

The bHLH gene *Hes1* is essential for expansion of early T cell precursors

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Mice mutant for the bHLH gene *Hes1*, which is known to keep cells in a proliferative state, mostly lack thymus. Transfer of *Hes1*-null fetal liver cells into *RAG2*-null host mice normally reconstitutes B cells but fails to generate mature T cells in the thymus. In the reconstituted thymus, T cell differentiation is arrested at the CD4⁺CD8⁺ double positive (DP) stage. Both the initial T cell receptor (TCR)-independent and the subsequent TCR-dependent selective expansion during the DP stage are severely affected. Thus, *Hes1* is essential for the earliest thymocyte expansion in a cell-autonomous manner.

[Key Words: bHLH; *Hes1*; T cell; thymocyte; thymus]

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T-cell precursors in the thymus are initially negative for expression of the surface antigens CD4 and CD8 [double negative (DN); Anderson et al. 1996; Shortman and Wu 1996; von Boehmer and Fehling 1997]. These DN cells then differentiate into CD4⁺CD8⁺ [double positive (DP)] cells, which finally become CD4⁺CD8⁺ or CD4⁺CD8⁺ mature single-positive (SP) cells. DN cells are subdivided further into four distinct differentiation stages based on changes in the surface expression of CD44 and CD25 in the following order: CD44⁺CD25⁺ → CD44⁺CD25⁺ → CD44⁺CD25⁺ → CD44⁺CD25⁺ (Godfrey and Zlotnik 1993; Godfrey et al. 1993, 1994; Pénit et al. 1995). At the first stage (CD44⁺CD25⁺) DN cells are resting and they start proliferation at the next stage (CD44⁺CD25⁺). At the third stage (CD44⁺CD25⁺), DN cells undergo rearrangement of T cell receptor (TCR) β locus and, only when the β locus is productively rearranged, these cells selectively proliferate and differentiate into DP cells. Thus, expansion of DN cells occurs at two distinct phases: the initial TCR-independent and the subsequent TCR-dependent selective proliferation (Pénit et al. 1995).

Although cell proliferation is very important for T-cell development because the majority of thymocytes eventually die after selection, the molecular mechanisms that control thymocyte proliferation are still largely unknown. The basic helix-loop-helix (bHLH) gene *Hes1* (Sasai et al. 1992), which is expressed in the developing thymus from very early stages, is one of the candidate genes that regulate thymocyte proliferation, as persistent expression of *Hes1* keeps cells in a proliferative

state (Ishibashi et al. 1994; Tomita et al. 1996). *Hes1* is a transcriptional repressor and functionally inhibits differentiation factors such as MyoD and Mash1 (Sasai et al. 1992; Ishibashi et al. 1995; Tomita et al. 1996; Chen et al. 1997). In the developing nervous system, *Hes1* is expressed in dividing precursor cells, and only after *Hes1* activity is lost do precursor cells stop cell division and start the differentiation program (Kageyama and Nakanishi 1997; Ström et al. 1997).

Here, we examined roles of *Hes1* in T cell development by reconstituting the lymphoid system of *RAG2*-null mice with *Hes1*-null fetal liver cells. We found that T cell development is arrested at the DN stage in the absence of *Hes1* and that *Hes1* is essential for both the initial TCR-independent and the subsequent TCR-dependent selective expansion of thymocytes.

Results and Discussion

Hes1 is essential for T cell development

Hes1 expression during normal thymic development was first determined by Northern blot analysis. *Hes1* was already expressed at a high level in the developing thymus at embryonic day 15 (E15), but the expression gradually decreased afterwards (Fig. 1A). To determine the cell types that expressed *Hes1*, thymocytes and thymic stroma were separately prepared. Thymic stroma was prepared by treating the thymus with deoxyguanosine (dGMO) and it was negative for the thymocyte-specific CD3 expression (Fig. 1A). *Hes1* was expressed in both thymocytes and thymic stroma (Fig. 1A), suggesting that *Hes1* functions in both cell types.

