Brief Definitive Report

IL-7 Receptor Signals Inhibit Expression of Transcription Factors TCF-1, LEF-1, and RORγt: Impact on Thymocyte Development

Qing Yu,[1] Batu Erman,[1] Jung-Hyun Park,[1] Lionel Feigenbaum,[2] and Alfred Singer[1]

[1]*Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892*
[2]*SAIC-Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702*

Abstract

Intrathymic T cell development depends on signals transduced by both T cell receptor and cytokine receptors. Early $CD4^-CD8^-$ (double negative) thymocytes require interleukin (IL)-7 receptor (IL-7R) signals for survival and proliferation, but IL-7R signals are normally extinguished by the immature single positive (ISP) stage of thymocyte development. We now demonstrate that IL-7R signals inhibit expression of transcription factors TCF-1, LEF-1, and RORγt that are required for the ISP to double positive (DP) transition in the thymus. In addition, we demonstrate that IL-7R signals also inhibit TCF-1 and LEF-1 expression in mature peripheral T cells. Thus, the present work has identified several important downstream target genes of IL-7R signaling in T cells and thymocytes that provide a molecular mechanism for the inhibitory influence of IL-7R signaling on DP thymocyte development. We conclude that IL-7R signals down-regulate transcription factors required for the ISP to DP transition and so must be terminated by the ISP stage of thymocyte development.

Key words: ISP thymocytes • IL-7Rα transgene • developmental arrest

Introduction

αβT cell differentiation in the thymus proceeds via an ordered series of steps resulting in the generation of functionally competent T cells. Early $CD4^-CD8^-$ (double negative [DN]) cells differentiate into $CD4^+8^+$ (double positive [DP]) thymocytes via a transitional intermediary referred to as immature single positive (ISP) cells (1). In turn, DP thymocytes differentiate into either $CD4^+$ or $CD8^+$ (single positive [SP]) T cells. Transition of thymocytes from one developmental stage to the next is dependent on signals transduced through components of the TCR complex. In addition to TCR signals, thymocyte differentiation is also critically dependent on signals transduced through cytokine receptors, especially IL-7Rs (2–7). In early thymocytes, IL-7R signals play a nonredundant role by supporting survival and proliferation of DN thymocytes that are $CD44^+25^+$ (so called DN2 cells; references 2, 6, 8–10). In contrast with the requirement for IL-7R signals in DN2 stage thymocytes, it is generally thought that IL-7R signals are not required for differentiation of DN thymocytes that have progressed beyond the DN2 stage of development (11, 12). In fact, IL-7Rα chain (IL-7Rα) expression declines after the DN2 stage and is normally terminated by the ISP stage of development so that IL-7R signals are absent during the transition of ISP into DP thymocytes (13, 14). There are indications that prolongation of IL-7R signals interferes with thymocyte development (15), but the role of IL-7R signaling in late stage DN thymocytes remains uncertain, as it has recently been suggested that IL-7R signals are required by DN4 thymocytes for survival and further differentiation (16).

The ISP to DP transition is an actively regulated differentiation step that leads to the generation of a large pool of DP thymocytes. The ISP to DP transition is regulated by HMG domain containing transcription factors TCF-1 and LEF-1, as well as by the orphan nuclear receptor RORγ and its isoform RORγt (17–20). TCF-1 and LEF-1 are both members of the TCF family of transcription factors that are expressed at various levels throughout T cell development (17, 21). Disruption of both TCF-1 and LEF-1 ex-

Address correspondence to Alfred Singer, Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bldg. 10, Rm. 4B36, Bethesda, MD 20892. Phone: (301)-496-5461; Fax: (301)-496-0887; email: SingerA@nih.gov

pression in double knockout mice results in a complete block of thymocyte development at the ISP stage, which is not observed in single knockout mice, demonstrating that TCF-1 and LEF-1 are redundant and critical for the ISP to DP transition (17, 18). RORγ and RORγt are both members of the orphan nuclear receptor family of proteins and are isoforms generated by usage of different promoters. They are both expressed at low levels in DN thymocytes and at high levels in DP thymocytes (20, 22). Deficiency of both RORγ and RORγt results in a partial block of thymocyte development at the ISP stage, suggesting an additional role of these two factors in the ISP to DP transition (19). Notably, TCF-1 and RORγ/RORγt are important for survival of DP thymocytes by their regulation of Bcl-xL expression, although this function does not account for the requirement for TCF-1 and RORγ/RORγt during the ISP to DP transition, as forced expression of Bcl-2 or Bcl-xL does not reconstitute the ISP to DP transition in either TCF-$1^{-/-}$ or ROR$\gamma^{-/-}$ROR$\gamma t^{-/-}$ mice (19, 21).

