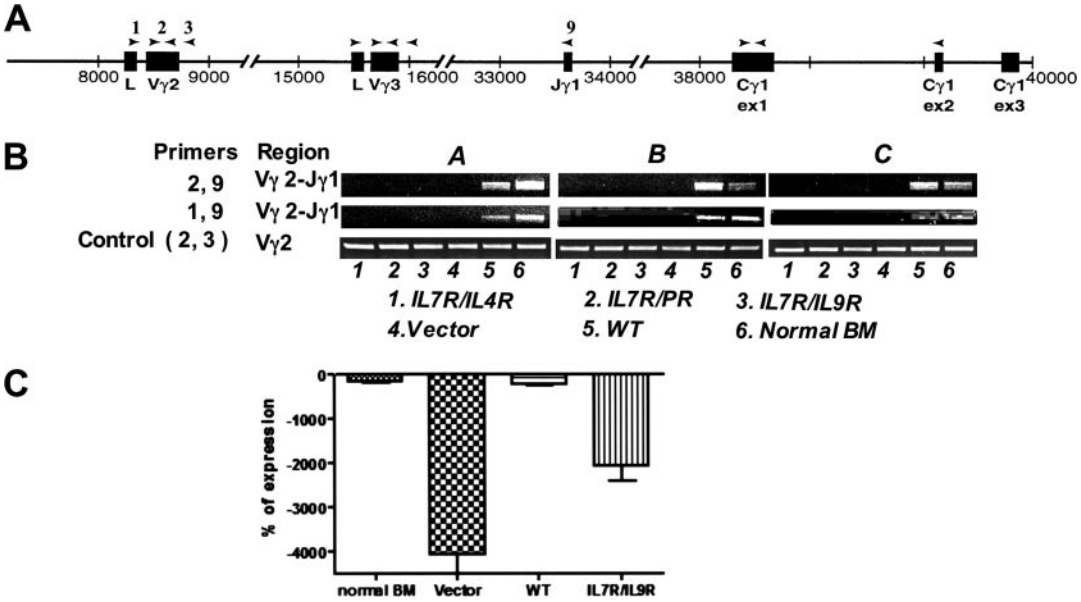

FIGURE 6. Rearrangement of the *TCRγ* locus in thymocytes. *A*, Diagram of the *TCRγ* locus and the primers (1, 2, 3, 9) used to amplify the gene fragments. *B*, PCR analysis for rearrangement of the *TCRγ* locus in IL-7Rα$^{-/-}$ thymocytes reconstituted with chimeric IL-7Rs. Samples of individual mice from three experiments (*A–C*) are shown. *C*, Q-PCR detection of *TCRγ* rearrangement using primer 1 and primer 9. Quantification (percentage of expression) was achieved by normalizing a particular sample to a reference sample (normal bone marrow reconstitution thymus) and endogenous control (primer 2 and 3).

IL-7R/IL-9R chimeric receptor in peripheral CD4 cells was verified by analyzing phosphorylation of Stat5. As shown in Fig. 8, the IL-7R/IL-9R chimeric receptor was about as effective in signaling as WT receptor in these cells.

Discussion

IL-7 is uniquely required for thymocyte development and peripheral T cell survival and proliferation. We examined whether this requirement is due to unique signaling from the IL-7R. The intracellular domain of IL-7R was exchanged with domains from related cytokine receptors, introduced into IL-7R$^{-/-}$ progenitors, and tested for reconstitution of T cell development. The IL-9R intracellular domain effectively replaced that of IL-7R in reconstituting αβ T cell development and peripheral survival. IL-4R or PRLR also restored a low level of αβ T cell development but far below the magnitude of cellularity imparted by IL-7R. Thus, IL-7R signals are not uniquely required in the αβ T cell lineage. In contrast, rearrangement of the TCRγ locus and development of the γδ lineage also require IL-7, and IL-9R failed to provide this IL-7R function.

IL-7Rα-chain is a member of a family of cytokine receptors that include receptors for IL-2, -4, -9, -15, and -21, all of which form heterodimers with γ_c and can induce T cell proliferation. From this family of intracellular domains to potentially swap with IL-7R, we chose IL-4R because its signaling largely depends on a different Stat (Stat6) and IL-9R because it activated the same Stat (Stat5) as well as Stats 1 and 3. We did not test IL-2R or IL-15R because these have three chains. We chose the PRLR because, like IL-7R, it activates Stat5; however, it does not pair with γ_c but rather forms a homodimer, and because it is primarily required in nonlymphoid cells.