To determine the *Hes1* function in T cell develop-

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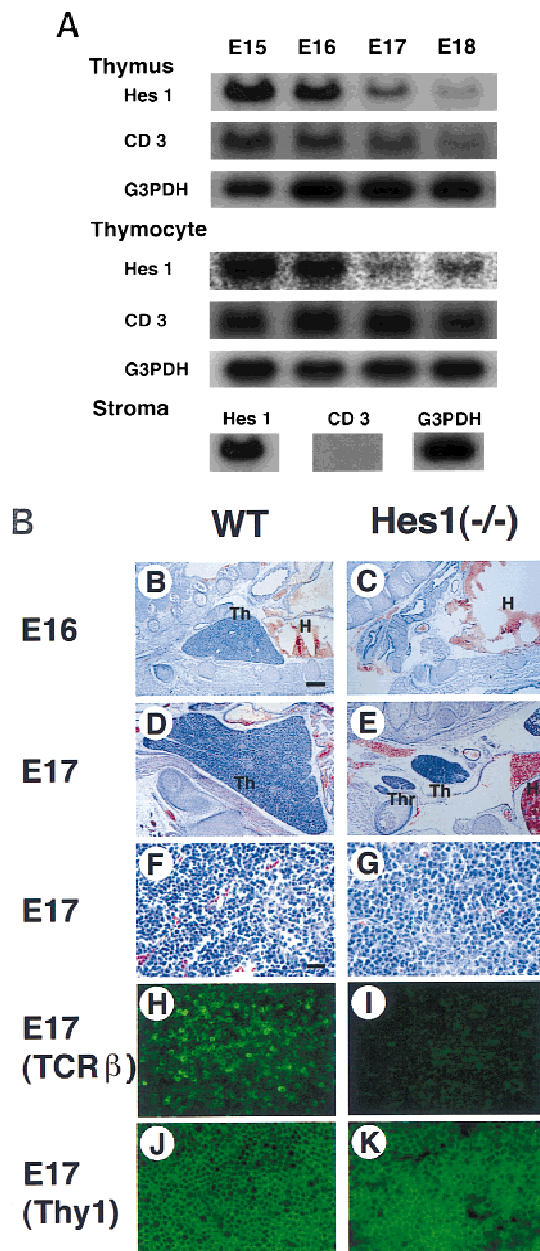


Figure 1. *Hes1* expression in the developing thymus and the thymic defects of mice mutant for *Hes1*. (A) *Hes1* expression in the thymus was determined by Northern blot analysis. *Hes1* was already expressed at a high level at E15, but expression decreased gradually afterward. *Hes1* was expressed by both thymocytes and stroma while CD3 was expressed only by thymocytes. G3PDH expression was examined as a control. (B–K) Thymic sections were prepared from wild-type (B,D,F,H,I) and *Hes1*-null (C,E,G,I,K) embryos and subjected to HE staining (B–G) and immunohistochemistry with anti-TCRβ (H,I) and anti-Thy1 antibodies (J,K). *Hes1*-null embryos lacked thymus (C) or had a very small thymus (E) compared to wild type (D). (F,G) A higher magnification of D and E. (H,I) Even when the thymus existed, αβ TCR⁺ thymocytes were absent in *Hes1*-null embryos (I), whereas they were present in wild-type thymus (H). (J,K) In the *Hes1*-null thymus, there were Thy1⁺ cells (K) as in the wild-type thymus (J). Scale bar, 150 μm (B–E); 25 μm (F–K). (H) Heart; (Th) thymus; (Thr) thyroid.

ment, we analyzed the mice mutant for *Hes1*. *Hes1*-null mice exhibit severe defects of neural tube and eye morphogenesis and die during gestation or just after birth (Ishibashi et al. 1995; Tomita et al. 1996). Surprisingly, >90% of *Hes1*-null embryos completely lacked the thymus (Fig. 1C), and in the rest the thymus was much smaller in size than that of the wild type (Fig. 1D,E). In the thymus of *Hes1*-null mice, there were some thymocytes (Thy1⁺, Fig. 1G,K) but TCRβ (Fig. 1I) and TCRγδ expression (data not shown) was not detectable, suggesting that mature T cells were virtually absent. These results demonstrate that *Hes1* is essential for T cell development.

Because *Hes1* is also expressed in embryonal liver and spleen (Sasai et al. 1992), other lymphohematopoietic lineages in *Hes1*-null mice were examined by studying fetal liver cells. The number of the recovered *Hes1*-null fetal liver cells $[(2.0 \pm 0.4) \times 10^7]$ was comparable to that of the wild type cells $[(2.5 \pm 0.5) \times 10^7]$. Flow cytometric analysis revealed that erythroids (TER-119⁺, Fig. 2A), myeloids (Gr-1⁺, Fig. 2B), and monocytes (Mac-1⁺, Fig. 2C) were normally generated in the absence of *Hes1*. In addition, *Hes1*-null fetal liver cells cultured on PA6 stromal cells in the presence of IL-7 (Sudo et al. 1989; Rolink et al. 1993) generated B-lineage cells comparably to wild-type cells (IgM⁺, B220⁺, Fig. 2D,E). Thus, cells of lymphohematopoietic lineages other than thymocytes appeared to develop normally in mice mutant for *Hes1*.

Hes1 is critical for thymocyte expansion in a cell-autonomous manner

Because *Hes1* is expressed in both stromal cells and thymocytes, the thymic phenotypes of *Hes1*-null mice could be due to abnormality of stromal cells and/or thymocytes. To determine whether *Hes1*-null thymocytes are intrinsically affected, we made bone marrow chimeras using *RAG2*-null mutant mice, which have no mature T or B lymphocytes (Shinkai et al. 1992). Fetal liver cells from *Hes1*-null or wild-type donor embryos were injected intravenously into irradiated *RAG2*-null mice of C57BL/6 background to allow differentiation of donor progenitors in the environment of normal thymic stromal cells. Lymphoid cells (Thy1⁺) of the donor origin were distinguished by Ly5.2 expression from those of possible host origin that are negative for Ly5.2 (Fig. 3B). This analysis could also help determine more precisely the stage at which T cell development is impaired in the absence of *Hes1*.