The present work was undertaken to examine the potential effect of IL-7R signaling on differentiation of DN cells into DP thymocytes, and to elucidate its molecular basis. We demonstrate that IL-7R signals inhibit expression of TCF-1, LEF-1, and RORγt transcription factors that are required for the ISP to DP transition and so impair the differentiation of ISP into DP thymocytes. In addition, we demonstrate that IL-7R signals also inhibit expression of TCF-1 and LEF-1 in mature T cells. Thus, the present work identifies several important downstream target genes of IL-7R signaling in T cells and thymocytes, providing a molecular mechanism for the inhibitory influence of IL-7R signaling on DP thymocyte development.

Materials and Methods

Animals. C57BL/6 (B6) mice were obtained from The Jackson Laboratory. ZAP$70^{-/-}$ mice were bred in our own colony (23). The IL-7Rα transgene encoded IL-7Rα cDNA driven by human CD2 (hCD2) enhancer-promoter elements. IL-7Rα cDNA was provided by W. Leonard (National Heart, Lung, and Blood Institute, Bethesda, MD) and S. Durum (National Cancer Institute, Frederick, MD).

Antibodies and Reagents. Antibodies with the following specificities were used for staining: CD4 (GK1.5, RM4.5); CD8α (53-6.7); TCRβ (H57-597); TCRδ (GL3); B220; Thy1.2; CD25 (7D4); CD44 (IM7); Mac-1 (M1/70); Gr-1 (RB6-8C5); NK1.1 (PK136); anti–IL-7Rα (A7R34) (13). Murine IL-7 was obtained from R&D Systems.

Immunofluorescence and Flow Cytometry. Stained cells were analyzed on a FACSVantage SE. Dead cells were excluded by forward light scatter gating and propidium iodide staining. Lineage-negative DN thymocytes were defined as $CD4^-CD8^-TCR\beta^-TCR\delta^-B220^-Gr\text{-}1^-Mac\text{-}1^-NK1.1^-$ thymocytes.

Cell Purification and Electronic Cell Sorting. To purify ISP, thymocytes from ZAP$70^{-/-}$ or IL-7RαTg ZAP$70^{-/-}$ mice were enriched for $CD4^-$ cells, stained, and sorted for $CD4^-8^+$ cells. To fractionate lymph node T cells (LNTs) from WT mice, LN cells were incubated with magnetic beads to which anti–mouse Ig had been adsorbed.

Fetal Thymic Organ Culture (FTOC). Embryonic day 15.5 thymus lobes from IL-7RαTg mice were placed in organ culture in medium or with 60 ng/ml murine IL-7 and harvested on day 6.

Quantitative Real-Time RT-PCR. Total RNA was reversed transcribed using poly(dT) and Superscript II reverse transcriptase (Invitrogen). The cDNA was subjected to real-time PCR amplification for 40 cycles, each cycle containing 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C.

Northern Blot Analyses. Total RNA was resolved and blotted onto nylon membranes. Radioactive probes were generated from cloned cDNA fragments of the indicated genes and hybridized overnight with RNA-blotted membranes. Membranes were washed and exposed to a PhosphorImager screen and analyzed.

Immunoprecipitation and Immunoblotting. Cells were lysed in RIPA buffer, and cell lysates were incubated with rabbit anti–TCF-1 (a gift from H. Kawamoto, Riken Research Center for Allergy and Immunology, Yokohama, Japan; reference 24) and protein A-Sepharose. The precipitated proteins were resolved on 10% SDS-PAGE and transferred to PVDF membrane. Blots were incubated with anti–TCF-1, followed by horseradish peroxidase–conjugated protein A. Reactivity was revealed by enhanced chemiluminescence.

