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Suppression of IL7Rα Transcription by IL-7 and Other Prosurvival Cytokines: A Novel Mechanism for Maximizing IL-7-Dependent T Cell Survival

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Summary

Survival of naive T cells is dependent upon IL-7, which is present in vivo in limiting amounts with the result that naive T cells must compete for IL-7-mediated survival signals. It would seem imperative during T cell homeostasis that limiting IL-7 be shared by the greatest possible number of T cells. We now describe a novel regulatory mechanism that specifically suppresses IL7Rα transcription in response to IL-7 and other prosurvival cytokines (IL-2, IL-4, IL-6, and IL-15). Consequently, IL7R expression is reduced on T cells that have received cytokine-mediated survival signals so they do not compete with unsignaled T cells for remaining IL-7. Interestingly, cytokine-mediated suppression of IL7Rα transcription involves different molecular mechanisms in $CD4^+$ and $CD8^+$ T cells, as $CD8^+$ T cells utilize the transcriptional repressor GFI1 while $CD4^+$ T cells do not. We suggest that this homeostatic regulatory mechanism promotes survival of the maximum possible number of T cells for the amount of IL-7 available.

Introduction

Effective immune protection requires a diverse T cell antigen receptor repertoire to insure the presence of T cells reactive against potential invaders. αβT cells, each bearing a unique antigen receptor, are generated in the thymus and exported to the periphery where they must survive for future encounters with antigen (Goldrath and Bevan, 1999). Survival of naive T cells in the periphery is referred to as T cell homeostasis (Jameson, 2002; Marrack et al., 2000) and is primarily mediated by interleukin-7 (IL-7) (Fry and Mackall, 2001; Khaled and Durum, 2002; Schluns et al., 2000; Tan et al., 2001), a 25 kDa glycoprotein that is produced by stromal cells, monocytes, and some epithelial cells (Hofmeister et al., 1999). IL-7 is now understood to be the major component that limits T cell "space" in vivo by limiting the number of T cells that can be maintained in the periphery (Seddon and Zamoyska, 2002; Stockinger et al., 2004). Because IL-7 is limiting relative to the number of T cells present in the periphery, naive T cells must continually compete with one another for IL-7-induced survival signals, with unsignaled T cells doomed to die (Geiselhart et al., 2001; Maraskovsky et al., 1996; Seddon and Zamoyska, 2002). It has been suggested that lymphocyte competition for a single survival source inevitably results in diminished clonal diversity, a problem that has been referred to as the competition/diversity paradox (De Boer and Perelson, 1994; Freitas and Rocha, 2000). Thus, it is important for effective T cell immunity that limiting IL-7 be shared by the greatest possible number of naive T cells, but homeostatic mechanisms that would do so have not yet been described.

To understand how IL-7 might be shared by the greatest possible number of peripheral T cells, we have examined the effects of IL-7 and other cytokines on IL-7 receptor (IL7R) expression in resting T cells. The IL7R is a heterodimeric complex composed of an IL-7 specific α chain (IL7Rα) and the common cytokine γ chain (γc), which is shared by receptors specific for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Ozaki and Leonard, 2002). Signaling by γc-dependent cytokines generally upregulates surface expression of their cytokine receptor (Depper et al., 1985). However, we reasoned that this might not be the case for IL7R, as upregulation of IL7R by IL-7 would severely constrain IL-7 availability, as T cells that had already received IL-7-mediated survival signals would then express higher IL7R levels than unsignaled T cells and would outcompete unsignaled T cells for any remaining IL-7, exacerbating clonal loss. Consequently, we considered that IL-7 might signal naive T cells to reduce, rather than increase, IL7R expression.

The present study identifies a novel regulatory mechanism that downregulates IL7R expression by suppressing IL7Rα transcription in resting $CD4^+$ and $CD8^+$ T cells in response to IL-7 and other prosurvival cytokines. Interestingly, $CD4^+$ and $CD8^+$ T cells differ in the precise molecular mechanism by which prosurvival cytokines suppress IL7Rα transcription, as $CD8^+$ T cells utilize and require the transcriptional repressor Growth Factor Independence-1 (GFI1) whereas $CD4^+$ T cells do not. We suggest that cytokine induced suppression of IL7Rα expression is an important homeostatic mechanism for maximizing IL-7 availability to naive T cells during T cell homeostasis and antigen driven clonal expansion, so that T cells that have already received survival signals do not outcompete unsignaled T cells for remaining IL-7.

Results

Suppression of IL7Rα Expression by Its Cognate Cytokine, IL-7

IL7R expression on peripheral T cells is somewhat heterogeneous (Figure 1A, left). To determine if IL7R expression is affected by the cytokine-rich in vivo environment, lymph node T cells (LNT) were placed in overnight (O/N) culture with medium and assessed for expression of IL7Rα and IL4Rα, two proteins that associate with surface γc chains to form functional cytokine receptors

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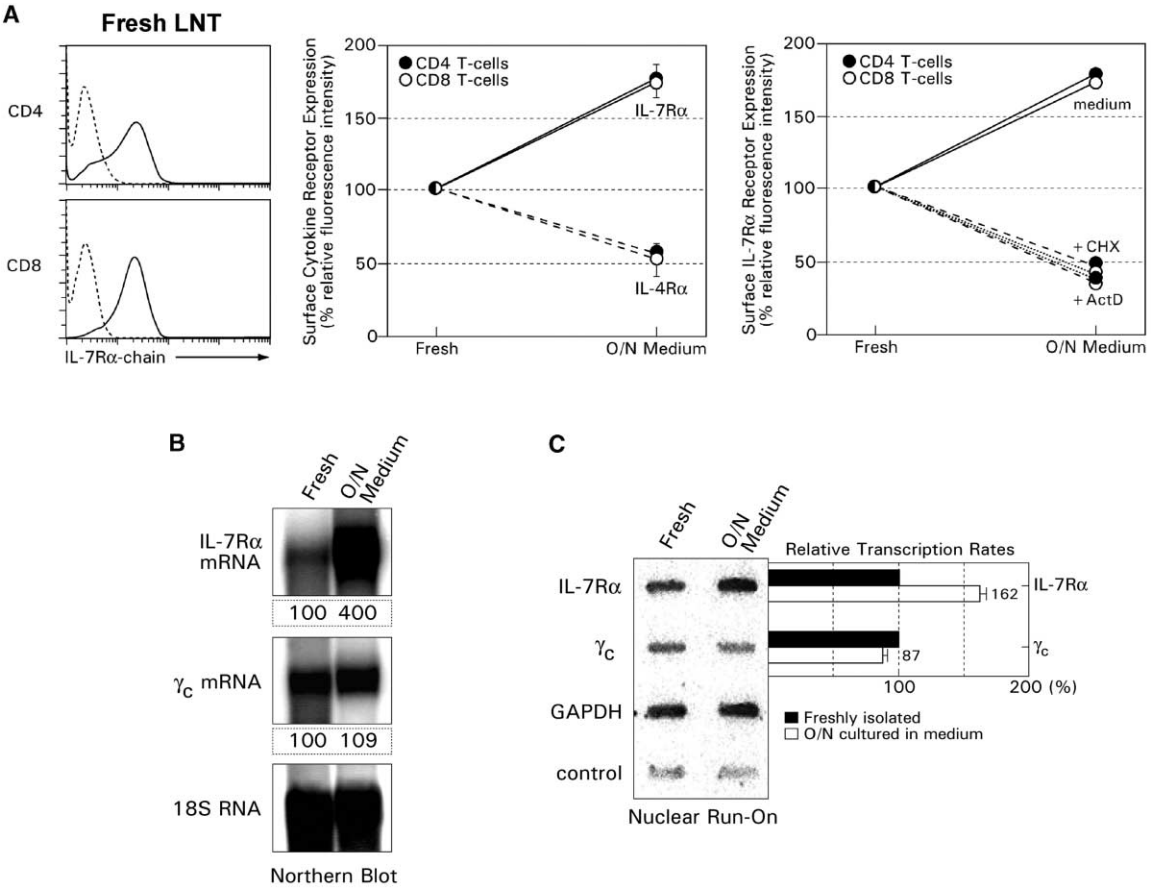

Figure 1. IL7 Receptor Expression In Vivo and In Vitro

(A) IL7Rα is expressed on fresh LNT (left). To assess the effect on cytokine receptor expression of removing T cells from their cytokine-rich in vivo environment, LNT were cultured O/N in medium (middle). T cell survival in O/N cultures averaged 92% ± 1.4%. Expression of IL4Rα (dashed line) and IL7Rα (solid line) on O/N cultured T cells was normalized to that on the same T cell subpopulations prior to culture, which was set to 100% (middle). Upregulation of surface IL7Rα expression on LNT during in vitro culture was inhibited by ActD (dotted line) and CHX (dashed line) (right).

(B) Effect of O/N culture on IL7Rα mRNA expression. Northern blots of total RNA from freshly isolated or O/N cultured T cells were probed for IL7Rα, γc, and 18S RNA. Single blots were sequentially stripped and hybridized with the indicated probes. Signal intensities of IL7Rα and γc mRNA bands were determined by a PhosphorImager and were normalized to band intensities from fresh T cells, which were set at 100.

(C) Effect of O/N culture on IL7Rα transcription. Nuclei from fresh or O/N cultured T cells were assessed by nuclear run-on assays for transcription of specific RNA's. Newly synthesized RNA's for the indicated genes were hybridized to corresponding probes and quantitated by a PhosphorImager. Signal intensities were calculated relative to GAPDH, and transcription rates of each gene was normalized to that in fresh T cells, which was set at 100%.

