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*J Immunol* 2004; 173:2307-2314; doi: 10.4049/jimmunol.173.4.2307

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STAT5 Is Required for Thymopoiesis in a Development Stage-Specific Manner

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Diverse cytokines necessary for normal lymphopoiesis and lymphocyte homeostasis activate STAT5 in responder cells. Although STAT5 has been suggested to be a central molecular effector of IL-7 function, its essential role during IL-7-dependent T cell development in vivo remained unclear. Using Stat5\textsuperscript{−/−} mice we now show that STAT5 is essential for various functions ascribed to IL-7 in vivo. STAT5 is required for embryonic thymocyte production, TCR\gamma gene transcription, and Peyer’s patch development. In sharp contrast, normal STAT5 is dispensable for adult thymopoiesis. In peripheral lymphocytes, STAT5 is primarily required for the generation and/or maintenance of γδ T cells and TCRγδ\textsuperscript{+} intraepithelial lymphocytes. Collectively, these results demonstrate that STAT5 is critical for many, but not all, aspects of steady state lymphoid lineage development and maintenance and suggest the existence of previously undocumented cytokine signaling traits and/or cytokine milieu during adult thymopoiesis. The Journal of Immunology, 2004, 173: 2307–2314.

Interleukin-7 belongs to the common γ-chain (γc)\textsuperscript{3} receptor family (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) and is essential for normal T cell development and homeostasis. IL-7R (composed of IL-7Rα and γc) signaling controls thymic T precursor cell proliferation, survival, and differentiation (1–4). Defective IL-7R signaling in vivo results in a severe depletion of early lymphocyte precursors and defects in adaptive immunity (5, 6). One differentiation-associated genetic event specifically controlled by IL-7R signaling is the V(D)J rearrangement at the TCR and Ig loci, as evidenced by specific impairments in TCRγ IgH gene rearrangement in IL-7R\textsuperscript{−/−} mice (2, 7). Subsequent studies have provided correlative evidence at the chromatin level that IL-7 induces epigenetic modifications of the TCRγ locus conducive for gene rearrangement (8–10).

Although IL-7R signaling can activate or augment the activation of several kinases (4, 11), only the JAK-STAT pathway has been implicated as the candidate modulator of TCRγ locus accessibility (12). IL-7R signaling primarily activates highly related STAT5A and STAT5B molecules via JAK3 in adult thymocytes, and functional links between IL-7 and STAT5 in fetal thymocytes have also been reported (4, 10, 12, 13). Consistent with the proposed role of STAT5 in modulating TCRγ expression, STAT binding sites are found in several regulatory DNA regions of the TCRγ locus (14). In addition, overexpression of a constitutively active version of STAT5 in IL-7R\textsuperscript{−/−} fetal thymocytes results in a switch from an inactive to an active TCRγ locus (10, 12). However, evidence against a central role for STAT in TCRγ gene rearrangement exists (15), and data supporting the requirement for STAT5 in IL-7 functions in vivo are lacking, because Stat5a\textsuperscript{−/−} (16), Stat5b\textsuperscript{−/−} (17), and Stat5a\textsuperscript{−/−}b\textsuperscript{−/−} mice all have been reported to undergo normal T cell development (18, 19) despite other notable impairments in hemopoiesis (20–22). Thus, the exact requirement for STAT5 during T cell development and TCRγ locus regulation remains unclear.

We set out to determine the contribution of STAT5 to IL-7 function and TCRγ gene regulation by examining lymphopoiesis in Stat5\textsuperscript{−/−} (compound Stat5a\textsuperscript{−/−}b\textsuperscript{−/−}) mice. Our results demonstrate that STAT5 is required for many, but not all, functions associated with IL-7 in vivo. STAT5 is critical for fetal thymocyte production and is essential for optimal TCRγ gene transcription, but not for gene rearrangement. Furthermore, STAT5 is required for Peyer’s patch (PP) genesis, an organ whose development depends on the activities of fetal intestine IL-7R\textsuperscript{+} precursor cells (23). In contrast, adult T cell development in the absence of STAT5 is normal, demonstrating that the requirement for STAT5 is distinct during fetal vs adult thymopoiesis.