Results and Discussion

The early phase of intrathymic T cell differentiation consists of an ordered series of developmental stages, referred to as DN1, DN2, DN3, DN4, ISP, and DP (25), during which IL-7Rα expression is dynamically regulated as revealed by IL-7Rα staining of B6 thymocytes (Fig. 1 A). The differentiation of ISP into DP thymocytes occurs after IL-7Rα expression has been extinguished and requires expression of the transcription factors TCF-1, LEF-1, RORγ, and RORγt (19, 21). To investigate the possibility that the ISP to DP transition might be affected by IL-7R signaling if such signals were generated in ISP, we added IL-7 to embryonic day 15.5 FTOCs from normal B6 mice (Fig. 1 B, left). On day 6 of culture, thymic lobes were harvested and αβ thymocytes were analyzed by electronically excluding $\gamma\delta^+$ cells. Relative to FTOC without IL-7, addition of IL-7 to WT FTOC markedly interfered with DP thymocyte generation as indicated by reduced frequencies of DP and CD4SP thymocytes and increased frequencies of DN and ISP (distinguished from mature CD8SP T cells by low surface TCRβ expression; Fig. 1 B, left). The relatively low ratio of DP/ISP in WT FTOC with IL-7 compared with medium (DP/ISP ratio of 2.1 vs. 14.6) suggested that IL-7 impaired the ISP to DP transition (Fig. 1 B, left), whereas the relatively low ratio of DP/DN cells with IL-7 compared with medium (DP/DN ratio of 0.5 vs. 5.2) was consistent with interference by IL-7 of the further differentiation of DN3 thymocytes as reported previously (15).

To increase IL-7R signaling in ISP, we constructed IL-7Rα transgenic (IL-7RαTg) mice in which IL-7Rα expression was driven by hCD2 enhancer-promoter elements so that IL-7Rα surface expression remained high on all thymocytes, including ISP. Expression of transgenic IL-7Rα did not itself qualitatively alter thymocyte differentiation either in vivo (26) or in day-6 FTOC (Fig. 1 B, com-