When reconstituted with wild-type or *Hes1*-null fetal liver cells, the *RAG2*-null hosts had a comparable number of mature B cells in the spleen (IgM⁺, B220⁺, Fig. 3C,D), indicating that reconstitution was successful and that B cells develop normally in the absence of *Hes1*. In contrast, the absolute number of thymocytes of the *Hes1*-null origin was by far less than that of the wild-type origin in the reconstituted *RAG2*-null thymus (Fig. 3A). When 1×10^6 and 1×10^7 fetal liver cells of wild-type origin were injected, an average of 1.6×10^8 ($n = 13$) and 1.0×10^8 ($n = 6$) thymocytes, respectively, was re-

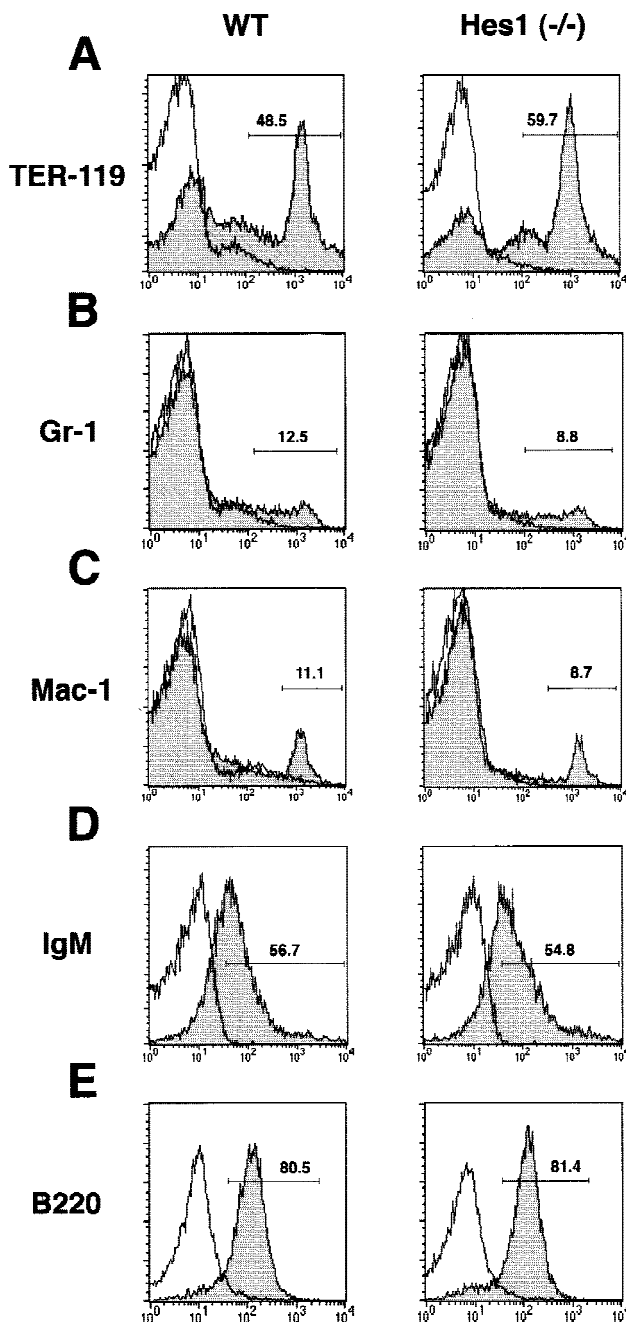


Figure 2. Flow cytometric analysis of fetal liver cells. Flow cytometric analysis was performed to determine the surface expression of TER-119 (A), Gr-1 (B), and Mac-1 (C). For B cell lineage analysis, fetal liver cells were cultured on PA6 stromal cells in the presence of IL-7 and examined for expression of IgM (D) and B220 (E). Cells were analyzed after incubation in the presence (shaded area) or absence (bold line only) of FITC-conjugated antibodies. No abnormality was detected in *Hes1*-null cells.

covered. In contrast, when 1×10^6 *Hes1*-null cells were injected, only 1.7×10^5 thymocytes were recovered ($n = 9$); and even when 1×10^7 *Hes1*-null cells were injected, only 4.1×10^5 thymocytes were recovered ($n = 8$), indicating that the thymocyte number of the *Hes1*-null

origin was 200- to 1000-fold less than that of wild-type origin. The thymocyte number of the *Hes1*-null origin was still 10- to 20-fold less than that of the nonreconstituted *RAG2*-null host mice (3.2×10^6 cells; $n = 10$) (Fig. 3A). These results demonstrate that thymocytes are intrinsically affected by *Hes1* mutation and that *Hes1* is critical for expansion of thymocytes.