Materials and Methods

Mice

Stat5\textsuperscript{−/−} (18) and B6 IL-15\textsuperscript{−/−} (24) mice have been described. We observed an increased frequency of death in Stat5\textsuperscript{−/−} mice after five or six backcrosses to B6 mice. Hence, nearly all mice used were from four or five B6 backcrosses. For peripheral T cell analysis, young healthy mice (3–4 wk old) with normal peripheral lymphoid organs were used. Stat5\textsuperscript{−/−} fetuses were identified by PCR-mediated typing of tail DNA (18) and by detecting decreased proportions of TCRγδ\textsuperscript{+} thymocytes using FACS analyses.

Cell preparation and culture

Dendritic epidermal T cells (DETCs) and intraepithelial lymphocytes (IELs) were obtained using published protocols, with minor modifications. For IEL preparation, PP were removed from the intestines, flushed with 25 ml of cold RPMI 1640 medium, and then incubated for 60–90 min in RPMI 1640 at 4°C. Semi-detached cells were then gently flushed using...
5–10 ml of 10% FCS/HBSS containing 1 mM dithioerythritol (37°C), followed by expulsion of remaining contents by squeezing down the length of the intestine. Collected mucus, debris, and cells were mixed and passed through a 70-µm pore size filter. Lymphocytes (both IELs and splenic T cells) were isolated using a nylon wool column (nonadherent fraction). The IEL yield using this relatively mild condition was 1–2 × 10^6/B6 mouse (~8 wk old). For fetal thymic organ culture (FTOC), embryonic day 16 (E16) or E17 thymic lobes were cultured for 4–7 days in Transwell plates (Corning Glass, Corning, NY). Single cell preparations were then analyzed by FACS. Standard culture medium (RPMI 1640 with 10% FCS, 50 µM 2-ME, 2 mM L-glutamine, 20 mM HEPEs, and antibiotics) was used without additional cytokines.

**Abs, FACS, and immunohistochemistry**

The following Abs were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA): Abs specific for CD4 (Cy5 conjugated), CD8 (Cy5), CD3e (Cy5), TCRβ (FITC), CD19 (FITC), CD24 (FITC), B220 (FITC), TCRγδ (biotin), Vγ2TCR (FITC), Vγ3TCR (FITC), CD122 (biotin), CD127 (biotin), and VCAM-1 (biotin). Streptavidin-PE was purchased from BD Pharmingen. Anti-TCRγ2-biotin, anti-TCRβ (H57)-biotin were prepared in our laboratory. FACS analyses were performed on an EPICS XL cytometer (Coulter, Hialeah, FL), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Histological analysis of frozen intestine tissue sections was performed as previously described (25). For Stat5c−/− intestines, sections near the visible PPs were examined for VCAM-1 and IL-7Rα expression, whereas for Stat5f−/− intestines, ~20 consecutive sections in the lower intestine were analyzed. Biotinylated mAb staining was visualized by HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA), followed by peroxidase substrate-conjugated 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO).

**PCR**

cDNA was generated using oligo(dT) and reverse transcriptase (Roche, Indianapolis, IN). The TCRγ and TCRδ gene-specific primers used were described previously (26, 27).

**Results**

**Fetal-specific T cell developmental defects in STAT5c−/− mice**

The initial characterization of adult Stat5c−/− (Stat5a/Sb double-deficient) mice indicated that, similar to Stat5a−/− or Stat5b−/− mice (16, 17), STAT5 is not essential for T cell development (18, 19), with the rather unexpected implication that IL-7 does not require STAT5 for signaling. Our analyses of Stat5c−/− mice described in this report suggest that this inference was premature because various aspects of T lymphopoiesis reported to be dependent on IL-7 examined, with the sole exception of the regulation of adult intrathymic T precursor differentiation, are abnormal when STAT5 is defective.

In accordance with published results (19), we observed that the thymic composition, development of αβ and γδ lineage thymocytes, and TCR repertoire were not significantly different in 4- to 10-wk-old Stat5c−/− (three to five times backcrossed to C57BL/6) compared with littermate wild-type (WT) mice (data not shown). However, thymic cellularity was marginally reduced by ~2-fold in Stat5c−/− mice (Fig. 1A). This result sharply contrasts with the severe defect in thymocyte development in adult mice deficient in any one of the membrane-proximal IL-7R signaling components, including IL-7Rα, JAK3, and γc (7). Global gene expression profiles of adult Stat5c−/− thymocyte subsets also indicated that the immature thymocyte subsets were remarkably unperturbed in the absence of STAT5 (K. Narayan and J. Kang, manuscript in preparation), suggesting that for adult thymocyte maturation, STAT5 is replaceable or is not required.

