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Overexpression of the Runx3 Transcription Factor Increases the Proportion of Mature Thymocytes of the CD8 Single-Positive Lineage

Kazuyoshi Kohu,* Takehito Sato,§ Shin-ichiro Ohno,§ Keitaro Hayashi,* Ryuji Uchino,* Natsumi Abe,* Megumi Nakazato,* Naomi Yoshida,* Toshiaki Kikuchi,† Yoichiro Iwakura,¶ Yoshihiro Inoue,‡ Toshio Watanabe,* Sonoko Habu,§ and Masanobu Satake2*

The Runx family of transcription factors is thought to regulate the differentiation of thymocytes. Runx3 protein is detected mainly in the CD4⁺⁻ subset of T lymphocytes. In the thymus of Runx3-deficient mice, CD4 expression is de-repressed and CD4⁺⁻ thymocytes do not develop. This clearly implicates Runx3 in CD4 silencing, but does not necessarily prove its role in the differentiation of CD4⁺⁻ thymocytes per se. In the present study, we created transgenic mice that overexpress Runx3 and analyzed the development of thymocytes in these animals. In the Runx3-transgenic thymus, the number of CD4⁺⁻ cells was greatly increased, whereas the numbers of CD4⁺⁻ and CD4⁺⁻ cells were reduced. The CD4⁺⁻ transgenic thymocytes contained mature cells with a TCRhighHSAlow phenotype. These cells were released from the thymus and contributed to the elevated level of CD4⁺⁻ cells relative to CD4⁺⁻ cells in the spleen. Runx3 overexpression also increased the number of mature CD4⁺⁻ thymocytes in mice with class II-restricted, transgenic TCR and in mice with a class I-deficient background, both of which are favorable for CD4⁺⁻ lineage selection. Thus, Runx3 can drive thymocytes to select the CD4⁺⁻ lineage. This activity is likely to be due to more than a simple silencing of CD4 gene expression.

Recent advances in our understanding of gene regulation in thymocyte differentiation have involved the roles of the Runx family of transcription factors (6). Expression of Runx1 protein is detected in immature, CD4⁺⁻ double-negative (DN) and premature DP thymocytes, as well as in mature SP thymocytes (8–11). As expected from this expression pattern, Runx1 appears to exert its function at each step of thymocyte differentiation. For example, both the transition of DN cells to the DP stage and the maturation of postselected SP cells are significantly perturbed if the endogenous Runx1 activity in thymus is reduced by artificially expressing a dominant interfering form of Runx1 (8, 11). Each of these steps is normally accompanied by a tremendous amount of cell proliferation, for which Runx1 function is necessary. Conditional targeting of Runx1 has also revealed that it has an indispensable role in the initial emergence of T-committed cells from stem cells (12).

In contrast to Runx1, the expression of Runx3 protein is detected mainly in the CD4⁺⁻ subset of thymocytes and splenocytes (9, 10). In accordance with this protein expression profile, CD4⁺⁻ thymocytes do not develop in the Runx3⁻/⁻ thymus (13, 14). Based on an analysis of CD4 gene regulation, Tanouchi et al. (13) proposed that Runx3 binds to the Runx elements in the CD4 silencer and represses CD4 expression. Use of a Morpholino antisense oligonucleotide in an in vitro thymocyte differentiation system also supported the requirement for Runx3 in the generation of CD4⁺⁻ cells (10).

These studies clearly implicate Runx3 in the regulation of CD4 expression, but do not necessarily prove its role in the differentiation of CD4⁺⁻ thymocytes per se. Loss-of-function experiments provide information about what Runx3 does but not about everything it can do. In the present study, we overexpressed a transgenic Runx3 specifically in the T lineage and analyzed the development of the transgenic thymocytes. Runx3 can actively drive thymocytes to the CD4⁺⁻ lineage, which implies that it does more than simply silencing CD4 gene expression.