Thymocyte expansion at the DN stage is impaired by Hes1 mutation

To determine the stage at which T cell development is impaired in the absence of *Hes1*, thymocytes of the donor origin were analyzed further with various surface markers. Whereas both $\alpha\beta$ and $\gamma\delta$ T cells were generated from wild-type donors, neither mature T cells were detected in the thymus reconstituted with *Hes1*-null donor cells (Fig. 4A). Furthermore, the vast majority of thymocytes of *Hes1*-null origin were negative for expression of the surface antigens CD4 and CD8 (Fig. 4B) but highly expressed the early marker heat-stable antigen (HSA) in the virtual absence of CD3 (Fig. 4C). Thus, in the absence of *Hes1*, T cell development is arrested at the CD3⁻DN stage. Essentially the same abnormality was observed irrespective of the number of the injected cells (1×10^6 or 1×10^7 , Fig. 4A–C).

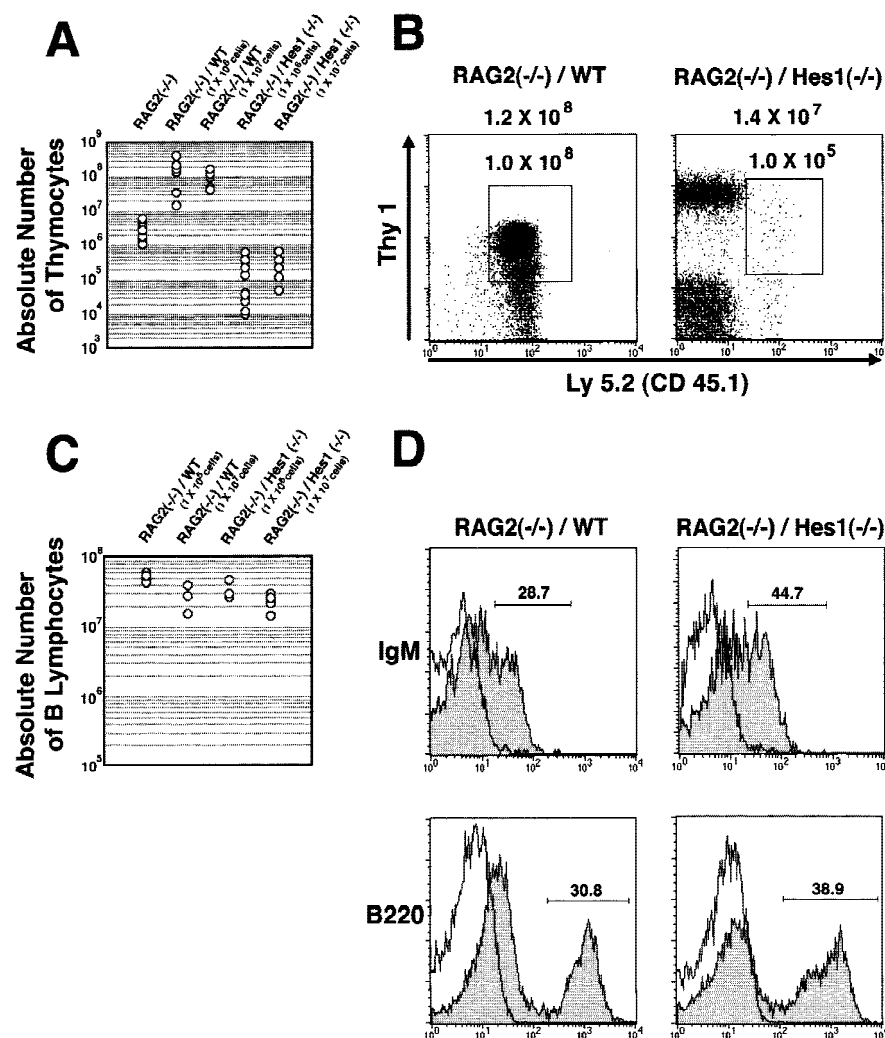
Because DN cells are further divided into four distinct differentiation stages based on the profiles in the surface expression of CD44 and CD25 (Godfrey and Zlotnik 1993; Godfrey et al. 1993, 1994; Pénit et al. 1995), donor DN thymocytes were analyzed with these markers (Fig. 4D). At the first stage (CD44⁺CD25⁻) DN cells are normally resting and the absolute number of *Hes1*-null DN thymocytes was, on an average, 1.3×10^5 cells ($n = 4$) and 0.6×10^5 cells ($n = 4$) when 1×10^6 and 1×10^7 cells, respectively, were injected. These numbers were comparable to those of wild-type DN cells (1.2×10^5 cells; $n = 9$). However, when 1×10^6 *Hes1*-null cells were injected, at the subsequent stages the number of *Hes1*-null thymocytes was by far less than that of the wild-type cells, which are known to proliferate TCR independently first and then TCR dependently during the DN stages (Fig. 4D). When 1×10^7 *Hes1*-null cells were injected, DN thymocytes increased at the CD44⁻CD25⁺ stage (2.2×10^5 cells; $n = 4$), but the number was still much less than the wild type (1.5×10^6 cells; $n = 9$) (Fig. 4D). Thus, proper expansion of DN thymocytes was severely impaired in the absence of *Hes1*.

The spleen reconstituted with *Hes1*-null donors contained virtually no $\alpha\beta$ T cells, but interestingly it had some $\gamma\delta$ T cells (Fig. 4E). Because the *RAG2*-null host mice did not have such T cells (Fig. 4F), they were derived from *Hes1*-null fetal liver cells. Thus, some $\gamma\delta$ T cells may develop normally in the absence of *Hes1*, although it remains to be determined whether they differentiate at the intrathymic or extrathymic environments.

