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Reduction of Runx1 Transcription Factor Activity Up-Regulates Fas and Bim Expression and Enhances the Apoptotic Sensitivity of Double Positive Thymocytes

Natsumi Abe,*‡ Kazuyoshi Kohu,* Hidetaka Ohmori,* Keitaro Hayashi,* Toshio Watanabe,* Katsuto Hozumi, ‡ Takehito Sato, ‡ Sonoko Habu, ‡ and Masanobu Satake*‡

The death or survival of double positive (DP) thymocytes is determined by the strength of their TCR signaling. Of the three Runx family proteins, the DP cells only express the Runx1 transcription factor. We introduced and expressed in murine thymocytes the Runx domain of Runx1, which antagonizes the activity of endogenous Runx1. The Runx transgenic DP thymocytes expressed higher levels of the proapoptotic molecules Fas and Bim compared with the wild-type cells. Furthermore, the Runx transgenic cells were more susceptible to apoptosis induced by the artificial cross-linking of the TCR by the anti-CD3 Ab. This susceptibility was partially abrogated by the lpr/lpr background. In addition, Runx1:HY-TCDR double transgenic DP thymocytes were resistant to the apoptosis induced by the endogenously presented HY Ag. We propose that Runx1 functions to suppress the apoptotic sensitivity of DP thymocytes in the context of TCR signaling. The Journal of Immunology, 2005, 175: 4475–4482.

To explore the role Runx1 plays in T cell development, we generated two lines of transgenic mice in which Runx1 itself or the Runx domain of Runx1 is overexpressed in a T lineage-specific manner (6, 8). These mice were designated as Runx1 transgenic and Runx1 transgenic mice, respectively. The Runx domain on its own antagonizes the functions of the endogenously expressed Runx proteins (9). Analysis of the mice led to the following observations. First, Runx transgenic mice had significantly fewer CD8 SP thymocytes (8). In contrast, Runx1 transgenic mice had substantially greater numbers of CD8 SP thymocytes compared with the wild type (6). Therefore, Runx1 appears to function to drive DP thymocytes to the CD8 SP lineage. The comparative analysis of Runx1+/-;Runx3-/- and Runx1+/-;Runx3-/- thymocytes that was performed by Woolf et al. (4) also revealed the involvement of Runx1 in the commitment of DP cells to the CD8 SP lineage. Second, Runx transgenic mice also showed a remarkable decrease in the number of CD4 SP thymocytes (8). The postselected CD4 SP thymocytes showed enhanced apoptosis and a retarded growth rate and maturation. Third, the numbers of DP thymocytes in a double transgenic thymus that expresses Run and produces thyocytes bearing a MHC class II-restricted TCR were significantly decreased compared with the TCR single transgenic thymus (7). A synchronous culture of the Run and TCR double transgenic thyocytes also revealed that cell division at the DN to DP transition was reduced. Thus, Runx1 functions at various stages of thyocyte differentiation.

Whether DP thyocytes survive or die is critical in lymphocyte development because it is linked to T cell selection (10, 11). The DP cells that recognize self-Ags with a high affinity are eliminated by the mechanism known as negative selection, whereas the DP cells that cannot recognize self-MHC are killed by a process called death by neglect. Only the DP cells that interact with self-MHC with a lower affinity can survive. This is a process that is termed positive selection. The surviving DP cells can then progress to the SP stage. In this study, we have examined the role that the Runx1 transcription factor plays in the survival or death of DP thyocytes.

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3 Abbreviations used in this paper: SP, single positive; BH, Bcl-2 homology domain; DN, double negative; DP, double positive; TSST-1, toxic shock syndrome toxin-1.
because this has not yet been addressed by our previous studies. It is possible that Runx1 may be involved in this process because we found earlier that overexpression of Runx1 can render a T cell hybridoma resistant to TCR-mediated apoptosis (12). We show here that the reduction of endogenous Runx1 activity up-regulates Fas and Bim expression and enhances the apoptotic sensitivity of DP thymocytes.