Given the seemingly normal thymopoiesis in adult Stat5c−/− mice, it was important to determine whether the observed STAT5-independent IL-7R signaling is a general rule at all stages of thymopoiesis. To address this issue, we first examined fetal T cell development in the absence of STAT5, particularly because distinct TCRγδ+ thymocyte populations are generated in fetal development from fetal stage-specific T precursors (28), and one exclusive function of IL-7 is to modulate TCRγ locus accessibility (9). In contrast to the near-normal range of thymocytes generated in
adult Stat5−/− mice, fetal thymic cellularity was reduced by 5- to 10-fold in the absence of STAT5 (Fig. 1A). Among individual cytokine-signaling-deficient mice, similar reductions in thymic cellularity have been observed only in IL-7−/− and IL-7R−−/− fetuses (29). The most severe reductions in thymocyte numbers occurred on E15 and E16, when precursors start maturing into TCR+ immature thymocytes (Fig. 1A). There was an ∼2-fold or a >3-fold increase in dead cells in ex vivo fetal thymocytes or in short term Stat5−/− FTOC, respectively, affecting all thymocyte subsets, as determined by dye uptake and/or FACS analysis (Fig. 1C and data not shown). In addition, the proportions of precursor cells (CD3−CD4−CD8−, triple negative (TN)) were increased 2- to 3-fold in Stat5−/− fetuses compared with littermate controls on E16–18, and Stat5−/− thymus were especially enriched for IL-7RhighCD4−CD8− thymocytes, representing the earliest precursor subsets, with a concomitant decrease in more differentiated IL-7Rneglow preT cells (Fig. 1D) (30). Although there was a consistent increase in the frequency of pro-T cells (CD25+CD44+TN) in Stat5−/− fetal thymocytes (Fig. 1E), there was not a significant developmental block at this stage, as is observed in adult IL-7−/− thymocytes. Collectively, these results indicate that the decrease in thymocyte number in Stat5−/− fetuses is, in part, a result of increased cell death combined with inefficient maturation of thymic precursor cells.

Although both fetal γδ and αβ subsets were affected by STAT5 insufficiency (Fig. 1B), the development of γδ cells was more severely affected throughout ontogeny, resulting in a >20- to 100-fold reduction in numbers. After birth, both the αβ and γδ thymocytes increased rapidly in number (Fig. 1A and data not shown), although reductions of ∼2- and 4-fold, respectively, remained in Stat5−/− mice compared with the controls. The selective accentuated deficit in γδ thymocyte development in Stat5−/− fetuses probably arose due to insufficient generation of functional TCRγ chains, as is the case in IL-7R−/− mice (2, 31). Expression of the Vγ3 chain is normally detected on the cell surface on E16, and the Vγ3+ thymocytes migrate to the skin, where they are referred to as DETCs (28). The most striking defect in fetal thymocyte development in Stat5−/− mice was the virtual absence of Vγ3+ γδ thymocytes (Fig. 2A). A consequence of this paucity of Vγ3+ thymocytes in Stat5−/− fetuses is the absence of DETCs in adult Stat5−/− mice (Fig. 2A). As IL-7, and not IL-2 or IL-15 (32), primarily regulates intrathymic γδ T cell development, these results indicate that IL-7R-STAT5 signaling is absolutely critical for intrathymic DETC differentiation.

A defect in Vγ2+ fetal thymocyte development was also observed in Stat5−/− mice. Reduced numbers (15- to 40-fold) and proportions (3- to 5-fold) of Vγ2+ thymocytes were found in E17–19 Stat5−/− fetuses compared with WT littermates. In contrast to the fetal γδ thymocyte defects, examination of the TCRVβ repertoire revealed no differences between E18/19 Stat5−/− fetuses and littermate controls (data not shown), although the total αβ thymocyte cellularity was reduced at this stage (Fig. 1B).
results show that in the absence of STAT5 there is a specific, severe block in fetal γδ TCR+ thymocyte development and significant, but less dramatic, defects in fetal αβ thymocyte generation.