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3 Abbreviations used in this paper: DP, double positive; SP, single positive; DN, double negative; HA, hemagglutinin; β₂m, β₂-microglobulin; ISP, immature single positive; E, embryonic day; HSA, heat-stable Ag.
Materials and Methods

Plasmids

The hemagglutinin (HA) tag that represents the epitope of flu virus HA was fused to the N terminus of the murine Runx3 coding region by the PCR method as follows. PCR was performed using a murine Runx3 cDNA (15) as a template. The sequences of the sense and antisense primers were 5'-GCC GGA TCC GTA ACC ACC TAT CCA TAT GAT CTC GAT TAT GCT ATG CTT CCC GTA GAC GAC CC-3' and 5'-GCC GGA TCC GAA TTC TTA GTA GGG CCG CCA CAC-3', respectively. The PCR product was digested with BamHI and subcloned into the BamHI site of pLck (p1017), which harbors the proximal promoter region of the murine Lck gene and a poly(A) addition sequence derived from the human growth hormone gene (16). The resulting plasmid was designated pLck-HA/Runx3. The accuracy of the modified sequences in the plasmid was confirmed by sequencing. Immunohistochemical staining of cDNA-transfected HeLa cells confirmed the nuclear localization of HA-tagged Runx3 protein (data not shown).

RT-PCR

Total cytoplasmic RNA was isolated from cells using the ISOGEN reagent (Nippon Gene). cDNAs were synthesized from the RNAs by reverse transcription using Superscript II reverse transcriptase (Invitrogen Life Technologies). The cDNAs were PCR-amplified (25 cycles for each gene) with LA-Taq polymerase (Takara), using the following sense and antisense primers to detect transcripts: for CD4, 5'-CCG AGA GTG TCG GCC AGA AGA AGA TCA CAG-3' and 5'-TGA TAG TCT TGC TCT TAA AAC CCA GCA CTG-3'; for CD8α, 5'-GGT GAG TCG ATT ATC CTT CGG AGT GGA GAA-3' and 5'-ACA ACA TTT TCT CTT AGG AAG TCC GGC GTC TGT GCT-3'; for perforin, 5'-CAA GGA GAA GAA CAA GTT GCG CTT GCT-3' and 5'-CCG GGT GGA GAA GAG AAA GAG TGC CTC-3'; and for G3PDH, 5'-ACC ACA GTC GAT GCC ATC AC-3' and 5'-TCC ACC ACC TTG CTG TA-3'. The PCR products were run through agarose gels and visualized with ethidium bromide staining.

Chromatin immunoprecipitation assay

A chromatin fraction was prepared from thymocytes, fixed and immunoprecipitated with the anti-Rnx or anti-HA Ab, respectively. The procedures were as recommended by the manufacturer of the assay kit (Upstate Cell Signaling Solutions). DNA was purified from the precipitate and processed as a template for PCR to amplify the CD4 silencer-specific sequence. The primers for PCR were 5'-TGG AGC CAC CCG AGA CAA AG-3' and 5'-GTT GCA GCA GAG CAG CCC CA-3'. The amplified product was run through agarose gels and transferred to nylon membranes. The membranes were hybridized with 32P-labeled, CD4 silencer-specific oligonucleotide, 5'-ATA CGA AGC TAG GCA ACA GA-3'.

Results

Overexpression of Runx3 in the T lineage cells

Endogenous expression of Runx3 protein is detected mainly in the CD4-8 subset of T lymphocytes (9, 10). To artificially overexpress Runx3 in the T cell lineage, we placed the Runx3 coding
Runx3 was also detected in the wild-type, CD4+pared from transgenic thymi as well as spleens. The endogenous (Fig. 1A). The 52-kDa Runx3 band was clearly detected in the A was examined by immunoblot analysis using an anti-Runx Ab mouse lines were established and the expression of Runx3 protein region under the control of the proximal Lek gene promoter. This promoter is known to be active in immature as well as mature T cells and in thymic as well as peripheral T cells (16). Transgenic mouse lines were established and the expression of Runx3 protein was examined by immunoblot analysis using an anti-Runx Ab (Fig. 1A). The 52-kDa Runx3 band was clearly detected in the extract of both CD4+8" and CD4+8" fractions, which were prepared from transgenic thymi as well as spleens. The endogenous Runx3 was also detected in the wild-type, CD4+8" thymocytes and splenocytes but to a much lesser degree compared with the transgenic cells. Thus, the magnitude of Runx3 overexpression in the transgenic vs wild-type cells was roughly 5-fold in the case of thymi and 3-fold in the case of spleens. A very faint band seen in the CD4+8" wild-type cells represents the nonspecific reaction of the Ab, because the band was not abolished by the preabsorption of the Ab with the Ag peptide. The endogenous Runx1 protein of 56 kDa was detected in all the fractions tested.