Materials and Methods

Mice

The transgenic mouse lines that express Runx1 or the Runt domain have been described previously (6, 8). HY mice were provided by Dr. H. von Boehmer (Institut National de la Santé et de la Recherche Médicale Unité 373, Institut Necker, Paris, France) (13) and lpr/lpr mice were purchased from Japan SLC. The anti-CD3 Ab was purified from the tissue culture supernatant of the 145-2C11 hybridoma as described previously (12). The Ab solution containing 100 μg of protein was injected i.p. into individual mice. I-A<sup>+</sup> restricted OVA<sub>323-339</sub>-specific TCR transgenic mice (7) were backcrossed with RAG-2-deficient mice (I-A<sup>+</sup>). Toxic shock syndrome toxin-1 (TSST-1; Toxin Technology) (20 μg) was injected i.p. into individual mice.

Flow cytometric analysis

Cells were liberated from the thymus and suspended in PBS containing 0.2% (w/v) BSA. The single cell suspensions were incubated with appropriately diluted mAbs. The fluorescein-conjugated Abs used were anti-CD4-Cy5 (RM4-5), anti-CD8a-PE (53–6.7), and anti-Fas-FITC (Jo2) (BD Pharmingen). The T3.70 mAb was provided by Dr. H. von Boehmer. To detect apoptotic cells, the cells were stained with annexin V according to the procedure supplied by the manufacturer (Medical and Biological Laboratories). The analytical flow cytometer used was EPICS-XL (Beckman Coulter), and the data were analyzed by the software MacLas. Necrotic cells or cells at the late stage of apoptosis were excluded by propidium iodide gating.

Cell culture

Cells were liberated from the thymus, suspended in RPMI 1640 medium containing 10% (v/v) FCS, 10 mM HEPES-KOH (pH 7.4), and 50 μM 2-ME, and cultured at a density of 2×10<sup>6</sup> cells/well in a 96-well plate. In some cases, cells were incubated for 30 h in a plate that was precoated with 0.1 or 1 μg/ml anti-CD3 Ab together with 5 μg/ml anti-Fas-ITC (BD Pharmingen). The T3.70 mAb was provided by Dr. H. von Boehmer. To detect apoptotic cells, the cells were stained with annexin V according to the procedure supplied by the manufacturer (Medical and Biological Laboratories). The analytical flow cytometer used was EPICS-XL (Beckman Coulter), and the data were analyzed by the software MacLas. Necrotic cells or cells at the late stage of apoptosis were excluded by propidium iodide gating.

Immunoblot analysis

Total thymocytes and their DP fraction purified by using autoMACS (Miltenyi Biotec) were processed for immunoblot analysis. The procedures of protein extraction, electrophoresis, transfer to a filter, and immunoreaction that were used have been described previously (6). An equal amount (10 μg) of protein was loaded into each lane of the SDS-8% (w/v) PAGE. The anti-Bim, anti-Bcl-2, anti-Bcl-x<sub>L</sub>, and anti-β-actin Abs were purchased from Santa Cruz Biotechnology. The intensity of each band on immunoblot was measured with the NIH Image 1.63 program.

Results

Runt transgenic DP thymocytes are sensitive to apoptosis induced by TCR stimulation

In this study, we examined the fate of DP thymocytes in Runt transgenic mice. Because of the three Runx family proteins only Runx1 is detected in the wild-type DP thymocytes (6, 7) and Runt antagonizes Runx1 function (9), it is likely that any observed phenotypes of the Runt transgenic DP cells are attributable to the reduced activity of endogenous Runx1. We first examined Runt transgenic thymi for the proportion of DP cells they constitute of the total number of thymocytes. The percentage of DP thymocytes in Runt transgenic thymus was similar to that of wild-type thymus. This is indicated by the 0 h flow cytometric data shown in Fig. 1A.