Defective TCRγ gene transcription in STAT5−/− fetal thymocytes

STAT5 activation by IL-7R signaling has been implicated as the initial event critical for establishing a permissive chromatin state for V(D)J recombination at the TCRγ locus (10). Hence, it is possible that the reduction in TCRγ+ thymocyte numbers during embryonic development in Stat5−/− mice is due to impaired TCRγ gene rearrangement. To test this proposal, we examined TCRγ locus activity in fetal thymocytes on different days of gestation. Semiquantitative PCR analysis of E15–18 total thymocyte genomic DNA showed only marginally diminished Vγ3 gene rearrangement in Stat5−/− fetuses (<2- to 3-fold; Fig. 2B, top panels), especially during E15 and E16. Similar small decreases in Vγ4 gene rearrangement levels were also observed. The δ gene rearrangements were not affected in Stat5−/− fetuses (see below), consistent with the normal TRδ gene rearrangement in IL-7R−/− mice (2). These results indicate that, contrary to expectation (10, 12), the effects of STAT5 insufficiency on TCRγ gene rearrangement in vivo are minimal, suggesting that STAT5 is not a nonredundant chromatin modifier necessary for the initial steps of TCRγ gene rearrangement.

Given that γ locus gene rearrangement was not severely compromised in Stat5−/− fetuses, an alternative explanation for the temporal block in γδ lineage development is that the transcription of the rearranged γ genes was impaired (13, 31). Results from semiquantitative RT-PCR assays strongly support this possibility. As shown in Fig. 2B (bottom panels), rearranged Vγ3 gene-specific RNA was substantially reduced (>16-fold) in Stat5−/− fetal thymocytes on E15 and E16. On E15, >95% of thymocytes were TN, and the relative levels of γ gene transcripts observed were not affected by different proportions of αβ and γδ thymocyte subsets found in WT and Stat5−/− mice. The transcription levels increased on E17, but the transcription of rearranged Vγ3 genes was lower in Stat5−/− fetuses than in littermate controls at all stages of fetal thymopoiesis. Similarly, expression of the rearranged Vγ2 gene was also diminished during Stat5−/− fetal development (Fig. 2C), but to a lesser extent than that of the Vγ3 gene.

Transcription of germline (unrearranged) TCR γ gene segments correlates with the onset of V(D)J recombinase activity and/or transcriptional efficiency (26). When Stat5 was absent, germline transcription of the Jγ1 gene segment was diminished ~4-fold in E15–16 thymocytes (Fig. 2D), consistent with the proposal that STAT5 activates Jγ1 gene segment transcription in fetal thymocytes (12).

In contrast to defects in TCRγ gene transcription, levels of the early wave of rearranged TRα5 gene transcription were largely unaltered by the absence of STAT5 (Vβ1-Jβ1, shown in Fig. 2E), indicating that STAT5 specifically modulates transcription levels of the γ locus, and importantly, that there are precursors with the potential to produce early γδ thymocyte subsets in Stat5−/− fetal thymus, and the extent of decrease in TCRγ gene transcription cannot be attributed to a corresponding reduction in these precursors.

Collectively, the results indicate that STAT5 is required specifically for optimal transcription of the TCRγ locus during fetal development and suggest that insufficient TCRγ chain expression in Stat5−/− fetuses causes the block in γδ T cell development. Significantly, the absence of STAT5 does not abrogate TCRγ gene rearrangement. Previous studies have shown that overexpression of the constitutively active version of STAT5 in FTOC results in histone acetylation at the TCRγ locus (10) and permits some TCRγ gene rearrangement to occur in the absence of IL-7R signaling (12). However, our analyses cast doubts on one possible interpretation from these studies that STAT5 is the initial activator of TCRγ locus accessibility per se downstream of IL-7R. Furthermore, because γδ thymocyte development in Stat5−/− mice was restored to near-normal levels subsequent to fetal thymopoiesis, we infer that IL-7R can activate, or can be complemented by, transcription activators other than normal STAT5 to regulate TCRγ gene transcription.

Abnormal PP formation in STAT5−/− mice

IL-7R−/− and Jak3−/− mice have defects in secondary lymphoid organogenesis, including the development of PP and lymph nodes (LNs). The requirement for IL-7R signaling in PP gene expression appears to be at the level of IL-7R+ lymphoid tissue-inducing cells found in PP and LN anlage during fetal ontogeny (25). Importantly, it has been shown that IL-7R signaling is necessary before E18 for PP development (23). No γc cytokines, other than IL-7, or any other cytokine that activates STAT5 are required for lymphoid organogenesis.