(Olosz and Malek, 2002; Ozaki and Leonard, 2002). Removal of T cells from their in vivo environment markedly reduced surface IL4Rα expression on both $CD4^+$ and $CD8^+$ T cells (Figure 1A, middle), indicating that IL4Rα surface expression may be actively upregulated by in vivo cytokines. Unlike its effect on IL4Rα, removal of T cells from their in vivo environment markedly increased surface IL7Rα expression (Figure 1A, middle), an increase that was inhibited by actinomycin D (ActD) and cycloheximide (CHX) and so required new transcription and new protein synthesis (Figure 1A, right). In fact, ActD and CHX reduced IL7Rα expression on T cells to ~45% of initial levels (Figure 1A, right), demonstrating that even the in vivo level of IL7Rα on T cells was highly dependent on continual transcription and protein synthesis. Molecularly, T cells removed from their in vivo environment increased both IL7Rα mRNA levels (Figure 1B) and IL7Rα transcription rates (Figure 1C), increases that were specific for IL-7Rα as γc mRNA and γc transcription remained unchanged. Thus, IL7Rα expression on both $CD4^+$ and $CD8^+$ T cells quantitatively reflected IL7Rα transcription, which increased upon removal of T cells from their in vivo environment.

We considered that IL-7 might be the factor suppressing IL7Rα transcription in vivo as naive T cells must be signaled by IL-7 to survive. To examine the effect of IL-7 on IL7Rα expression, LNT were placed in O/N culture to allow them to fully upregulate IL7Rα surface levels and then were exposed for 6 hr to either IL-7 or IL-4 (Figure 2). IL-4 upregulated surface expression of IL4Rα (Figure 2A), as expected. In contrast, IL-7 dramatically reduced surface IL7Rα expression (Figure 2A). The inhibitory effect of IL-7 on IL7Rα expression was not limited to IL7Rα protein, as IL7Rα mRNA levels were also drastically reduced (Figure 2B). The inhibitory effect of IL-7 on IL7Rα mRNA was specific, as bcl-2 mRNA

levels increased and γc mRNA levels were unchanged (Figure 2B). To determine the duration of IL-7's inhibitory effect on IL7Rα gene expression, T cells that had been incubated O/N with IL-7 were allowed to recover in medium for various times and then assessed for IL7Rα mRNA content (Figure 2C). IL7Rα mRNA levels increased within 3 hr and returned to undepleted levels by 6–12 hr after release from IL-7 signaling (Figure 2C).

Most remarkably, IL-7 treatment specifically reduced IL7Rα transcription (Figure 2D). Purified $CD4^+$ and $CD8^+$ T cells were placed in O/N culture to recover from in vivo suppression, treated for 6 hr with IL-7, and then assessed for transcription of various genes. IL-7 treatment reduced IL7Rα transcription, even as it increased bcl-2 transcription and left γc transcription essentially unchanged (Figure 2D). These results demonstrate that IL-7 signaling reduces IL7Rα surface protein expression, decreases IL7Rα mRNA content, and significantly suppresses IL7Rα transcription in both $CD4^+$ and $CD8^+$ T cells.

To determine if endogenous IL-7 had a similarly suppressive effect in vivo, we performed two different experiments. In the first experiment, we injected neutralizing anti-IL-7 mAb (M25) into normal B6 mice and found that it upregulated IL7Rα expression on LNT (Figure 2E), demonstrating that endogenous IL-7 actively suppresses IL7Rα expression on in vivo T cells. In the second experiment, B6 LNT were adoptively transferred into either IL-7 replete $RAG^{-/-}$ or IL-7-deficient $RAG^{-/-}$ $IL\text{-}7^{-/-}$ host mice, which were identically devoid of lymphocytes (Figure 2F). Examination of these mice 16h after transfer (which was too early for donor T cells to have begun proliferating [Moses et al., 2003]) revealed that T cells transferred into $RAG^{-/-}$ hosts expressed significantly lower IL7Rα surface levels than prior to transfer, whereas B6 T cells transferred into $RAG^{-/-}$ $IL\text{-}7^{-/-}$ hosts expressed significantly higher IL7Rα surface levels than prior to transfer (Figure 2F). In fact, B6 LNT in IL-7-deficient hosts expressed >2.5-fold more surface IL7Rα than did the same B6 T cells in IL-7 replete hosts (Figure 2F). Thus, these in vivo experiments precisely paralleled our in vitro experiments and further document that endogenous IL-7 suppresses IL7R expression on in vivo T cells. That surface IL7Rα expression on B6 T cells was reduced from initial levels by transfer into IL-7 replete $RAG^{-/-}$ hosts indicated that greater amounts of IL-7 were available in $RAG^{-/-}$ host mice than in donor B6 mice, presumably because $RAG^{-/-}$ host mice lacked endogenous $IL7R^+$ T cells that bound and sequestered much of the in vivo IL-7.

One mechanism by which IL-7 might suppress IL7Rα transcription would be to upregulate transcriptional repressor proteins. In this case, IL-7's suppression of IL7Rα transcription might require new protein synthesis and would be abrogated by inhibitors of protein synthesis, such as CHX. To assess whether CHX affected IL-7's suppression of IL7Rα transcription, T cells that had recovered from in vivo suppression by O/N culture were treated for 6 hr with either CHX + IL-7 or CHX + medium (Figures 3A–3C). Six hour treatment with CHX by itself reduced IL7Rα surface levels (Figure 3A, compare lanes 1 and 3 to 5 and 7). More importantly, CHX-treated T cells were resistant to IL-7-induced downregulation of IL7Rα surface expression (Figure 3A) and were resistant to IL-7 induced reductions in IL7Rα mRNA content (Figure 3B). Notably, CHX treatment did not interfere with IL-7 signaling since IL-7 still upregulated bcl-2 mRNA transcripts in the very same CHX-treated T cells (Figure 3B). We then assessed the effect of CHX treatment on IL-7-signaled downregulation of IL7Rα transcription (Figure 3C). In fact, IL-7 failed to downregulate IL7Rα transcription in CHX-treated cells, even though it downregulated IL7Rα transcription in the same cell population treated with medium (Figure 3C, compare top and bottom panels). It is important to emphasize that CHX treatment did not interfere with IL7Rα transcription, which was equivalent in CHX- and medium-treated T cells (Figure 3C, compare top and bottom), but it specifically blocked IL-7's suppression of IL7Rα transcription. Notably, even as CHX blocked IL-7's suppression of IL7Rα transcription, CHX did not interfere with IL-7's induction of bcl-2 transcription (Figure 3C, compare top and bottom). Thus, suppression of IL7Rα transcription by IL-7 requires protein synthesis, suggesting involvement of newly synthesized transcriptional repressor proteins.

In addition to suppressing IL7Rα transcription, we considered that IL-7 might also destabilize preexisting IL7Rα mRNA transcripts, as has recently been reported in activated T cells for IL-2 (Xue et al., 2002). Consequently, we treated T cells after O/N culture with ActD to block synthesis of new transcripts and performed a kinetic analysis to determine the half-life of preexisting IL7Rα mRNA transcripts in both unsignaled and IL-7-signaled T cells (Supplemental Figure S1). In unsignaled T cells the half-life of IL7Rα mRNA transcripts was 1.2 ± 0.1 hr and was essentially identical to that in IL-7-signaled T cells even when T cells were pretreated with IL-7 for 2.5 hr (prior to addition of ActD) to allow IL-7 sufficient time to potentially induce destabilizing ribonucleases (Supplemental Figure S1). We conclude that IL-7 signals do not measurably affect IL7Rα mRNA stability and that IL-7's inhibition of IL7Rα gene expression is predominantly the result of IL-7's suppression of IL7Rα transcription.

Induction of IL4Rα Gene Expression by IL-7

We next wished to determine if IL-7 also suppressed other cytokine receptor genes such as IL4Rα (Figure 4). To examine this question, LNT were rested in O/N culture, treated with IL-7 for 6 hr, and then assessed for expression of IL4Rα and IL7Rα. Northern blot analysis of LNT revealed that IL-7 markedly upregulated IL4Rα mRNA in the same cells in which it downregulated IL7Rα mRNA (Figure 4A). In fact, IL-7 upregulated IL4Rα expression in both $CD4^+$ and $CD8^+$ T cells as determined by surface protein staining and Northern blot analyses of IL4Rα mRNA (Figures 4B and 4C). IL-7's upregulation of IL4Rα mRNA was quantitatively less than that induced by the cognate cytokine IL-4 even though both cytokines were equally suppressive of IL7Rα mRNA (Figure 4C). We conclude that IL-7 specifically suppresses IL7Rα expression even as it upregulates IL4Rα expression.