We assessed the contribution of overexpressed Runx3 to the Runx-specific DNA binding activity using EMSA (Fig. 1B). The endogenous activity detected in a thymocyte extract from wild-type mice was mainly due to the Runx1 protein. The extract from transgenic thymocytes gave rise to a band that migrated slightly faster, reflecting the smaller size of the Runx3 protein compared to the endogenous activity detected in a thymocyte extract from wild-type mice. Thus, the magnitude of Runx3 overexpression in the transgenic vs wild-type cells was roughly 5-fold in the case of thymi and 3-fold in the case of spleens. A very faint band seen in the CD4+8" wild-type cells represents the nonspecific reaction of the Ab, because the band was not abolished by the preabsorption of the Ab with the Ag peptide. The endogenous Runx1 protein of 56 kDa was detected in all the fractions tested.

The percentage of CD4+8" cells increased and the percentage of CD4+8" and CD4+8" cells simultaneously decreases in the Runx3-transgenic thymus

After confirming the protein expression of transgenic Runx3, we evaluated its effect on T cell differentiation. Flow cytometry was used to analyze CD4 and CD8 in thymocytes and splenocytes (Fig. 2). In the Runx3-transgenic thymi, the percentage of CD4+8" cells increased to 80% of the total population, whereas the percentage of CD4+8" cells decreased to only 9%; the percentage of CD4+8" cells also decreased substantially. The unusual profile of CD4 and CD8 expression in the transgenic thymocytes was reflected in the transgenic splenocytes as well. In the transgenic spleen, the percentage of CD4+8" cells was higher than that of CD4+8" cells, whereas the opposite was true in the wild-type spleen.

We counted the number of cells that were recovered from the thymi and spleens of several individual adult mice (Table I). The number of transgenic thymocytes was ~60% of that of wild-type

Table I. The numbers and percentages of SP cells in wild-type and Runx3-transgenic thymi and spleens

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<tr>
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<th>Thymus</th>
<th>Spleen</th>
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<tr>
<td></td>
<td>Total cells (×10^6)</td>
<td>CD4+8&quot; (%)</td>
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<tr>
<td>Wild type (n = 9)</td>
<td>2.20 ± 0.67</td>
<td>80.8 ± 1.5</td>
</tr>
<tr>
<td>Runx3-transgenic (n = 9)</td>
<td>1.33 ± 0.46</td>
<td>10.9 ± 1.7</td>
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a The means and SD are presented. n, The number of individual mice examined.
thymocytes. As a result, the number of cells in the CD4^8^ fraction was higher, and the number in the CD4^8^ and CD4^8^ fractions was lower, in the transgenic thymi compared with the wild-type thymi. The total number of splenocytes did not differ significantly between the two genotypes.

Decrease in the CD4 expression in the Runx3-transgenic thymocytes

The increase in the CD4^8^ fraction in the Runx3-transgenic thymus could be due either to an increase in CD8 expression or a decrease in CD4 expression. To distinguish these two possibilities, the CD8 and CD4 expression profiles were displayed for the wild-type and the Runx3-transgenic thymocytes (Fig. 3A). The relative ratios of CD8^+^ and CD8^−^ cells were not different between the two genotypes. In contrast, the number of CD4^+^ cells was greatly increased and the number of CD4^−^ cells was decreased in the transgenic thymus compared with the wild-type thymus.

