Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection

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Death by neglect requires that $CD4^+8^+$ double-positive (DP) thymocytes avoid cytokine-mediated survival signals, which is presumably why DP thymocytes normally extinguish IL-7R gene expression. We report that DP thymocytes before positive selection (preselection DP thymocytes) fail to transduce IL-7 signals even when they express high levels of transgenic IL-7R on their surface, because IL-7R signal transduction is actively suppressed in preselection DP thymocytes by suppressor of cytokine signaling (SOCS)–1. SOCS-1 is highly expressed in preselection DP thymocytes, but it is down-regulated by T cell receptor–mediated positive selection signals. Interestingly, we found that the uniquely small cell volume of DP thymocytes is largely the result of absent IL-7 signaling in preselection DP thymocytes. We also report that, contrary to current concepts, preselection DP thymocytes express high levels of endogenously encoded IL-4Rs. However, their ability to transduce cytokine signals is similarly suppressed by SOCS-1. Thus, despite high surface expression of transgenic or endogenous cytokine receptors, cytokine signal transduction is actively suppressed in preselection DP thymocytes until it is restored by positive selection.

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Abbreviations used: DP, double positive; EMSA, electrophoretic mobility shift assay; γ_c, common gamma chain; LNT cell, lymph node T cell; p-Stat5, phospho-Stat5; SP, single positive; SOCS, suppressor of cytokine signaling; TFU, total fluorescence unit.

Development and survival of T cells in the thymus depend on signals transduced by both TCRs and cytokine receptors, especially cytokine receptors specific for IL-7 (1–5). Although signaling by TCRs is known to be critical for thymocyte development, it is now appreciated that signaling by prosurvival cytokines, especially IL-7, also contributes to various stages of thymocyte development. In early thymocytes that have not yet reached the double-positive (DP) stage of differentiation, IL-7 signaling opens the TCRγ gene locus, induces expression of prosurvival factors, and promotes cell proliferation (6–13). In thymocytes later than the DP stage of differentiation, IL-7 induces survival factors required by mature T cells in both the thymus and periphery (14–16). And it has been recently appreciated that IL-7 signaling plays an important role during positive selection, as positively selected thymocytes up-regulate IL-7Rα expression and receive IL-7 signals that promote their survival and maturation into $CD8^+$ T cells (17). Thus, IL-7 signaling is important for survival and development of thymocytes before and immediately after the DP stage of differentiation.

In contrast, preselection DP thymocytes have terminated IL-7Rα gene expression and are impaired in their ability to be signaled by IL-7 (9, 18–21). In fact, DP thymocytes are thought to express few, if any, receptors for IL-7 or for any other prosurvival cytokine, although they do express the common gamma chain (γ_c) that is unable by itself to initiate cytokine signaling (22, 23). It has also been suggested that the thymic cortex where DP thymocytes reside is relatively deficient in IL-7–producing cells (24). One important consequence of deficient cytokine signaling in DP thymocytes is that, unlike other thymocytes, DP thymocytes do not express the prosurvival factor Bcl-2 and the trophic factor glucose transporter–1 (17, 25, 26) and are thus destined to die in the thymic cortex without TCR-mediated positive selection signals. The ability of unsignaled DP thymocytes

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to die of neglect ensures that only DP thymocytes with appropriate TCR specificities to undergo positive selection can continue to survive and differentiate. Thus, it is important for appropriate repertoire selection in the thymus that DP thymocytes avoid signals from IL-7 and other prosurvival cytokines.

We initiated this study to examine the concept that prosurvival signals transduced by cytokine receptors on DP thymocytes might perturb thymocyte selection and development. Consequently, we examined thymocyte development in IL-7Rα transgenic mice whose preselection DP thymocytes do not down-regulate transgenic IL-7Rα expression. Despite high surface expression of transgenic IL-7Rs, we found that IL-7R$^+$ DP thymocytes were unable to transduce cytokine signals even in response to exogenously added IL-7. We found that signal transduction by highly expressed IL-7Rs was actively suppressed in preselection DP thymocytes by suppressor of cytokine signaling (SOCS)–1 and was reversed by TCR signaling. Because it seemed unlikely that a sophisticated mechanism would exist in DP thymocytes solely to suppress IL-7 signaling when DP thymocytes did not express surface IL-7Rs at more than barely detectable levels, we assessed normal DP thymocytes for expression of surface receptors to other prosurvival cytokines. Remarkably, we found that normal preselection DP thymocytes do express high surface levels of endogenous IL-4Rs but that their ability to transduce IL-4 signals was similarly suppressed by SOCS-1. Consequently, this paper demonstrates that DP thymocytes do express high surface levels of endogenous receptors for at least one prosurvival cytokine (IL-4) but also that transduction of prosurvival cytokine signals is actively suppressed in preselection DP thymocytes that have not received TCR-mediated positive selection signals.

RESULTS

Impaired IL-7R signal transduction in IL-7R$^+$ DP thymocytes

Unlike other thymocytes that actively express genes encoding receptors for IL-7 and other prosurvival cytokines, preselection DP thymocytes extinguish expression of genes encoding receptors for IL-7 and presumably for other prosurvival cytokines as well. Indeed, Northern blot analysis revealed that preselection DP thymocytes are devoid of IL-7Rα mRNA, although they do express mRNA encoding the common cytokine receptor γ_c, which is the other component of the IL-7R complex (Fig. 1 A). Consequently, to assess cytokine signal transduction in DP thymocytes, we used mice expressing an IL-7Rα transgene whose DP thymocytes express transgenically encoded IL-7Rα proteins on their surface (Fig. 1 B) (21). In IL-7Rα transgenic (IL-7RαTg) mice, preselection DP thymocytes express the same high-surface IL-7Rα level as other thymocytes (Fig. 1 B) and peripheral T cells (not depicted). Because DP thymocytes constitutively express endogenously encoded γ_c (Fig. 1), DP thymocytes from IL-7RαTg mice expressed the two IL-7R components (IL-7Rα and γ_c) that are required to initiate IL-7 signal transduction. Nevertheless, analysis of IL-7RαTg thymocytes provided no indication that preselection IL-7R$^+$ DP thymocytes had been signaled by IL-7 in vivo, as their differentiation into single-positive (SP) T cells was identical to that in WT B6

