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Keitaro Hayashi,* Waka Natsume,* Toshio Watanabe,* Natsumi Abe,* Naomi Iwai,* Hitoshi Okada,† Yoshiaki Ito,§ Masahide Asano,§ Yoichiro Iwakura,§ Sonoko Habu,¶ Yousuke Takahama,‖ and Masanobu Satake‡

In the thymic cortex, T lymphocytes are positively selected to survive and committed either to the CD4 single-positive (SP) or the CD8 SP lineage. The SP cells then pass through a step of maturation in the medulla and are delivered to peripheral lymphoid tissues. We examined the role of AML1, the gene encoding a transcription factor, in the above processes by using the transgenic mice expressing a dominant interfering form of AML1 as well as mice targeted heterozygously for AML1. One phenotypic change seen in the AML1-diminished mice was the reduction in the numbers of both CD4 SP and CD8 SP thymocytes, reflecting the partial impairment of the transition from the double-positive to SP stage. In addition, distinct from the above abnormality, perturbed were several aspects of SP cells, including the maturation of SP thymocytes, the recent thymic emigration, and the proliferative responsiveness of peripheral T cells to TCR stimulation. Interestingly, the AML1 diminution caused inhibitory and enhancing effects on the CD4 SP and CD8 SP cells, respectively. These differential effects are most likely related to the reduction in the peripheral CD4 SP/CD8 SP ratio observed in the AML1-diminished mice. The AML1 transcription factor thus maintains the homeostasis of each SP subset by functioning at the later stages of T lymphocyte differentiation. The Journal of Immunology, 2000, 165: 6816–6824.

The development of T lymphocytes in thymus proceeds through multiple, defined steps, each of which is characterized by expression and/or down-regulation of distinct cell surface markers, TCRs, and their associated molecules. How this process is regulated at the level of gene expression remains a fascinating challenge in developmental biology as well as in immunology. Accumulated evidence indicates that differentiation of T lymphocytes is regulated by the function(s) of a set of transcription factors (reviewed in Ref. 1). This notion is mainly based on the analysis of gene-targeted and/or transgenic mice. For example, several transcription factors such as Ikaros, GATA-3, c-Myb, and T cell-specific factor-I have been listed as regulators of the early steps of T lymphocyte development. However, far less is known about the mechanism of gene regulation involved in the maturation of single-positive (SP)3 cells in the thymus and their maintenance in peripheral lymphoid tissues such as spleen and lymph nodes.

Human and murine AML1, also known as Pebp2ab, Cbfa2, or Runx1, encode the DNA-binding subunit of the heterodimeric transcription factor, polyomavirus enhancer binding protein 2/core binding factor (PEBP2/CBF) (2, 3). The DNA binding domain of AML1, called the Runt domain, shows homology to the products of Drosophila developmental genes, runt and l Zheng. The AML1 gene is known to be involved in human leukemogenesis (reviewed in Ref. 4) as well as in the physiological development of definitive hemopoiesis in the murine fetal liver (5–7).

There have been some observations suggesting that AML1 plays a significant role in the T lymphocytes. First, expression of AML1 is detected abundantly in embryonic and adult thymuses. Based on the in situ hybridization and morphological observation, the cells positive for AML1 expression represent T lymphocytes rather than stromal cells (8). Second, PEBP2/CBF has been implicated in the T cell-specific expression of several genes including TCRs. The enhancer elements of the TCRα, TCRβ, TCRγ, and TCRδ genes harbor PEBP2/CBF binding sites (reviewed in Ref. 9). PEBP2/CBF and Ets-1 or c-Myb bind to their adjacent sites in the enhancer elements of TCRs and activate transcription synergistically (10–14). PEBP2/CBF was also identified as a binding factor for the enhancer core of a T cell tropic strain of murine leukemia virus (15, 16). Finally, we reported recently that overexpression of AML1 protein can render a T cell hybridoma resistant to TCR-mediated apoptosis (17). This is achieved by down-regulating the expression of the apoptotic Fas-ligand gene and simultaneously up-regulating IL-2Ra expression. We proposed that AML1 might

3 Abbreviations used in this paper: SP, single-positive; BrdU, bromodeoxyuridine; DP, double-positive; HA, hemagglutinin; HSA, heat-stable Ag; NLS, nuclear localization signal; PEBP2/CBF, polyomavirus enhancer binding protein 2/core binding factor.
play a pivotal role in TCR-mediated growth and/or the death of T lymphocytes.