We also performed a semiquantitative RT-PCR analysis of CD4 and CD8 transcripts (Fig. 3B). RNA was prepared from the thymocytes, and increasing amounts of the cDNAs were processed for PCR. Although the relative amount of CD8 transcript did not differ significantly between the two types of cells, many fewer CD4 transcripts were present in the Runx3-transgenic thymocytes compared with the wild-type cells.

The CD4 silencer is proposed to be a main target by a Runx3 transcription factor (13, 14). We checked this by chromatin immunoprecipitation analysis (Fig. 3C). An increasing amount of chromatin fraction-derived DNA that was precipitated by the anti-Runx or anti-HA Ab was processed for PCR and hybridized by a CD4 silencer-specific oligonucleotide. Both Abs precipitated a significantly greater amount of CD4 silencer sequence from the Runx3-transgenic thymocytes compared with the wild-type cells. The results in Fig. 3 thus suggest that the phenotypic alteration seen in the transgenic thymocytes in Fig. 2 can be at least partly explained by the down-regulation of CD4 expression.

The increased CD4^8^ fraction of transgenic thymocytes includes immature, premature, and mature subpopulations

We next characterized in detail the CD4^8^ fraction of transgenic thymocytes. As described below, this fraction was found to contain three different subpopulations: immature, premature, and mature cells.
The first subpopulation in the CD4−8− fraction was recognized as immature single-positive (ISP) cells, which can be easily seen by following the ontogeny of thymocyte development (Fig. 4A). In wild-type thymus, only CD4−8− cells were detected at embryonic day (E)15.5. CD4−8− ISP cells transiently appeared at E16.5, CD4−8− cells at E17.5, and CD4−8− cells at day 2 after birth. In the Runx3-transgenic thymus, immature CD4−8− cells first appeared at E16.5 and remained as the main population until after birth. The persistence of ISP cells is probably due to the down-regulation of CD4 by Runx3. This CD4 repression appeared to be partial, because some CD4−8− and CD4+8+ cells emerged at day 2 after birth in transgenic mice.

Immature CD4+8− cells were also prominent in thymi from adult transgenic mice. To further characterize this population, flow cytometry was first used to select the CD4−8− fraction of the thymocytes, and then the expression profiles of TCRβ (hereafter TCR) and heat-stable Ag (HSA) were displayed for this fraction (Fig. 4B). The immature TCRlowHSAhigh fraction made up 27% of the wild-type and 57% of the transgenic CD4−8− thymocytes. Therefore, overexpression of Runx3 increased the number of ISP cells.

Another characteristic of the transgenic CD4−8− fraction was the presence of an aberrant TCRmedHSAhigh subpopulation that was not as apparent in the wild-type fraction (33 vs 4%; Fig. 4B). The medium degree of TCR expression indicates that this second subpopulation should be categorized as representing the premature DP stage rather than the ISP stage. We further confirmed this point by staining the thymocytes with CD69, a marker of positive selection (Fig. 4C). In the case of wild-type cells, the TCRmedCD69− cells exhibited a CD4+8− phenotype, whereas the TCRmedCD69+ exhibited both the CD4+8− and CD4+8+ phenotypes. The TCRmedCD69− population could also be detected in the transgenic thymus, but the apparent phenotype of this population was CD4+8−, not CD4+8+. The CD4+8− fraction persisting in the developing transgenic thymus (Fig. 4A) may contain these TCRmed cells as well. Thus, the second subpopulation can be summarized as the premature, “CD4-repressed DP” cells.

The transgenic CD4−8− fraction also contained a third subpopulation of mature, TCRhighHSAlow cells (see 10% in Fig. 4B). We next evaluated the effect of Runx3 overexpression on these mature CD4−8− cells. To do so, we first obtained a TCR expression profile for the total thymocyte population (Fig. 5A). Both the wild-type and Runx3-transgenic thymi contained TCRlow, TCRmed, and TCRhigh subpopulations to a comparable degree. Because the TCRhigh subpopulation corresponds to mature cells, overexpression of Runx3 did not appear to arrest or block thymocyte differentiation. We gated the TCRhigh subpopulation and then displayed the CD4+8+ profile (Fig. 5B). In the TCRhigh thymocytes from the wild-type, the percentage of CD4+8+ cells was one-third that of CD4−8− cells, whereas in the transgenic TCRhigh thymocytes, the percentage of CD4+8+ cells was three times that of CD4−8− cells. We also counted the cell numbers constituting each fraction and found that the absolute number of CD4+8+ TCRhigh cells in the transgenic thymi was approximately twice that in the wild-type thymi.

