Stat5a/b are essential for normal lymphoid development and differentiation

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Cytokines that use the common gamma chain γc are critical for lymphoid development and function. Mutations of the IL-7 receptor, γc, or its associated kinase, Jak3, are the major cause of human severe combined immunodeficiency. Although activated by IL-7, Stat5a/b (Stat, signal transducer and activator of transcription) have been thought to play limited roles in lymphoid development. However, we now show that mice completely deficient in Stat5a/b have severely impaired lymphoid development and differentiation. Absence of Stat5 also abrogates T cell receptor γ rearrangement and survival of peripheral CD8⁺ T cells. Thus, deficiency of Stat5 results in severe combined immunodeficiency, similar in many respects to deficiency of IL-7R, γc, and Jak3.

cytokine | jak | lymphocyte | severe combined immunodeficiency | interleukin

The development and homeostasis of lymphoid cells are tightly regulated by cytokines such as interleukin (IL)-7 (1). Its receptor comprises a ligand-specific subunit (IL-7R) associated with a shared receptor subunit designated the cytokine common gamma chain (γc), which binds the Janus kinase 3 (Jak3). Importantly, mutations of IL-7R, γc, or Jak3 underlie the majority of cases of human severe combined immunodeficiency and mouse models in which these genes are deleted also have severe combined immunodeficiency phenotypes (2–8).

Activated Jaks phosphorylate cytokine receptors, providing docking sites that recruit Signal transducers and activators of transcription (Stats), which are also phosphorylated. Stats then dimerize, bind DNA, and regulate gene transcription (9, 10). The predominant Stat activated by IL-7 and other γc cytokines is Stat5 (11–13). Encoded by two separate genes, the two isoforms of this transcription factor, Stat5a and Stat5b, have distinct physiological functions (14). Deficiency of Stat5a results in impaired prolactin-dependent mammary cell differentiation (15), whereas deficiency of Stat5b results in impaired growth (16).

With respect to T and B cell development, deficiency of Stat5a or Stat5b individually does not have severe consequences (13, 17–19). Furthermore, analysis of mice in which both genes were targeted also led to the conclusion that Stat5 was not essential for T or B cell development (20, 21). Peripheral B cells and bone marrow precursors were reduced but not eliminated and it was suggested that Stat5 is differentially required for T and B cell development (22–26). More recently, a 5- to 10-fold reduction in thymocytes was demonstrated in this model during fetal development, but after birth, the number of thymocytes normalized (21, 27, 28).

Thus, the differences in phenotypes between Stat5a/b knockout mice and mice lacking IL-7R, Jak3, or γc were striking, suggesting that γc cytokines like IL-7 must employ Stat5-independent mechanisms to direct lymphocyte development. However, the gene targeting strategy used in the original Stat5 knockout mice encodes a N-terminally truncated and partially functional Stat5 protein (Stat5ΔN) (ref. 29; see also Fig. 7, which

is published as supporting information on the PNAS web site). We therefore revisited the role of Stat5 in lymphoid development by analyzing mice in which the entire Stat5a/b locus was deleted (30) and compared these mice to Stat5ΔN mice and mice lacking IL-7R, Jak3, and γc. The present study demonstrates that Stat5 is more critical for lymphoid development and function than previously appreciated.

Results

Complete Stat5 Deficiency Results in a Severe Combined Immunodeficiency Phenotype. Deletion of Stat5a/b resulted in >99% perinatal lethality; therefore, we examined embryonic day 18.5 (E18.5) fetuses from timed pregnancies in which Stat5⁺/⁻ mice were interbred. Stat5⁻/⁻ fetuses were anemic, leukopenic, and had small spleens and thymi with disordered thymic architecture (Fig. 1A) (30). A >98% reduction in thymocyte number was noted, similar in magnitude to IL-7R- and γc-deficient fetuses (Fig. 1B; refs. 31 and 32), whereas Stat5ΔN E18.5 fetuses had significantly more thymocytes (27). The number of splenocytes was also reduced in E18.5 Stat5⁻/⁻ fetuses (Fig. 1C); in fact, the deficits were more severe than in IL-7R- or γc-deficient fetuses possibly due to the fact that Stat5 is activated even by cytokines other than γc cytokines. We confirmed the absence of Stat5a and Stat5b mRNA but found that Stat3 levels were unaffected (Fig. 7). Prominent bands representing Stat5a and Stat5b proteins were present in Stat5⁺/⁻ and Stat5⁺/⁺ mice but were absent in Stat5⁻/⁻ splenocytes. Residual Stat5 protein of smaller molecular weight was detected in Stat5ΔN cells (discussed below). Stat3 protein levels were normal in Stat5⁻/⁻ splenocytes, whereas slight reduction in Stat3 protein was observed in Stat5ΔN cells; however, this lane was underloaded and was not a consistent finding.