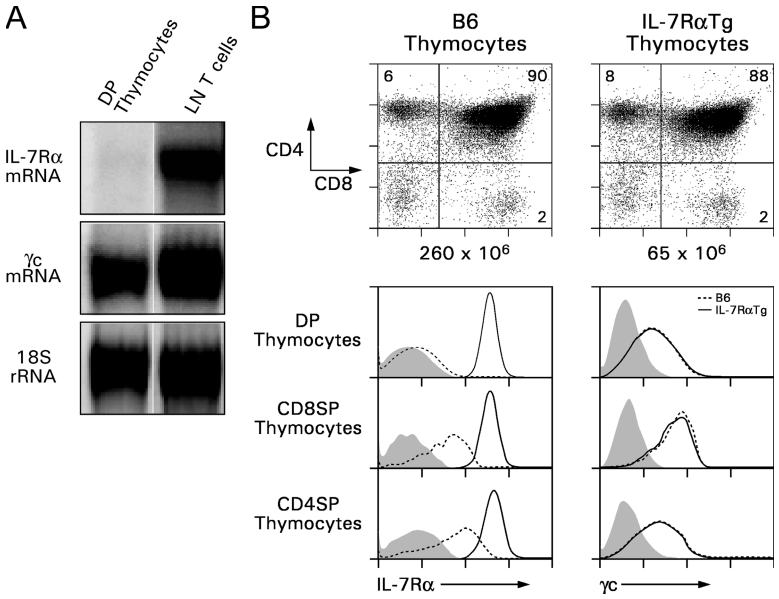

Figure 1. Expression of IL-7Rα and γ_c in thymocytes. (A) Purified DP thymocytes and LN T cells from B6 mice were analyzed for IL-7Rα and γ_c mRNA. 18S rRNA is shown as a loading control. (B) Thymocytes from B6 mice and IL-7RαTg mice were stained for CD4, CD8, and either IL-7Rα or γ_c. Total thymocyte numbers are indicated below the CD4/CD8 dot plots. In histograms, IL-7RαTg thymocytes (continuous line), B6 thymocytes (dashed line), and control staining (shaded histogram) are shown. The numbers in the quadrants indicate the frequency of cells falling into that quadrant. Data are representative of five independent experiments.

mice (Fig.1 B) (21). Indeed, the only observable impact of transgenic IL-7Rα expression was a reduction in overall thymocyte numbers (Fig. 1 B) that has been ascribed to arrested development of double-negative thymocytes before the DP stage of development (27). One explanation for absent IL-7 signaling in IL-7RαTg DP thymocytes is the relative deficiency of IL-7–producing cells in the thymic cortex where DP thymocytes reside (24). However, we also considered that cytokine signal transduction might be impaired in IL-7RαTg DP thymocytes despite their high expression of surface IL-7Rs.

Because IL-7 is a prosurvival cytokine, we first assessed the ability of exogenous IL-7 to promote survival of preselection DP thymocytes. We placed DP thymocytes from IL-7RαTg and B6 mice in single cell suspension cultures in the presence of exogenous IL-7 and assessed survival over time (Fig. 2 A). Exogenous IL-7 failed to promote the in vitro survival of either DP population, including IL-7R$^+$ DP thymocytes from IL-7RαTg mice (Fig. 2 A, left), although exogenous IL-7 did dramatically enhance the in vitro survival of lymph node T cells (LNT cells) from IL-7RαTg mice (Fig. 2 A, right). To understand IL-7's failure to enhance the survival of IL-7R$^+$ DP thymocytes, we examined IL-7's ability to up-regulate expression of the anti-apoptotic protein Bcl-2. Thymocytes and T cells from IL-7RαTg and B6 mice were cultured for 18 h with and without IL-7 and then stained for intracellular Bcl-2 protein (Fig. 2 B, right). IL-7 did not up-regulate Bcl-2 protein expression in normal B6 DP thymocytes, as these cells expressed few if any IL-7Rs (Fig. 2 B, right). However, IL-7 did up-regulate Bcl-2 protein expression in IL-7R$^+$ DP thymocytes from IL-7RαTg mice, but the levels of Bcl-2 protein expression in DP thymocytes were only ~10% of those induced in either CD8SP thymocytes or CD8 LNT cells (Fig. 2 B, right). Thus, IL-7R$^+$ DP thymocytes could be signaled by IL-7 to up-regulate Bcl-2 protein expression, but their response to IL-7 was considerably blunted relative to CD8SP thymocytes and CD8 LNT cells and was insufficient to promote cell survival.

To examine proximal IL-7R signaling events, we assessed IL-7–induced Stat5 phosphorylation by briefly exposing cells to exogenous IL-7 for 20 min and then staining for intracellular phospho-Stat5 (p-Stat5; Fig. 2 B, left). Because they are deficient in surface IL-7Rs, IL-7 did not induce significant p-Stat5 in B6 DP thymocytes (Fig. 2 B, left). Importantly, however, IL-7 did induce p-Stat5 in IL-7R$^+$ DP thymocytes from IL-7RαTg mice, but the amount of p-Stat5 induced in IL-7R$^+$ DP thymocytes was only 10–25% of that induced by IL-7 in CD8SP thymocytes and CD8 LNT cells (Fig. 2 B, left). Because the diminished amount of p-Stat5 induced in DP thymocytes might have been caused by less Stat5 protein in DP thymocytes than in mature T cells, we examined the amount of Stat5 protein present in DP and LNT cells purified from IL-7RαTg mice by protein immunoblotting (Fig. 2 C). In fact, protein immunoblotting revealed that the amounts of Stat5 protein in DP and LNT cells were essentially identical even though the p-Stat5 induced by IL-7 in DP thymocytes was <25% of that induced in LNT cells (Fig. 2 C). Thus, IL-7 is substantially less efficient in inducing Stat5 phosphorylation in IL-7R$^+$ DP thymocytes than in CD8SP thymocytes and LNT cells.

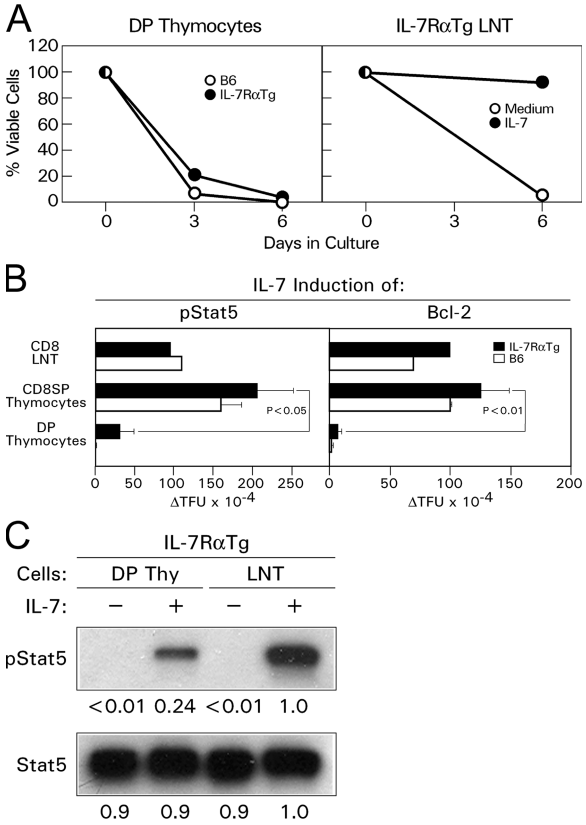

Figure 2. IL-7R signaling in DP thymocytes. (A) Purified DP thymocytes from B6 mice or IL-7RαTg mice were cultured with 6 ng/ml IL-7 and assessed for viable cell numbers by trypan blue exclusion on days 3 and 6 (left). In parallel, purified LNT cells from IL-7RαTg mice were cultured with either medium or 6 ng/ml IL-7 (right). Viable cell numbers were normalized to culture day 0, which was set at 100%. Data are representative of three independent experiments. (B) Thymocytes or purified LNT cells from B6 or IL-7RαTg mice were stimulated with medium or 6 ng/ml IL-7 and stained for CD4, CD8, intracellular p-Stat5, or Bcl-2 to assess p-Stat5 and Bcl-2 expression levels in different cell populations. For p-Stat5, cells were stimulated for 20 min; for Bcl-2, cells were stimulated for 18 h. Levels of p-Stat5 and Bcl-2 were quantified into linear TFU so that expression levels could be directly compared between samples. △TFU were obtained by subtracting fluorescence levels in cells cultured in medium from those cultured in IL-7. Data are representative of seven independent experiments. Values represent means ± SEM. (C) DP thymocytes were purified from IL-7RαTg mice by electronic sorting. DP thymocytes and LNT cells were stimulated with IL-7 for 20 min, lysed, analyzed by SDS-PAGE, and blotted with antibodies specific for either p-Stat5 or Stat5. Band intensity for each lane was expressed relative to that of LNT cells stimulated with IL-7, which was set at 1. Data are representative of three independent experiments.