Unlike IL-7R−/− mice, adult Stat5−/− mice have normal LNs, but they specifically lack PP. Whereas the littermate controls (n = 9) have six to nine PPs per mouse, Stat5−/− mice (n = 12) have no visible PP. A typical PP from the WT intestine stained with VCA1-specific mAb to identify the PP stromal cells (33) is shown in Fig. 3. In Stat5−/− intestines, no such organized histological structures were found, although small clusters of lymphocytes in mucosal layers of intestine were occasionally detectable. These clusters were only weakly positive for VCA1 (Fig. 3). Lymphoid tissue-inducing cells were detected in E17/18 Stat5−/− fetal intestines, but were reduced in number by ~3-fold compared with those in WT controls (data not shown). This PP-specific defect has not been observed with any other cytokine or chemokine mutation. Given that PP development requires IL-7R signaling before E18 (23), and that no other cytokines that can activate STAT5 affect PP development, the absence of PP in Stat5−/− mice further supports the proposal that STAT5 is an essential mediator of IL-7R function during fetal lymphoid lineage maturation.

**STAT5 is essential for TCRγδ+ IELs and γδ T cells**

Our analyses of T cell development in Stat5−/− mice showed that although normal STAT5 is dispensable for adult T cell development, it is required during ontogeny. We next examined aspects of...
extrathymic IL-7 function to discern whether the difference in STAT5 requirement is rooted in global differences in fetal vs adult lymphoid compartments (34) or if it is adult thymopoiesis-specific. Stat5\(^{-/-}\) mice lack functional NK cells and exhibit defects in CD8\(^{+}\) T cell maintenance, a phenotype similar to that of IL-15\(^{-/-}\) and IL-15\(R\alpha^{-/-}\) mice, indicating that IL-15 signaling in the periphery is dependent on STAT5 (data not shown) (19). IL-7 has been implicated in IEL generation in the gut, particularly for TCR\(\gamma\delta^{+}\) IELs (35) and in peripheral \(\alpha\beta^{+}\) T cell homeostasis (36). The latter function is difficult to examine in Stat5\(^{-/-}\) mice, because \(\alpha\beta^{+}\) T cells are in an aberrantly activated state (19).

IL-7 and IL-15 cooperatively regulate the extrathymic IEL population. It has been shown that IL-15\(^{-/-}\) (24) and IL-15R\(\alpha^{-/-}\) mice (37) have significantly diminished numbers of extrathymically derived IELs (Thy1\(^{+}\)CD8\(\alpha\alpha\)), whereas IL-7R\(^{-/-}\) mice lack TCR\(\gamma\delta^{+}\) IELs, but maintain \(\alpha\beta^{+}\) IELs (38). The requirement for STAT5 in IEL development was not known. Examination of IELs in Stat5\(^{-/-}\) mice revealed that total IELs were decreased in number by 2- to 3-fold (Table I). Strikingly, \(\gamma\delta^{+}\) IELs were dramatically reduced by \(\sim 10\)-fold in Stat5\(^{-/-}\) mice, whereas the reduction in \(\alpha\beta^{+}\) IELs was modest (Fig. 4A and Table I). CD8\(\alpha\alpha^{-/-}\) (CD4\(^{neg}\)) IELs were reduced to a similar level in Stat5\(^{-/-}\) and IL-15\(^{-/-}\) mice (Fig. 4A, bottom panels, and Table I). The majority (average, 65%; \(n = 7\)) of the remaining Stat5\(^{-/-}\) CD8\(\alpha\alpha^{+}\) IELs (Fig. 4A, bottom) coexpressed CD4\(^{+}\) (Table I). CD4\(^{+}\)CD8\(^{+}\) IELs constitute a minor population in normal mice. A more severe loss of \(\gamma\delta^{+}\) IELs in Stat5\(^{-/-}\) compared with IL-15\(^{-/-}\) (or IL-2R\(\beta^{-/-}\)) (39) mice combined with a comparable reduction in CD8\(\alpha\alpha^{+}\) IELs in Stat5\(^{-/-}\) and IL-15\(^{-/-}\) mice suggest that IL-7R-STAT5 and IL-15-STAT5 signaling selectively regulate the development and/or maintenance of \(\gamma\delta^{+}\) IELs and CD8\(\alpha\alpha^{+}\) IELs, respectively.