Whether DP thymocytes survive or die is determined by the potency with which their TCR recognizes Ag/MHC. We therefore examined the degree of intrathymic cell death that is induced by artificially cross-linking the TCRs of the thymocytes. To do this, an anti-CD3 Ab was injected i.p. into mice. At 12 and 24 h after Ab administration, the percentages of DP thymocytes in Runt transgenic mice were greatly decreased, whereas those in wild-type mice were only slightly decreased. This indicates a massive depletion of DP cells from the Runt transgenic thymus (see the legend of Fig. 1A for the decline of cell numbers).

FIGURE 1. Effect of anti-CD3 Ab-mediated TCR cross-linking on the survival of DP thymocytes. A, Flow cytometric analysis of wild-type and Runt transgenic thymocytes before and 12 and 24 h after an i.p. injection of anti-CD3 Ab. The thymocytes were harvested, stained for CD4 and CD8 and processed for flow cytometry. The numbers indicate the percentages of DP cells. The absolute (and relative) numbers of total thymocytes recovered from the various thymi at 0, 12, and 24 h after anti-CD3 Ab administration were as follows: wild-type thymus, 1.05×10<sup>7</sup> (100), 3.3×10<sup>6</sup> (31), and 2.0×10<sup>6</sup> (19), respectively, and Runt transgenic thymus, 7.6×10<sup>7</sup> (100), 1.0×10<sup>6</sup> (13), and 1.0×10<sup>5</sup> (1.3), respectively. B, Profiles of annexin V-stained DP thymocytes. Thymocytes were prepared before and 7 h after the injection of anti-CD3 Ab, stained for CD4, CD8, and annexin V, and processed for flow cytometry. The cells in the DP gate were analyzed for their intensity of annexin V staining and the numbers indicate the percentages of annexin V-high cells. C, Effect of anti-CD3 Ab incubation in vitro on the apoptosis of DP thymocytes. Thymocytes were prepared from untreated wild-type and Runt transgenic mice, respectively, and incubated with the indicated concentration of anti-CD3 Ab in vitro. The cells were stained for CD4, CD8, and annexin V, and the numbers indicate the percentages of annexin V-high cells in the DP fraction. A, 0hr 12hr 24hr

   Wild type
   Runt-tg
   CD4
   CD8
   0hr 12hr 7hr
   Wild type
   Runt-tg
   DP
   Annxin V
   3.60
   3.26
   3.96
   20.10
   4.20
   1.0
   C
   anti-CD3: (μg/ml)
   0
   0.1
   1.0
   31.11
   29.11
   54.00
   11.33
   38.54
   56.95

   FIGURE 1.
We then examined whether the decrease of DP cells in the Runt transgenic thymi is due to a high incidence of apoptosis (Fig. 1B). Cells that are stained by annexin V are undergoing apoptosis. Thus, thymocytes harvested before or 7 h after anti-CD3 Ab administration were stained for annexin V. Before the Ab was administered, the proportion of annexin V-high DP thymocytes was low in both the wild-type and Runt transgenic mice. However, 7 h after the Ab injection, the proportion of annexin V-high DP thymocytes was 20% in the Runt transgenic mice but only 4% in the wild-type mice. This was reproducibly observed in several Runt transgenic thymi (Table I). In addition, thymocytes were taken from untreated mice and incubated with various concentrations of an anti-CD3 Ab in vitro (Fig. 1C). The DP fraction from the Runt transgenic thymi consistently contained a larger percentage of annexin V-high cells, compared with that from the wild-type thymi. Thus, the reduction of Runx1 activity renders DP thymocytes more sensitive to apoptosis, at least when the cells are exposed to an artificial TCR stimulation.