Having determined that IL-7 induced less Stat5 phosphorylation in IL-7R$^+$ DP thymocytes than other cell types, we considered the possibility that p-Stat5 in DP thymocytes

might be further impaired in translocating from cytosol to nucleus or that p-Stat5 in DP thymocytes might be inhibited in some other way from binding to Stat5 target DNA sequences. Consequently, we assessed nuclear extracts from IL-7–stimulated DP and LNT cells for nuclear proteins able to bind Stat5 target DNA sequences. We did this by performing electrophoretic mobility shift assays (EMSAs) with a labeled Stat5/6 consensus oligonucleotide (Fig. 3). Nuclear extracts from unstimulated DP and LNT cells failed to bind to the labeled oligonucleotide (Fig. 3, lanes 1 and 2). However, nuclear extracts from IL-7–stimulated DP and LNT cells both bound to the labeled oligonucleotide, indicating that IL-7 had induced Stat5 nuclear translocation in both cell types (Fig. 3, lanes 3 and 7). The protein–DNA complexes formed were specifically dependent on Stat5 target DNA sequences, as protein binding to the labeled oligonucleotide could be inhibited by competitor oligonucleotides with intact, but not mutated, Stat5 target binding sites (Fig. 3, lanes 3–5 and 7–9). Moreover, the protein–DNA complexes contained Stat5 proteins as they were supershifted by anti-Stat5 antibody (Fig. 3, lanes 6 and 10). And, finally, it was evident from their relative band intensities that nuclear extracts from IL-7–stimulated DP thymocytes bound quantitatively less labeled oligonucleotide than nuclear extracts from IL-7–stimulated LNT cells (Fig. 3, compare lane 3 with 7), commensurate with quantitatively less p-Stat5 induced by IL-7 stimulation of DP versus LNT cells. These data reveal that p-Stat5 induced in IL-7–signaled DP thymocytes does translocate to the nucleus and bind to Stat5 target DNA sequences. Collectively, these data demonstrate that proximal IL-7R signal transduction is impaired in IL-7R$^+$ DP thymocytes as revealed by inefficient Stat5 phosphorylation.

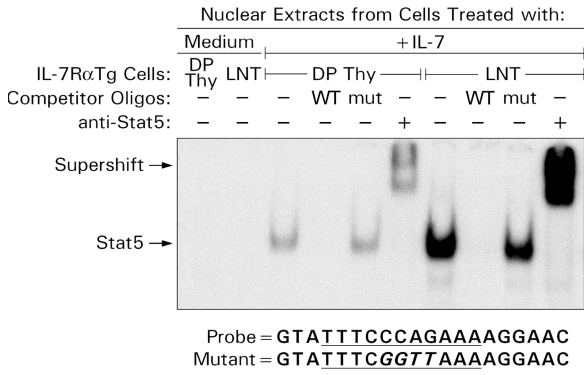

Figure 3. IL-7–induced p-Stat5 DNA binding activity in DP thymocytes and LNT cells. DP thymocytes were purified from IL-7RαTg mice by electronic sorting. DP thymocytes and LNT cells were stimulated with IL-7 for 20 min, nuclear extracts were made, and p-Stat5 DNA binding activity was determined by EMSA. Where indicated, 50× cold competitor oligos (WT or mutant) and 1 μl anti-Stat5 antibody were added to the reaction. Sequences of the Stat5/6-labeled probe and mutant competitor oligonucleotide are indicated at the bottom of the figure; italicized letters represent nucleotides that are mutated from the WT consensus binding sequence. Data are representative of two independent experiments.

Role of SOCS-1

Because SOCS-1 is known to inhibit phosphorylation of Stat proteins in cytokine-stimulated T cells (28–30) and SOCS-1 is constitutively expressed in preselection DP thymocytes (31), inefficient Stat-5 phosphorylation in IL-7R$^+$ DP thymocytes was likely caused by SOCS-1. Consistent with previous observations (31), quantitative real-time RT-PCR revealed that DP thymocytes contained 10× the number of SOCS-1 transcripts as unstimulated LNT cells (Fig. 4 A). To determine whether constitutive expression of SOCS-1 was responsible for impaired IL-7 signaling in DP thymocytes, we bred the IL-7RαTg into SOCS-1$^{-/-}$ mice (which were necessarily also IFN-γ$^{-/-}$ to survive SOCS-1 deficiency) (32, 33), generating three strains of mice that differed in their number of SOCS-1 alleles: IL-7RαTgSOCS-1$^{+/+}$, IL-7RαTgSOCS-1$^{+/-}$, and IL-7RαTgSOCS-1$^{-/-}$ (all of which were selected to be IFN-γ$^{-/-}$, as well). DP thymocytes from these mice contained graded levels of SOCS-1 transcripts, with DP thymocytes from SOCS-1$^{+/+}$ mice containing twice the number of SOCS-1 transcripts as DP thymocytes from SOCS-1$^{+/-}$ mice (Fig. 4 B). Thymocyte profiles of these mice are shown (Fig. 4 C). We stimulated thymocytes from these three mouse strains with either medium or IL-7 for 1 h and stained the cells for intracellular p-Stat5 (Fig. 4 D, left). Indeed, the amount of p-Stat5 induced by IL-7 in DP thymocytes increased in a dose-dependent fashion as SOCS-1 expression decreased, with the highest p-Stat5 levels in DP thymocytes from SOCS-1$^{-/-}$ mice (Fig. 4 D, left). Thus, SOCS-1 expression clearly interferes with p-Stat5 induction in IL-7R$^+$ DP thymocytes. Even so, complete removal of SOCS-1 did not completely restore IL-7 signaling in IL-7R$^+$ DP thymocytes, as p-Stat5 levels in cells from SOCS-1$^{-/-}$ mice were still quantitatively lower in DP than CD8SP thymocytes (note different scales for DP and CD8SP thymocytes; Fig. 4 D, left). It might be noted that, unlike SOCS-1's inhibitory effect in DP thymocytes, p-Stat5 and Bcl-2 levels in CD8SP thymocytes were unaffected by the presence or absence of SOCS-1 (Fig. 4 D, left), which was consistent with reduced constitutive SOCS-1 expression in postselection T cells relative to preselection DP thymocytes (31, 33).