Cytokine Crosstalk and *trans* Suppression of IL7Rα Expression by Prosurvival Cytokines

To determine if IL7Rα expression could be suppressed by cytokines other than IL-7, we cultured T cells O/N with various cytokines and assessed their effect on surface expression of IL7Rα (Figure 5A). Interestingly, IL-7 was

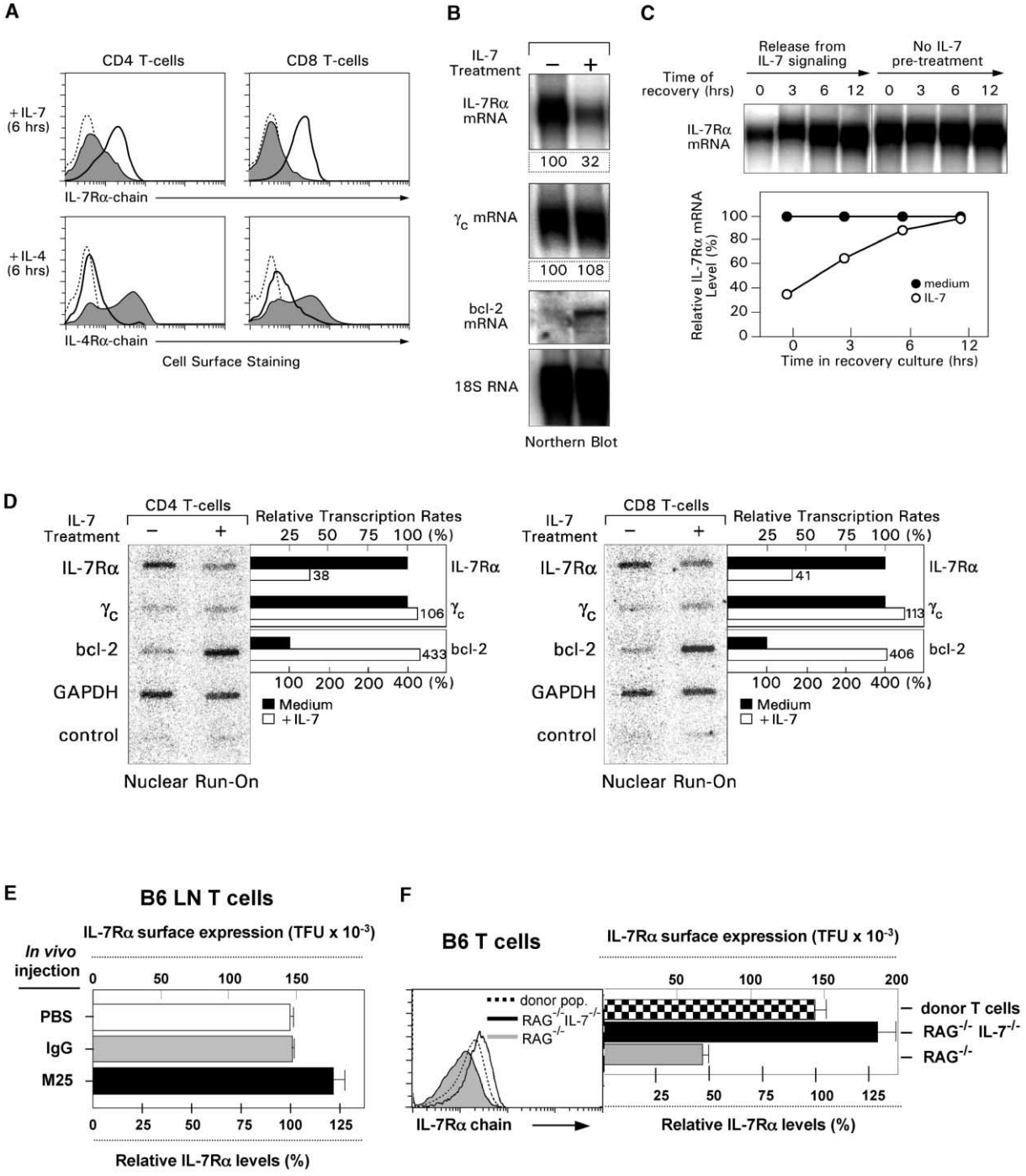

Figure 2. IL-7 Downregulates IL7Rα Expression and IL7Rα Transcription

(A) Effect of cytokines on surface expression of their cognate receptor. LNT were rested O/N in medium cultures and then treated for 6 hr with either IL-7 or IL-4. Histograms show IL7Rα and IL4Rα expression on $CD4^+$ and $CD8^+$ T cells after 6 hr treatment with cytokine (filled curve) or medium (open curve). Solid line = cytokine receptor staining; dotted line = control staining.

(B) IL-7 induces a rapid loss of IL7Rα mRNA. LNT were rested O/N in medium and then treated for 6 hr with either IL-7 or medium. Northern blots of total RNA were probed for IL7Rα, γc, bcl-2, and 18S RNA. Single blots were sequentially stripped and hybridized with the indicated probes. Intensities of IL7Rα and γc RNA bands were normalized to corresponding band intensities from cultured T cells exposed only to medium, which were set at 100.

(C) IL7Rα mRNA reexpression after release from IL-7 signaling. LNT were cultured O/N in either IL-7 or medium. The next day, cells were washed and cultured for 12 hr in only medium and their content of IL7Rα mRNA determined in Northern blots. The line graph displays IL7Rα mRNA content of LNT that had been initially cultured in IL-7 relative to that of LNT that had only been cultured in medium, which was set at 100.

(D) IL-7 downregulates IL7Rα transcription. Purified $CD4^+$ or $CD8^+$ T cells were rested O/N in medium and then treated for 6 hr with either IL-7 or medium. Nuclei were isolated and assessed by nuclear run-on assays for transcription of specific RNA's. Signal intensities were calculated relative to GAPDH, and transcription rates of each gene in IL-7 signaled T cells were normalized to that in medium cultured T cells, which was set at 100%.

not unique in suppressing IL7Rα expression, as IL7Rα surface expression was also reduced by IL-2, IL-4, IL-6, and IL-15. Note that IL-4, IL-6, and IL-7 suppressed IL7Rα expression equally on $CD4^+$ and $CD8^+$ T cells but that IL-2 and IL-15 were significantly more suppressive on $CD8^+$ T cells than on $CD4^+$ T cells (Figure 5A), presumably because signaling by IL-2 and IL-15 requires CD122 (IL2Rβ), which is expressed on $CD8^+$, but not $CD4^+$, naive T cells. In addition to cytokines that reduced IL7Rα surface expression, we identified one cytokine (TNF-α) that upregulated IL7Rα expression, and two cytokines that only minimally affected IL7Rα surface expression (IL-9 and IFN-γ) on both $CD4^+$ and $CD8^+$ T cells (Figure 5A).

To determine if these *trans* cytokines affected IL7Rα gene expression in addition to affecting IL7Rα surface protein expression, we performed Northern blots on RNA from unfractionated T cells that had been cultured O/N with various cytokines. The same blot was probed sequentially for IL7Rα, γc, and bcl-2 mRNAs (Figure 5B). TNF-α significantly increased IL7Rα mRNA levels, whereas IFN-γ only minimally affected IL7Rα mRNA levels, paralleling their effect on IL7Rα surface expression (Figure 5B). More important, each of the cytokines that reduced IL7Rα surface expression (IL-2, IL-4, IL-6, IL-7, and IL-15) also significantly reduced IL7Rα mRNA levels, with IL-2 and IL-15 having diminished effects presumably because of the absence of CD122 on naive $CD4^+$ T cells (Figure 5B). Suppression of IL7Rα gene expression by these cytokines was specific in that γc mRNA levels were unaffected. It should be appreciated that each of the suppressive cytokines promotes survival of resting T cells (Rathmell et al., 2001). With the notable exception of IL-6, each of these cytokines markedly upregulated bcl-2 mRNA (Figure 5B). We presume that IL-6's function as a prosurvival cytokine (Teague et al., 1997) results from its upregulation of survival factors other than bcl-2. To determine if *trans* cytokines suppressed IL7Rα gene transcription, we performed nuclear run-on assays on T cells that had been treated with IL-4, which we used as a representative *trans* cytokine (Figure 5C). We found that IL-4 treatment of T cells was indistinguishable from that of IL-7 in that both cytokines suppressed IL7Rα transcription while upregulating bcl-2 transcription (Figure 5C).

We conclude that T cells respond to multiple cytokines by specifically suppressing IL7Rα gene expression and that the cytokines that suppress IL7Rα gene expression (IL-2, IL-4, IL-6, IL-7, and IL-15) have the common feature of being prosurvival cytokines.

Involvement of the Transcriptional Repressor Protein GFI1

Having identified a cytokine-mediated mechanism suppressing IL7Rα gene expression in T cells, we wished to determine its molecular basis. The fact that cytokine-mediated suppression of IL7Rα transcription was dependent upon new protein synthesis strongly suggested a role for cytokine-induced transcriptional repressor proteins. We immediately considered the possible involvement of GFI1 (Gilks et al., 1993) because IL7Rα surface expression is reduced in mice transgenic for GFI1B (Doan et al., 2003), a transcriptional repressor protein with identical DNA binding specificities to GFI1 but which is not expressed in peripheral T cells. We analyzed IL7Rα gene sequences from both mouse and human and found that introns 2 and 4 in both species contained putative GFI1 binding sites, and we confirmed that GFI1 actually bound to the IL7Rα gene locus in living cells by chromatin immunoprecipitation (data not shown).

However, GFI1 in resting T cells is expressed at very low levels (Doan et al., 2003), although GFI1 expression can be increased in activated T cells by IL-4 (Zhu et al., 2002). By Northern blot analyses we found that *Gfi1* mRNA was present in low but significant amounts in fresh $CD8^+$ T cells but in undetectable amounts in fresh $CD4^+$ T cells (Figure 6A). *Gfi1* mRNA remained undetectable in $CD4^+$ T cells even after O/N culture and subsequent addition of IL-7 (Figure 6A, top right). In contrast, *Gfi1* mRNA was present in $CD8^+$ T cells and was modulated by IL-7 signals reciprocally to that of IL7Rα mRNA (Figure 6A, left).