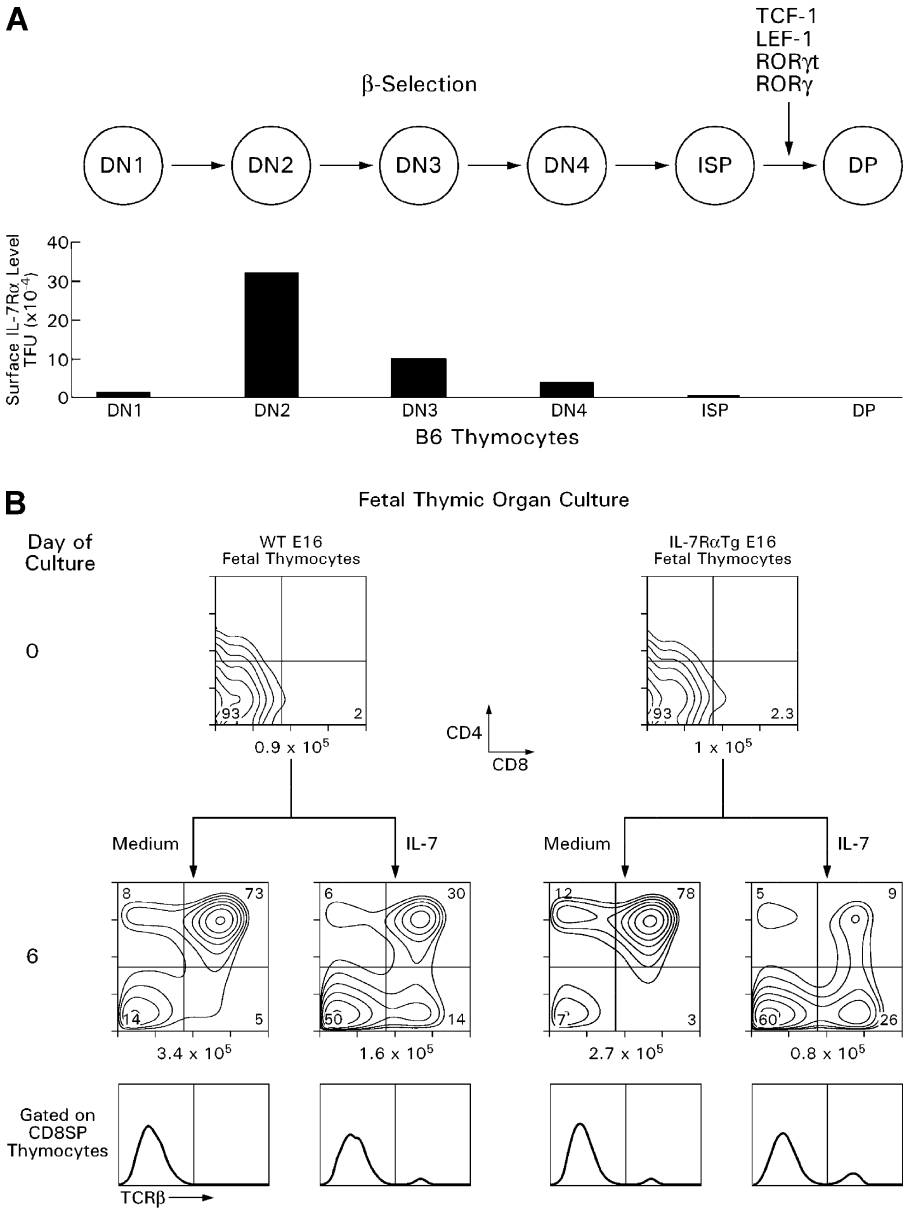

Figure 1. Effect of IL-7R signaling on thymocyte differentiation. (A) Surface expression of IL-7Rα in thymocyte subpopulations from B6 mice. Thymocytes from B6 mice were stained for multiple lineage markers including CD4, CD8, TCRβ, TCRδ, B220, Gr-1, Mac-1, and NK1.1. DN thymocytes were defined as thymocytes lacking all of these markers and were further stained for CD25, CD44, and IL-7Rα to determine expression of IL-7Rα on different DN subsets. IL-7Rα surface expression was quantitated into total fluorescence units (TFU). (B) Effect of IL-7 on thymocyte differentiation in FTOC. Fetal thymic lobes (embryonic day 15.5) from WT or IL-7RαTg mice were placed in fetal thymic organ cultures (FTOCs) in medium or with additional IL-7 (60 ng/ml) starting on day 0 for 6 d. On day 6, the differentiation of αβT cells was specifically examined by electronically excluding TCRδ$^+$ cells. The vast majority of CD8SP thymocytes generated in these cultures were ISP as >95% were TCRβ^{lo}. Results are representative of four experiments.

pare columns 1 and 3). However, addition of IL-7 to FTOC of IL-7RαTg thymi resulted in an even more profound block in the ISP to DP transition than we observed in WT FTOC (Fig. 1 B).

To directly examine the effect of IL-7R signaling on the ISP to DP transition, we introduced the IL-7Rα transgene into ZAP70$^{-/-}$ mice because ZAP70$^{-/-}$ mice are devoid of mature TCR^{hi} CD8SP thymocytes (23) so that the only CD8SP thymocytes they contain are TCR^{lo} ISP. In fact, we obtained highly purified populations of TCR^{lo} ISP from both nontransgenic ZAP70$^{-/-}$ and IL-7RαTg ZAP70$^{-/-}$ mice by simply sorting their thymocytes for CD4$^-$8$^+$ cells (Fig. 2 A). The purified ISP from both nontransgenic ZAP70$^{-/-}$ and IL-7RαTg ZAP70$^{-/-}$ mice were placed in overnight suspension cultures with either medium or IL-7. ISP from nontransgenic ZAP70$^{-/-}$ mice were IL-7R$^-$ and spontaneously differentiated into DP thymocytes whether they were cultured in medium or IL-7 (Fig. 2 A, left). In contrast, ISP from IL-7RαTg ZAP70$^{-/-}$ mice were IL-7R$^+$ and differentiated into DP thymocytes when cultured in medium but remained largely CD4$^-$8$^+$ when cultured in IL-7, demonstrating that IL-7 significantly impaired their differentiation into DP thymocytes (Fig. 2 A, right). This inhibitory effect of IL-7 on the ISP to DP transition of IL-7R$^+$ thymocytes was strictly dose dependent, with greater concentrations of IL-7 causing greater inhibition (Fig. 2 B).

To understand why IL-7R signaling in ISP impaired their differentiation into DP thymocytes, we assessed the effect of IL-7R signaling on transcription factors known to be required for the ISP to DP transition (19, 21). Purified ISP from IL-7RαTg ZAP70$^{-/-}$ mice were placed in over-