We also assessed the effect of SOCS-1 on up-regulation of a downstream target of IL-7 signaling, Bcl-2. We cultured thymocytes from IL-7RαTg SOCS-1$^{+/+}$, IL-7RαTg SOCS-1$^{+/-}$, and IL-7RαTg SOCS-1$^{-/-}$ mice for 18 h with either medium or IL-7 and stained the cells for intracellular Bcl-2 protein (Fig. 4 D, right). In parallel with p-Stat5, Bcl-2 was up-regulated in DP thymocytes in a dose-dependent fashion as SOCS-1 expression decreased, with maximal Bcl-2 expression in DP thymocytes from SOCS-1$^{-/-}$ mice (Fig. 4 D, right). As with p-Stat5, Bcl-2 up-regulation in DP thymocytes from SOCS-1$^{-/-}$ mice that were devoid of SOCS-1 was still lower in DP than CD8SP thymocytes (Fig. 4 D, right).

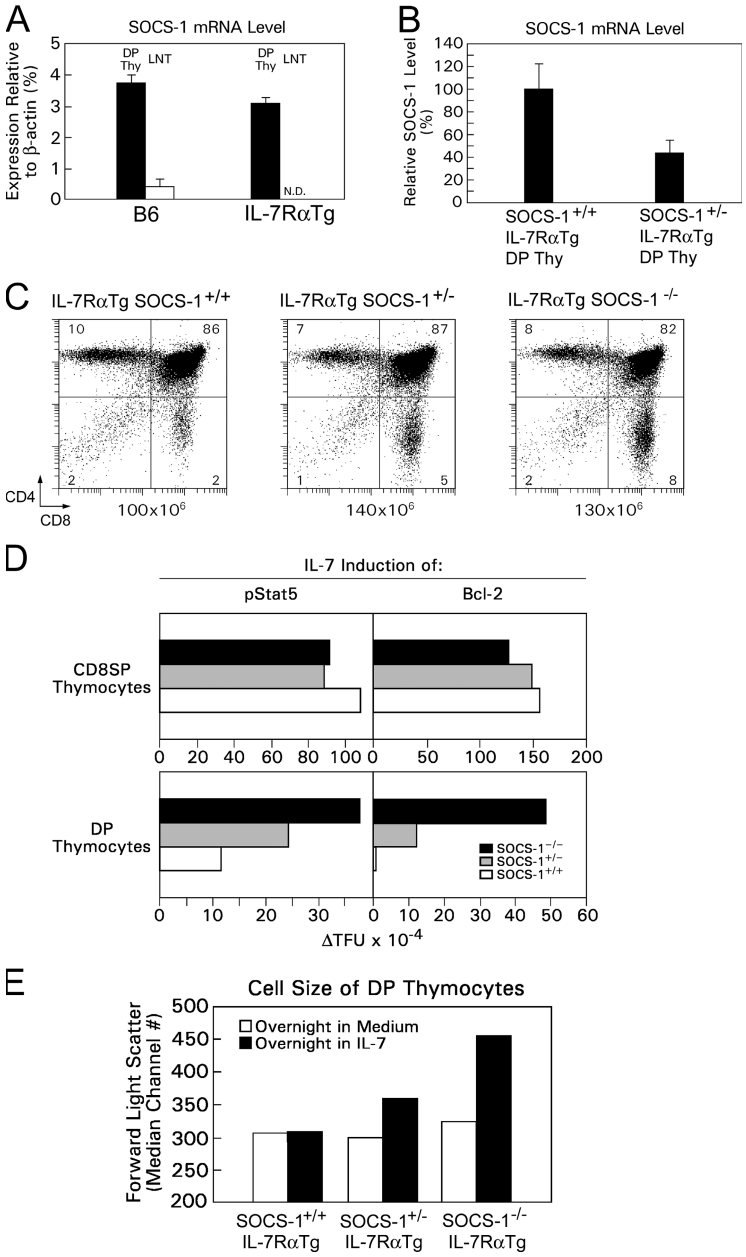

Figure 4. SOCS-1 inhibits IL-7R signaling in DP thymocytes. (A) Total RNAs from purified DP thymocytes and LNT cells were subjected to quantitative real-time RT-PCR for SOCS-1 expression. mRNA expression levels of SOCS-1 were determined relative to β-actin as an internal control. N.D., not done. (B) Total RNAs from purified DP thymocytes from SOCS-$1^{+/+}$ or SOCS-$1^{+/-}$ mice were subjected to real-time RT-PCR for SOCS-1 expression. mRNA expression levels of SOCS-1 were determined relative to β-actin and normalized to SOCS-$1^{+/+}$ DP thymocytes, which was set at 100%. Values in A and B represent means ± SEM. (C) Thymocyte profiles of IL-7RαTgSOCS-$1^{+/+}$, IL-7RαTgSOCS-$1^{+/-}$, and IL-7RαTgSOCS-$1^{-/-}$ mice. Thymocytes were assessed for CD4 versus CD8 surface expression. Total thymocyte numbers are comparable, but CD8SP thymocyte numbers increase as SOCS-1 expression decreases, as previously reported (31). (D) Thymocytes from IL-7RαTgSOCS-$1^{+/+}$, IL-7RαTgSOCS-$1^{+/-}$, or IL-7RαTgSOCS-$1^{-/-}$ mice were stimulated with either medium or IL-7 and stained for intracellular p-Stat5 or Bcl-2. For p-Stat5, cells were stimulated for 20 min; for Bcl-2, cells were stimulated for 18 h. Levels of p-Stat5 and Bcl-2 were quantified into linear TFU so that expression levels could be directly compared between samples. △TFU were obtained by subtracting fluorescence levels in cells cultured in medium from those cultured in IL-7. Data are representative of three independent experiments. (E) Thymocytes from IL-7RαTgSOCS-$1^{+/+}$, IL-7RαTgSOCS-$1^{+/-}$, or IL-7RαTgSOCS-$1^{-/-}$ mice were cultured for 18 h in vitro in either medium or IL-7. Median channel numbers for forward light scatter (FSC) of DP thymocytes were determined by flow cytometry. Data are representative of three independent experiments.

IL-7 signaling not only activates Stat5 but also activates PI3 kinase, which is responsible for IL-7–induced increases in cell size (34). Because SOCS-1 directly binds to JAKs and inhibits all further downstream signaling events, we reasoned that constitutive SOCS-1 expression would also prevent IL-7 from signaling an increase in the cell size of IL-7RαTg DP thymocytes. Indeed, overnight stimulation with IL-7 failed to signal IL-7R$^+$ DP thymocytes from IL-7RαTg SOCS-1$^{+/+}$ mice to increase cell size (Fig. 4 E). In contrast, IL-7R$^+$ DP thymocytes with decreased SOCS-1 expression responded to IL-7 by increasing their cell size, as IL-7 induced the forward light scatter of DP thymocytes from IL-7RαTg SOCS-1$^{-/-}$ mice to increase by ~50% (Fig. 4 E). Thus, constitutive SOCS-1 expression contributed to suppression of IL-7–mediated PI3 kinase activation in DP thymocytes. Interestingly, these data also reveal that the uniquely small cell volume of DP thymocytes is due, at least in part, to absent IL-7 signaling. We conclude that high SOCS-1 expression impairs IL-7 signal transduction in preselection DP thymocytes, although other factors also contribute.