Based on the circumstances and the rationales described above, we were motivated to examine the role of AML1 in the later stages of T lymphocyte development in vivo. Unfortunately, AML1−/− embryos die on embryonic day 12.5 due to the concomitant and massive hemorrhage of primitive erythrocytes in the CNS (5–7) and, thus, cannot provide any information on the role of AML1 in T lymphocyte development. Therefore, we used transgenic mice expressing a dominant interfering form of AML1 as well as mice targeted heterozygously for AML1. The results obtained reveal a new and unexpected aspect of the gene regulation involved in the fate of SP T cells. The AML1 gene plays a key role in the maturation of SP thymocytes, and furthermore functions differentially to maintain the pool of CD4 SP (CD4+CD8−) vs CD8 SP (CD4−CD8+) cells in peripheral lymphatic tissues.

Materials and Methods

Plasmids

The expression plasmid pCX2neoBS harbors the Runt domain of murine AML1 fused to an epitope tag, as previously described (18). The hemagglutinin (HA) tag that represents the epitope of influenza virus HA and the nuclear localization signal (NLS) of SV40 were fused to the amino terminus of the 9-mer (CCGGAATTCTACACCTTACGCCTGGTAGTACTAGCTGACCACTTGCTACGCTCTTTGCGACCCATGATGTTGGAGGTTACCTAGCTGACCAC) that represents the HA epitope and the codon for the initiating methionine, respectively. The sense primer was 5′-ccggattcgccacacagctgctttgttgtctgcttcggtttgtgtttgtagcagt-3′, in which the singly and doubly underlined sequences represent the HA epitope and the codon for the initiating methionine, respectively. The antisense primer was 5′-ccggattcgccacacagctgctttgttgtctgcttcggtttgtgtttgtagcagt-3′, in which the singly and doubly underlined sequences represent the NLS in tandem and the terminating codon, respectively. The PCR product was digested by EcoRI and subcloned into the EcoRI site of human pCD2 minigene (19). The resulting plasmid was designated pCD2-HA/Runt/NLS. The authenticity of the modified sequences in the plasmid was confirmed by sequencing.

Mice

Transgenic mouse lines expressing the Runt protein were generated as follows. The DNA of pCD2-HA/Runt/NLS was digested by SalI and the modified sequences in the plasmid was confirmed by sequencing. The DNA of pCD2-HA/Runt/NLS was digested by SalI and the modified sequences in the plasmid was confirmed by sequencing. The DNA of pCD2-HA/Runt/NLS was digested by SalI and the modified sequences in the plasmid was confirmed by sequencing. The DNA of pCD2-HA/Runt/NLS was digested by SalI and the modified sequences in the plasmid was confirmed by sequencing. The DNA of pCD2-HA/Runt/NLS was digested by SalI and the modified sequences in the plasmid was confirmed by sequencing. Mice were bred with C57BL/6J mice. The presence or absence of the transgene was determined by PCR using genomic DNA as a template. The sense and antisense primers were 5′-ctgctttgttgtctgcttcggtttgtgtttgtagcagt-3′ and 5′-ccggattcgccacacagctgctttgttgtctgcttcggtttgtgtttgtagcagt-3′, respectively, and a 410-bp fragment was amplified from the transgene. The targeting of the AML1 gene was described previously (20). In case of splenocytes, cells were purified from the spleen, and the purified fragment containing the insert was microinjected into fertilized eggs of C3H/HeN mice. Litters possessing the transgene were backcrossed to C57BL/6J to generate transgenic mice lines expressing the Runt protein. Three independent transgenic lines, namely 48, 65, and 81, were established. Targeting of the AML1 gene was described previously (7). In case of splenocytes, cells were purified from the spleen, and the purified fragment containing the insert was microinjected into fertilized eggs of C3H/HeN mice. Litters possessing the transgene were backcrossed to C57BL/6J to generate transgenic mice lines expressing the Runt protein. 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Results

The expression of AML1 in SP T lymphocytes

Because the study was mainly designed to follow the fate of SP T lymphocytes, we examined the expression of AML1 polypeptides in such cells by immunoblot analysis (Fig. 1a). The CD4 SP and CD8 SP cells were purified from thymocytes and splenocytes, and their protein extracts were probed by the anti-AML1 peptide Ab. One band was detected at a position corresponding to a protein of 56 kDa (lanes 1–4). The bands represent specific immunocomplexes, because they were abolished by including an excessive amount of the corresponding peptide in the incubation mixture (lanes 5–8). The 56-kDa species comigrated with the AML1 polypeptide purified from a bacterial lysate and was missing from a protein extract of the AML1+/− embryo (22). Thus, we were able to confirm the expression of AML1 polypeptides in thymic and splenic CD4 SP and CD8 SP subsets.

Establishment of transgenic mouse lines expressing a dominant interfering form of AML1

To explore the role of AML1 in the development of T lymphocytes, we generated transgenic mouse lines expressing a dominant interfering form of AML1.
Because the DNA binding domain of AML1, the Runt domain, is known to dominantly interfere with PEBP2/CBF-dependent transactivation (18), it was placed downstream of the CD2 promoter and upstream of the CD2-poly(A) addition sequence in the microinjected plasmid (19). Three transgenic mouse lines, namely lines 48, 65, and 81, were established. The expression of the Runt domain in thymuses and spleens of transgenic mice was examined by immunoblot analysis (Fig. 1b).