Both TCR-independent and -dependent thymocyte expansion is affected by Hes1 mutation

DN cells normally undergo rearrangement of TCR β lo-

Figure 3. Reconstitution of the lymphoid system of *RAG2* mutant mice by transfer of wild-type and *Hes1*-null fetal liver cells. Either wild-type or *Hes1*-null fetal liver cells (1×10^6 or 1×10^7 , indicated in parentheses above the panel) were transferred intravenously into *RAG2* mutant mice to reconstitute the lymphoid system, and after 4–6 weeks of transfer, the lymphoid cells were examined. (A) The absolute numbers of total thymocytes. From the reconstituted thymuses, only Ly5.2⁺ cells were counted. The average thymocyte number with standard error is *RAG2*^{-/-}, $(3.2 \pm 0.6) \times 10^6$ ($n = 10$); *RAG2*^{-/-}/WT (1×10^6 cells injected), $(1.6 \pm 0.3) \times 10^8$ ($n = 13$); *RAG2*^{-/-}/WT (1×10^7 cells injected), $(1.0 \pm 0.2) \times 10^8$ ($n = 6$); *RAG2*^{-/-}/*Hes1*^{-/-} (1×10^6 cells injected), $(1.7 \pm 0.7) \times 10^5$ ($n = 9$); *RAG2*^{-/-}/*Hes1*^{-/-} (1×10^7 cells injected), $(4.1 \pm 1.0) \times 10^5$ ($n = 8$). The number of *Hes1*-null thymocytes averaged 200- to 1000-fold less than that of wild-type thymocytes and 10- to 20-fold less than that of irradiated and nonreconstituted *RAG2*-null thymocytes. (B) Flow cytometric analysis of the reconstituted thymus with α -Ly5.2 and α -Thy1. The absolute number of total cells is indicated above each panel; the number of Ly5.2⁺Thy1⁺ cells is shown above the gated region. (C) The absolute numbers of B cells in the spleen. The B cell number of *Hes1*-null origin was normal. (D) Flow cytometric analysis of the reconstituted spleen with α -IgM and α -B220. B cells developed normally in the absence of *Hes1*.



cus at the CD44⁺CD25⁺ stage, and only when the β locus is productively rearranged are these cells allowed to selectively proliferate and mature into the last stage (CD44⁺CD25⁺) (Godfrey and Zlotnik 1993; Godfrey et al. 1993, 1994; Pénit et al. 1995). Because the thymocyte number at the CD44⁺CD25⁺ and CD44⁺CD25⁻ stages was reduced significantly by *Hes1* mutation, it is possible that TCR gene rearrangement is affected in *Hes1*-null thymocytes. We therefore examined rearrangement of TCR genes by amplifying the genomic DNA of the reconstituted thymi (Anderson et al. 1992; Itohara et al. 1993; Maki et al. 1996). Because in reconstituted mice *Hes1*-null thymocytes were, on an average, 200- to 1000-fold less in number than wild-type thymocytes, we used a 500-fold dilution of the wild-type DNA as a template to normalize the cell numbers. In the thymus and spleen of *RAG2* mutant mice reconstituted with wild-type cells, TCR gene rearrangements clearly occurred (+/+ in Fig. 5A–H). In contrast, when *RAG2* mutant mice were reconstituted with 1×10^6 *Hes1*-null cells, complete rearrangement was not detected at the TCR α or β locus (–/– in Fig. 5A,E), although only a low level of D–J recombi-

nation could be detected at the TCR β locus (–/– in Fig. 5C). TCR γ gene rearrangement was not detected either in the thymus reconstituted with 1×10^6 *Hes1*-null cells (–/– in Fig. 5G). These results demonstrated that the *Hes1* mutation severely affected expansion of immature thymocytes before the initiation of TCR gene rearrangement. However, when *RAG2* mutant mice were reconstituted with 1×10^7 *Hes1*-null cells, TCR gene rearrangements became detectable (–/– in Fig. 5B,D,F,H), suggesting that *Hes1* is not a prerequisite for the TCR gene rearrangements per se. Even when TCR gene rearrangements could take place, *Hes1*-null thymocytes did not expand properly at the CD44⁺CD25⁺ and CD44⁺CD25⁻ stages (see above), suggesting that TCR-dependent selective proliferation seemed to be disturbed in the absence of *Hes1*. However, the amount of TCR gene rearrangements was still low in the absence of *Hes1*; therefore, future experiments with TCR transgenic in the *Hes1*^{-/-} background will be required to determine the role of *Hes1* in TCR-dependent expansion and differentiation.