The requirement for STAT5 in the peripheral \(\gamma\delta^{+}\) T cell maintenance was not restricted to the mucosal epithila. Splenic \(\gamma\delta^{+}\) T cells were severely reduced in number in young (3–4 wk old), healthy, Stat5\(^{-/-}\) mice, but not in IL-15\(^{-/-}\) (or IL-2R\(\beta^{-/-}\)) mice (Fig. 4B) (40). Stat5\(^{-/-}\) mice were especially devoid of IL-7R\(^{+}\) \(\gamma\delta^{+}\) T cells (Fig. 4B). Similar results were obtained in B6 chimeras generated using Stat5\(^{-/-}\) bone marrow (BM) cells (data not shown). Because TCR\(\gamma\delta^{+}\) thymocytes were generated in adult Stat5\(^{-/-}\) mice, and cytokines other than IL-7 do not exhibit a paucity of \(\gamma\delta^{+}\) T cells in spleen or LNs, the absence of TCR\(\gamma\delta^{+}\) cells in the peripheral lymphoid organs of adult Stat5\(^{-/-}\) mice is probably the result of a defect in IL-7R signals required for \(\gamma\delta^{+}\) T cell maintenance. The possibility that the loss of splenic \(\gamma\delta^{+}\) T cells is a result of nonspecific attrition induced by activated \(\alpha\beta^{+}\) T cells found in Stat5\(^{-/-}\) mice is unlikely because \(\gamma\delta^{+}\) T cells persist in other mouse models with aberrantly activated CD4\(^{+}\) \(\alpha\beta^{+}\) T cells such as IL-2\(^{-/-}\) and CTLA-4\(^{-/-}\) mice (40, 41). Moreover, although the number of B cells is reduced in peripheral blood (42), splenic B cells are normal in number in Stat5\(^{-/-}\) mice (data not shown), indicating that the loss of splenic \(\gamma\delta^{+}\) T cells is unlikely to be part of a generalized peripheral lymphocyte abnormality of Stat5\(^{-/-}\) mice. Collectively, these results show that with the exception of the adult thymic population, all major subsets of \(\gamma\delta^{+}\) T cells are severely and selectively reduced when STAT5 is absent, suggesting that outside of the adult thymus, the IL-7R signaling necessary for \(\gamma\delta^{+}\) T cell development and maintenance requires STAT5.

**Discussion**

**STAT5 functions downstream of IL-7R and IL-15**

IL-7 and IL-15 regulate nearly all facets of T and NK lymphocyte development and homeostasis. STAT5 is now shown to be critical for most functions associated with IL-7 and IL-15. First, STAT5 is

### Table I. Composition of IELs in Stat5\(^{-/-}\) mice

<table>
<thead>
<tr>
<th>Total T Cell No. (CD3(^{+})) ((\times 10^{4}))</th>
<th>No. of TCR(\gamma\delta^{+}) Cells ((\times 10^{4}))</th>
<th>No. of TCR(\alpha\beta^{+}) Cells ((\times 10^{4}))</th>
<th>No. of CD8(\alpha\alpha^{+}) (\times 10^{4}) ((\text{range of } % \text{CD8(\alpha\alpha^{+})}\text{CD4(^{neg})}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5(^{-/-}) ((n = 6))</td>
<td>11.3 ± 1.9</td>
<td>29.4</td>
<td>81.7</td>
</tr>
<tr>
<td>Stat5(^{-/-}) ((n = 7))</td>
<td>4.6 ± 1.4</td>
<td>3.2</td>
<td>44.0</td>
</tr>
<tr>
<td>IL-15(^{-/-}) ((n = 4))</td>
<td>5.8 ± 1.2</td>
<td>12.4</td>
<td>46.5</td>
</tr>
</tbody>
</table>

*Eight- to 10-wk-old female mice were used for analysis.

Numbers were calculated by total intestinal cell numbers \(\times\) proportion of cells in live lymphocyte gate according to forward and side scatter \(\times\) %CD3\(^{+}\).*
essential for fetal lymphocyte development; second, STAT5 is required for the development and/or maintenance of lymphocytes in the mucosal epithelia; and third, STAT5 is required for the maintenance of peripheral γδ T cells. STAT5 functions uncovered by this study combined with the previously documented roles in NK cell function and maintenance of activated CD8+ T cells (17, 19) indicate that normal STAT5 is an obligate factor for most, but not all, the biological activities of IL-7 and IL-15.