EMSA was performed using the PEBP2/CBF-binding sequence as a probe (Fig. 1c). Endogenous PEBP2/CBF DNA-binding activity was detected in the thymus and spleen of nontransgenic mouse (lanes 1 and 3), whereas Runt domain-derived DNA-binding activity was detected in the tissues of transgenic line 81 (lanes 2 and 4). DNA-binding specificity was confirmed for both complexes by competition analysis (data not shown). Absence of slower migrating complex indicates that the Runt domain indeed functioned to interfere with the endogenous PEBP2/CBF DNA-binding activity in the tissues of transgenic mice. In the following sections, only the data obtained for the transgenic line 81 will be presented, but essentially similar results were obtained for the other two lines as well.

**Generation of SP thymocytes is partially impaired in Runt-transgenic mice**

The profiles of T lymphocytes in the thymus, spleen, and lymph nodes were examined using 8-wk-old transgenic mice (Fig. 2a). When thymocytes were analyzed by two-color flow cytometry, the percentages of CD4 SP and CD8 SP cells in the transgenic mouse (3.6 and 0.8%, respectively) were reduced compared with those of the nontransgenic mouse (9 and 3.4%, respectively). The total number of cells present in the thymus did not vary significantly between the nontransgenic and transgenic mouse lines, when a
number of individuals from each group were examined (Table I). Therefore, the mean numbers of CD4 SP and CD8 SP thymocytes in the transgenic line were 40 and 28%, respectively, of those seen in the nontransgenic mouse. The mean percentage and cell number of CD4+CD8- (double-positive, DP) thymocytes in the transgenic line were slightly larger than those seen in the nontransgenic mouse (Fig. 2a). Actually, the mean numbers of CD4 SP cells in spleens and lymph nodes of transgenic mouse were significantly smaller than those seen in the nontransgenic mouse (Fig. 2a). Actually, the mean numbers of CD4 SP cells in spleens and lymph nodes of transgenic line were not 40%, as simply extrapolated from the situation in the thymus, but were 17 and 16%, respectively, of the mean numbers in the nontransgenic mouse (Table I). Therefore, in the transgenic line, the decrease in the proportion of peripheral CD4 SP cells was more severe than that of CD4 SP thymocytes.

In contrast, we encountered the different situation in the case of CD8 SP cells. The percentages of CD8 SP cells in the spleen and lymph node of transgenic mouse were similar to and rather larger, respectively, than those seen in the nontransgenic mouse (Fig. 2a). The mean numbers of CD8 SP cells in spleens and lymph nodes of the transgenic line were 37 and 33%, respectively, of the mean numbers in the nontransgenic line (Table I). Therefore, in the transgenic line, the decrease in the peripheral lymphoid organs. This phenotype is considered to be distinct from the above-described alteration in thymocytes, in which the differential profiles of each SP subset were more apparent, when the ratios of CD4 SP to CD8 SP cells were calculated. The mean numbers of CD4 SP cells in the nontransgenic line were 37 and 33%, respectively, of the mean numbers in the nontransgenic line. Thus, unlike the case of the CD4 SP cells, the proportion of CD8 SP cells remained unchanged or became slightly larger in the peripheral lymphoid tissues of transgenic mice. The differential profiles of each SP subset were more apparent, when the ratios of CD4 SP to CD8 SP cells were calculated. The mean numbers of CD4 SP cells in the spleen and lymph node of transgenic mouse were similar to and rather larger, respectively, than those seen in the nontransgenic mouse (Fig. 2a). The mean numbers of CD8 SP cells in spleens and lymph nodes of the transgenic line were 37 and 33%, respectively, of the mean numbers in the nontransgenic line (Table I). Therefore, in the transgenic line, the decrease in the peripheral lymphoid organs. This phenotype is considered to be distinct from the above-described alteration in thymocytes, in which both SP subsets were decreased in the transgenic line. The CD4 SP/CD8 SP ratios of thymuses were rather higher in transgenic mice.

**The proportions of CD4 SP and CD8 SP cells are perturbed in peripheral lymphatic tissues of Runt-transgenic mice**

Analysis of T lymphocytes in peripheral lymphoid tissues revealed additional alterations in the transgenic mice. As described above, these were a further decrease in the proportions of CD4 SP, but not of CD8 SP cells, resulting in the overall reduction of the CD4 SP/CD8 SP ratio. The percentages of CD4 SP cells in the spleen or lymph node of transgenic mouse were significantly smaller than those seen in the nontransgenic mouse (Fig. 2a). Actually, the mean numbers of CD4 SP cells in spleens and lymph nodes of the transgenic line were not 40%, as simply extrapolated from the situation in the thymus, but were 17 and 16%, respectively, of the mean numbers in the nontransgenic mouse (Table I). Therefore, in the transgenic line, the decrease in the proportion of peripheral CD4 SP cells was more severe than that of CD4 SP thymocytes.