To determine whether GFI1 actually regulates IL7Rα expression in either T cell subset in vivo, we assessed IL7Rα expression on GFI1-deficient T cells from *Gfi1*$^{-/-}$ mice (Hock et al., 2003; Karsunky et al., 2002) and on GFI1 replete T cells from either *Gfi1*$^{+/-}$ or *Gfi1* transgenic mice (Figure 6B). To compare IL7Rα expression between T cell subsets and between groups of mice, we quantitated IL7Rα immunofluorescence in linear total fluorescence units (TFU). IL7Rα was expressed in equal amounts on $CD4^+$ and $CD8^+$ T cells from wild-type *Gfi1*$^{+/-}$ mice (Figure 6B). In contrast, IL7Rα expression on GFI1-deficient T cells from *Gfi1*$^{-/-}$ mice was markedly skewed in that IL7Rα expression on GFI1-deficient $CD8^+$ T cells was 2.5-fold higher than that on $CD4^+$ T cells from the same animals and was nearly 2-fold higher than IL7Rα expression on T cells from wild-type *Gfi1*$^{+/-}$ mice (Figure 6B). Thus, IL7Rα expression was markedly increased on GFI1-deficient $CD8^+$ T cells but was not increased on $CD4^+$ T cells from the same *Gfi1*$^{-/-}$ mice (Figure 6B). Consistent with GFI1's selective effect on $CD8^+$ T cells, constitutive expression of a *Gfi1* transgene in either wild-type (*Gfi1*$^{+/-}$) or *Gfi1* knockout (*Gfi1*$^{-/-}$) mice markedly reduced IL7Rα expression on $CD8^+$ T cells without significantly reducing IL7Rα expression on $CD4^+$ T cells (Figure 6B). The minimal reduction in IL7Rα expression observed on GFI1-deficient $CD4^+$ T cells from *Gfi1*$^{-/-}$ knockout mice was probably not

(E) Acute depletion of in vivo IL-7 upregulates IL7Rα expression. B6 mice were injected i.p. three times at 12 hr intervals with 0.5 mg of neutralizing anti-IL-7 mAb (clone M25), 0.5 mg of control IgG, or PBS and analyzed 12 hr after the last injection. Bar graphs quantitate IL7Rα expression on T cells in linear TFU, which was significantly higher on T cells from mice injected with M25 ($p < 0.05$).

(F) IL7Rα expression on LNT adoptively transferred into IL-7 replete or IL-7-deficient host mice. B6 LNT (2×10^7) were injected iv into RAG$^{-/-}$ or RAG$^{-/-}$IL-7$^{-/-}$ host mice, harvested from host spleens 16 hr later, and analyzed. Histograms show IL7Rα expression on donor T cells prior to transfer (dotted line) or 16 hr after transfer (solid line) into either RAG$^{-/-}$ (open curve) or RAG$^{-/-}$IL-7$^{-/-}$ (filled curve) host mice. Bar graphs quantitate surface IL7Rα expression in linear TFU.

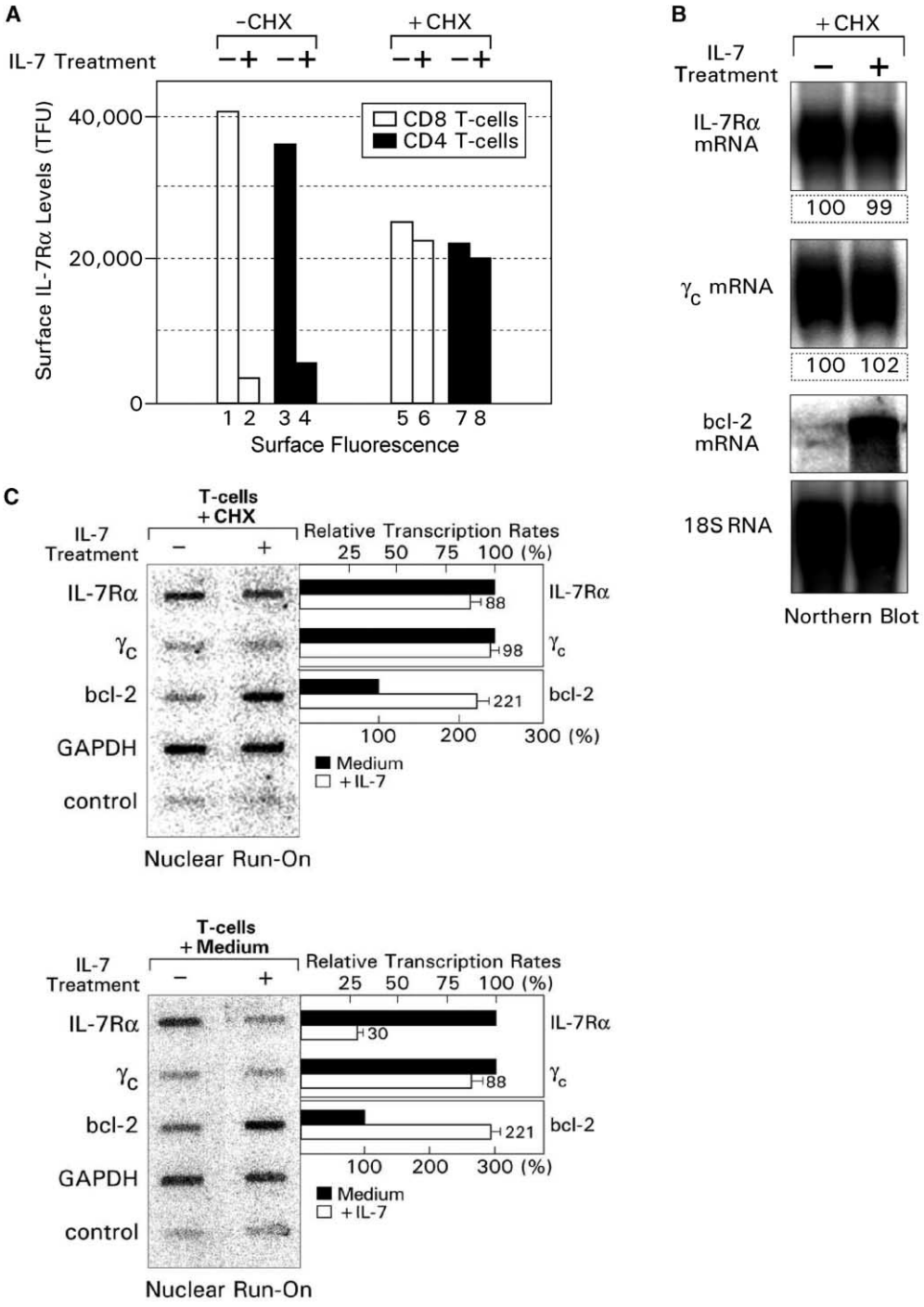

Figure 3. IL7R Downregulation Requires Protein Synthesis

(A) CHX treatment inhibits IL-7-induced downregulation of surface IL7Rα expression. O/N rested LNT were treated for 6 hr with either IL-7 or medium ± CHX. Fluorescence intensities of surface IL7Rα staining were quantitated in linear TFU. Lanes 1–4, cells were stimulated in the absence of CHX; lanes 5–8, cells were stimulated in the presence of CHX.

(B) CHX treatment prevents IL-7 from downregulating IL7Rα mRNA. LNT were rested O/N in medium and then treated for 6 hr with either IL-7 or medium in the presence of CHX. Northern blots of total RNA were probed for IL7Rα, γc, bcl-2, and 18S RNA. Single blots were sequentially stripped and hybridized with the indicated probes. Intensities of IL7Rα and γc RNA bands were normalized to corresponding band intensities from T cells exposed only to medium, which were set at 100. Northern blots of the same T cells without CHX treatment are displayed in Figure 2B.

(C) CHX treatment prevents IL-7-induced suppression of IL7Rα mRNA transcription. Purified LNT were rested O/N in medium and then treated for 6 hr with either IL-7 or medium in the presence of CHX (top), after which nuclei were assessed by nuclear run-on assays for transcription of specific RNA's. Lower panel shows the transcriptional regulation of IL7Rα mRNA by IL-7 under the same conditions but without CHX.