To further verify the differentiation stage of the apparently mature CD4−8− cells that were generated in Runx3-transgenic thymi, we examined the marker expression in the HSA−low cells by RT-PCR analysis (Fig. 5C). A transcript of perforin1, a CD4+8− marker (21), was clearly detected in the wild-type as well as Runx3-transgenic CD4−8− cells, but detected only in a subtle amount in the CD4−8− cells of both genotypes. In contrast, a transcript of GATA3, a CD4+8− marker (21), was expressed more abundantly in the CD4−8− cells than in the CD4−8− cells irrespective of genotypes of cells. The results in Fig. 5 indicate that the overexpressed Runx3 in fact promoted the differentiation and maturation of thymocytes toward the CD8 lineage.

**FIGURE 5.** Effect of the Runx3 transgene on the differentiation of mature, TCRhigh cells. A, The thymocytes from wild-type and Runx3-transgenic mice were stained for TCR, and their expression profiles were analyzed. The cells were classified into three subpopulations: TCRlow, TCRmed, and TCRhigh. B, The wild-type and Runx3-transgenic thymocytes were processed for three-color flow cytomterical analysis. The mature, TCRhigh subpopulation was selected, and its CD8 and CD4 expression profile was analyzed. The numbers in the individual quadrants indicate the percentage of cells of each type. C, Semiquantitative RT-PCR analysis of perforin1, GATA3, and G3PDH transcripts. RNA was isolated from the CD4+8− HSA−low and CD4+8+ HSA−low thymocytes’ fractions and converted to cDNA. An increasing amount of cDNA synthesized from the wild-type and Runx3-transgenic cells, respectively, was processed for PCR.

The mature CD4−8− cells are released into periphery of Runx3-transgenic mice

Promotion of thymocyte differentiation toward the CD8 lineage by Runx3 was also reflected in the cell composition in the spleen (Fig. 6, A and B). Among the TCRhighHSA−low mature T cells, the ratio of CD4−8− cells to CD4+8− cells was 0.5 in the spleens from the wild type, but was 1.4 in the transgenic splenocytes.

We wondered whether the increase in mature CD8− cells reflected the preferential expansion of a specific repertoire of TCR. We therefore examined the usage of Vβ regions by the TCR−highCD8− splenocytes using flow cytometry (Fig. 6C). The pattern of the Vβ repertoire was essentially similar between the transgenic and wild-type cells. Therefore, in the Runx3-transgenic mice, apparently normal, multicolonial, mature CD8− cells were generated in the thymus and released into periphery as in the wild-type mice.
Overexpression of Runx3 can drive originally CD4-oriented thymocytes toward the CD8 lineage

The results shown in Figs. 5 and 6 indicate that the overexpressed Runx3 can drive thymocytes to select and mature along the CD8 lineage. We then examined whether this effect of Runx3 is dependent on the TCR signaling elicited from proper MHC interactions. The TCR transgene, which is restricted to MHC class II, was introduced into Runx3 transgenic mice (Fig. 7A). Thymi from TCR single-transgenic mice showed a skew of cell differentiation to the CD4 lineage (33% CD4+/8− compared with 3.6% CD4+/8−). In contrast, in the TCR and Runx3 double-transgenic thymi, the CD4+/8− cells constituted the major population (73%), just as in the case of Runx3 single-transgenic thymi. When only the mature cells were selected by gating the HSA low fraction (and by gating the transgene-specific TCRhigh fraction as well [data not shown]), it was clear that the Runx3 transgene switched the differentiation of class II-restricted cells to the CD8 lineage.