Interestingly, TCR γ 2 and TCR γ 4 gene rearrangements

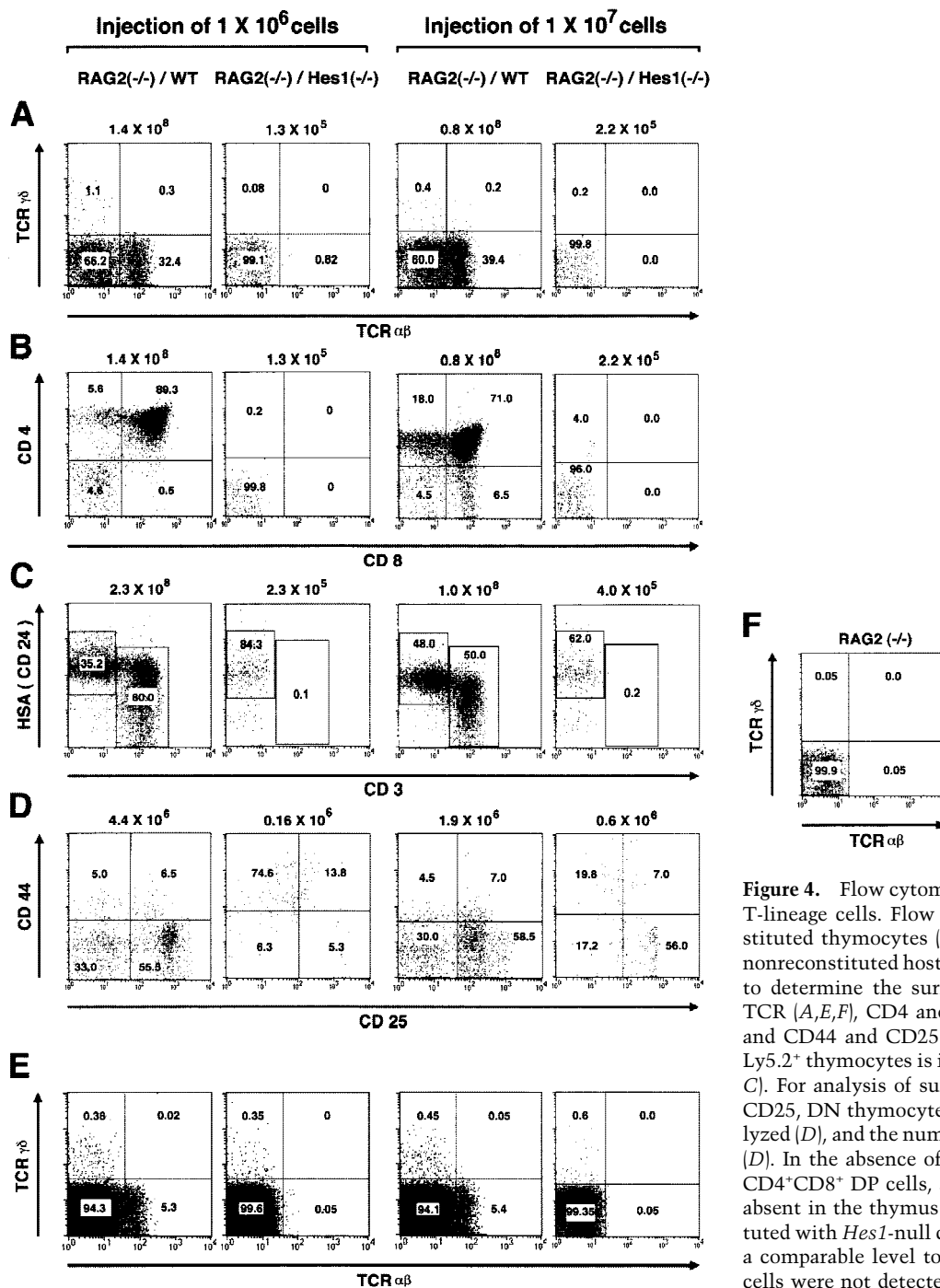


Figure 4. Flow cytometric analysis of reconstituted T-lineage cells. Flow cytometric analysis of reconstituted thymocytes (A–D) and spleen cells (E) and nonreconstituted host spleen cells (F) was performed to determine the surface expression of $\alpha\beta$ and $\gamma\delta$ TCR (A,E,F), CD4 and CD8 (B), HSA and CD3 (C), and CD44 and CD25 (D). The absolute number of Ly5.2⁺ thymocytes is indicated above each panel (A–C). For analysis of surface expression of CD44 and CD25, DN thymocytes from donor origin were analyzed (D), and the number is shown above each panel (D). In the absence of *Hes1*, $\alpha\beta$ and $\gamma\delta$ TCR⁺ cells, CD4⁺CD8⁺ DP cells, and CD3⁺ cells were virtually absent in the thymus (A–C). In the spleen reconstituted with *Hes1*-null cells, $\gamma\delta$ T cells were present at a comparable level to the wild type, whereas $\alpha\beta$ T cells were not detected (E).

were consistently and selectively detectable in the spleen but not in the thymus when *RAG2* mutant mice were reconstituted with 1×10^6 *Hes1*-null fetal liver cells (–/– in Fig. 5G). Thus, $\gamma\delta$ T cells with restricted repertoire could develop normally in the absence of *Hes1*.