Defective B Cell Development in Stat5⁻/⁻ Fetuses. We next examined the proportion of B cells in spleens of Stat5⁻/⁻ fetuses and observed that there was both an absolute and relative reduction in the number of CD19⁺B220⁺ cells (0.09 × 10⁴) compared to wild-type fetuses (1.3 × 10⁴) (Fig. 2A). We also noted a severe reduction in fetal liver B cells in Stat5⁻/⁻ fetuses (0.25 × 10⁵) compared to controls (4.9 × 10⁵), similar to Il7r⁻/⁻ fetuses (Fig.
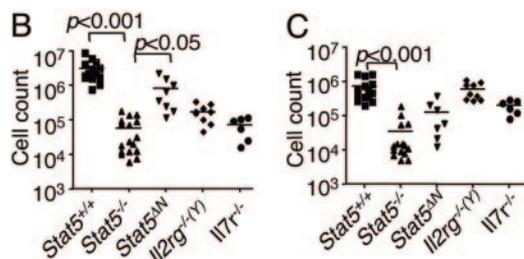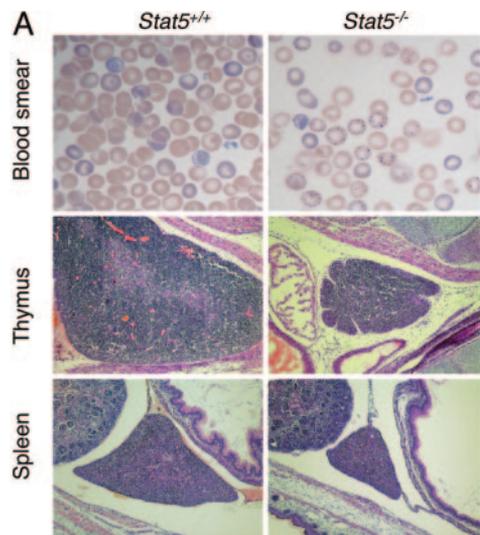
Fig. 1. Stat5 deficiency results in a severe combined immunodeficiency phenotype. (*A*) Mice in which one Stat5 allele had been deleted by expression of MMTV-Cre (30) were intercrossed, and E18.5 fetuses were obtained for analysis. Peripheral blood smears and hematoxylin/eosin-stained sagittal sections of thymus and spleen were prepared from $Stat5^{-/-}$ fetuses (*Right*) and $Stat5^{+/+}$ littermate controls (*Left*). The number of viable cells in thymi (*B*) and spleens (*C*) from E18.5 $Stat5^{-/-}$ fetuses (triangles) was determined and compared to E18.5 wild-type ($Stat5^{+/+}$, squares), $Stat5^{\Delta N}$ (inverted triangles), γc- ($IL2rg^{-/-}$, diamonds) and IL-7R- ($IL7r^{-/-}$, circles) deficient fetuses. Wild-type fetuses had $3.1 \pm 0.6 \times 10^6$ (mean $\pm$ SE) thymocytes ($n = 15$), whereas $Stat5^{-/-}$ fetuses had $0.05 \pm 0.01 \times 10^6$ thymocytes ($n = 17$, $P < 0.001$ compared to wild type). $Stat5^{\Delta N}$ fetuses had $0.8 \pm 0.3 \times 10^6$ ($n = 8$, $P < 0.05$ compared to $Stat5^{-/-}$ fetuses). $IL2rg^{-/-}$ fetuses had $0.07 \pm 0.01 \times 10^6$ thymocytes and $IL7r^{-/-}$ fetuses had $0.2 \pm 0.03 \times 10^6$ thymocytes. Enumeration of splenocytes was as follows: wild type, $7 \pm 1 \times 10^5$; $Stat5^{-/-}$, $0.3 \pm 0.1 \times 10^5$; $Stat5^{\Delta N}$, $1 \pm 0.5 \times 10^5$; $IL2rg^{-/-}$, $5 \pm 1 \times 10^5$; $IL7r^{-/-}$, $2 \pm 0.3 \times 10^5$.
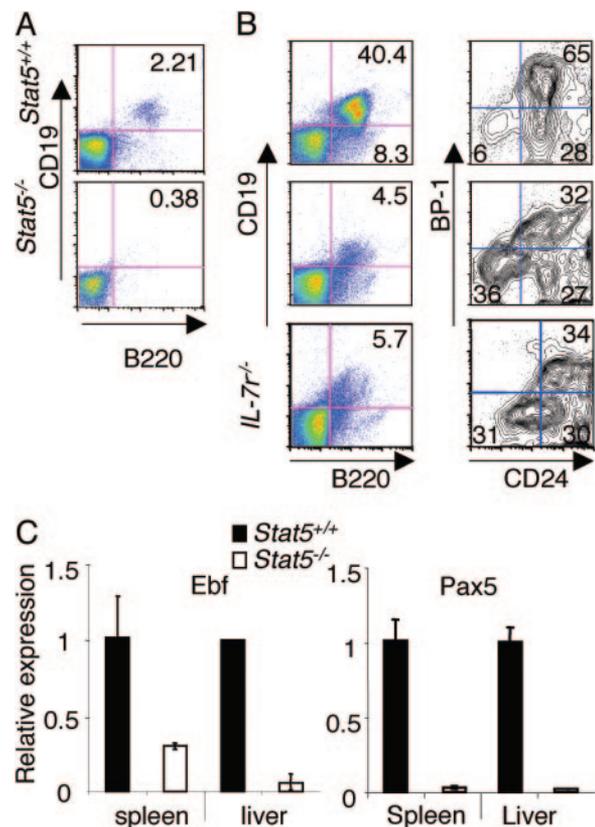