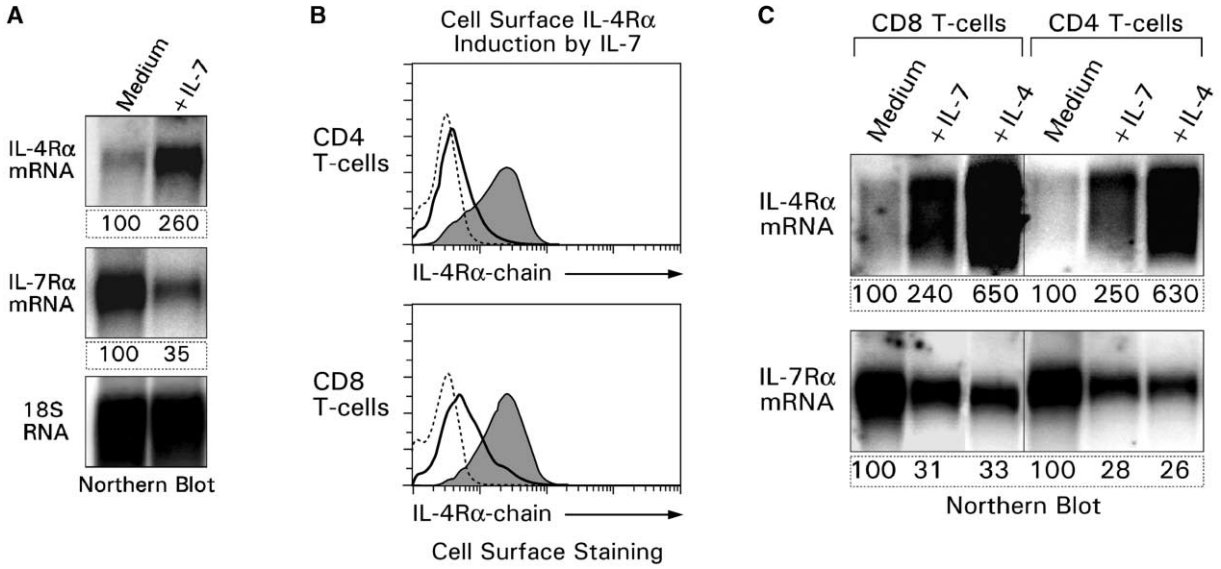

Figure 4. IL-7 Differentially Affects IL4Rα and IL7Rα Expression

(A) IL-7 upregulates IL4Rα mRNA expression. LNT were rested in medium O/N, treated for 6 hr with either medium or IL-7, and then assessed by Northern blot for IL7Rα, IL4Rα, and 18S RNA. Single blots were sequentially stripped and hybridized with the indicated probes.

(B) IL-7 upregulates IL4Rα surface expression on both $CD4^+$ and $CD8^+$ T cells. LNT were rested in medium O/N and then treated for 6 hr with either IL-7 or medium. Histograms show IL4Rα expression levels after 6 hr treatment with either IL-7 (filled curve) or medium (open curve). Solid line = IL4Rα staining; dotted line = control staining.

(C) Cytokines differentially affect IL4Rα and IL7Rα mRNA expression. Purified $CD4^+$ and $CD8^+$ LNT were cultured O/N with IL-4, IL-7, or medium as indicated. The next day, cells were assessed for IL4Rα and IL7Rα mRNA. Single blots were sequentially stripped and hybridized with the indicated probes.

due to GFI1 deficiency as it was not reversed to any significant extent by the *Gfi1* transgene (Figure 6B). We conclude that GFI1 does suppress IL7Rα expression in $CD8^+$ T cells but that it does not suppress IL7Rα expression in $CD4^+$ T cells.

We further analyzed the effect of GFI1 on IL7Rα expression by culturing T cells O/N, which increases IL7Rα surface expression and mRNA content in both $CD4^+$ and $CD8^+$ T cells (Figures 6C and 6D). While GFI1-deficient $CD4^+$ T cells behaved like normal $CD4^+$ T cells in O/N cultures, IL7Rα expression in GFI1-deficient $CD8^+$ T cells was significantly dysregulated relative to normal $CD8^+$ T cells in that GFI1-deficient $CD8^+$ T cells did not increase surface IL7Rα expression and did not upregulate IL7Rα mRNA (Figures 6C and 6D). In fact, fresh GFI1$^{-/-}$ $CD8^+$ T cells already expressed IL7Rα at high levels that were equivalent to those achieved by normal $CD8^+$ T cells after O/N culture (Figures 6C and 6D).

We also wished to assess the effect of endogenous IL-7 on $CD4^+$ and $CD8^+$ T cells from GFI1$^{-/-}$ mice and so utilized the adoptive transfer system described in Figure 2F. Purified T cells from GFI1$^{-/-}$ or control B6 mice were adoptively transferred into IL-7 replete RAG$^{-/-}$ or IL-7-deficient RAG$^{-/-}$IL-7$^{-/-}$ host mice and were assessed 16 hr later for IL7Rα expression (Figure 6E). $CD4^+$ and $CD8^+$ T cells from control B6 mice behaved identically in that both expressed higher IL7Rα levels in IL-7-deficient than in IL-7 replete host mice. In contrast, $CD4^+$ and $CD8^+$ T cells from GFI1$^{-/-}$ mice behaved quite differently from one another: GFI1-deficient $CD4^+$ T cells, like control B6 T cells, expressed significantly higher IL7Rα levels in IL-7-deficient than in IL-7 replete host mice, whereas GFI1-deficient $CD8^+$ T cells expressed nearly identical levels of IL7Rα in both IL-7 deficient and IL-7 replete host mice. These results confirm in vivo that IL-7 induced suppression of IL7Rα expression involves GFI1 in $CD8^+$ T cells, but not $CD4^+$ T cells.

Effect of IL7Rα Sppression on In Vivo T Cell Numbers

Finally, we considered that cytokine-mediated transcriptional suppression of IL7Rα expression might function to maximize utilization of endogenous IL7 and thereby expand the peripheral T cell pool that can be maintained in vivo. To assess this possibility, we examined IL7Rα transgenic (IL7RαTg) mice that expressed a transgene encoding human CD2 (hCD2) enhancer-promoter elements driving mouse IL7Rα cDNA (Yu et al., 2004). T cell development in such IL7RαTg mice is entirely normal (Porter et al., 2001; Yu et al., 2004). Because IL7RαTg T cells expressed IL7Rα molecules transcribed from both endogenous IL7Rα genes and the hCD2-driven IL7Rα transgene, IL7RαTg T cells expressed 4.6 times as much IL7Rα surface protein as nontransgenic B6 T cells (Figure 7A, left). Endogenous and transgene encoded IL7Rα mRNA could be distinguished on Northern blots, revealing that >75% of the IL7Rα mRNA in IL7RαTg T cells were transgene encoded (Figure 7A, middle). Removal of IL7RαTg T cells from their cytokine-rich in vivo environment by placement in O/N culture was found not to affect IL7Rα transgene expression but only upregulated endogenous IL7Rα gene expression (Figure 7A, middle), indicating that

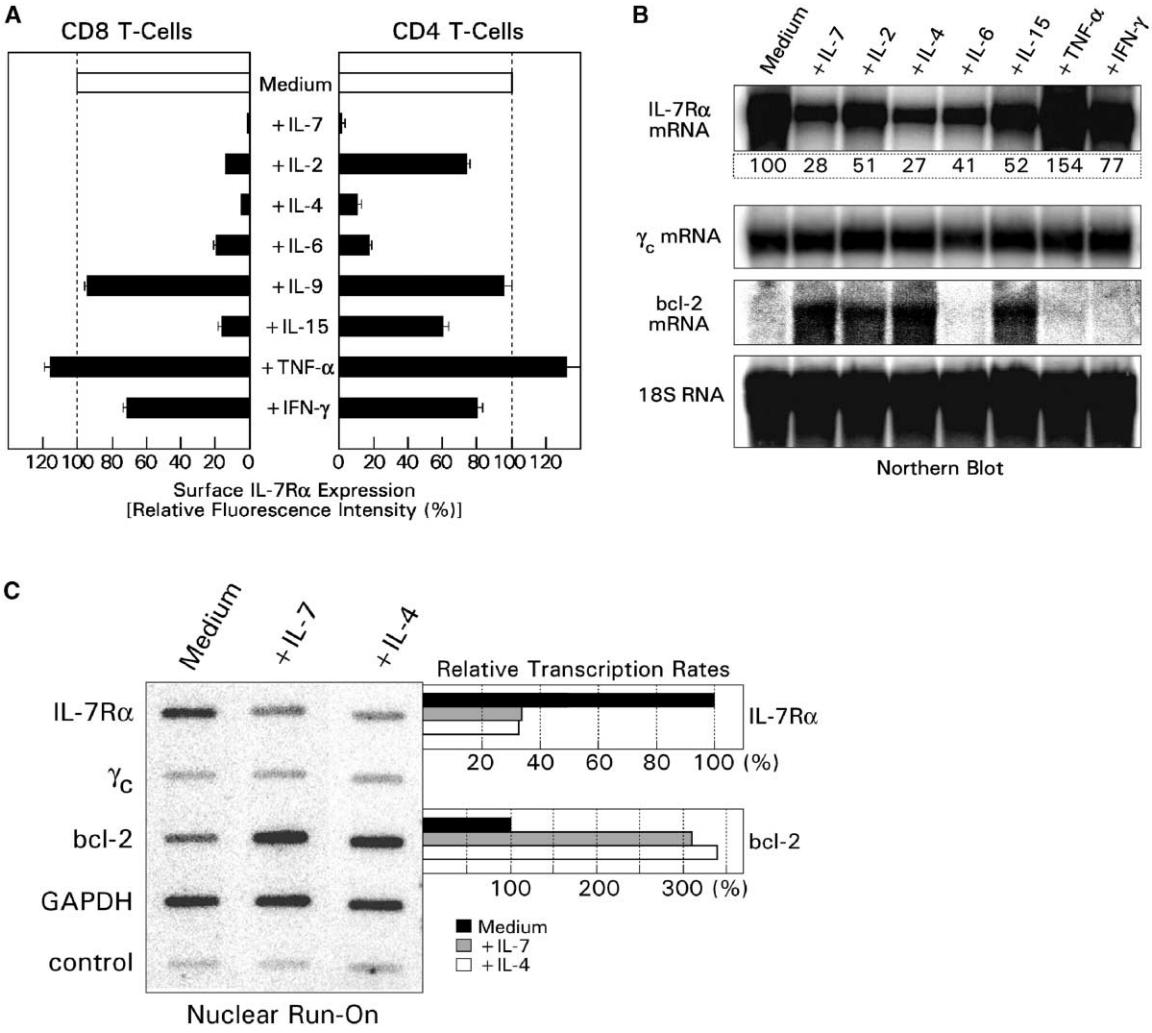

Figure 5. Effect of Multiple Cytokines on IL7Rα Expression

(A) Surface IL7Rα expression is downregulated by multiple cytokines. LNT were cultured O/N with either cytokines or medium. IL7Rα surface fluorescence on $CD4^+$ and $CD8^+$ T cells was assessed in four independent experiments, quantified into linear TFU, and normalized relative to medium-cultured T cells, which were set at 100%.