The IL-4R and PRLR gave very weak responses, whereas the IL-9R intracellular domain substantially replaced IL-7R. The negative IL-4R and PRLR responses may reflect a true difference in second messenger pathways. However, we cannot rule out low

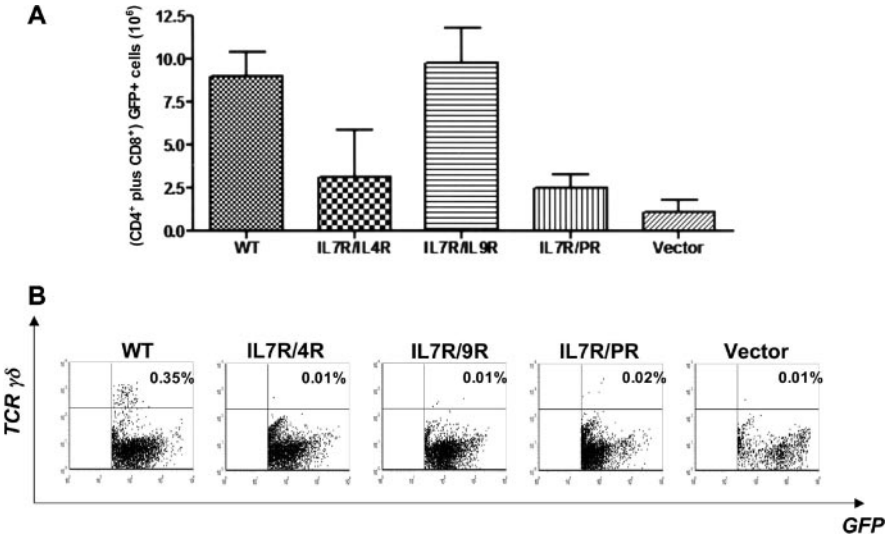

FIGURE 7. Reconstitution of splenic T cells. IL-7Rα$^{-/-}$ bone marrow cells were reconstituted with chimeric IL-7Rs and injected into Rag2$^{-/-}$ mice. Four weeks later spleen cells were analyzed by flow cytometry. *A*, Splenic CD4$^+$ and CD8$^+$ were quantified, gating on GFP. Data are from three experiments of six individual mice. *B*, Spleen cells were stained for γδ TCR, gating on GFP. Representative samples from individual mice are shown from three experiments of six mice per group.

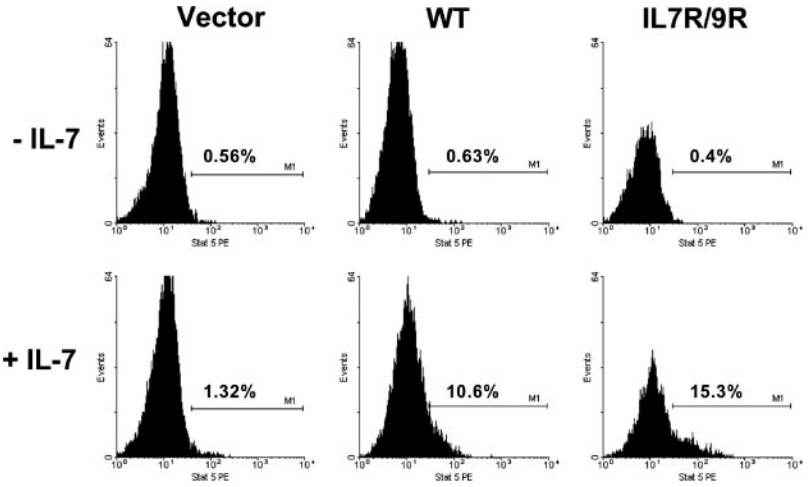

FIGURE 8. Stat5 phosphorylation in peripheral CD4 T cells: IL-7R vs IL-9R intracellular domains. Spleen cells were prepared from $Rag2^{-/-}$ mice that received IL-$7R^{-/-}$ bone marrow progenitors reconstituted with WT or IL-7R/IL-9R chimeric receptors. Cells were stimulated 20 min with IL-7, then surface stained with anti-CD4 and intracellular stained with anti-phospho-Stat5. Analysis was gated on $CD4^+$ cells and shows a similar degree of Stat5 phosphorylation comparing WT with IL-7R/IL-9R groups. Spleen cells were pooled from three mice per group, and the experiment was repeated three times.

expression or misfolding in thymocytes despite their ability to function in transduced bone marrow cells (Fig. 3). For this reason, the following discussion will focus on the robust response to the IL-9R because this clearly shows that the IL-7R does not deliver unique signals in $\alpha\beta$ T cell development.

IL-9 knockout mice have no thymic deficiency (28), whereas IL-7 (1) or IL-7R (2) knockout mice are markedly deficient. Thus, although we show that the IL-9R intracellular domain can mimic IL-7R in $\alpha\beta$ T cell development, the IL-9 pathway apparently is not operational in normal conditions, presumably for lack of ligand and/or receptor expression. There is a report that exogenously added IL-9 can promote human thymic development in vitro (29), which might suggest that the receptor is present but the ligand is unavailable. Whereas IL-7 is produced by thymic stromal cells, IL-9 is primarily produced by activated T cells that would make it generally unavailable to thymic progenitors. However, the knockouts of IL-7 or IL-7R are somewhat "leaky" in that most mice show small, variable but detectable thymopoiesis compared with an absolute block in, for example, Rag knockout mice. Because γ_c serves both IL-7R and IL-9R and the knockout of γ_c is somewhat more severe than in IL-7/IL-7R knockouts, the latter may develop some thymocytes via the IL-9 pathway. A combined IL-7/IL-9 knockout would therefore be of interest.