Unique and essential functions of *Hes1* in T cell development

In this study, we have demonstrated that early events of

$\alpha\beta$ and $\gamma\delta$ T cell development in the thymus, such as cell expansion and TCR gene rearrangement, are severely impaired in *Hes1*-null mice. Particularly, *Hes1* seems to be essential for cell expansion at the very early stages, as *Hes1*-null cells did not expand at the CD44⁺CD25⁺ stage in the reconstituted thymus when 1×10^6 fetal liver cells were injected. Homing of *Hes1*-null progenitors to the thymus appeared unaffected, as the number of *Hes1*-null thymocytes at the CD44⁺CD25[–] stage was comparable with that of wild-type cells. It is likely that *Hes1*-null thymocytes might remain as resting cells or be more

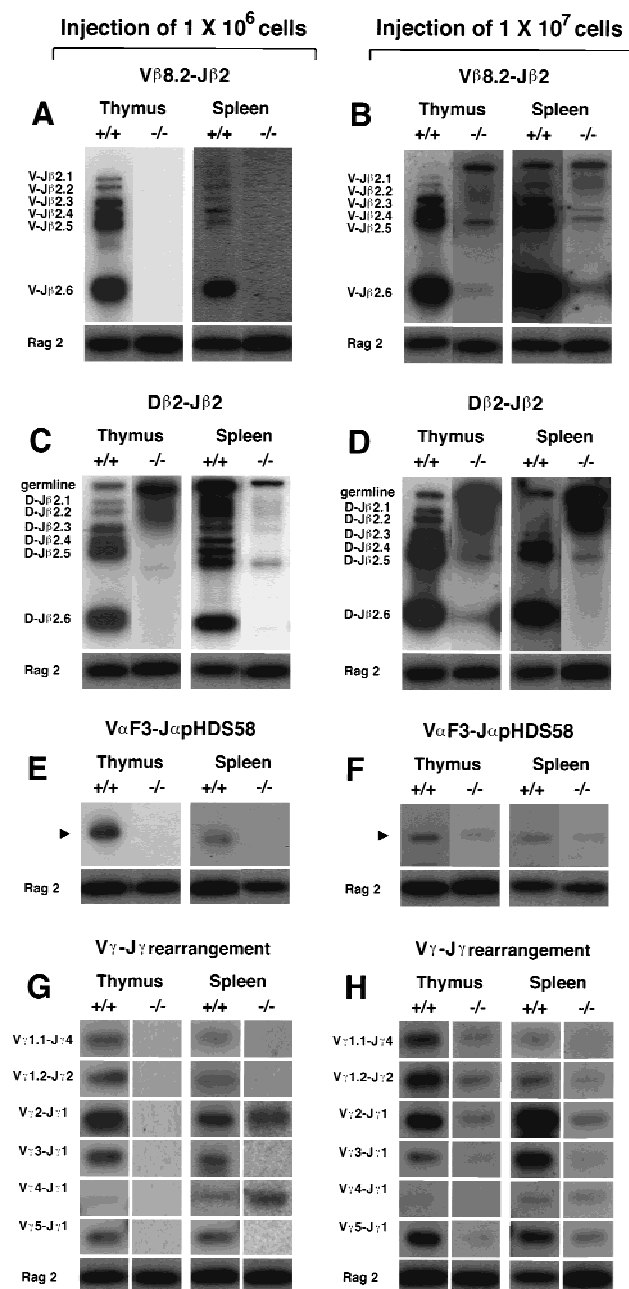


Figure 5. Rearrangement of TCR genes. Either 1×10^6 (A,C,E,G) or 1×10^7 (B,D,F,H) fetal liver cells of wild-type (+/+) and *Hes1*-null origin (-/-) were transferred intravenously into *RAG2* mutant mice, and rearrangements of TCR genes were examined by PCR followed by Southern blot analysis with ³²P-labeled oligonucleotide probes. In the thymus reconstituted with 1×10^6 *Hes1*-null cells, no apparent rearrangement was detected in the TCR α and TCR β loci (A,E), although only a low level of D-J recombination could be detected (C). Whereas TCR γ gene rearrangement was not detected in the thymus reconstituted with 1×10^6 *Hes1*-null cells, TCR γ 2 and TCR γ 4 genes (Maki et al. 1996) were rearranged in the spleen in the absence of *Hes1* (G). When 1×10^7 *Hes1*-null cells were injected, TCR gene rearrangement was detected (B,D,F,H). As an internal control for PCR, the *RAG2* gene was amplified. The *RAG2* signal was derived only from the donor cells.

susceptible to apoptosis. This defect of expansion might contribute to the lack of or a small-sized thymus in *Hes1* mutant mice, although it is possible that certain functional defects of the *Hes1*-null stromal cells additionally contribute to the thymic aplasia.