A formal possibility exists that the defects seen in fetal T cell development in Stat5−/− mice might be explained by abnormalities in early fetal liver (FL) progenitors, unrelated to IL-7R signaling defects. This issue is of concern because other reports have shown that in mixed BM or FL chimeras, Stat5−/− stem cells cannot produce lymphoid and myeloid progenies in the presence of WT cells, suggesting impairments at the lymphomyeloid progenitor level when STAT5 is absent (21, 22, 43) (our unpublished observations). However, whether lymphoid or T cell lineage-committed Stat5−/− progenitors exhibit similar competitive reconstitution defects has not been tested. Moreover, it should be noted that the kinetics of thymic reconstitution of lethally irradiated mice by Stat5−/− BM or FL cells, without competitor WT stem cells, are similar to those of WT counterparts (data not shown). Hence, there is no direct evidence to indicate that STAT5 specifically influences lymphoid or thymic progenitor production, migration, or viability. The fact that Stat5−/− FL cells exhibit normal reconstitution capability in adult hosts, that Stat5−/− fetal thymocytes in culture are highly sensitive to cell death (Fig. 1C), that the frequency of the fetal immature thymic precursor subset is increased relative to that of other thymic subsets (Fig. 1D), and that γδ thymocyte development is more dependent on STAT5 than αβ thymocyte development (Figs. 1B and 2), all suggest that defects seen in fetal T cell development in Stat5−/− mice are specific to intrathymic differentiation events and are not simply a consequence of inefficient thymic seeding of T progenitors. Moreover, given the similarity in phenotype of Stat5−/− fetuses and various IL-7R signaling-defective fetuses, it is highly probable that STAT5 is the critical downstream signal transducer of IL-7 during fetal T cell development.

**Defects in fetal thymocyte development and TCRγ gene transcription in Stat5−/− mice**

A role for IL-7 in controlling TCRγ locus activity has been well documented (2, 9, 10, 12, 31), and STAT5 has been implicated as a general chromatin modifier of the TCRγ locus upon IL-7 signaling (10, 12). Direct in vivo demonstration of STAT5’s function in γδ T cell development had been lacking. We have now shown that there are deficiencies in fetal γδ thymocyte development and inefficient transcription of TCRγ genes in Stat5−/− mice. The severe reduction in the early Vγ3+ γδ thymocyte subset results in the absence of DETCs in adult Stat5−/− mice. This fetal γδ thymocyte deficiency is most likely caused by an IL-7-specific signaling defect because abnormalities in other cytokines do not lead to a block in fetal γδ thymocyte development. However, in contrast to IL-7R−/− thymocytes in which the Cγ1 locus is completely inactive (9, 31), Stat5−/− fetal thymocytes have significant levels of TCRγ gene rearrangements, indicating that normal STAT5 is not essential for the regulation of γ locus chromatin accessibility per se.

The data indicate that the 3′Ey and locus control region-like DNase hypersensitive site A (HsA) cis regulatory regions control the levels of transcription (14, 43). STAT binding sites are found in Ey and HsA, but not in the upstream Vγ gene segment transcription promoters. STAT5 binding to 3′Ey has been shown in cell lines (10). Critically, deletion of both 3′Ey and HsA elements exhibit abnormalities in γδ thymocyte development that are strikingly similar to those observed in Stat5−/− mice; Ey−/− HsA−/− mice essentially lack Vγ3+ thymocytes and show diminished transcription of rearranged TCRγ genes, but TCRγ gene rearrangement remains normal (43). Hence, we propose that STAT5 interacts with Ey and HsA cis-acting elements to regulate the Cγ1 locus, but factors other than STAT5 control TCRγ locus accessibility by interacting with sites other than, or in addition to, the 3′Ey and HsA. Collectively, these results indicate that IL-7R controls transcription of the fetal TCRγ locus via STAT5 in vivo, but in adults, IL-7R can potentially recruit transcription activators other than STAT5 to modulate the TCRγ locus.