Fig. 2. Defective B cell development in $Stat5^{-/-}$ fetuses. (*A*) Spleens from E18.5 $Stat5^{+/+}$ and $Stat5^{-/-}$ fetuses were obtained and analyzed by flow cytometry with antibodies against CD19 and B220. (*B*) Fetal liver cells were obtained from E18.5 $Stat5^{+/+}$, $Stat5^{-/-}$, and $Il7r^{-/-}$ fetuses. B cells were assessed by staining with antibodies against CD19 and B220 (*Left*). B cell precursors were delineated by gating on B220$^+$ cells and staining with antibodies against BP1 and CD24 (*Right*). (*C*) The expression of the B cell transcription factors Ebf and Pax5 in splenocytes and fetal liver cells was determined by quantitative PCR. Filled bars, $Stat5^{+/+}$; open bars, $Stat5^{-/-}$.

2B Left). Large proportions of early pro (CD24$^+$BP-1$^-$) and late pro (CD24$^+$BP-1$^+$) B cells were detected in normal E18.5 fetal liver (Fig. 2*B Right*). In contrast, most cells in $Stat5^{-/-}$ and $Il7r^{-/-}$ fetal livers had markers of prepro (CD24$^-$BP-1$^-$) B cells (33). Accordingly, the B cell transcription factors Ebf and Pax5 were reduced in expression in $Stat5^{-/-}$ fetal liver (Fig. 2*C*). In contrast, $Stat5^{\Delta N}$ had a more modest reduction in B cells and B cell precursors (ref. 22 and data not shown). We conclude from these experiments that Stat5 is essential for normal B cell development, likely related to its requirement in IL-7 signaling, and $Stat5^{\Delta N}$ mice underestimated the critical functions of these transcription factors.

Defective T Cell Development in Stat5 Null Fetuses. We next examined T cell development in $Stat5^{-/-}$ fetuses. Although the absolute number of thymocytes was drastically reduced, CD4 single positive (SP), CD8 SP, and CD4/CD8 double-positive (DP) thymocytes were all present in roughly normal percentages (Fig. 3*A Left*), as in γc- and Jak3-deficient mice (31, 34, 35). Cells lacking CD4 and CD8 [double-negative (DN) thymocytes] are

subdivided based on expression of CD44 and CD25 (Fig. 3*A Right*). A large proportion of DN thymocytes from wild-type fetuses had low expression of CD44 and CD25, indicative of the DN4 stage (36%) (Fig. 3*B*). However, in $Stat5^{-/-}$ fetuses, the proportion of DN4 cells was reduced to 5% and the proportion of less mature cells was increased, whereas $Stat5^{\Delta N}$ mice had modest alterations in their proportion of DN cells (ref. 27; see also Fig. 8, which is published as supporting information on the PNAS web site).