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**Heterozygous disruption of AML1 causes similar phenotypes to those seen in Runt-transgenic mice**

In addition to AML1, two other mammalian runt genes are known to exist, namely AML3/PEBP2a/Chf1 and AML2/PEBP2a/Chf3. The Runt domain of AML1 should interfere with the DNA-binding and transcriptional activation potential of all three of these mammalian proteins. A PEBP2/CBF DNA-binding activity detected in the protein extract of thymus is reported to be 100%.

When thymocytes were analyzed by flow cytometry, the percentages of CD4 SP and CD8 SP cells were slightly lower in the transgenic line were 40 and 28%, respectively, of those seen in the nontransgenic mouse. The mean percentage and cell number of CD4+CD8- (double-positive, DP) thymocytes in the transgenic line were slightly larger than those seen in the nontransgenic mouse (Fig. 2a). Actually, the mean numbers of CD4 SP cells in spleens and lymph nodes of transgenic mouse were significantly smaller than those seen in the nontransgenic mouse (Fig. 2a). Actually, the mean numbers of CD4 SP cells in spleens and lymph nodes of the transgenic line were not 40%, as simply extrapolated from the situation in the thymus, but were 17 and 16%, respectively, of the mean numbers in the nontransgenic mouse (Table I). Therefore, in the transgenic line, the decrease in the proportion of peripheral CD4 SP cells was more severe than that of CD4 SP thymocytes.

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**Table I. The numbers of SP T lymphocytes and the CD4+SP/CD8+SP ratios in the wild-type and AML1-diminished mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild-type</th>
<th>Runt-tg</th>
<th>AML1&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>AML1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>9.8 ± 2.5</td>
<td>10 ± 1.1</td>
<td>7.5 ± 2.3</td>
<td>7.1 ± 1.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>11.8 ± 6.1</td>
<td>14.3 ± 6.1</td>
<td>6.7 ± 2.3</td>
<td>4.1 ± 1.3</td>
</tr>
<tr>
<td>Lymph Node</td>
<td>4.4 ± 2.7</td>
<td>5.2 ± 1.4</td>
<td>3.7 ± 0.6</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td><strong>CD4 SP</strong></td>
<td>40 ± 2.7</td>
<td>40 ± 1.1</td>
<td>27 ± 0.6</td>
<td>27 ± 0.6</td>
</tr>
<tr>
<td><strong>CD8 SP</strong></td>
<td>27 ± 0.7</td>
<td>27 ± 0.9</td>
<td>15 ± 0.6</td>
<td>15 ± 0.6</td>
</tr>
</tbody>
</table>

*Eight-week-old mice were used for analysis. The mean medians of cell numbers and their standard deviations are presented. Also presented are the percentages of cell numbers in the AML1-diminished mice, taking those in the wild-type and Runt-transgenic mice as 100%.

**AML1<sup>+/−</sup>** mice apparently suffer from no developmental abnormalities and survive to adulthood (7).
AML1+/- mouse compared with those in the wild-type mouse (Fig. 2b). Reflecting this, the mean numbers of each SP thymocyte in the heterozygotes were ~70% of those of the wild-type mice (Table I). Therefore, the decrease in the number of both SP subsets can be seen in the AML1 heterozygous thymocytes as well, although this decrease was less severe compared with the decrease seen in transgenic mice.

As for the SP cells in the peripheral lymphatic tissues of the AML1 heterozygous mice, changes similar to those of the Runt-transgenic mice were observed. This can be readily seen by comparing the ratios of CD4 SP to CD8 SP cells. The mean ratios in the AML1+/- mice (0.6) were half of those of the wild-type mice (1.3), reflecting the additional decrease in the proportion of CD4 SP cells and the slight increase in that of CD8 SP cells in peripheral tissues of heterozygous mice.

It must be noted that the surface expression of TCR was not altered significantly in the AML1-diminished, CD4 SP splenocytes (Fig. 3). TCR expression in the CD8 SP splenocytes was not altered by AML1 dysfunction, either (data not shown). Thus, the reduction in the peripheral CD4 SP/CD8 SP ratios was observed for their TCRhigh subpopulations. In addition, almost all of peripheral CD8 SP cells in the AML1+/- mice were of the CD8αβ phenotype, indicating their thymic origin (data not shown).