Role of TCR signals

During differentiation of normal DP thymocytes into mature T cells, IL-7Rα expression is up-regulated and IL-7 responsiveness is restored (17, 19). Consequently, we considered that TCR-mediated positive selection signals in DP thymocytes might down-regulate SOCS-1 gene expression and improve IL-7 signaling. To examine the effect of TCR signaling on SOCS-1 expression, we placed purified DP thymocytes from B6 and IL-7RαTg mice in culture overnight with immobilized anti-TCR and anti-CD2 mAbs and determined their SOCS-1 mRNA levels by quantitative RT-PCR. Indeed, TCR signaling reduced SOCS-1 gene expression in DP thymocytes from both B6 and IL-7RαTg mice (Fig. 5 A). Importantly, this experiment necessarily underestimates the amount that SOCS-1 gene expression is actually reduced by TCR signaling of DP thymocytes, as 30% of DP thymocytes do not express surface TCR complexes and so would not have received any TCR signals at all (Fig. 5 A). Consequently, the actual level of SOCS-1 mRNA in DP thymocytes that could have received TCR signals in this experiment would only be 10% of starting levels, a 90% reduction.

To determine whether TCR signaling improved IL-7 signaling in DP thymocytes, we initially prestimulated IL-7RαTg ZAP70$^{-/-}$ DP thymocytes for 3 h with the pharmacologic mimic of TCR signaling, PMA, and ionomycin (P+I) and then assessed their subsequent ability to respond to IL-7 by up-regulating expression of Bcl-2 mRNA and Bcl-2 protein. Because in vitro P+I stimulation for <12 h is insufficient to signal positive selection (17, 19), prestimulation of DP thymocytes with P+I for 3 h in our present experiment did not itself up-regulate either Bcl-2 mRNA or Bcl-2 protein (Fig. 5 B). However, prestimulation with P+I substantially augmented the ability of DP thymocytes to subsequently respond to IL-7, as indicated by a 3–4-fold increase in both Bcl-2 mRNA and Bcl-2 protein expression (Fig. 5 B). Thus,

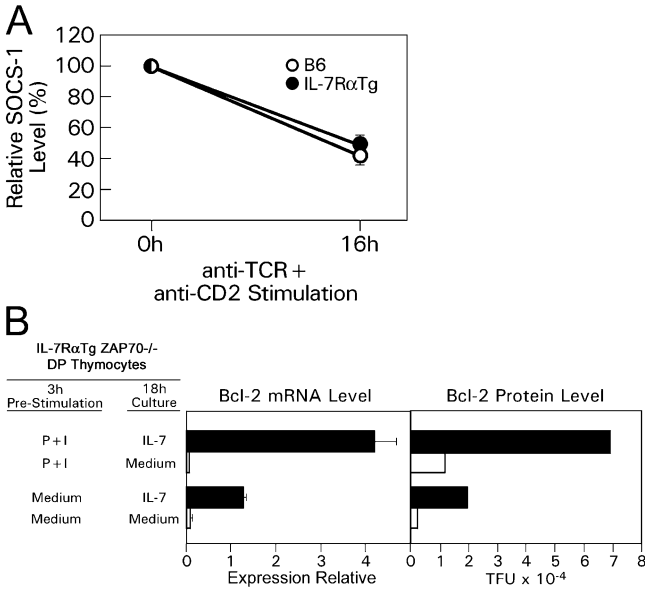

Figure 5. TCR stimulation of DP thymocytes down-regulates SOCS-1 and improves IL-7R signaling. (A) DP thymocytes from B6 or IL-7RαTg mice were stimulated in vitro with 10 μg/ml plate-bound anti-TCR and 10 μg/ml anti-CD2 for 16 h. mRNAs for SOCS-1 were examined by real-time RT-PCR. mRNA expression levels were determined relative to β-actin and were normalized to cells at 0 h of culture, which was set at 100%. (B) Purified DP thymocytes from IL-7RαTg ZAP70$^{-/-}$ mice were cultured in vitro in medium alone or stimulated with 0.3 ng/ml PMA plus 0.3 ng/ml ionomycin for 3 h and further cultured in either medium alone or with IL-7 for 18 h. mRNA levels for Bcl-2 were examined by real-time RT-PCR and determined relative to β-actin. Bcl-2 protein levels were examined by intracellular staining and quantified into linear TFU so that expression levels could be directly compared between samples. Data are representative of three independent experiments. Because ZAP70 deficiency prevents positive selection signaling, all IL-7RαTg ZAP70$^{-/-}$ DP thymocytes are preselection thymocytes. Values represent means ± SEM.

TCR signaling down-regulates SOCS-1 expression and improves IL-7 signaling in preselection DP thymocytes.

DP thymocytes that have received TCR-mediated positive selection appear in vivo as CD4$^+$8^{lo} cells, and it has previously been shown that SOCS-1 gene expression is down-regulated in CD4$^+$8^{lo} thymocytes relative to DP thymocytes (31). Consequently, we examined IL-7 induction of p-Stat5 in preselection DP and postselection CD4$^+$8^{lo} thymocytes by culturing IL-7RαTg thymocytes with either medium or IL-7 for 20 min (Fig. 6). Neither DP nor CD4$^+$8^{lo} thymocytes contained substantial amounts of p-Stat5 in the absence of IL-7, and exposure to exogenous IL-7 induced p-Stat5 in both cell populations. More importantly, IL-7 stimulation induced ~5× more p-Stat5 in postselection CD4$^+$8^{lo} than in preselection DP thymocytes (Fig. 6), demonstrating that IL-7 signaling was considerably improved in CD4$^+$8^{lo} thymocytes. We also examined Bcl-2 protein levels after 18 h of stimulation with either medium or exogenous IL-7 (Fig. 6). In parallel with the p-Stat5, IL-7 stimulation induced considerably more Bcl-2 protein in CD4$^+$8^{lo} than DP thymocytes (Fig. 6).

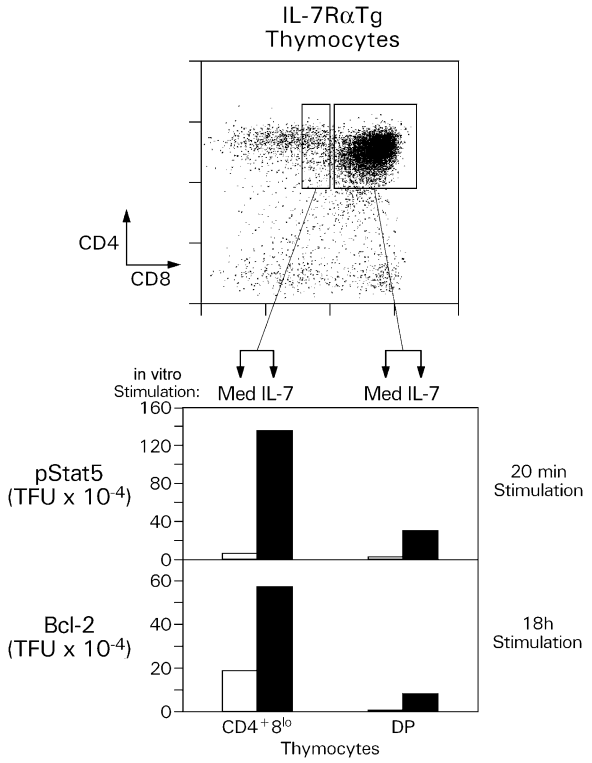

Figure 6. In vivo positive selection signals improve IL-7R signaling. Thymocytes from IL-7RαTg mice were cultured in vitro in medium alone or with IL-7 and assessed for intracellular p-Stat5 or Bcl-2. Data are representative of seven independent experiments. Levels of p-Stat5 and Bcl-2 in $CD4^+8^{lo}$, which are thymocytes that have recently received in vivo positive-selecting TCR signals, and DP thymocytes were quantified into linear TFU.