DN thymocytes rapidly proliferate and undergo rearrangement of T cell receptor (TCR) loci to generate $\alpha\beta$ and $\gamma\delta$ TCRs. Consistent with the presence of DP and SP thymocytes, Rag2 and IL-7R expression were not impaired in $Stat5^{-/-}$ thymocytes (data not shown), whereas Bcl-2 and cyclin D2 expression were decreased (Fig. 3*B*). IL-7 is thought to be dispensable for TCR $\alpha\beta$ rearrangement but critical for TCRγ rearrangement (36–38). Although Stat5 has been implicated, TCRγ rearrangement occurs in $Stat5^{\Delta N}$ mice, leading to the conclusion that this process is Stat5-independent (27). We analyzed TCR rearrangement by amplifying genomic DNA by using primers that detect the juxtaposed rearranged segments (Fig. 3*C*). Amplified products indicative of TCR gene rearrangement were detected in $Stat5^{+/+}$ fetuses, but in $Stat5^{-/-}$ fetuses, TCRγ rearrangement was not detected, whereas TCR $\alpha\beta$ rearrangement was apparent. Accordingly, $Stat5^{-/-}$ fetuses lacked TCR$\gamma\delta$ cells (Fig. 3*D*).
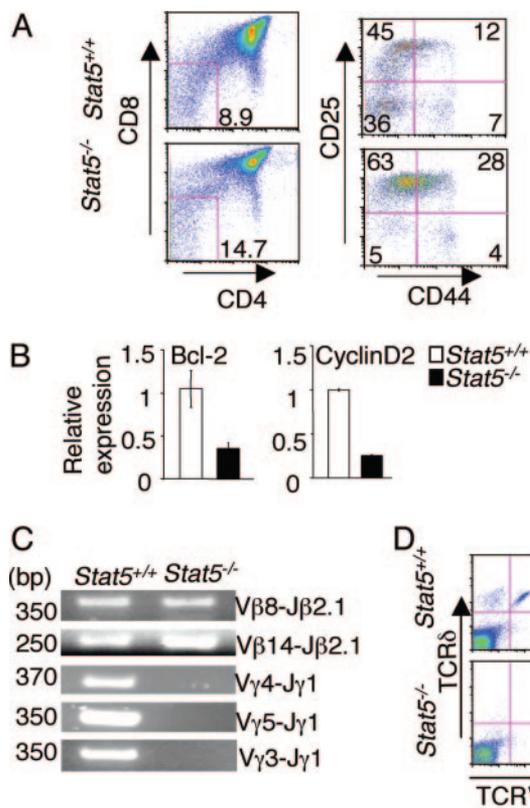
Fig. 3. Altered T cell development in *Stat5*$^{-/-}$ fetuses. (*A* and *B*) *Stat5*$^{-/-}$ fetuses produce CD4$^+$ and CD8$^+$ SP T cells but have increased proportions of immature DN thymocytes. Cells were obtained from thymi of E18.5 *Stat5*$^{+/+}$ *or Stat5*$^{-/-}$ fetuses. Single-cell suspensions were prepared and stained for surface expression of CD4, CD8, CD44, and CD25. (*A*) Expression of CD4 and CD8 is shown at *Left*. Gating on the CD4$^-$CD8$^-$ population, the proportion of CD25$^-$CD44$^+$ (DN1), CD25$^+$CD44$^+$ (DN2), CD25$^+$CD44$^-$ (DN3), and CD25$^-$CD44$^-$ (DN4) cells is depicted (*Right*) and summarized in Fig. 8. (*B*) Impaired expression of Bcl-2 and cyclin D2. RNA was isolated from thymocytes of *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ E18.5 fetuses, and the levels of Bcl-2 and cyclin D2 were assessed by real-time PCR. Open bars, *Stat5*$^{+/+}$; filled bars, *Stat5*$^{-/-}$. (*C* and *D*) TCRγδ, but not αβ, rearrangement depends on Stat5 (*C*). Genomic DNA was isolated from thymi from wild-type and mutant mice. Products corresponding to V β8-J β2.1, V β14-J β2.1, Vγ4-Jγ1, Vγ5-Jγ1, and Vγ3-Jγ1 rearrangement were identified by PCR. (*D*) Thymocytes were stained with antibodies against TCRVγ3 and TCRδ and analyzed by flow cytometry.

Abnormal Lymphoid Development in Viable *Stat5*$^{-/-}$ Mice. Although complete Stat5 deficiency typically resulted in perinatal lethality, a few mice survived after weaning. Viable *Stat5*$^{-/-}$ mice also had small, atrophic thymi and few thymocytes, equivalent to *Jak3*$^{-/-}$ and *Il2rg*$^{-/-}$ mice of the same age (≈3 weeks) (Fig. 4*A*). Although the absolute number was very low, *Stat5*$^{-/-}$ mice generated SP thymocytes (Fig. 4*B Left*). As was noted in the *Stat5*$^{-/-}$ fetuses, the proportion of DN4 cells was reduced (Fig. 4*B Right*).

Assessment of lymphoid populations in spleens of viable Stat5-deficient mice also revealed that T cells were present (Fig. 4*C*), although, again, the absolute numbers were greatly reduced (Fig. 4*A*). Interestingly, there was marked reduction in the proportion of CD8$^+$ T cells as in *Jak3*$^{-/-}$ mice (Fig. 4*C*). In addition, the proportion of B cells was profoundly reduced (Fig. 4*D*) as has been noted in *Jak3*$^{-/-}$ mice (data not shown). Of note, the proportion of B cells can recover somewhat with time although the absolute number is greatly reduced (Fig. 4*D Right*). *Stat5*$^{\Delta N}$, *Il2rg*$^{-/-}$, and *Jak3*$^{-/-}$ have previously been reported to have major deficits in natural killer (NK) cells (18, 34, 35, 39, 40);
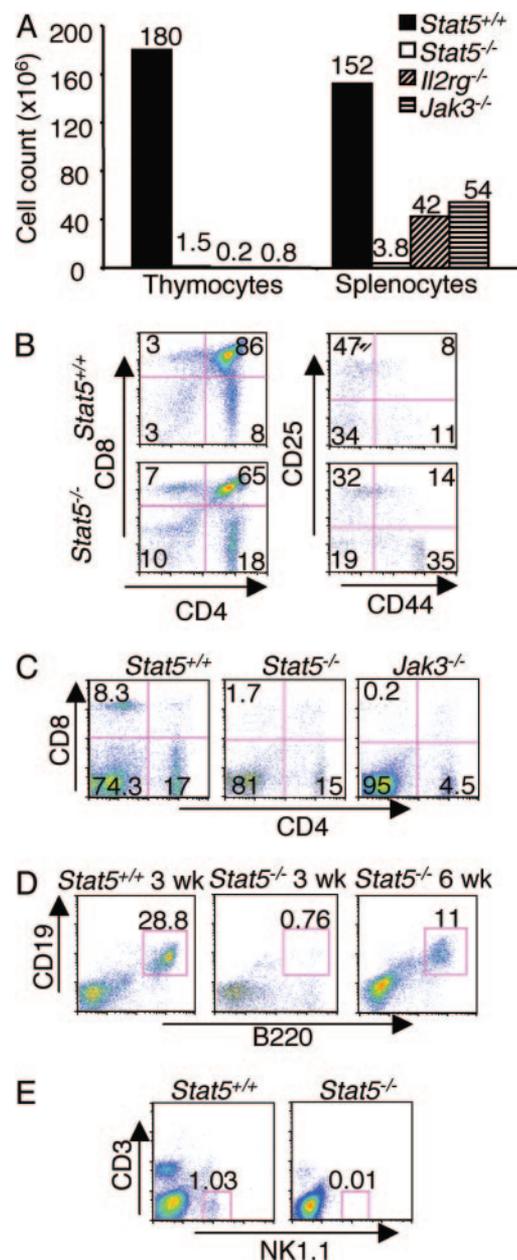