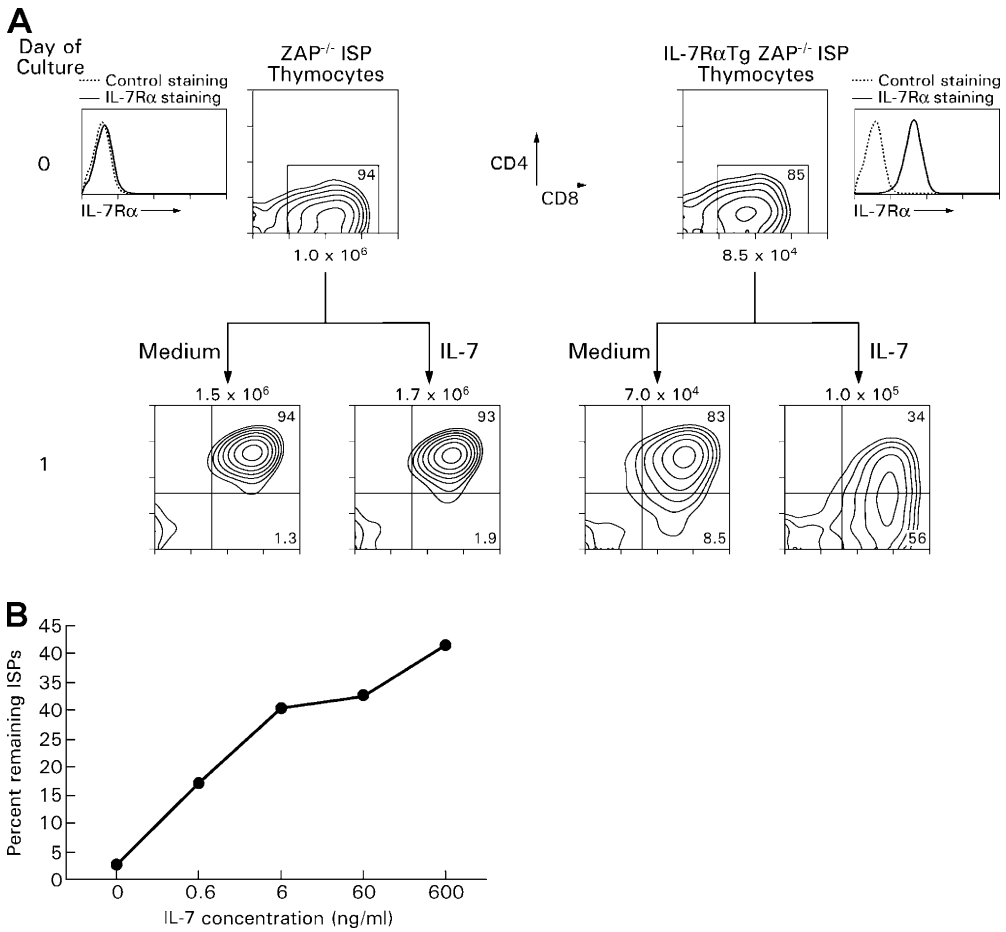

Figure 2. Inhibitory effect of IL-7 on in vitro differentiation of ISP. (A) $CD4^-8^+$ thymocytes were purified from $ZAP70^{-/-}$ or IL-7RαTg $ZAP70^{-/-}$ mice by electronic sorting. Such $CD4^-8^+$ thymocytes were exclusively ISP because $ZAP70^{-/-}$ mice have no mature CD8SP thymocytes (reference 23) and were documented to be $TCR\beta^{lo}$ (not depicted). The ISP were placed in culture with medium or with IL-7 (60 ng/ml). After 1 d of culture, cells were analyzed for CD4 and CD8 expression. Results are representative of three experiments. (B) Inhibitory effect of IL-7 is dose dependent. Purified ISP were cultured overnight in medium or with various doses of IL-7. Cells were analyzed for CD4 and CD8 expression, and the percentage of cells that failed to differentiate into DP thymocytes by remaining $CD4^-8^+$ was plotted against the IL-7 dose.

night (14 h) suspension cultures with either medium or IL-7, and assessed for expression of various genes by quantitative real-time RT-PCR (Fig. 3). Freshly isolated ISP (time, 0 h) contained few, if any, detectable RNA transcripts for either CD4 or Bcl-2, but did contain transcripts for TCF-1, LEF-1, RORγt, and RORγ (Fig. 3). After 14 h in medium, ISP phenotypically differentiated into DP thymocytes and was accompanied by increased numbers of transcripts for CD4 but not Bcl-2, as would be expected because DP thymocytes express CD4 protein but do not express Bcl-2 protein. With regard to transcription factor expression, differentiation of ISP into DP thymocytes was accompanied by increased numbers of transcripts for TCF-1, LEF-1, RORγt, and RORγ, as has been reported previously (17, 20) (Fig. 3).

However, IL-7R signaling in ISP markedly altered this pattern of gene expression. IL-7R signals prevented up-regulation of CD4 RNA, but promoted up-regulation of Bcl-2 RNA in ISP (Fig. 3). More importantly for the present work, IL-7 completely blocked up-regulation of RNAs for TCF-1, LEF-1, and RORγt to a degree that was highly statistically significant (Fig. 3) and appeared to also block up-regulation of RNA transcripts for RORγ, but this reduction did not achieve statistical significance (Fig. 3). Because the ISP to DP transition has been shown to require TCF-1, LEF-1, and RORγt expression (19, 21), IL-7's inhibition of TCF-1, LEF-1, and RORγt expression is sufficient to explain the inhibitory effect of IL-7R signaling on the ISP to DP transition. However, it is possible to argue that the inhibitory effect of IL-7R signaling on transcription factor expression in ISP is the result, not the cause, of their impaired differentiation into DP thymocytes.