In summary, at least two stages of T lymphocyte development in the thymus and peripheral tissues appear to be disturbed in both the Runt-transgenic and AML1 heterozygously targeted mice. The two phenotypes described above are due to diminution of AML1 function and most likely to be intrinsic to T lymphocytes rather than to their microenvironment because Runt is expressed in the T cell lineage.

The peripheral CD4 SP/CD8 SP ratio is perturbed at the level of recent thymic emigrants in AML1-diminished mice

In all the experiments above, mice with an age of 8 wk were used. At, or soon after birth, positively selected SP thymocytes start to emigrate from the thymus and to settle in the peripheral lymphoid tissues. We next examined whether a similar phenotypic change would be observed in the peripheral T lymphocytes of neonatal mice, which resemble recent thymic emigrants. As can be seen in Table II, the CD4 SP and CD8 SP cells in the spleens of wild-type neonates represent only a small proportion of total splenocytes. Interestingly, the mean percentages of splenic CD4 SP cells in the AML1-diminished neonates were significantly lower than those of the corresponding SP subset in the wild-type neonates. Thus, the CD4 SP/CD8 SP ratios in spleen of AML1-diminished mice were roughly half of those in spleen of wild-type mice. This suggests that the event leading to the reduction of the CD4 SP/CD8 SP ratio by AML1 diminution occurs probably at the time, or soon after the SP cells are released from the thymus.

The above observations then led us to analyze recent thymic emigrants themselves. Adult AML1+/- mice were given BrdU in the drinking water continuously. After labeling, thymocytes and splenocytes were processed for three-color (CD4, CD8, and BrdU) flow cytometry (Fig. 4a). The BrdU<sup>low</sup> subpopulation in spleen corresponded to 11% in the CD4 SP and 9% in the CD8 SP subsets, respectively. Because this BrdU<sup>low</sup> fraction disappears from spleens of thymectomized mice, the cells in the splenic BrdU<sup>low</sup> subpopulation are considered to have incorporated BrdU when they are staying in thymus as SP cells (25). Therefore, the splenic BrdU<sup>low</sup> subpopulation represents recent thymic emigrants (see the figure legend for the BrdU<sup>high</sup> subpopulation).

The numbers of BrdU<sup>low</sup> cells appearing in spleen after BrdU drinking were compared between the wild-type and Runt-transgenic mice (Fig. 4b). In the wild-type mice, the numbers of BrdU<sup>low</sup> cells in the CD4 SP subset were consistently larger than those in the CD8 SP subset, whereas, in the transgenic mice, a converse situation was observed in most of the mice examined. Essentially similar as above results were obtained when comparing the AML1+/- and AML1+/- mice (Fig. 4c). Thus, the larger numbers of recent thymic emigrants in the CD4 SP subset than those in the CD8 SP subset most likely underlie the basis of CD4 SP/CD8 SP ratio seen in peripheral lymphatic tissues of wild-type mice. Likewise, the smaller ratio of peripheral CD4 SP/CD8 SP observed in the AML1-diminished mice appears to correlate with the reduced number of emigrants in the CD4 SP subset compared with that in the CD8 SP subset (see the figure legend for the mean numbers of BrdU<sup>low</sup> cells).

The results presented in Table II and Fig. 4 suggest that the reduction of the peripheral CD4 SP/CD8 SP ratio seen in the AML1-diminished mice is at least partially due to the perturbation of recent thymic emigration.

Maturation of SP thymocytes is affected in AML1-diminished mice

After SP cells are positively selected and before they are released into the circulation, the cells pass through a step called maturation in the thymic medulla. The more mature SP cells are, the more efficiently they proliferate and emigrate (reviewed in Ref. 26). To correlate maturation with the recent thymocyte emigration described above, we examined the degree of maturation of SP thymocytes by measuring the expression of the HSA (27).

Thymocytes from the wild-type and AML1-diminished mice were processed for four-color flow-cytometrical analysis (CD4, CD8, TCR, and HSA). Fig. 5 depicts the profiles of HSA expression in the CD4<sup>+</sup>CD8<sup>-</sup> TCR<sup>high</sup> and CD4<sup>+</sup>CD8<sup>-</sup> TCR<sup>low</sup> fractions. Characteristically, the HSA<sup>low</sup> population in the CD4 SP

![FIGURE 3.](http://www.jimmunol.org/DownloadedFrom/3365763a.png)

**FIGURE 3.** Expression profiles of TCR in the CD4 SP splenocytes. The representative profiles are presented here, but essentially similar results were obtained for several individual mice. Splenocytes were prepared from the wild-type, Runt-transgenic (line 81), and AML1+/- mice and processed for three-color flow cytometric analysis (CD4, CD8, and TCRβ). The cells in the CD4<sup>+</sup>CD8<sup>-</sup> gates were analyzed for TCR fluorescence intensity.