Fig. 4. Severe disruption of lymphoid development in viable *Stat5*$^{-/-}$ mice. (*A*) Thymus and spleen cell counts for 3-week-old *Stat5*$^{+/+}$ (filled bars), *Stat5*$^{-/-}$ (open bars), *Il2rg*$^{-/-}$ (diagonal hatched bars), and *Jak3*$^{-/-}$ (horizontal hatched bars) mice. (*B*) Thymocytes were stained for surface expression of CD4 and CD8 (*Left*). In addition, CD4/CD8 double-negative cells were gated and stained for surface expression of CD25 and CD44 to distinguish between four developmental stages within that population (*Right*; DN1, CD44$^+$CD25$^-$; DN2, CD44$^+$CD25$^+$; DN3, CD44$^-$CD25$^+$; DN4, CD44$^-$CD25$^-$). (*C–E*) Splenocytes were stained for CD4 and CD8 (*C*), B220 and CD19 (*D*), and NK1.1 and CD3 (*E*).

accordingly, NK cells were absent in the viable *Stat5*$^{-/-}$ mice (Fig. 4*E*).

Requirement for Stat5 in Peripheral T Cells. To ensure that the findings in *Stat5*$^{-/-}$ viable mice truly reflect the importance of Stat5 in T cell biology, we generated mice in which *Stat5a/b* genes were selectively deleted in T cells. Breeding *Stat5*$^{fl/-}$ mice with transgenic mice expressing cre recombinase under the control of the *CD4* gene promoter resulted in deletion of the floxed Stat5 allele in DP thymocytes and loss of Stat5 mRNA
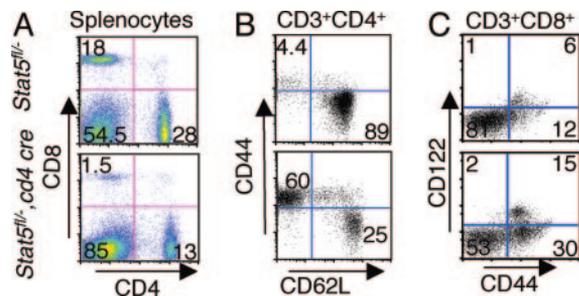
Fig. 5. Requirement for Stat5 in peripheral T cells. (*A*) Splenocytes from 6-week-old *Stat5*^{fl/−}/*CD4cre* and *Stat5*^{fl/−} mice were stained for CD3, CD4, and CD8 expression. (*B* and *C*) The expression of memory markers on CD4^{+} T cells (*B*; CD62L and CD44) and CD8^{+} T cells (*C*; CD44 and CD122) were assessed.

(ref. 41; see also Fig. 9, which is published as supporting information on the PNAS web site). There was no reduction in the overall number of thymocytes in Stat5^{fl/−}/CD4-Cre mice and variable reduction in the proportion of CD8^{+} SP T cells (data not shown). However, *Stat5*^{fl/−}/CD4-cre mice exhibited marked peripheral reduction of CD4^{+} T cells and CD8^{+} T cells, although the latter were more profoundly affected (Fig. 5*A*). Interestingly, most CD4^{+} T cells (Fig. 5*B*) and many of the residual CD8^{+} T cells (Fig. 5*C*) expressed markers of memory cells. A possible explanation for this phenotype could be the absence of regulatory T cells, a subset dependent on IL-2 and noted to be dysregulated in *Stat5*[ΔN] mice (42, 43). *Stat5*^{fl/−}/CD4-cre mice had marked reduction in thymic CD4^{+}CD25^{+} T cells and poor expression of the transcription factor FoxP3 (Fig. 10, which is published as supporting information on the PNAS web site). Thus, we conclude that Stat5 is critical not only for early T cell development but also for the proper homeostasis of mature T cells, especially CD8^{+} T cells.