However, it is interesting to note that even without exogenous IL-7, unstimulated $CD4^+8^{lo}$ thymocytes expressed both elevated p-Stat5 and Bcl-2 protein levels relative to DP thymocytes (Fig. 6), suggesting that postselection $CD4^+8^{lo}$ thymocytes had been stimulated in vivo by intrathymic IL-7. Thus, IL-7 signal transduction is suppressed in preselection DP thymocytes by SOCS-1 and other factors but is substantially improved by TCR-mediated positive selection signaling.

DP thymocyte expression of endogenous IL-4Rs

Active suppression of cytokine signal transduction in preselection DP thymocytes by constitutive expression of SOCS-1 seemed unnecessary if preselection DP thymocytes were in fact relatively deficient in surface receptors for prosurvival cytokines, expressing mainly isolated γ_c as is currently thought. Consequently, we reconsidered the possibility that normal preselection DP thymocytes might actually express substantial quantities of surface receptors specific for some prosurvival cytokine. In fact, we found that DP thymocytes from normal B6 mice do express high levels of endogenous IL-4Rα proteins on their surface that, together with surface γ_c proteins, should form intact IL-4Rs (Fig. 7 A). Although IL-4R expression has not previously been observed on DP thymocytes, we found that expression of endogenously encoded IL-4Rα proteins on the surface of DP thymocytes was higher than on any other thymocyte population, with levels of IL-4Rα expression slightly exceeding those on CD4SP and CD8SP thymocytes (Fig. 7 A).

To confirm that DP thymocytes produced the IL-4Rα protein they expressed, we performed Northern blot analyses of purified DP thymocytes and of LNT cells from normal B6 mice (Fig. 7 B). Northern blots revealed that DP thymocytes contained mRNA for both IL-4R components, IL-4Rα and γ_c but did not detectably contain mRNA for IL-7Rα or SOCS-3 (Figs. 1 A and 7 B). In addition, these Northern blots confirmed that DP thymocytes constitutively expressed SOCS-1 mRNA, whereas unstimulated LNT cells did not (Fig. 7 B).

Finally, we assessed surface IL-4Rs for their ability to transduce signals in DP thymocytes and whether constitutive SOCS-1 expression interfered with that signaling. We used thymocytes from SOCS-$1^{+/+}$, SOCS-$1^{+/-}$, and SOCS-$1^{-/-}$ (containing 2, 1, or 0 SOCS-1 alleles), stimulated them with IL-4 for 1 h, and then stained the cells for intracellular p-Stat5 and -6, as IL-4 can induce phosphorylation of both Stat molecules (Fig. 7 C, left and center). IL-4 stimulation was unable to induce either p-Stat5 or -6 in SOCS-$1^{+/+}$ DP thymocytes despite high surface levels of IL-4Rs, whereas IL-4 induced both p-Stat molecules in CD8SP thymocytes present in the same culture wells (Fig. 7 C, left and center). Importantly, as SOCS-1 expression decreased, IL-4's ability to induce p-Stat5 and -6 in DP thymocytes increased, with SOCS-$1^{-/-}$ DP thymocytes achieving the highest levels of both p-Stat5 and -6 (Fig. 7 C, left and center). In fact, p-Stat5 and -6 levels induced by IL-4 stimulation of SOCS-$1^{-/-}$ DP thymocytes approached levels induced in CD8SP thymocytes (note different scales for DP and CD8SP thymocytes; Fig. 7 C, left and center). Continuing IL-4 stimulation for 18 h permitted us to also examine IL-4 induction of Bcl-2 protein expression (Fig. 7 C, right). In parallel with p-Stat5 and -6 induction, IL-4 failed to induce Bcl-2 protein expression in SOCS-$1^{+/+}$ DP thymocytes but induced increasing amounts of Bcl-2 protein as SOCS-1 expression decreased in DP thymocytes, with SOCS-$1^{-/-}$ DP thymocytes achieving the highest Bcl-2 protein levels (Fig. 7 C, right). These results demonstrate that DP thymocytes express endogenously encoded IL-4Rs on their surface, but their ability to transduce IL-4 signals is suppressed in DP thymocytes largely by constitutively expressed SOCS-1.

DISCUSSION

This paper has assessed cytokine signaling in DP thymocytes by both transgenic and endogenous cytokine receptors. By using an IL-7Rα transgene to circumvent the down-regulation of IL-7Rα gene expression that normally occurs in preselection DP thymocytes, this study demonstrates that IL-7R signal transduction is actively suppressed in DP thymocytes and is unable to promote DP thymocyte survival even when IL-7Rs are highly expressed on the cell surface, in large part