(B) Downregulation of IL7Rα mRNA expression by *cis* and *trans* cytokines. LNT were incubated O/N with either medium or the indicated cytokines and then assessed by Northern blot analysis. Individual blots were stripped and sequentially hybridized with the indicated probes. Relative band intensities were quantitated as previously described.

(C) Downregulation of IL7Rα transcription by both *cis* and *trans* cytokines. LNT were rested O/N in medium and then treated for 6 hr with IL-4, IL-7, or medium. Nuclei were then isolated and assessed by nuclear run-on assays for transcription of specific RNA's. Signal intensities were calculated relative to GAPDH, and transcription rates were normalized to T cells that had been cultured only in medium.

hCD2-driven IL7Rα transgene expression was not transcriptionally suppressed in vivo. Remarkably, while higher IL7Rα expression levels might have been predicted to confer a survival advantage to individual IL7RαTg T cells, assessment of spleen T cell numbers in IL7RαTg and age-matched nontransgenic littermates revealed that IL7RαTg mice contained significantly fewer peripheral T cells than their nontransgenic littermate controls (Figure 7A, right). Thus, the peripheral T cell pool is smaller in IL7Rα transgenic mice than in nontransgenic mice, suggesting that the ability to downregulate IL7Rα gene expression is important for expanding the overall number of T cells that can be maintained in vivo by endogenous IL-7.

Discussion

The present study has identified a cytokine-mediated mechanism that regulates IL7Rα transcription in resting $CD4^+$ and $CD8^+$ T cells and expands the size of the peripheral T cell pool that can be maintained during T cell homeostasis. Naive T cells are dependent on IL-7-induced survival signals, but we found that their transcription and expression of IL7Rα was paradoxically suppressed by IL-7 and other prosurvival cytokines. Interestingly, IL7Rα transcription was not suppressed by identical molecular mechanisms in $CD4^+$ and $CD8^+$ T cells, as $CD8^+$ T cells required the transcriptional repressor protein GFI1, while $CD4^+$ T cells did not. Be-

cause IL7Rα transcription and expression were suppressed specifically by prosurvival cytokines, we think that this regulatory mechanism functions during T cell homeostasis and antigen stimulation to reduce IL7Rα expression precisely on those T cells that have already received survival signals, effectively removing them from competition with unsignaled T cells for any remaining IL-7. As a result, limiting IL-7 would be made available to the greatest possible number of T cells, maximizing the size of the peripheral T cell pool and receptor diversity. Thus, the present findings help resolve the competition/diversity paradox for IL-7-dependent T cells.

Biological Implications

The competition/diversity paradox was initially recognized by De Boer and Perelson and further described by Freitas and Rocha, who noted that continual competition among peripheral lymphocytes for in vivo survival factors (i.e., cytokines) necessarily results in winners and losers (De Boer and Perelson, 1994; Freitas and Rocha, 2000), with the losers failing to survive. Many, if not all, T cells require intermittent TCR signals derived from engagement of peripheral self-antigens to be receptive to cytokine-induced survival signals (Tanchot et al., 1997), but it is competition among T cells for limiting cytokines, not self-antigens, that results in the competition/diversity paradox. Indeed, each T cell potentially expresses a unique antigen receptor so that clonal loss due to continual in vivo competition for limiting cytokine results in progressive narrowing of the T cell antigen receptor repertoire. Different T cell subsets (e.g., naive and memory, resting and antigen-stimulated, etc.) might, in theory, utilize different survival factors, so that different T cell subsets could coexist without competing with one another for survival (Freitas and Rocha, 2000; Sprent and Surh, 2003). However, it has not been understood how clonal loss might be minimized within a single T cell subset whose members compete with one another for the same survival factor.

The present study identifies a possible solution to this problem for naive T cells. As depicted in Figure 7B, the periphery is populated by the maximum number of naive T cells that can be maintained by the amount of IL-7 that is present. Importantly, the diverse clonal make-up of peripheral T cells is stably maintained because IL-7-induced signals transiently downregulate IL7Rα expression, ensuring that T cell clones that have already received IL-7-induced survival signals do not express sufficient amounts of IL7Rα to compete for remaining IL-7 with T cell clones that have not yet received IL-7-induced survival signals (Figure 7B). In this way, the size of the peripheral T cell pool is increased and the chance for survival of each naive T cell clone is maximized.

The present study also provides a possible explanation for how antigen-stimulated expansion of antigen-specific T cell clones occurs without massive clonal loss of unstimulated T cells (Figure 7C). Since the periphery contains the maximal number of T cells that can be maintained by the amount of IL-7 that is present, antigen encounters that lead to acute expansion of antigen-specific T cell clones might be expected to increase IL-7 requirements, resulting in increased competition, increased loss of antigen-unstimulated T cell clones, and marked narrowing of the naive T cell repertoire. However, antigen-stimulated T cells produce prosurvival cytokines (e.g., IL-2, IL-4, and IL-6), which provide them with survival and proliferative signals and which signal antigen-stimulated T cells to downregulate expression of IL7Rα. As a consequence, antigen-stimulated T cells express lower amounts of IL7Rα than naive resting T cells and so do not effectively compete for the limiting IL-7 that resting T cells require for survival (Figure 7C). Thus, acute expansion of antigen-stimulated T cell clones can occur without inducing significant loss of antigen-unstimulated T cell clones.

Regulation of IL7Rα Transcription

Beyond the fact that PU.1 is a transcriptional activator that promotes IL7Rα transcription in B cells and early T cell precursors, little is known about IL7Rα transcription, especially in T cells (DeKoter et al., 2002). The present study now identifies a negative regulatory mechanism that suppresses IL7Rα transcription in both $CD4^+$ and $CD8^+$ T cells and that is activated by the *cis* cytokine IL-7 as well as the *trans* cytokines IL-2, IL-4, IL-6, and IL-15. IL-7 signals suppress transcription and expression of its cognate receptor, while these other cytokines upregulate expression of their cognate receptors. Importantly, these other cytokines also suppress expression of IL7Rα even as they upregulate expression of their own receptors. Receptors for cytokines IL-2, IL-4, IL-7, and IL-15 utilize the γc chain but the receptor for the cytokine IL-6 does not (Ozaki and Leonard, 2002), so there is no known common signaling pathway for these cytokines even though they all suppress IL7Rα transcription.

IL7Rα transcription and expression is not suppressed by all cytokine signals, as TNF-α upregulated IL7Rα expression in both $CD4^+$ and $CD8^+$ T cells, a point that will be pursued in future studies. The common feature of the cytokines that do suppress IL7Rα transcription (IL-2, IL-4, IL-6, IL-7, and IL-15) is that they function as prosurvival cytokines, upregulating expression of anti-apoptotic genes and proteins in T cells (Rathmell et al., 2001). We think this point is key to the biological significance of their inhibitory effect on IL7Rα transcription and expression: antigen-stimulated T cells that have been signaled by a prosurvival cytokine no longer require IL-7 for survival, so suppression of IL7Rα transcription and expression prevents them from unnecessarily consuming limiting IL-7, which is critical for survival of antigen-unstimulated T cells (see Figure 7C).

Cytokine-induced suppression of IL7Rα transcription has not been previously appreciated. However, IL-2 was previously recognized to downregulate IL7Rα mRNA content in activated T cells, and it apparently did so by destabilizing IL7Rα mRNA transcripts (Xue et al., 2002). In the present study, we found that IL-2, along with other prosurvival cytokines, reduced IL7Rα mRNA content, but the mechanism in resting T cells does not involve destabilization of IL7Rα mRNA. Direct measurements of mRNA stability revealed that IL7Rα mRNA transcripts had a relatively short half-life of 1.2 hr in unsignaled T cells and that this half-life was not affected by IL-7 signals. Instead of significantly destabilizing IL7Rα