Stem Cells Are Present in *Stat5*^{−/−} Fetal Liver but Inefficiently Generate T and B Cells. One factor underlying the defective lymphoid development associated with Stat5 deficiency could be reduced numbers or function of lymphoid stem cells in *Stat5*^{−/−} fetuses. Previous studies employing grafts of *Stat5*[ΔN] bone marrow and fetal liver cells have documented the reduced function of these precursor cells in repopulating irradiated recipients, especially in a competitive setting (28). We therefore enumerated fetal liver lineage negative Sca-1^{+} c-Kit[+] (LSK) cells in E14.5 *Stat5*^{−/−} fetuses (Fig. 6*A*). We found the proportion of LSKs in *Stat5*^{−/−} mice to be greater than or equal to littermate controls, although the absolute number of stem cells was reduced (*Stat5*^{+/+}, 1.7 × 10⁵; *Stat5*^{−/−}, 5.6 × 10⁴). We also examined LSKs in bone marrow from *Stat5*^{−/−} viable mice and found a similar phenotype. That is, although the percentage of LSKs was increased 2.4-fold in the bone marrow of the *Stat5*^{−/−} survivor mice (*Stat5*^{+/+}, 0.4%; *Stat5*^{−/−}, 1%), the absolute number of stem cells (LSKs) was still reduced overall because of the greatly reduced total cell number in the bone marrow (*Stat5*^{+/+}, 3 × 10⁴; *Stat5*^{−/−}, 6 × 10³).

Although the absolute numbers of stem cells was modestly reduced, it was also important to examine the function of these cells. To this end, we transplanted equal numbers of fetal liver cells from E14.5 fetuses into irradiated *Rag2*^{−/−} CD45.1 congenic mice to assess whether lymphoid and myeloid cells could develop in this setting. Cell counts showed that despite the higher proportions of LSKs transplanted, *Stat5*^{−/−} stem cells failed to repopulate thymic, splenic, or lymph node compartments (Fig. 6*B*). Examination of thymi of transplanted mice showed that CD4^{+} and CD8^{+} single-positive thymocytes were detected in mice transplanted with wild-type fetal liver cells (Fig. 6*C Lower*). In contrast, irradiated mice transplanted with *Stat5*^{−/−} cells
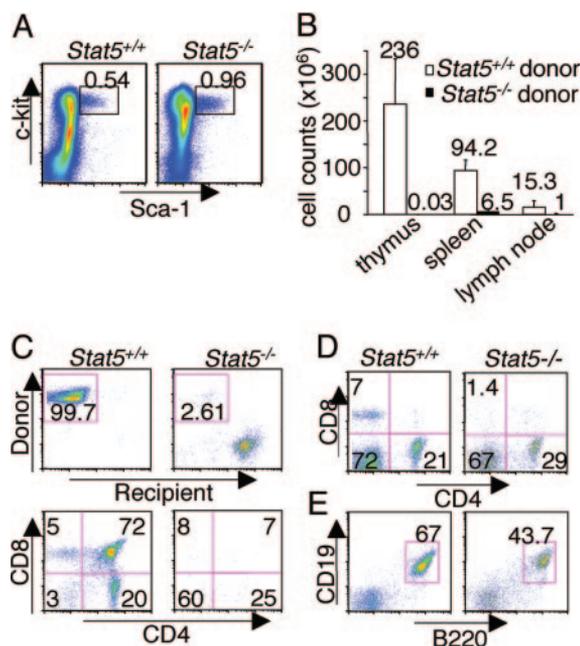

Fig. 6. *Stat5*^{−/−} fetuses have reduced numbers of lymphoid stem cells with poor repopulating function. (*A*) Fetal liver cells from E14.5 fetuses were stained for lineage negative Sca-1^{+} c-Kit[+] (LSK) stem cells and analyzed by flow cytometry. Lineage negative cells were gated and analyzed for c-Kit and Sca-1 expression. Although the proportion of LSKs in *Stat5*^{−/−} mice is greater than or equal to that in wild-type littermates, the absolute number of LSKs is reduced in *Stat5*^{−/−} fetuses (Stat5^{+/+}, 16.7 × 10⁴± 0.07 × 10⁴; Stat5^{−/−}, 5.6 × 10⁴± 0.5 × 10⁴). (*B–E*) Irradiated C57BL/6 rag2^{−/−} CD45.1[+] congenic mice were reconstituted with 2 × 10⁶ total fetal liver cells from CD45.2[+] wild-type or Stat5^{−/−} mice and housed in pathogen-free conditions on acidified water. Eight weeks after reconstitution, mice were killed; spleens, thymi, and lymph nodes were harvested, counted, and stained for various cell lineages along with antibodies against CD45.1 and CD45.2 to detect recipient and donor cells, respectively. (*B*) Donor-derived cell counts are shown. (*C*) The proportion of donor-derived thymocytes is shown in *Upper*. Donor-derived thymocytes were stained for CD4 and CD8 populations (*Lower*). (*D* and *E*) Donor-derived splenocytes were stained for T cell populations (*D*) or B cell populations (*E*). *Stat5*^{−/−} fetal liver cells were unable to repopulate T and B cell lineages to any significant degree.

developed few donor-derived (CD45.2^{+}) thymocytes of any kind (Fig. 6*C Right*). Furthermore, reduced absolute numbers of splenic CD4^{+} T cells and almost no CD8^{+} T cells were detected in recipients of *Stat5*^{−/−} fetal liver cells (Fig. 6*D Right*). The peripheral T cells in mice transplanted with *Stat5*^{−/−} fetal liver had a marked increase in the number of apoptotic cells and the proportion of memory cells (data not shown). The proportion and absolute number of splenic B cells developing from *Stat5*^{−/−} donor cells were also reduced (Fig. 6*E*), confirming that Stat5 is critical for the development of both T and B cells.