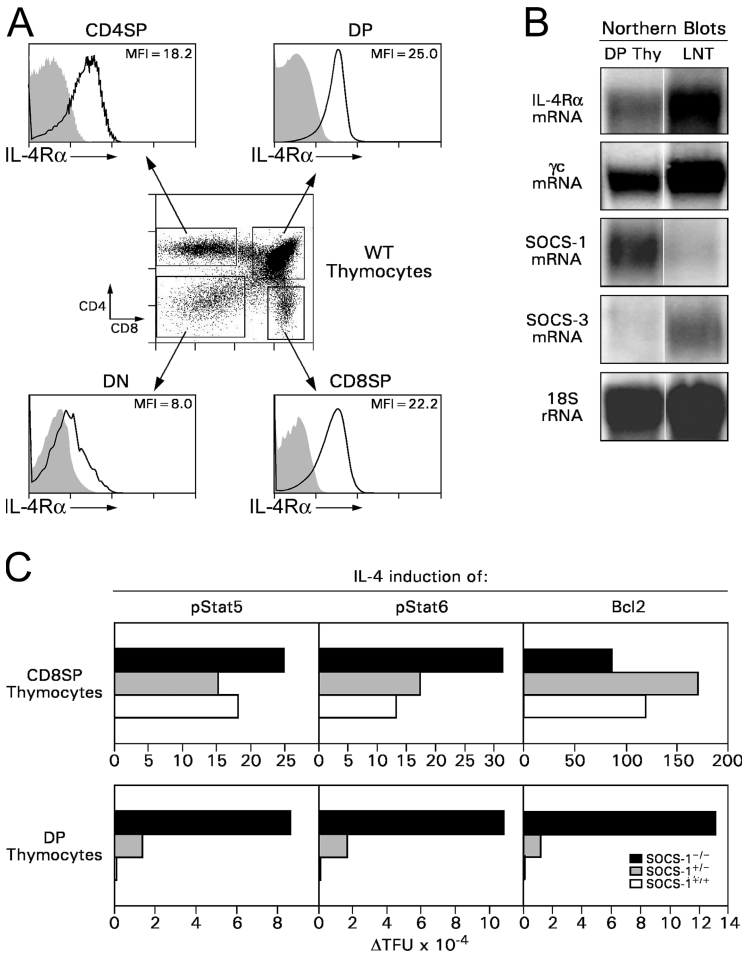

Figure 7. DP thymocytes express signaling-competent surface IL-4R complexes but are impaired in IL-4 signal transduction. (A) Freshly isolated thymocytes from B6 mice were stained for surface IL-4Rα, CD4, and CD8 expression. In histograms, IL-4Rα staining (continuous line) and control staining (shaded histogram) are shown. Levels of surface IL-4Rα expression are indicated for each subpopulation in mean fluorescence intensities (MFIs), as the particular flow cytometer used had not been calibrated for TFU. (B) DP thymocytes express mRNA for IL-4Rα, γ_c, and SOCS-1. Northern blot analysis of total RNA from purified DP thymocytes and LNT cells from nontransgenic mice show that both cell populations express IL-4R mRNAs but that only DP thymocytes concomitantly express SOCS-1. (C) Thymocytes from SOCS-$1^{+/+}$, SOCS-$1^{+/-}$, or SOCS-$1^{-/-}$ mice, which were not transgenic for IL-7Rα, were stimulated with either medium or IL-4. Intracellular p-Stat5 or -6 levels were determined after 1 h of stimulation, and Bcl-2 levels were determined after 18 h of stimulation. Levels of p-Stat5, p-Stat6, and Bcl-2 were quantified into linear TFU so that expression levels could be directly compared between samples. △TFU were obtained by subtracting fluorescence levels in cells cultured in medium from those cultured in IL-4. Data are representative of three independent experiments.

as a result of SOCS-1, which is constitutively expressed in DP thymocytes. Interestingly, poor IL-7R signal transduction in DP thymocytes is responsible for the uniquely small cell volume of these cortical thymocytes. IL-7R signal transduction in DP thymocytes is substantially restored by TCR-mediated positive selection signals that down-regulate SOCS-1 expression and enhance IL-7 induction of prosurvival molecules such as Bcl-2. Finally, this study demonstrates that normal DP thymocytes express high levels of endogenous IL-4Rs on their cell surface but that IL-4R signal transduction is similarly suppressed by SOCS-1 in DP thymocytes. Thus, DP thymocytes do express high levels of surface receptors for the prosurvival cytokine IL-4, but signal transduction by γ_c cytokine receptors is actively suppressed in preselection DP thymocytes and restored by TCR-signaled positive selection signaling.

The vast majority of DP thymocytes in the thymus fail to receive TCR-mediated positive selection signals and, thus, "die by neglect." The ability of unselected DP thymocytes to die by neglect permits thymocytes with appropriate TCR specificities to be the only ones to survive and to differentiate into functionally mature T cells. However, the ability of DP thymocytes to die by neglect requires the absence of prosurvival factors such as Bcl-2 and glucose transporter–1, which can be induced by signals from prosurvival cytokines (IL-2, -4, -6, -7, and -15) (34). As a result, death by neglect requires that DP thymocytes fail to be signaled by prosurvival cytokines.

Several mechanisms for avoiding cytokine induction of prosurvival factors in DP thymocytes have been suggested. One obvious mechanism is the down-regulation of IL-7Rα gene expression in DP thymocytes so that DP thymocytes are relatively deficient in IL-7Rα surface expression (20). However, low levels of residual IL-7Rα protein may remain on the surface of DP thymocytes, albeit in barely detectable quantities, with the possibility that some DP thymocytes might still be able to bind IL-7. A second mechanism that would reduce the possibility of IL-7 signaling in DP thymocytes is for DP thymocytes to arise from precursor cells that are themselves unable to respond to IL-7. Indeed, IL-7 signals have recently been shown to inhibit the expression of transcription factors required for differentiation of immature SPs into DP thymocytes, so that DP thymocytes only arise from immature SPs that have not been signaled by intrathymic IL-7 (21). A third mechanism for minimizing IL-7 signaling in DP thymocytes is the relative deficiency of IL-7–producing cells in the thymic cortex where DP thymocytes reside (24). Importantly, all three mechanisms only minimize and do not eliminate the possibility of IL-7 signaling, and none prevents potential signaling of DP thymocytes by other prosurvival cytokines. Indeed, because normal DP thymocytes constitutively express γ_c, it was possible that they might express receptors for prosurvival cytokines other than IL-7. In fact, we found that DP thymocytes do express endogenously encoded receptors for IL-4 and they express IL-4Rs at higher levels than any other cell population in the thymus. We do not yet know why DP thymocytes highly express IL-4Rs, especially because their ability to transduce IL-4 signals is suppressed by constitutively expressed SOCS-1. Speculatively, it may simply be that no molecular mechanism exists to dynamically regulate IL-4R gene expression during thymocyte development the way that IL-7Rα gene expression is dynamically regulated during thymocyte development, with the result that once IL-4R gene expression is turned on, IL-4R gene expression remains on in preselection DP thymocytes. By whatever mechanism, high endogenous IL-4R expression would require that DP thymocytes contain a suppressive mechanism to prevent IL-4Rs from transducing prosurvival signals that would prevent them from undergoing death by neglect.

Consequently, the inability of surface cytokine receptors to transduce signals in DP thymocytes represents a fail-safe mechanism to prevent signaling of DP thymocytes by any prosurvival cytokine, and an important component of this suppression is SOCS-1. In mature T cells, SOCS-1 expression is induced by cytokine signals as a feedback mechanism to limit further cytokine signaling (28–30, 35). In contrast, SOCS-1 expression in DP thymocytes is constitutive (31, 33). The concept of constitutive SOCS-1 expression as a fail-safe mechanism to prevent any possibility of cytokine signaling in preselection DP thymocytes is made all the more plausible by our finding that DP thymocytes do in fact highly express endogenous IL-4Rs on their surface. This concept is further supported by the fact that constitutive SOCS-1 expression in preselection DP thymocytes is down-regulated by TCR signals in vitro and by positive selection signals in vivo, so that positive selection restores cytokine signal transduction. Thus, TCR-mediated positive selection signals restore IL-7 responsiveness to DP thymocytes in two ways: by down-regulating constitutive SOCS-1 expression and by up-regulating IL-7Rα gene expression (17, 19).

This study also provides an explanation for the uniquely small cell volume of DP thymocytes. We found that IL-7R$^+$ DP thymocytes from SOCS-1$^{-/-}$ mice responded to IL-7 by increasing their cell size, suggesting that the uniquely small size of most DP thymocytes results, at least in part, from absent IL-7 signaling. From this perspective, it is interesting that DP thymocytes were identically small in IL-7R$^+$SOCS-1$^{+/+}$, IL-7R$^+$SOCS-1$^{+/-}$, and IL-7R$^+$SOCS-1$^{-/-}$ mice (see Fig. 4 E), even though the latter were able to considerably increase their cell size in response to exogenous IL-7, inferring that endogenous IL-7 was not present in sufficient amounts in the thymic cortex to increase DP thymocyte size.