Consequently, we wished to assess the effect of IL-7R signaling on transcription factor expression in terminally differentiated T cells. As TCF-1 and LEF-1 are expressed in both immature thymocytes and mature T cells, we examined the effect of IL-7R signaling on TCF-1 and LEF-1 expression in normal LNTs from nontransgenic mice, all of which express endogenously encoded IL-7R's on their surface. It should be appreciated that, because IL-7 provides critical survival signals for T cells in vivo, freshly explanted T cells would likely have been recently signaled by in vivo IL-7. We purified LNTs from WT mice and either cultured them overnight in medium (to allow them to recover from potential in vivo signals) or overnight in IL-7. Real-time PCR analysis showed that both TCF-1 and LEF-1 RNA levels were relatively low in freshly explanted T cells, but were both markedly up-regulated during overnight culture in medium. Importantly, the marked up-reg-

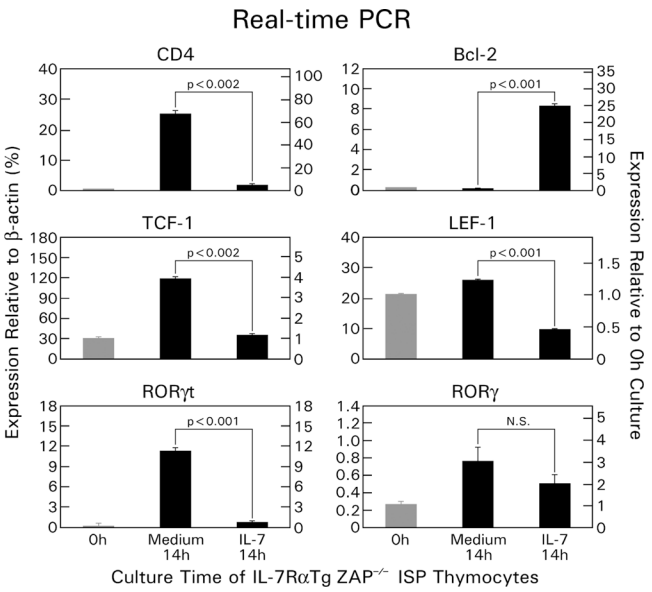

Figure 3. Effect of IL-7R signaling on transcriptional regulators of the ISP to DP transition. ISP were purified from IL-7RαTg ZAP70$^{-/-}$ mice by electronic sorting and were cultured in medium or with 60 ng/ml IL-7. Total RNAs from ISP at 0 h, after a 14-h culture in medium, or after a 14-h culture in IL-7 were reverse transcribed into cDNA and subjected to quantitative real-time PCR. mRNA expression levels were determined either relative to β-actin as an internal control (left, y axes) or relative to fresh ISP at 0 h of culture (right, y axes). The values are from two individual experiments. Quantitative differences in gene expression levels were subjected to the two-tailed Student's *t* test and p-values are indicated. Differences were considered NS when $P > 0.01$.

ulation of TCF-1 and LEF-1 RNA in overnight culture was significantly inhibited by IL-7 (Fig. 4 A). Conversely, Bcl-2 mRNA levels, which remained low in T cells after culture in medium, were dramatically up-regulated by addition of IL-7 (Fig. 4 A), demonstrating that LNTs were indeed responsive to IL-7R signals.

To confirm these real-time PCR results, we also performed Northern blot analyses for TCF-1 gene expression (Fig. 4 B). Northern blot analysis revealed that TCF-1 RNA expression was low in freshly explanted T cells, doubled during overnight culture in medium, but was prevented from increasing by addition of IL-7 to the overnight culture (Fig. 4 B). These Northern blot results paralleled those obtained with real-time PCR, and indicate that IL-7R signals can inhibit TCF-1 gene expression. To determine if IL-7R signals can reduce TCF-1 gene expression after it has been up-regulated, purified CD4 and CD8 LNTs were first released from in vivo suppressive signals and allowed to up-regulate TCF-1 expression by overnight culture in medium, and signaled with IL-7 for 6 h. In both T cell subsets, IL-7 significantly down-regulated TCF-1 mRNA levels even as it up-regulated expression of the prosurvival gene Mcl-1 (Fig. 4 C and reference 27). Finally, to document that IL-7 induced changes in TCF-1 gene expression resulted in changes in TCF-1 protein expression, we performed protein immunoblots for TCF-1 in IL-7 signaled T cells. In precise accord with changes in TCF-1 gene expression, we found that TCF-1 protein levels were up-regulated in T cells after overnight culture in medium, and this up-regulation of TCF-1 protein was inhibited by IL-7 (Fig. 4 D).