<table>
<thead>
<tr>
<th>CD4 SP (%)</th>
<th>CD8 SP (%)</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>Wild type</td>
<td>7.1 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Runt-tg</td>
<td>4.3 ± 0.97</td>
</tr>
<tr>
<td>Spleen</td>
<td>Wild type</td>
<td>4.3 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>Runt-tg</td>
<td>0.9 ± 0.29</td>
</tr>
<tr>
<td>Thymus</td>
<td>AML1+/-</td>
<td>5.0 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>AML1+/-</td>
<td>4.4 ± 0.32</td>
</tr>
<tr>
<td>Spleen</td>
<td>AML1+/-</td>
<td>2.7 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>AML1+/-</td>
<td>1.6 ± 0.16</td>
</tr>
</tbody>
</table>

*Thymocytes and splenocytes were prepared from 7-day-old mice with the respective genotype and processed for two-color (CD4 and CD8) flow cytometry. The numbers of mice used were five for each genotype. The mean medians and SD are presented.*
The splenic CD4 SP subset were 10.2 × 10^6 AML1 bbers of BrdU low cells in the splenic CD8 SP subset were 8.8 × 10^6. Comparison of numbers of BrdU low splenocytes between the wild-type and Runt-transgenic (line 81) mice. The mean numbers of BrdU low cells in the splenic CD4 SP subset were 9.5 × 10^6, 1.6 × 10^6, and 3.5 × 10^6, respectively, in the wild-type and transgenic mice. In contrast, the mean numbers of BrdU low cells in the CD8 SP subset were 7.3 × 10^6 and 6.6 × 10^6, respectively, in the wild-type and transgenic mice. A minor subpopulation that incorporated BrdU to a higher degree is reported elsewhere.

FIGURE 4. Analysis of recent thymic emigrants in AML1-diminished mice. a, Profiles of BrdU incorporation into thymocytes and splenocytes of wild-type mouse. A mouse was given BrdU in the drinking water for 4 days continuously. Thymocytes and splenocytes were analyzed by three-color (CD4, CD8, and BrdU) flow cytometry. The cells in the CD4 SP and CD8 SP gates were further analyzed for BrdU fluorescence intensity. Numbers represent the percentages of BrdUlow and BrdUhigh subpopulations in each gate. A minor subpopulation that incorporated BrdU to a higher degree is reported to express markers of memory/activated T cells (25). This BrdUhigh subpopulation was detected in spleen, but not in thymus. b, Comparison of numbers of BrdUlow splenocytes between the wild-type and Runt-transgenic (line 81) mice. Mice were given BrdU in the drinking water for 2 days, and splenocytes were analyzed as in a. The numbers of BrdUlow cells in the CD4 SP (open boxes) and CD8 SP subsets (hatched boxes) are displayed. Each pair of open and hatched boxes represents an individual mouse examined. The mean numbers of BrdUlow cells in the splenic CD4 SP subset were 9.5 ± 1.7 × 10^6 and 3.5 ± 0.83 × 10^6, respectively, in the wild-type and Runt-transgenic (line 81) mice. In contrast, the mean numbers of BrdUlow cells in the splenic CD8 SP subset were 7.2 ± 2.1 × 10^6 and 4.7 ± 1.4 × 10^6, respectively, in the wild-type and transgenic mice. c, Comparison of numbers of BrdUlow splenocytes between the AML1^+/+ and AML1^−/− mice. Details were the same as in b, except that the AML1^+/+ and AML1^−/− mice were used. The mean numbers of BrdUlow cells in the splenic CD4 SP subset were 10.2 ± 1.9 × 10^6 and 6.6 ± 1.9 × 10^6, respectively, in the AML1^+/+ and AML1^−/− mice. In contrast, the mean numbers of BrdUlow cells in the splenic CD8 SP subset were 8.8 ± 2.5 × 10^6 and 8.3 ± 1.6 × 10^6, respectively, in the wild-type and heterozygous mice.

The responsiveness of SP thymocytes to TCR stimulation was evaluated in vitro as well (Fig. 6). Thymocytes were incubated with the varying concentration of anti-CD3 Ab and processed for three-color flow cytometry (CD4, CD8, and annexin V). Fig. 6a depicts the percentages of annexin V-positive cells in the CD4 SP fraction. The Runt-transgenic CD4 SP subset contained more apoptotic cells after the Ab treatment than the wild-type subset. In the case of CD8 SP cells, they were first purified from thymocytes, incubated in the presence of anti-CD3 Ab, and processed for three-color flow cytometry (CD8, HSA, and annexin V; Fig. 6b). The Runt-transgenic CD8^+ HSA^low-medium subset (this gating excluded the immature CD8^+ SP cells) contained less apoptotic cells than the wild-type subset. Thus, the CD4 SP and CD8 SP thymocytes from the Runt-transgenic mice were more sensitive and less sensitive, respectively, to TCR-mediated apoptosis than the corresponding wild-type cells. A differential effect of anti-CD3 treatment in vitro was marginal when comparing the AML1^+/+ and AML1^−/− thymocytes (data not shown).
These altered qualities of AML1-diminished, SP thymocytes, as exemplified by the HSA expression (Fig. 5) and TCR sensitivity (Table III and Fig. 6), are considered to be reflected in the perturbation of recent thymic emigration described in the previous section.