It has been shown that the selective proliferation of DN cells that express functional TCRs involves various transcription factors such as HMG-box factors LEF-1 and TCF-1 and zinc finger transcription factor GATA-3 (Ting et al. 1996; Okamura et al. 1998). However, none of these factors appears to be involved in cell expansion before TCR gene rearrangement. Thus, *Hes1* is unique in regulating TCR-independent expansion of thymocytes at the earliest stage. Interestingly, in the absence of IL-7 receptor, thymocyte expansion is severely disturbed before TCR gene rearrangement (Peschon et al. 1994; Candéas et al. 1997), suggesting that IL-7 is one of the major growth factors involved in initial TCR-independent thymocyte proliferation. It is therefore tempting to speculate that *Hes1* functions downstream of the IL-7 signaling pathway. In addition to TCR-independent expansion, *Hes1* seems to be involved in expansion at later stages, as—even though TCR gene rearrangement occurred in the thymus of *RAG2*-null mice reconstituted with as many as 1×10^7 fetal liver cells—*Hes1*-null thymocytes were still confined exclusively to the DN fraction with negligible increase in cell number. In the developing nervous system, *Hes1* is known to keep cells in a proliferative state and down regulation of *Hes1* expression leads to transition to a nonproliferative differentiation stage (Kageyama and Nakanishi 1997). Present results have indicated that similarly in the thymus *Hes1* promotes the initial expansion of immigrant progenitor cells, which is essential for the extensive clonal diversification and selection to generate mature T cells.

Hes1 is also known as a target gene of the Notch signaling (Jarriault et al. 1995; Nishimura et al. 1998). It has been shown that both Notch and *Hes1* regulate CD4 SP versus CD8 SP fate choice of T cell development (Robey et al. 1996; Kim and Siu 1998). Although this function could not be examined here, as T cell development was arrested much earlier by *Hes1* mutation, collectively these data suggest that *Hes1* might function at multiple steps of T cell development.

Materials and methods

Hes1 expression in the thymus

Northern blot analysis was done as described previously (Sasai et al. 1992). The thymic stroma devoid of thymocytes was prepared as reported (Ikuta et al. 1990), with some modifications: Fetal thymic lobes (E15) were cultured at 37°C for 6 days in 7% CO₂ on filters in 12-well plates containing RPMI1640 supplemented with 10% fetal calf serum, 1.35 mM dGMO, and 50 μ M β -mercaptoethanol.

Histological analysis of mouse embryos

Whole embryos were fixed with 4% paraformaldehyde in PBS at 4°C for 1 hr and incubated in 30% sucrose in PBS at 4°C over-

night. Frozen sections were prepared at 16 μ m thickness and subjected to hematoxylin–eosin (HE) staining and immunohistochemistry. For immunohistochemistry, sections were incubated with biotinylated anti-TCR β (Pharmingen, H57-597) and anti-Thy1 antibody (Pharmingen, 53-2.1) followed by visualization with avidin-labeled fluorescein (Vector).

Fetal liver cell analysis

Single cell suspensions from fetal livers of E17 wild-type or *Hes1*-null embryos were prepared and subjected to flow cytometric analysis (Becton-Dickinson). The antibodies used were as follows: anti-TER-119 (Pharmingen, TER-119), anti-Gr-1 (Pharmingen, RB6-8C5), and anti-Mac-1 (Pharmingen, M1/70). Cultured fetal liver cells on PA6 stromal cells with IL-7 were subjected to immunostaining with anti-IgM (Pharmingen, R6-60.2), and anti-B220 antibodies (Pharmingen, RA3-6B2). Detailed culture conditions were described previously (Sudo et al. 1989; Rolink et al. 1993).

Reconstitution of RAG2 mutant mice

Fetal liver cells (1×10^6 to 10×10^6) from E13–E16 wild-type and *Hes1*-null embryos (Ly5.2⁺) were injected intravenously into 4 Gy irradiated RAG2 mutant mice (Ly5.2⁻). After 4–6 weeks, single cell suspensions were prepared from thymuses and spleens of the reconstituted RAG2 mutant mice and subjected to flow cytometric analysis (Becton-Dickinson). Only Ly5.2⁺ cells (the donor origin) were analyzed. The antibodies used were as follows: anti-TCR β (Pharmingen, H57-597), anti- $\gamma\delta$ TCR (Pharmingen, GL-3), anti-CD4 (Pharmingen, RM4-5), anti-CD8 (Pharmingen, 53-6.7), anti-CD25 (Pharmingen, 7D4), anti-CD44 (Pharmingen, IM7), anti-CD24 (HSA) (Pharmingen, M1/69), anti-CD3 (Pharmingen, 145-2C11), anti-Thy1 (Pharmingen, 53-2.1), and anti-CD45.1 (Ly5.2) (Pharmingen, A20).

PCR analysis for TCR gene rearrangement

The genomic DNA extracted from the reconstituted thymuses was amplified by PCR and subjected to Southern blot analysis with ³²P-labeled oligonucleotide probes as described previously (Anderson et al. 1992; Itohara et al. 1993; Maki et al. 1996). Because the numbers of *Hes1*-null thymocytes were 200- to 1000-fold less than those of wild-type thymocytes, we used a 500-fold dilution of the wild-type samples to normalize the cell numbers. As a PCR control, we amplified the RAG2 gene, which was derived only from donor cells. In each experiment, a similar intensity of the RAG2 band was detected, indicating that the number of donor cells was normalized.

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The bHLH gene *Hes1* is essential for expansion of early T cell precursors

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