Discussion

Since the discovery of Stat5a and Stat5b, these transcription factors have proven to be critical in numerous biological processes. However, previous studies using *Stat5*[ΔN] mice, which express partially functional Stat5a and Stat5b proteins, led to the conclusion that these Stat5 transcription factors have limited roles in immune cell development and function (29). However, using mice completely deficient of Stat5a/b, we now provide data indicating that Stat5 is essential for the normal development of all lymphoid lineages; the trends noted in *Stat5*[ΔN] mice are far more profound when the entire Stat5 locus is deleted. *Stat5*[ΔN] mice were generated by targeting exons encoding the initiation methionines (Fig. 7*C*), which preserves downstream in-frame

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methionines (Fig. 7D). This strategy likely explains the existence of the truncated Stat5-immunoreactive polypeptides noted in these cells (Fig. 7B). Although we have not unequivocally established the nature of these polypeptides, it is seems likely that these species are partially functional given the disparate phenotypes of $Stat5^{\Delta N}$ mice and Stat5 null mice. These differences are important because the putatively limited effects observed in $Stat5^{\Delta N}$ mice have been interpreted to imply that significant Stat5-independent pathways must exist. Instead, the current data emphasize the need to understand exactly how Stat5 participates in many phases of lymphoid development and function.

IL-7 is known to be critical for B cell development in mice (but not humans), and previous work has indicated that expression of a gain-of-function Stat5 allele rescues B cell development, proliferation, and Ig gene rearrangement in $Il7r^{-/-}$ mice (23, 24). IL-7-activated Stat5 was shown to bind the Igh gene and promote chromatin remodeling, and Stat5 has been argued to be necessary and sufficient for germline transcription (26). However, $Stat5^{\Delta N}$ mice have a reduction but not elimination of B cell precursors in the bone marrow. Furthermore, B cell proliferation, Ig production, and class switching are reportedly normal in $Stat5^{\Delta N}$ mice (21, 22). These paradoxical results are clarified by the findings in the present study, which clearly place Stat5 in a more central role with respect to B cell development and function; B cell development is profoundly affected by complete Stat5 deficiency and appears to be blocked at a prepro stage, congruent with the phenotype of $Il7r^{-/-}$ mice (33). $Stat5^{\Delta N}$ mice have clearly been useful in documenting important roles of this transcription factor (26), but they are less reliable in defining Stat5-independent events. With respect to B cell development, the present studies clearly point to Stat5 as a major mediator of IL-7 signals.

Similarly, although IL-7 is also critical for T cell thymic development, a requirement for Stat5 was not evident in $Stat5^{\Delta N}$ mice; a reduction but not a profound effect on thymocytes was observed (27). Moreover, transgenic expression of a constitutively active Stat5 allele was noted to have little effect on thymocyte number, a result that was interpreted to indicate that Stat5 is differentially required for T and B cell development (23). However, we found that $Stat5^{-/-}$ fetuses, $Stat5^{-/-}$ viable mice, and recipients of $Stat5^{-/-}$ fetal liver cells all had profound reductions in thymocyte numbers. We also found that deletion of Stat5 in DP thymocytes by using CD4-cre did not affect this population. This result is expected because IL-7R expression is extinguished in DN4 and DP thymocytes; IL-7 activation of Stat5 would not be predicted to be necessary for the survival of this thymic subset. Of note, however, a discrete, stage-specific block in T cell development was not apparent in $Stat5^{-/-}$ fetuses and adults, similar to what is seen in $Il2rg^{-/-}$ and $Jak3^{-/-}$ mice. This finding contrasts with the phenotype of $Il7r^{-/-}$ mice in which a clearer (but not complete) block is present at the DN1 stage. These differences indicate that Stat5 deficiency accounts for some but not all of the alterations in T cell development seen in $Il7r^{-/-}$ mice and supports the need to identify IL-7-dependent, Stat5-independent signaling events in DN thymocytes.

Stat5 was reported not to be essential for $TCR\gamma$ rearrangement even though IL-7 is critical for this event (27). Yet, the studies presented here by using $Stat5^{-/-}$ mice demonstrate broader functions of Stat5 in thymic development and $TCR\gamma$ rearrangement, more consistent with what would be expected of impaired IL-7 signaling. Stat5 appears to be directly involved in Igh rearrangement (26); therefore, it is quite possible that Stat5 plays a similar role in $TCR\gamma$ rearrangement.