**Differences in fetal vs adult T cell development**

A number of studies have demonstrated that there are fundamental differences between fetal and adult lymphopoiesis (34, 44). Conspicuously, one function of IL-7 not mediated by STAT5 in mice is the survival and differentiation of adult thymic T precursors (Fig. 1) (K. Narayan and J. Kang, manuscript in preparation). This unique exception illustrates previously unsuspected differences in IL-7R signaling requirements and/or the manner in which the cells can respond to IL-7 during fetal vs adult lymphopoiesis as well as in intrathymic vs extrathymic lymphopoiesis. Whether the difference is rooted in the quantitative or qualitative changes in microenvironments, cell-intrinsic biochemical circuits, or a combination of both is unclear.

It is possible that the adult thymus provides unique trans-acting factors conducive for T cell development independently of STAT5. These putative factors may simply constitute an increased bioavailability of IL-7 that activates IL-7R-JAK-regulated, non-STAT signal transducers that can substitute for STAT5. Alternatively, it is possible that other cytokines in adult thymus can modulate the levels of active STAT1 and/or -3, which can substitute for STAT5. Stat1 and Stat3 genes are expressed during ontogeny and in diverse adult lymphocyte subsets (J. Kang, unpublished observation), ruling out the possibility that it is simply differences in the expression pattern of these genes in fetal vs adult developmental stages that is the mechanism of compensation. Support for changes in STAT protein levels as a consequence of the STAT5 defect does exist. STAT1 protein levels are reportedly increased, whereas STAT3 is decreased in Stat5−/− liver tissue samples compared with WT controls (18). We are currently testing the activity of STAT1/3 in fetal and adult Stat5−/− thymocytes and generating mice deficient for STAT1/3/5 in thymocytes to definitively resolve this issue. Preliminary studies indicate that Stat1−/− Stat5−/− mice do not exhibit adult T cell developmental defects (K. Pinault and J. Kang, unpublished observations), ruling out STAT1 as a redundant factor of STAT5. It should be emphasized, however, that even if another STAT family member is interchangeable for IL-7 function, this redundancy must operate only during adult lymphopoiesis of normal mice, a developmentally unique phenomenon for which a mechanistic explanation is required.

Conversely, the possibility that fetal and adult thymocytes are cell-intrinsically distinct needs to be addressed, as evidenced by extensive differences in global gene expression pattern between stem cells found in FL and adult BM (45), and between fetal (E18) vs adult αβ double-positive thymocytes, where ~20% of expressed genes are differentially (≥3-fold) expressed (S. Der and J. Kang, unpublished observations). Furthermore, it has been shown that cell-intrinsic responses to cytokines can be altered developmentally. For instance, fetal, but not adult, B cell precursors are responsive to thymic stromal lymphopoietin (TSLP) (46), the only other known cytokine that uses IL-7R α-chain as a component of its receptor. The possibility that complex activities of TSLP can
account for STAT5-independent adult thymopoiesis exists, but this is unlikely. Although TSLP activates STAT5 in a JAK-independent manner (47), it appears to have a limited role by itself in promoting fetal thymocyte or adult T precursor proliferation (48) and, importantly, cannot compensate for the absence of IL-7 in thymopoiesis.

In summary, we have demonstrated that during fetal γδ cell development, STAT5 is required for IL-7R-modulated optional TCRγ gene transcription, but it is not absolutely essential for TCRγ gene rearrangement, indicating that factors other than STAT5 activated by the IL-7R-JAK pathway control TCRγ locus accessibility to V(D)J recombinase. In contrast, normal STAT5 is not essential for IL-7R-regulated TCRγ gene transcription or for thymocyte production in adults, indicating that the requirement for STAT5 in thymopoiesis is developmental stage specific. This observation suggests that IL-7R signaling properties (cell-extrinsic environmental and/or cell-intrinsic parameters) are different during fetal vs adult T cell development. Developmental stage-dependent differences in erythropoietin receptor (49) and TSLP receptor (46) signaling have also been observed, suggesting that this variability is a common feature of a certain class of cytokines as a consequence of, or perhaps contributing to, the highly distinct nature of fetal and adult lymphopoiesis.

Acknowledgments

We are indebted to Drs. J. Ihle and J. Peschon for making Stat5−/− and IL−15−/− mice, respectively, available for this study. We thank Tammy Krumpoch for expert cell sorting, Kristin Wallace for microscopy. We also thank Dr. M. Bix, Dr. L. Berg, and H. Melicar for critical reading of the manuscript.

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