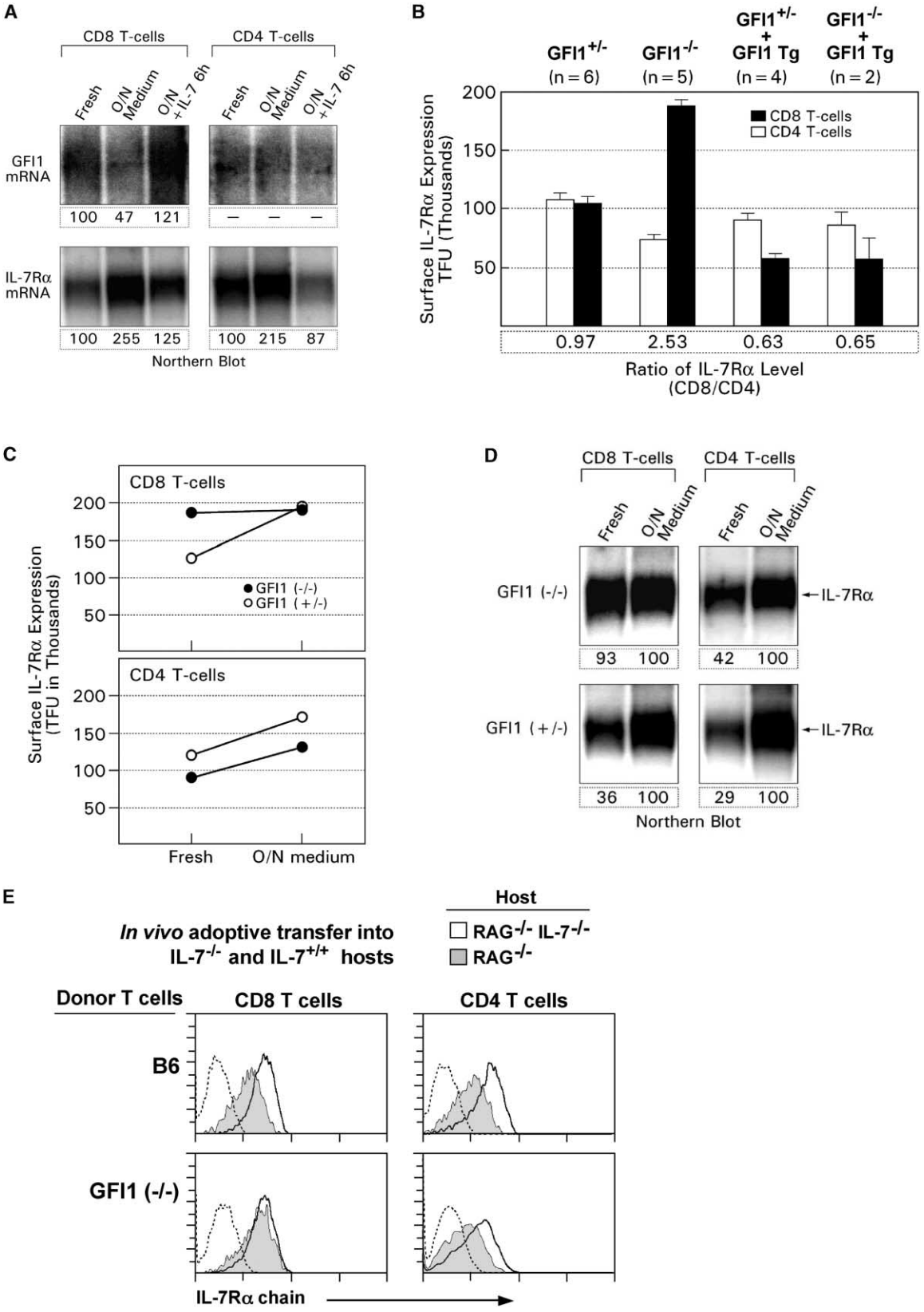

Figure 6. Selective Modulation of IL7Rα Expression in CD8$^+$ T Cells by the Transcriptional Repressor GFI1

(A) *Gfi1* mRNA expression in resting and IL-7-signaled T cells. Northern blot analyses were performed on total RNA from purified CD4$^+$ and CD8$^+$ LNT directly after isolation, after O/N culture, or after subsequent 6 hr stimulation with IL-7, as indicated. Individual blots were stripped and sequentially probed for *Gfi1* and IL-7Rα mRNA.

mRNA transcripts, we found that IL-7 induced the synthesis of proteins that suppressed IL7Rα transcription. Thus, prosurvival cytokines reduce IL7Rα mRNA content in primary T cells by suppressing IL7Rα transcription, not by destabilizing IL7Rα mRNA transcripts.

Selective Involvement of the Transcriptional Repressor GFI1 in CD8$^+$ T Cells

Cytokine-induced suppression of IL7Rα expression in CD8$^+$ T cells involves the transcriptional repressor factor GFI1. GFI1 is a nuclear protein that was originally described to confer cytokine-independent growth in an IL-2-dependent rat CD8$^+$ T cell line (Gilks et al., 1993). Molecular analysis revealed that the murine *Gfi1* gene encodes a 423 amino acid polypeptide that contains six C2H2-type C-terminal zinc-finger motifs. The amino-terminal 20 amino acids contain a nuclear localization signal coincident with a novel transcriptional repressor domain termed SNAG (for "Snail/GFI1"), which acts in a position-and orientation-independent manner (Grimes et al., 1996). GFI1 specifically binds to the DNA sequence TAAATCAC(A/T)GCA, but its binding to DNA is insufficient in itself to repress transcription. Rather, GFI1 that is bound to DNA must specifically interact with other nuclear proteins to repress transcription (Grimes et al., 1996). The identity of the interacting proteins required for GFI1-mediated transcriptional repression is not yet known, but recent studies have revealed that GFI1 complexes with ETO, a corepressor protein, and associates with histone deacetylase proteins (McGhee et al., 2003); and that GFI1 interacts with PIAS-3, a specific inhibitor of STAT-3 (Rodel et al., 2000). GFI1 is upregulated in activated T cells (Gilks et al., 1993; Rodel et al., 2000) and has been shown to regulate IL-4 driven Th2 CD4$^+$ T cell proliferation (Zhu et al., 2002). Mice with targeted disruption of the *Gfi1* gene have growth retardation, dramatically shortened life span (11 wks), blocked myeloid cell differentiation, and impaired early thymocyte development (Hock et al., 2003; Karsunky et al., 2002; Yucel et al., 2003).

The present study demonstrates that cytokine-mediated suppression of IL7Rα transcription utilizes GFI1 in CD8$^+$ T cells but occurs independently of GFI1 in CD4$^+$ T cells. Notably, both gain-of-function and loss-of-function mutations in GFI1 affected IL7Rα levels on CD8$^+$ T cells, while neither affected IL7Rα levels on CD4$^+$ T cells. This finding was surprising since it indicated that CD8$^+$ and CD4$^+$ T cells used different molecular mechanisms to achieve the same end. However, the fact that GFI1 was not detectably expressed in resting CD4$^+$ T cells, even after IL-7 signaling, strongly indicated that GFI1 did not mediate IL-7's suppression of IL7Rα transcription in CD4$^+$ T cells, a possibility subsequently confirmed in GFI1 knockout mice. Whether GFI1 could suppress IL7Rα transcription if it were expressed in naive CD4$^+$ T cells is uncertain. We found that when *Gfi1* was encoded by a human CD2-driven transgene that is expressed in both CD4$^+$ and CD8$^+$ T cells (Rodel et al., 2000), it still selectively downregulated IL7Rα expression in naive CD8$^+$ T cells, not CD4$^+$ T cells. Since GFI1 would be expected to bind to the IL7Rα gene in both CD4$^+$ and CD8$^+$ T cells, its failure to efficiently suppress IL7Rα transcription in CD4$^+$ T cells suggests that CD4$^+$ T cells may additionally lack a GFI1 interacting protein required for IL7Rα transcriptional repression. An analogous explanation may underlie the observation that GFI1 selectively affected Th2 cells without discernibly affecting Th1 cells, even though GFI1 was present in both (Zhu et al., 2002).

Conclusions

The present study has identified and characterized a novel transcriptional regulatory mechanism involving GFI1 that is induced by IL-7 and other prosurvival cytokines and that suppresses IL7Rα expression in peripheral T cells. These findings provide a molecular solution to a fundamental cellular problem—namely how to maximize the size of the peripheral T cell pool and minimize clonal loss during continuous competition among naive T cells for limiting IL-7.

Experimental Procedures

Animals

C57BL/6 (B6) mice were obtained from the Frederick Cancer Research and Development Center, Frederick, MD. *Gfi1*$^{-/-}$ mice were generously provided by Dr. Stuart Orkin, Harvard Medical School, Boston, MA. Mice transgenic for *Gfi1* were generously provided by Dr. Tarik Moroy, Universitaetsklinikum Essen, Germany, and *Gfi1* transgenic mice on *Gfi1*$^{-/-}$ background were bred in the Baxter Barrier animal care facility at the University of Louisville School of Medicine. RAG$^{-/-}$ and RAG$^{-/-}$IL-7$^{-/-}$ mice were kindly provided by Dr. Scott Durum (NCI-Frederick, MD). Mice expressing a mouse IL7Rα transgene under the control of hCD2-enhancer promoter elements were generated in our lab (Yu et al., 2004).

Cell Culture and Immunofluorescence Analysis

LNT were depleted of B cells with anti-mouse IgG beads. CD4$^+$ or CD8$^+$ LNT were further purified by depleting LNT with either anti-

(B) IL7Rα expression on T cells containing or lacking GFI1. Surface IL7Rα expression on CD4$^+$ and CD8$^+$ T cells was assessed by immunofluorescence and multicolor flow cytometry on LNT from the indicated mice. Surface fluorescence was quantitated in TFU. The bar graph displays surface IL7Rα expression, expressed as TFU ± SEM, on CD4$^+$ (open bar) and CD8$^+$ (closed bar) LNT from the indicated strains in multiple experiments.

(C) Effect of GFI1 deficiency on in vivo suppression of IL7Rα surface expression. Surface expression of IL7Rα, quantitated in TFU, was determined on CD4$^+$ and CD8$^+$ LNT from GFI-deficient (*Gfi1*$^{-/-}$) and GFI1 replete (*Gfi1*$^{+/-}$) littermate mice directly after explantation and after O/N culture.

(D) Effect of GFI1 deficiency on in vivo suppression of IL7Rα mRNA content. IL7Rα mRNA content in purified CD4$^+$ and CD8$^+$ LNT from GFI1-deficient (*Gfi1*$^{-/-}$) and GFI1 replete (*Gfi1*$^{+/-}$) littermate mice was determined by Northern blot analyses of cells either directly after explantation or after O/N culture, as indicated.