We further confirmed the cell-autonomous activity of Runx3 by altering the MHC background. The β2m (−/−), class I-deficient thymus provides an environment unfavorable for the selection of CD4+/8− cells (Fig. 7B). In the TCRhigh fraction, 90% of wild-type thymocytes were CD4+/8− cells. In contrast, the Runx3 transgene appeared to shift the differentiation of thymocytes toward the CD8 lineage even in the context of class I deficiency. Thus, overexpressed Runx3 can push a cell toward the CD8 lineage independently of the MHC-elicted TCR signaling.

Overexpression of Runx3 can drive thymocytes toward the CD8 lineage irrespective of the CD4 signaling

In thymocyte differentiation, the TCR signaling exerts its effect in concert with the signaling elicited from either the CD4 or CD8 molecule. We examined the activity of overexpressed Runx3 on thymocyte differentiation under the condition of either excess or deficiency of CD4 signaling. First, the Runx3 transgene was introduced into human CD4−transgenic mice (Fig. 8A). As seen, the level of human CD4 expression was not so high and therefore might be limited to compensate the endogenous, murine CD4, which should be silenced by the overexpressed Runx3. Under this limitation, a majority of mature TCRhigh cells possessed a CD4+/8− phenotype in Runx3−transgenic thymi. Second, the Runx3−transgenic mice were expressed in a CD4−deficient background (Fig. 8B). When a CD4+/8− fraction was displayed for its TCR expression, the mature TCRhigh cells corresponded to 27% of CD4−deficient and Runx3−transgenic thymocytes. In contrast, such mature cells occupied only 17% of simple CD4−deficient thymocytes. Collectively, neither an excess nor a lack of CD4 signaling appears to influence the extent of overproduction of mature CD4+/8− thymocytes, which is caused by the overexpressed Runx3. Thus, the activity of Runx3 to drive thymocytes toward the CD8 lineage is likely to be due to more than a simple silencing of CD4 gene expression.

Discussion

Whether DP thymocytes select the CD8 or CD4 lineage is determined by the strength and/or duration of the TCR signal the cells receive through their interactions with an MHC/peptide complex (7, 22, 23). The DP cells cease expressing either the CD4+ or CD8 gene, and thus eventually become committed to the CD8 SP or CD4 SP lineage, respectively. A CD4 silencer element and the Runx binding sites in it play a pivotal role in the cessation of CD4 expression (13, 24). Based on the analysis of thymocytes lacking Runx1 or Runx3, Taniuchi et al. (13) proposed that Runx1 functions as an active repressor of CD4 expression at the DN stage, whereas Runx3 is involved in the epigenetic silencing of the gene at the CD8 SP stage.

In the present study, we created Runx3−transgenic mice and found that the number of mature CD4+/8− thymocytes was increased. This result is opposite to that found in the Runx3 (−/−) thymus, in which the number of mature CD4+/8− cells is markedly

FIGURE 6. Effect of the Runx3−transgene on splenic T lymphocytes. A, The wild-type and Runx3−transgenic splenocytes were processed for three-color flow cytometrical analysis. The mature TCRhigh subpopulation was selected, and its CD8 and CD4 expression profile was analyzed. The numbers in the individual quadrants indicate the percentage of cells of each type. B, The cell number ratios of TCRhigh/HSA low/CD4+8− cells to TCRlow/HSA low/CD4+8− cells in the wild-type and Runx3−transgenic thymus and spleen. C, The Vβ repertoire used by the TCRs of splenic CD4−8+ cells. Wild-type (□) and Runx3−transgenic (■) cells were stained by an Ab mixture against various Vβ segments and processed for flow cytometrical analysis.
decreased (13, 14). Therefore, the present gain-of-function analysis complements the previous loss-of-function analysis. However, a close inspection of our results reveals a new aspect of Runx3 function as described below and as summarized in Fig. 9.