Altered proliferative response of AML1-diminished, peripheral T cells to TCR stimulation

We subsequently examined whether the peripheral T lymphocytes in the AML1-diminished mice would suffer from any functional abnormality, in addition to the perturbation of cell numbers. Splenocytes from the wild-type and Runt-transgenic mice were incubated in the presence of Con A. On following days after incubation, an aliquot of cells was removed and processed for two-color flow cytometry and the relative numbers of CD4 SP and CD8 SP cells were plotted, taking the cell numbers at the beginning of culture to be equal to 1 (Fig. 7). The increase in the number of Runt-transgenic CD4 SP cells (closed circles) was significantly greater than that of nontransgenic counterparts (open circles). Conversely, the number of Runt-transgenic CD8 SP cells increased more rapidly than the number of nontransgenic controls. Overall, the difference in the CD8 SP/CD4 SP ratio between the Runt-transgenic and wild-type splenocytes became larger. Similar results were obtained when splenocytes were taken from AML1+/– and AML1+/- mice or when splenocytes were treated with the anti-CD3 Ab (data not shown). The results indicate that the peripheral CD4 SP and CD8 SP cells respond to proliferate upon TCR stimulation with weak and enhanced sensitivity, respectively, when the function of AML1 is diminished. The altered TCR responsiveness of peripheral T lymphocytes is, thus, an additional phenotype seen in the AML1-diminished mice.

Finally, we note that the expression profiles of CD44 and CD62L, which are markers of activated and/or memory T cells, were not significantly different between splenocytes from the wild-type mice and those from the AML1-diminished mice. In addition, the percentages of apoptotic cells in spleen were not significantly different between the two types of mice, as judged by annexin V staining and flow cytometry (data not shown).

Discussion

Several phenotypic changes were observed in the AML1 heterozygous as well as in Runt-transgenic mice. First, the numbers of both CD4 SP and CD8 SP thymocytes were reduced. Second, the maturation of SP thymocytes as well as the recent thymic emigration were altered, and third, the homeostasis of peripheral T cell pool was perturbed, such that the proportion of CD4 SP cells decreased further, whereas that of CD8 SP cells remained unchanged or slightly increased, resulting in the overall reduction of the peripheral CD4 SP/CD8 SP ratio. Finally, the proliferative responsiveness to TCR stimulation was altered differentially in the peripheral CD4 SP and CD8 SP cells.

A role for AML1 in the generation of SP thymocytes

The numbers of both CD4 SP and CD8 SP thymocytes were reduced in the AML1-diminished mice. This reflects the partial impairment of transition of thymocytes from the DP to the SP stage, irrespective of the choice between the CD4 SP and the CD8 SP lineages. We confirmed this by introducing the class I- and class

Table III. Flow cytometrical analysis of thymocytes from mice administered by the anti-CD3 Ab

<table>
<thead>
<tr>
<th></th>
<th>DN</th>
<th>DP</th>
<th>CD4 SP</th>
<th>CD8 SP</th>
<th>CD4 SP/CD8 SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>64 ± 2.9</td>
<td>0.4 ± 0.1</td>
<td>28 ± 1.6</td>
<td>7.4 ± 1.5</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>Runt-tg</td>
<td>78 ± 9.6</td>
<td>0.5 ± 0.3</td>
<td>15 ± 5.9</td>
<td>5.9 ± 3.3</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>AML1+/-</td>
<td>60 ± 5.8</td>
<td>2.6 ± 0.8</td>
<td>23 ± 4.0</td>
<td>14 ± 2.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>AML1-/-</td>
<td>76 ± 8.5</td>
<td>3.9 ± 2.3</td>
<td>10 ± 4.2</td>
<td>9.6 ± 3.7</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

a The anti-CD3 Ab was injected i.p. into the 8-wk-old mice with the respective genotype. After 48 h, thymocytes were isolated and processed for two-color (CD4 and CD8) flow cytometry. Numbers shown represent mean percentages ± SD of each subpopulation. The numbers of mice analyzed were three for each genotype. DN means the CD4–CD8– subpopulation.
showed that the HSA<sub>low</sub> population decreased in the CD4 SP and their responsiveness to TCR stimulation (28, 29). We each other in their secreting cytokines (30, 31), their proliferating II-restricted, TCR transgenes, respectively, into the AML1