(E) IL7Rα expression on GFI1-deficient T cells adoptively transferred into IL-7 replete or IL-7-deficient host mice. LNT from GFI1-deficient or control B6 mice were injected i.v. into RAG$^{-/-}$ or RAG$^{-/-}$IL-7$^{-/-}$ host mice, harvested from host spleens 16 hr later, and analyzed for IL7Rα expression. Histograms show surface IL7Rα expression on CD4$^+$ and CD8$^+$ donor T cells transferred into either RAG$^{-/-}$ (open curves) or RAG$^{-/-}$IL-7$^{-/-}$ (filled curves) host mice. Dotted line = isotype control antibody.

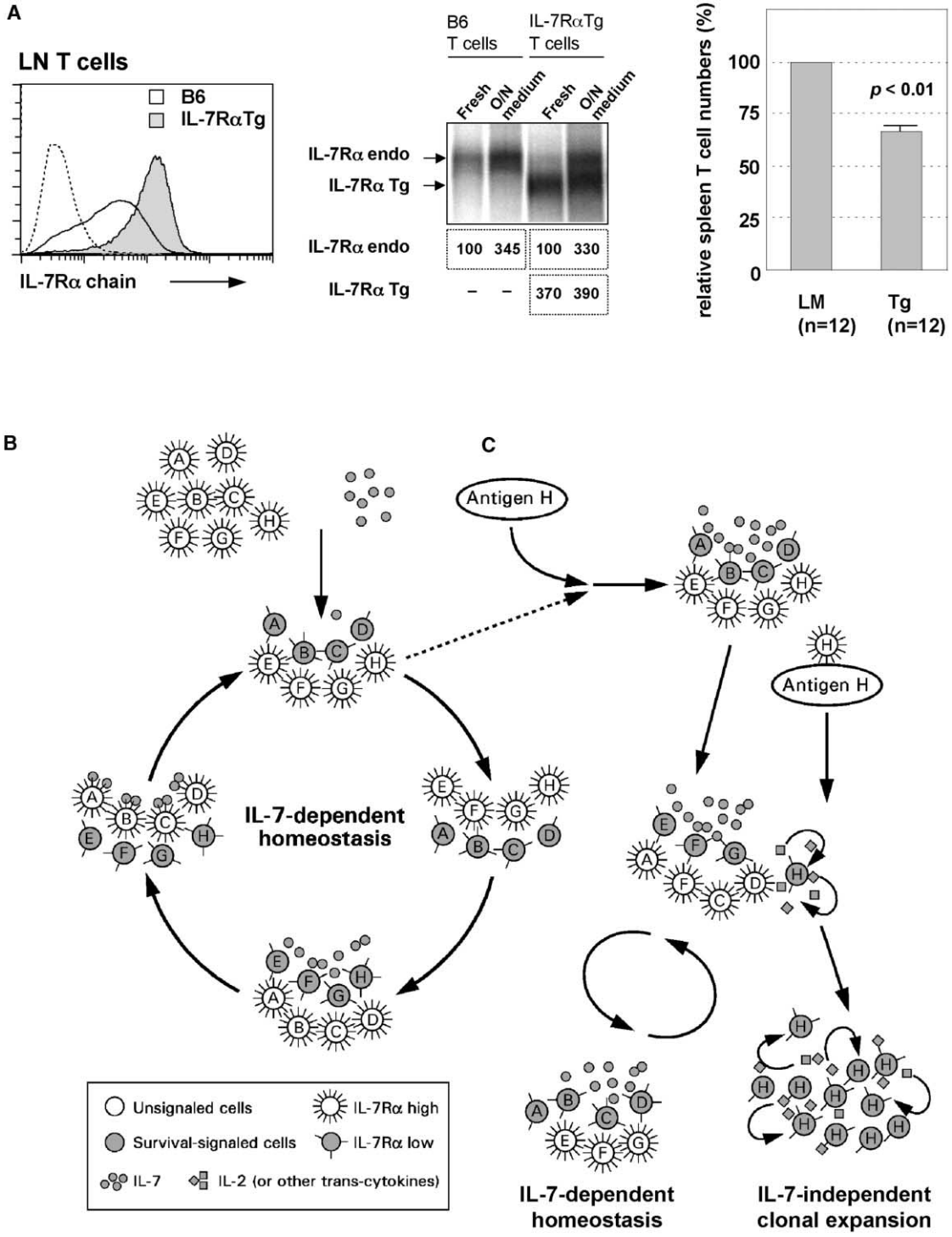

Figure 7. Cytokine-Mediated Regulation of IL7R Expression and Its Effect on T Cell Homeostasis

(A) IL7RαTg limits the number of peripheral T cells. Surface IL7Rα expression on fresh LNT (left) and RNA content on fresh and O/N cultured LNT (middle) from B6 and IL7RαTg mice were determined. Spleen T cell numbers in IL7RαTg mice were compared to that in littermate control mice, which were set at 100% (right). Twenty-four mice from four independent litters (12 mice in each group) were analyzed, and the bar graph displays the mean ± SEM of four independent litters.

(B) Schematic representation of T cell homeostasis in the absence of antigen stimulation. The lymphoid periphery is populated by the maximum number of naive T cells that can be maintained by the limiting amount of IL-7 that is present in vivo. As individual T cells bind IL-7 and receive survival signals, they downregulate IL7R expression. As a result, remaining IL-7 will be bound by T cells with the highest IL7R levels, which are precisely those T cells that have not yet received an IL-7 survival signal. In this way, limiting IL-7 can be shared by the greatest possible number of T cells, maintaining cell survival and clonal diversity.

(C) Schematic representation of T cell homeostasis in the presence of antigen stimulation. Antigen-induced activation of a specific T cell (as shown in the diagram for clone H and its cognate antigen H) induces the antigen-specific T cell to proliferate and secrete cytokines such as IL-2 or IL-4. IL-2/IL-4 provides activated T cells with both proliferation and survival signals, so that their survival is no longer dependent upon IL-7. Importantly, IL-2/IL-4 also downregulate IL-7R expression on antigen-activated T cells so they do not compete with antigen-unstimulated T cells for limiting IL-7. In this way, proliferation and survival of antigen-stimulated T cells can occur without inflicting clonal loss on antigen-unstimulated T cells.

CD8 or anti-CD4 mAbs. Cells (5×10^6 /ml) were cultured in 7.5% CO_2 at 37°C in medium with 10% FCS that had been steroid depleted by charcoal stripping. Where indicated, cells were treated with CHX or ActD (10 μg/ml final concentration). Neutralizing anti-IL-7 mAb (clone M25) (Grabstein et al., 1993) was kindly provided by Dr. Fred Finkelman, University of Cincinnati, with permission from Amgen, Thousand Oaks, CA. Recombinant cytokines were obtained from Pharmingen or R & D Systems and used at the final concentrations indicated: human IL-2 (100 units), IL-15 (100 ng/ml); mouse IL-4 (45 ng/ml), IL-6 (45 ng/ml), IL-7 (6 ng/ml), IFNγ (25 ng/ml), mouse TNFα (1.5 ng/ml). Immunofluorescence and flow cytometry were performed as previously described (Yu et al., 2003).

Northern Blot Analyses

Total RNA was isolated by using TriZol (Invitrogen) and equal amounts were resolved in a 1.5% agarose gel under denaturing conditions and blotted onto Hybond-N^+ nylon membranes (Amersham). Radioactive probes were generated from cloned cDNA fragments of the corresponding genes by using the EZ-strip DNA kit (Ambion) and hybridized O/N with RNA-blotted membrane in UltraHyb hybridization solution (Ambion) at 42°C. Next day, membranes were washed two times with 2× SSC/0.1% SDS for 30 min and two times with 0.1× SSC/0.1% SDS at 55°C. Membranes were exposed O/N or longer to a PhosphorImager screen and analyzed.

Nuclear Run-On Assays

Nuclear run-on assays were performed as described (Cibotti et al., 2000). In brief, $5–10 \times 10^7$ cells were washed in ice-cold wash buffer (150 mM KCl, 4 mM MgOAc, 10 mM Tris-HCl [pH 7.4]), and nuclei were isolated by detergent lysis (0.5% NP-40 in wash buffer) and dounce homogenization. In vitro transcription was performed in labeling buffer (20% glycerol, 10 mM Tris-HCl [pH 8.0], 140 mM KCl, 14.3 mM 2-mercaptoethanol, 0.2 mM $MgCl_2$, and 0.25 mM each ATP, CTP and GTP) in the presence of 0.3 mCi ^{32}P-UTP for 30 min at 31°C. After lysis of the labeled nuclei in high-salt buffer, genomic DNA was digested by DNase I treatment, and RNA was isolated by acidic phenol extraction. 1 μg of each linearized and denatured plasmid was transferred and crosslinked to a Hybond-N^+ nitrocellulose membrane by using a slot-blot apparatus. After 48 hr hybridization under continuous rotation at 42°C in UltraHyb hybridization buffer, blots were washed and treated with 10 μg/ml RNaseA in 2× SSC for 10 min at room temperature, and washed again. Blots were exposed for 2–3 days to a PhosphorImager screen.

In Vivo Adoptive Transfer

Donor LNT (2×10^7) were injected into the tail vein of either $RAG^{-/-}$ or $RAG^{-/-}IL\text{-}7^{-/-}$ host mice. 16 hours later, spleen cells from host mice were analyzed.

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