The cellular responses of lymphocytes to stimulation through IL-9R are similar to those for IL-7R, both of which can induce survival, proliferation, and lymphomagenesis. Thus, IL-9 was shown to induce potent antiapoptotic signals in murine thymic lymphomas (30). IL-9 induced proliferation in T cell lines (31) and thymic lymphomas (32). Overexpression of IL-9 as a transgene induced thymic lymphomas (33), although the phenotype of the lymphomas was not the IL-7-dependent DN stage but rather the later, double-positive stage that is no longer IL-7 dependent.

The survival effect of IL-7 has been shown by us and others to primarily involve a balance of Bcl-2 family members. Thus, IL-7 induces synthesis of Bcl-2 (4) and Mcl-1 (5) and represses post-translational activation of the death proteins Bax (8), Bad (34), and Bim (9). The proliferative activity of IL-7 is through posttranslational activation of Cdc25a (15) and $p27^{Kip1}$ (Refs. 16 and 35), which regulate Cdk2. Many of these effects have been shown to also be induced by IL-3 in lymphocyte cell lines, and they may be controlled by other receptors in lymphocytes, such as IL-9R.

IL-7 has been reported to activate several second messenger pathways (20) of which a key signal is Stat5, which docks to pY449 in the intracellular domain (18, 20). IL-9 signaling also appears to be mainly via Stat5, which docks to pY407 in the intracellular domain, although the same tyrosine residue is also part of a motif that docks Stats 1 and 3 (36). The PI3K pathway is activated by IL-7R (19), whereas IL-9R activates this pathway in some cell lines but not others (37).

Negative regulation of signaling may also differ among otherwise related receptors. For example, activated receptors can differentially induce production of suppressor of cytokine signaling (SOCS) proteins, which in turn differentially suppress receptor signaling: SOCS-1 suppresses IL-7R signaling (38), whereas SOCS-3 suppresses IL-9R signaling (39). Activation of phosphatases has been shown to differ between receptors and affect the duration of Stat5 signaling: Epo receptor signaling is more phosphatase inhibited than IL-2R or IL-9R (40). Differential ubiquitin-coupling to activated receptors could also affect the duration of their signaling: IL-9Rα, IL-2Rβ, Epo receptor (41), and PRLR (42) are ubiquitinated and degraded following activation, attenuating their signaling.

Our results in this study show that most IL-7R signals are not unique in that they can be mimicked by IL-9R. Conversely, we recently showed that IL-7R, which normally functions in the lymphocyte lineage, can also induce myelopoiesis when ectopically expressed in the myeloid lineage (26), perhaps mimicking a myeloid factor such as G-CSF. In this study, we also observed a myelopoietic effect (data not shown). Thus, in Fig. 6, we showed the number of splenic lymphocytes reconstituted by these receptors, but other data not shown in the figure indicated that the number of splenic neutrophils actually exceeded the number of lymphocytes in both WT IL-7R and IL-9R groups; thus, the IL-9R intracellular domain, like that of IL-7R, can also induce myelopoiesis.

The IL-9Rα intracellular domain failed to reconstitute rearrangement of the TCRγ locus in IL-7R$\alpha^{-/-}$ progenitors, despite its robust activity in supporting $\alpha\beta$ development and peripheral homeostasis and the fact that TCRγ locus is normally rearranged in many $\alpha\beta$ T cells. IL-7R has been shown to induce chromatin remodeling of the TCRγ locus, rendering it accessible to the recombinase complex resulting in gene rearrangement (10). Stat5 has been implicated in this process, having been shown to restore histone acetylation and locus accessibility in IL-$7R^{-/-}$ thymocytes (43), and we showed that the Stat5 docking site, Y449, is required for TCRγ locus rearrangement (17). We have attempted to reconstitute T cell development in IL-$7R^{-/-}$ progenitors with a retrovirus expressing active Stat5, and this process has not been successful as yet (Q. Jiang and S. K. Durum, unpublished data). There is new evidence that Stat5 is required for TCRγ rearrangement

(22), yet Stat5 is activated by both IL-7R and IL-9R. There are several potential mechanisms to account for the failure of IL-9R to induce TCRγ locus rearrangement. 1) There could be a unique signal emanating from Y449 or another site on IL-7R, perhaps working together with Stat5. 2) The nature of the Stat5 multimers could differ; for example, there are potentially different mixtures of Stat5a and -b dimers or tetramers (44). 3) There could be a quantitative difference; perhaps IL-7R is a stronger Stat5 activator in thymocytes because it also induces more cellularity than IL-9R. A more detailed functional comparison of the IL-7R vs the IL-9R intracellular domains may distinguish these hypotheses.

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Disclosures

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