The present work has demonstrated that the transcription factors TCF-1 and LEF-1 are downstream targets of IL-7R signaling in both mature T cells and developing thymocytes, and that the transcription factor RORγt is a downstream target of IL-7R signaling in immature thymocytes. We found that IL-7R signals in IL-7R$^+$ ISP from IL-7RαTg mice inhibited their differentiation into DP thymocytes and inhibited their expression of transcription factors critical for the ISP to DP transition: TCF-1, LEF-1, and RORγt. Indeed, inhibition of TCF-1, LEF-1, and RORγt expression by IL-7R signals is sufficient to account for IL-7's inhibitory affect on the ISP to DP transition in IL-7RαTg thymocytes, although cause and effect was not directly demonstrated in the present work. In addition, inhibition of TCF-1 and LEF-1 expression by IL-7 is sufficient to explain the partial inhibitory effect of IL-7 on differentiation of DN3 thymocytes, as TCF-1/LEF-1 deficiency also results in partial inhibition of DN3 thymocyte differentiation (18). Notably, IL-7's inhibition of the ISP to DP transition is not due to impaired cell survival as IL-7R signals up-regulate expression of the prosurvival gene Bcl-2, a conclusion that is consistent with previous findings that TCF-1 and RORγt regulate the ISP to DP transition independently of promoting cell survival (19, 21). The molecular targets of TCF-1, LEF-1, and RORγt transcription factors during the ISP to DP differentiation are not yet known, although TCF-1/LEF-1 deficiency has no apparent affects on pre-TCR signaling (18).

It is important to emphasize that IL-7R signals also inhibited TCF-1 and LEF-1 gene expression in mature LNTs from normal nontransgenic mice. In fact, TCF-1 and LEF-1 gene expression in normal LNTs immediately increased upon removal of T cells from their in vivo environment, suggesting that expression of these transcription factors in T cells is down-regulated by in vivo IL-7 signals that are required for T cell homeostasis and T cell survival (28); and the immediate increase in TCF-1 and LEF-1 gene expression in ex vivo T cells was inhibited by exposure to IL-7. IL-7 not only down-regulated TCF-1 gene expression, but also down-regulated TCF-1 protein expression in LNTs. Indeed, negative regulation of TCF-1 and LEF-1 expression by in vivo IL-7 signals may play an important role in normal T cell homeostasis, as overactivity of the Wnt-β–catenin–TCF–LEF pathway can result in tumorigenesis (29).

Finally, we would like to point out that termination of IL-7Rα signals in ISP during normal thymocyte differentiation is likely to be achieved in two complementary ways: (a) by termination of endogenous IL-7Rα expression, and (b) by the local absence of IL-7 where ISP are located in the thymus. Recent findings demonstrate that IL-7–producing cells are present in the subcapsular and medullary areas of the thymus, but are absent from the thymic cortex (30).