SOCS-1 deficiency leads to perinatal lethality resulting from both IFN-γ and T cell–dependent inflammation and necrosis in multiple organs (32, 33), so that survival of SOCS-1$^{-/-}$ mice requires that they be additionally IFN-γ$^{-/-}$ or Rag$^{-/-}$. However, SOCS-1 conditional knockout mice have been described in which conditional deletion of SOCS-1 in T lineage cells results in improved IL-7 signaling, increased positive selection of CD8$^+$ T cells (31), and decreased positive selection of CD4$^+$ T cells in an IFN-γ–dependent manner (36). Thus, SOCS-1 regulates cytokine responsiveness and importantly affects the lineage direction of positively selected thymocytes. This study now demonstrates that SOCS-1 prevents unselected DP thymocytes from transducing cytokine-mediated survival signals.

In this paper, complete SOCS-1 deficiency did not completely restore either IL-7 signaling in transgenic IL-7R$^+$ DP thymocytes or IL-4 signaling in normal DP thymocytes, although SOCS-1 deficiency appeared to improve IL-4 signaling more than IL-7 signaling. Consequently, we think that other factors in DP thymocytes in addition to SOCS-1 also contribute to impaired cytokine signaling. It is possible that other SOCS proteins in DP thymocytes contribute to decreased cytokine responsiveness, although we found little SOCS-3 mRNA in DP thymocytes; it is possible that the somewhat lower surface expression of γ_c on DP than mature T cells may also contribute. Future studies will assess the contribution of components other than SOCS-1 to impaired cytokine signal transduction in preselection DP thymocytes.

This study documents that signal transduction by highly expressed cytokine receptors is actively suppressed in preselection DP thymocytes and that SOCS-1 is an important component of the suppressive mechanism. Our current observations with highly expressed cytokine receptors for IL-7 and IL-4 are consistent with, and substantially extend, the study by Chong et al. (31) that found that weak IL-7 signaling by the few endogenously encoded IL-7Rs present on DP thymocytes was enhanced by removal of SOCS-1. In contrast, our current observations conflict with those of Munitic et al. (27),

who observed that IL-7 signals in DP thymocytes from IL-7RαTg mice up-regulated Bcl-2 expression. Finally, we would note that the finding by van De Wiele et al. (18) that normal nontransgenic DP thymocytes were unresponsive to in vivo IL-7 as assessed by Stat-5 phosphorylation did not necessarily implicate a SOCS-1–mediated suppressive mechanism as it was simply consistent with deficient IL-7R expression on DP thymocytes.

In conclusion, DP thymocytes fail to transduce signals from highly expressed cytokine receptors, and their impairment is partly the result of SOCS-1–mediated suppression. We think that suppression of cytokine signaling in preselection DP thymocytes is necessary for death by neglect and reinforces TCR-specific repertoire selection in the thymus.

MATERIALS AND METHODS

Animals. C57BL/6 (B6) mice were obtained from the Jackson Laboratory. ZAP70$^{-/-}$ mice were bred in our own colony. The IL-7Rα–transgenic construct was made by ligating an IL-7Rα cDNA into a human CD2 (hCD2) enhancer-promoter–based vector and was injected into fertilized B6 oocytes to generate IL-7Rα transgenic mice. SOCS-1$^{+/-}$IFN-γ$^{-/-}$ mice (33) were provided by J. Ihle (St. Jude Children's Research Hospital, Memphis, TN) and were bred with IL-7Rα transgenic mice in our own colony. Animal studies were approved by the Animal Care and Use Committee of the Laboratory of Animal Sciences Program at the NCI.

Immunofluorescence and flow cytometry. Cells were harvested, stained, and analyzed on a FACSVantage SE (Becton Dickinson). Dead cells were excluded by forward light scatter gating and propidium iodide staining. Data were analyzed using software designed by the Division of Computer Research and Technology at the National Institutes of Health (NIH). Total fluorescence units (TFU) were calculated with this software using the empirically derived formula for the FACSVantage: TFU = A × $10^{([B-516.94]/250.98)}$ × 10,000, where A is the percent positive population and B is the median channel for each corresponding FITC fluorescence using 4-log amplification. Antibodies (all obtained from BD Biosciences) with the following specificities were used for staining: CD4 (GK1.5 and RM4.5); CD8α (53-6.7), TCRβ (H57-597), γ_c (4G3), p-Stat5 (clone 47), and Bcl-2 (3F11). Anti–IL-7Rα (A7R34) was obtained from eBioscience, and PE-conjugated anti–IL-4Rα (M1) was obtained from Research Diagnostics, Inc.

Cell purification. DP thymocytes were purified by panning with anti-CD8 mAb. Where indicated in the figures, DP thymocytes were also purified by electronic sorting. Lymph node T cells were purified by incubating lymph node cells with magnetic beads to which anti–mouse Ig had been adsorbed and selecting the nonadherent cells.

In vitro suspension culture and stimulation. Purified DP thymocytes or LNT cells were placed in suspension cultures in medium alone or with 6 ng/ml IL-7 (R&D Systems) or with 20 ng/ml IL-4 (R&D Systems) for the times indicated in the figures. Where indicated in the figures, DP thymocytes were stimulated with 0.3 ng/ml PMA (Calbiochem) and 0.3 μg/ml ionomycin (Calbiochem) or with 10 μg/ml plate-bound anti-TCR (BD Biosciences) and 10 μg/ml anti-CD2 (BD Biosciences).

Intracellular staining. Cells were fixed and permeabilized first with 4% PFA and then with a 1:1 methanol/acetone mixture (vol/vol). Cells were stained with anti–Bcl-2 antibody followed by FITC-labeled secondary antibody. After intracellular staining, cells were further stained for surface proteins. For intracellular p-Stat5 staining, cells were incubated in medium alone or with IL-7 for either 20 min or 1 h. The cells were fixed and permeabilized and stained with FITC-labeled anti–p-Stat5 mAb, and then stained for surface proteins.

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

Immunoblotting. Cells were lysed in SDS sample buffer, and cell lysates were resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Blots were incubated with anti–p-Stat5 antibody (Zymed Laboratories) or a mixture of anti-Stat5a and anti-Stat5b antibodies (Santa Cruz Biotechnology, Inc.), followed by horseradish peroxidase–conjugated protein A. Reactivity was revealed by enhanced chemiluminescence.

EMSA. Equal amounts of nuclear extracts were incubated with ^{32}P-labeled ATP Stat5/6 consensus oligonucleotides (Santa Cruz) either without or with 50× cold oligonucleotide competitors. For supershift assays, anti-Stat5 gel supershift antibody (Santa Cruz Biotechnology, Inc.) was added to the binding reactions. Protein–DNA complexes were electrophoresed through a 5% nondenaturing polyacrylamide gel, which was subsequently dried and exposed to film for autoradiography.

Northern blot analysis. Total RNA was isolated from purified DP thymocytes and LNT cells using TriZol (Invitrogen). Equal amounts of RNA were resolved in a 3-(*N*-morpholino) propanesulfonic acid–buffered agarose gel under denaturing conditions and subsequently blotted onto Hybond-N+ membranes (GE Healthcare) by capillary transfer. Radioactive probes were generated from cloned cDNA fragments of the corresponding genes by using the EZ-strip DNA kit (Ambion) and hybridized for 16 h with RNA-blotted membranes in UltraHyb solution (Ambion) at 42°C. Unbound probes were washed off 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 then exposed to a PhosphoImager screen (GE Healthcare) and analyzed.

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