A role for Stat5 in CD8$^+$ T cell homeostasis has been suggested previously. Deficiency of Stat5a and Stat5b individually resulted in reduced numbers of CD8$^+$ T cells (25% and 50% reduction, respectively), whereas transgenic expression of Stat5 results in

expansion of CD8$^+$ T cells and lymphomagenesis (19, 23). The absence of CD8$^+$ cells in viable $Stat5^{-/-}$ mice, Stat5$^{fl/-}$/CD4-Cre mice, and transplant recipients is consistent with these findings and is also in line with the known effects of IL-7 on CD8$^+$ T cell survival (1, 44, 45). The homeostasis of CD4$^+$ T cells depends less on IL-7 (45, 46). Of note, CD4$^+$ T cells were present, albeit in very low numbers, in $Stat5^{-/-}$ and Stat5$^{fl/-}$/CD4-Cre mice. Previous studies using $Stat5^{\Delta N}$ mice also have pointed to a role for Stat5 in CD4$^+$ helper T cell differentiation (47–49). It will be of interest to address the role of Stat5 in CD4$^+$ T cell differentiation in the setting of selective Stat5 deletion in this subset. A complete lack of Stat5 is expected to have even more profound effects, although based on the present data, loss of Stat5 does affect CD4$^+$ T cell survival to some extent. Another complication is that the peripheral T cells generated in Stat5$^{fl/-}$/CD4-Cre mice have memory cell markers. Stat5 has also been implicated in the generation and survival of CD4$^+$CD25$^+$ regulatory T cells (T$_{reg}$). In Stat5$^{\Delta N}$ mice, this subset of T cells is greatly reduced, whereas transgenic expression of the constitutively active Stat5 allele expanded the number of T$_{regs}$ (23, 43, 50). It will be important to determine whether the abnormalities in peripheral T cells can be corrected by addition of T$_{regs}$ or if this abnormality is an intrinsic problem. In addition, assessing whether Stat5 directly or indirectly regulates FoxP3 is clearly of interest.

Finally, Stat5 is also evidently very important for the function of hematopoietic stem cells as $Stat5^{-/-}$ precursors failed to support the development of any lymphoid lineages. This deficit, no doubt, is a major contributor to the poor lymphoid development associated with Stat5 deficiency. The present data indicate that lineage negative precursor cells are present in $Stat5^{-/-}$ fetal liver; defining precisely how Stat5 contributes to stem cell function will be a critical issue.

In summary, analysis of mice that completely lack Stat5a/b documents the essential role of these key transcription factors in normal lymphoid development, likely due to their actions in transmitting signals from γc cytokines. Previous studies have pointed to important functions of Stat5 but, in general, have underestimated their essential roles in immune cells. Defects in stem cell function appear to be a major underlying factor in this phenotype, but impaired development and impaired survival and growth of mature cells also contributes to these aberrations. Systematically deleting Stat5a/b in T and B cells at various developmental stages should provide important insights into their roles and their target genes in lymphoid development and differentiation.

Materials and Methods

Generation of *Stat5*-Deficient Mice. The generation and screening of *Stat5*$^{fl/fl}$ and $Stat5^{-/-}$ mice has been described in ref. 30. Mice in which one Stat5 allele was deleted ($Stat5^{+/-}$) were intercrossed and E18.5 fetuses were obtained for analysis unless noted otherwise. Stat5$^{fl/-}$ mice were also crossed to transgenic mice expressing Cre under the control of the CD4 promoter to generate selective loss of *Stat5a/b* in T cells (41).

Antibodies and Flow Cytometry. Labeled antibodies were purchased (BD Pharmingen) and analyzed by using a FACSCalibur. Fetal liver cells were stained with phycoerythrin anti-mouse IL-7Rα (eBioscience), FITC anti-mouse Sca-1, APC anti-mouse c-Kit, and a mixture of biotinylated antibodies against murine lineage markers B220, CD11b, Gr-1, Ter-119, NK-1.1, CD3, CD8α, TCRβ, and $\gamma\delta$ TCR (Pharmingen). Cell counts were done by using a hemocytometer and verified by FACS with a bead standard for calibration.

RNA Isolation and Measurement. RNA was prepared with TRIzol reagent according to the manufacturer's protocol (Invitrogen). For real-time PCR, cDNA was generated by using a first-strand cDNA synthesis kit (Roche). The primers and probes were

purchased (Applied Biosystems). RT-PCR was performed on (ABI) PRISM 7700.

TCR Rearrangement. Genomic DNA was extracted from E18.5 fetal thymi by using a Qiagen DNA extraction kit, and 150 ng of DNA was used for each PCR. The primers for detecting DNA gene rearrangement were derived from sequences published in refs. 27 and 38.

Fetal Liver Cell Transplants. Single cell suspensions were generated from E14.5 fetal livers and cells (2×10^6) were injected into tail veins of irradiated Rag2$^{-/-}$ CD45.1 congenic recipient mice housed under pathogen-free conditions with medicated water.

Six to eight weeks later, tissues were harvested. Cells were counted and analyzed by flow cytometry for donor-derived CD45.2$^+$ cells of various lineages.

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