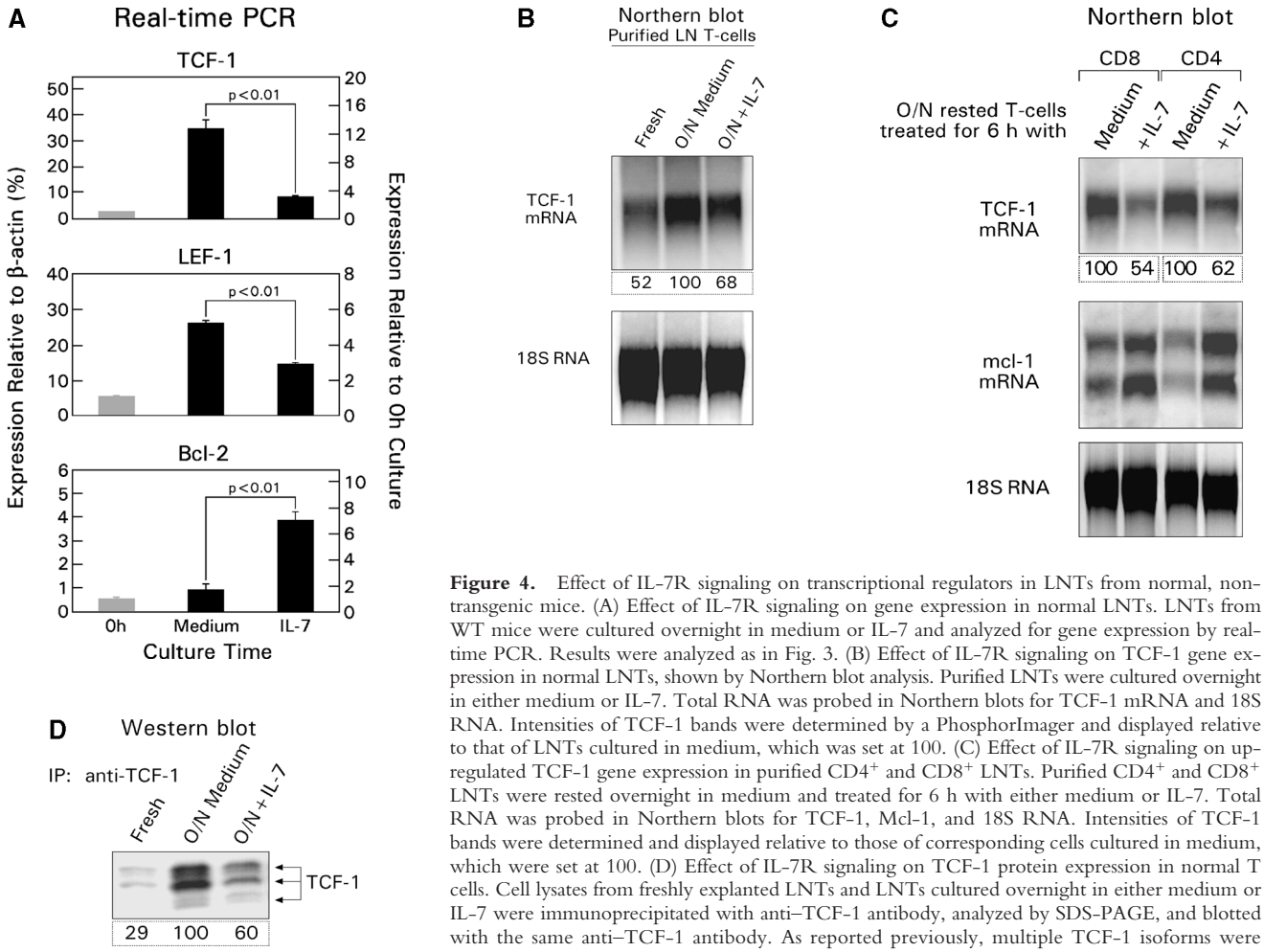

Figure 4. Effect of IL-7R signaling on transcriptional regulators in LNTs from normal, nontransgenic mice. (A) Effect of IL-7R signaling on gene expression in normal LNTs. LNTs from WT mice were cultured overnight in medium or IL-7 and analyzed for gene expression by real-time PCR. Results were analyzed as in Fig. 3. (B) Effect of IL-7R signaling on TCF-1 gene expression in normal LNTs, shown by Northern blot analysis. Purified LNTs were cultured overnight in either medium or IL-7. Total RNA was probed in Northern blots for TCF-1 mRNA and 18S RNA. Intensities of TCF-1 bands were determined by a PhosphorImager and displayed relative to that of LNTs cultured in medium, which was set at 100. (C) Effect of IL-7R signaling on up-regulated TCF-1 gene expression in purified $CD4^+$ and $CD8^+$ LNTs. Purified $CD4^+$ and $CD8^+$ LNTs were rested overnight in medium and treated for 6 h with either medium or IL-7. Total RNA was probed in Northern blots for TCF-1, Mcl-1, and 18S RNA. Intensities of TCF-1 bands were determined and displayed relative to those of corresponding cells cultured in medium, which were set at 100. (D) Effect of IL-7R signaling on TCF-1 protein expression in normal T cells. Cell lysates from freshly explanted LNTs and LNTs cultured overnight in either medium or IL-7 were immunoprecipitated with anti–TCF-1 antibody, analyzed by SDS-PAGE, and blotted with the same anti–TCF-1 antibody. As reported previously, multiple TCF-1 isoforms were detected (reference 21). The intensities of multiple TCF-1 bands in each lane were added together to determine total band intensity for each lane, and total band intensity for each lane were expressed relative to that of LNTs cultured overnight in medium, which was set at 100.

In conclusion, the present work identifies several transcription factors that are critical for the ISP to DP transition as downstream targets of IL-7R signaling. As a result, the present findings reveal that IL-7R signaling, which is critical for early thymocyte survival and differentiation, must be terminated by the ISP stage of thymocyte development for differentiation into DP thymocytes to proceed.

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