Proliferative potential of wild-type and Runt-transgenic, may reflect an additional possibility that the CD4 SP/CD8 SP ratios observed in the Runt-transgenic thymusesished mice (K. H. and M. S., unpublished observation). The higher cytes from individual mice. Splenocytes from one mouse were cultured in the initiation of culture to be 1. Each solid or dashed line represents spleno-

numbers of CD4 SP and CD8 SP cells were plotted, taking the number at and cultured in the presence of Con A. After 48- and 72-h incubation, the F

prepared from the wild-type (E

CD4 SP and CD8 SP splenocytes upon Con A treatment. Splenocytes were ing the fluctuations in the numbers of CD4 SP and CD8 SP thy-

-eliminution. AML1-diminished neo-

A role for AML1 in the maturation of SP thymocytes

SP thymocytes generated by positive selection pass through a maturation step in the thymic medulla. The down-regulation of HSA expression that accompanies this maturation does not merely signify a change of the differentiation surface marker. The HSA<sup>high</sup> and HSA<sup>low</sup> SP thymocytes, which correspond to less advanced and more mature stages of differentiation, respectively, differ from each other in their secreting cytokines (30, 31), their proliferating capacity, and their responsiveness to TCR stimulation (28, 29). We showed that the HSA<sup>low</sup> population decreased in the CD4 SP and increased in the CD8 SP thymocytes in the Runt-transgenic mice compared with the wild-type mice. In addition, we observed that the CD4 SP thymocytes from the AML1-diminished mice contained TCR-sensitive and thus going to die cells more abundantly. Thus, the maturation was impeded in the CD4 SP and accelerated in the CD8 SP thymocytes as a result of AML1 diminution. AML1 is the first example of a transcription factor that regulates the maturation of SP thymocytes.

A role for AML1 to maintain the homeostasis of the peripheral T cell pool

The reduction of the peripheral CD4 SP/CD8 SP ratio in the AML1-diminished mice cannot be explained by simply extrapolating the fluctuations in the numbers of CD4 SP and CD8 SP thymocytes. Based on the observations with AML1-diminished neocytates, we suggested that the event leading to the reduction of the peripheral CD4 SP/CD8 SP ratio most likely occurs at the time, or soon after the cells emigrate from thymus.

The thymic medulla is known to be the site in which the post-selection expansion takes place. Although this expansion is more prominent in embryonic thymuses (32, 33), it is detected at a sig-

nificant level in adult thymuses as well (34, 35). The proliferation tends to occur at a later and more mature stage of SP thymocyte differentiation and contributes to the homeostasis of peripheral T cell pool, because approximately half of the thymic emigrants are the progenies of proliferating, mature SP thymocytes (reviewed in Ref. 26). In this respect, our observation on the numbers of recent thymic emigrants appears particularly relevant. In the AML1<sup>−/−</sup>-mice, the mean numbers of BrdU<sup>low</sup> cells in the splenic CD4 SP subset decreased to 60% of those in the wild-type mice, whereas the BrdU<sup>low</sup> numbers in the AML1<sup>−/−</sup>-CD8 SP subset recovered to almost a similar level as those in the wild-type mice. Therefore, the reduction of the peripheral CD4 SP/CD8 SP ratio seen in the AML1-diminished mice appears at least partially due to the perturbation in the recent thymic emigration.

However, it should be pointed out that continual contact between the TCR and correctly restricted MHC molecules within the context of peripheral tissues contributes to the maintenance (survival and expansion) of peripheral T cell homeostasis as well, even in the absence of a specific Ag (36–40). We observed that diminution of AML1 weakened and enhanced, respectively, the proliferative response of splenic CD4 SP and CD8 SP cells upon TCR stimulation. The reduction of peripheral CD4 SP/CD8 SP ratio seen in the AML1-diminished mice might be also attributable to yet unidentified alteration in the in vivo proliferation of peripheral T lymphocytes.

AML1 and TCR signaling in the CD4 SP vs CD8 SP cells

Diminution of AML1 appears in general to have caused converse effects on the fates of CD4 SP vs CD8 SP cells. This was particularly evident when peripheral T cells were stimulated via their TCRs. The molecular mechanism how AML1 causes differential effects on respective SP subset is not known at present. In our recent report using the DO11.10 cell line as a model of Ag-induced cell death, we observed that overexpression of AML1 changed the outcome of TCR signaling from the Fas-ligand to IL-2R induction (17). It would be a challenge to elucidate how differentially AML1 coordinates with TCR signaling in the CD4 SP vs CD8 SP cells. In any case, the AML1 transcription factor most likely stands as one of the key molecules regulating the fates of SP T cells at the levels of thymus and peripheral lymphatic tissues.

Acknowledgments

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References

AML1 regulates the homeostasis of single-positive T cells