In the Runx3-transgenic thymus, the absolute number of mature CD4^-/8^- thymocytes was increased 2-fold compared with the non-transgenic thymus. This phenomenon cannot be explained solely by the effect of Runx3 on the CD4 silencer. If CD4 silencing had been overwhelming in the Runx3-transgenic mice, then the mature CD4^-/8^- thymocytes might also lose CD4 expression, and a significant number of CD4^-/8^- TCR^high^ thymocytes might have been generated. However, we did not see evidence of such a population in the transgenic thymus. Mice lacking the CD4 gene itself lost CD4 expression completely, and the number of mature CD4^-/8^-
thymocytes does not vary from that of wild-type mice (25). In contrast, we observed that overexpression of Runx3 could more efficiently convert the $CD4^{+}$ thymocytes to the mature $CD8^{+}$ cells. A similar result was obtained for the Runx3- and class II-restricted TCR double-transgenic mice as well as Runx3-transgenic: $\beta_{2}m^{-/-}$ mice. Taken together, Runx3 likely possesses the capacity not only to suppress $CD4$ gene expression but also to actively drive the thymocytes toward the $CD8$ lineage.

In the wild-type thymus, the endogenous Runx3 is likely involved in the selection of and commitment to the $CD8$ lineage in concert with TCR signaling. A short and/or weak TCR signal is somehow transduced to Runx3, which in turn regulates the gene expression necessary for the $CD8$ lineage determination. $CD4$ silencing is one target of Runx3 (13) and maintenance of $CD8$ expression is probably a target as well. Another possibility is that Runx3 is involved in the survival and/or maturation of thymocytes after they have selected the $CD8$ lineage.

At the DN stage, the $CD4$ silencer is reported to be “ON.” Transcription of the $CD4$ gene is initiated when the DN cells move to the DP stage, and the activity of the $CD4$ silencer is expected to be turned “OFF” during the transition from DN to DP (26). The mechanism of this “OFF” switch cannot be assessed by targeted deletions of Runx3 or $CD4$ silencer. In our Runx3-transgenic thymus, the percentage and number of $CD4^{+}8^{+}$ cells were
FIGURE 9. A model of T lymphocyte differentiation in the Runx3-transgenic thymus. Each step of differentiation is characterized by the specific expression patterns of CD4, CD8, TCR, and HSA. The cells usually start at the DN stage, go through the ISP and DP stages, and mature at the CD8 SP stage. The “CD4-repressed DP” stage is characterized by TCRmed expression; it is apparently categorized as a CD4-8 fraction; and is observed only in the Runx3-transgenic, but not the wild-type, thymus. The majority of mature TCRmedHSAhighCD4-8 cells are considered to be derived from the premature “CD4-repressed DP” cells. A minor pathway for CD8+ maturation in the transgenic animals would be through the usual “DP” stage.

Remarkably reduced, and an aberrant population of “CD4-repressed DP” cells with a CD4-8+TCRmedHSAhigh phenotype emerged instead. It is likely that exogenous expression of the transgene-derived Runx3 protein maintained the CD4 silencer in the “ON” position, thereby giving rise to the “CD4-repressed DP” thymocytes from the immature CD4-8+TCRlow cells. However, these premature cells do acquire a CD4-8+ phenotype, probably due to the strong repression of CD4 expression.

We previously reported the phenotype of Runx1-transgenic mice in which the numbers of both immature ISP and mature CD8 SP cells were increased (9). Even taking into consideration the differences between the Runx3- and Runx1-transgenic thymocytes in terms of the promoters used and/or the magnitude of transduced protein expression, it is interesting to note that overexpression of Runx1 did not generate the “CD4-repressed DP” cells as Runx3 did. Furthermore, the endogenous Runx1 protein is easily detected in the DP cells of wild-type thymus (10, 11), and Runx1 and Runx3 do not associate with each other in a communoprecipitation experiment (K. Kohu and M. Satake, unpublished data). These observations suggest both that Runx1 is not involved in the turning on the “ON” position, thereby giving rise to the “CD4-repressed DP” thymocytes from the immature CD4-8+TCRlow cells. However, these premature cells do acquire a CD4-8+ phenotype, probably due to the strong repression of CD4 expression.

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Disclosures
The authors have no financial conflict of interest.

References