An increased sensitivity of Runt transgenic DP thymocytes to apoptosis appears to be an intrinsic nature of themselves

To exclude a possible contribution of SP cell-derived cytokines to thymocytes’ apoptosis, we used TCR-neut mice. In these mice, the OVA-specific TCR transgene (I-Aβ) was introduced into a RAG-2-deficient background (I-Aβ) (7). Because of the absence of selecting MHC (I-Aβ), thymocytes were arrested at the DP stage and did not differentiate further to the SP stage (Fig. 2A). Runt transgenic mice were crossed with TCR-neut mice and TSST-1 was injected i.p. into TCR-neut and TCR-neut; Runt transgenic mice, respectively. This superantigen, TSST-1, recognizes Vβ3, 15, and 17 of TCR and the OVA-specific TCR represents Vβ15. Thymocytes were harvested before and 7 h after the administration and stained for annexin V (Fig. 2B). After the injection, the proportion of annexin V-high DP thymocytes was 13% in the TCR-neut; Runt transgenic mice but only 2% in the TCR-neut. A similar tendency was observed by injecting anti-CD3 Ab into respective mice (data not shown). Therefore, an enhanced sensitivity of Runt transgenic DP thymocytes to apoptosis appears to be an intrinsic nature of themselves.

**Fas and Bim expression is elevated in Runt transgenic thymocytes**

Fas is a member of the TNF receptor family and is involved in the apoptosis of activated peripheral T cells (14, 15). In the thymus, expression of Fas is low at the DN stage but it is up-regulated at the DP and SP stages (16–18). In addition, application of an anti-Fas Ab can induce DP thymocytes to apoptose both in vitro and in vivo (17, 18). We speculated that the enhanced sensitivity of the Runt transgenic DP thymocytes to TCR-mediated apoptosis could be due to their elevated expression of Fas. Thus, we prepared the thymocytes from wild-type and Runt transgenic mice that had or had not been injected with anti-CD3 Ab and processed them for flow cytometry (Fig. 3A). The Runt transgenic DP thymocytes expressed significantly higher levels of Fas compared with the wild-type cells regardless of whether they had been stimulated with anti-CD3 Ab or not (compare the peak intensities of fluorescence). This difference found between the wild-type and Runt transgenic cells was statistically significant at p < 0.05 by a t test. The peak intensities of Fas were as follows: at 0 h, 352 ± 3.1 for the wild-type and 368 ± 3.1 for the Runt transgenic, and at 7 h, 377 ± 5.1 for the wild-type and 392 ± 4.5 for the Runt transgenic, respectively, n = 3 in each case. In contrast, both DP thymocyte genotypes expressed Fas ligand at equivalently low levels (data not shown).

**Table I. Effect of anti-CD3 Ab on the percentages of annexin V-high, DP thymocytes in wild-type and Runt transgenic mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Anti-CD3 Ab (h)</th>
<th>n</th>
<th>Total Cells (×10⁶)</th>
<th>DP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>4</td>
<td>120 ± 38</td>
<td>79 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>104 ± 19</td>
<td>80 ± 1.7</td>
</tr>
<tr>
<td>Runt transgenic</td>
<td>0</td>
<td>4</td>
<td>55 ± 11</td>
<td>81 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>42 ± 9.0</td>
<td>77 ± 3.2</td>
</tr>
</tbody>
</table>

*Thymocytes were prepared from wild-type and Runt transgenic mice before and 7 h after the anti-CD3 Ab injection and processed for flow cytometry. The mean medians and SD are presented for the total cell numbers, the percentages of DP, and annexin V-high DP cells, respectively; n, the number of individual mice examined.*
We also subjected the anti-CD3 Ab-treated or untreated thymocytes to an RNase protection analysis that assessed the expression of various apoptosis-related genes. The Runt transgenic and wild-type thymocytes did not differ significantly in their levels of anti-apoptotic bcl-2 and bcl-xL transcripts (data not shown). The same analysis also demonstrated that the two cell genotypes did not differ in the transcript levels of the proapoptotic genes TNF-α, trail, Bax, Bad, and caspase 8 (data not shown). However, when we examined the expression of Bim, which is another proapoptotic Bcl-related protein (19), by immunoblot analysis, we observed that the Runt transgenic thymus showed a remarkable increase in the levels of Bim protein compared with the wild-type thymus (Fig. 3B). This up-regulation of Bim expression in the Runt transgenic cells was observed both before and after the TCR stimulation. Furthermore, a similar tendency was observed when the DP fraction was purified and processed for immunoblot analysis (data not shown). In contrast, the protein levels of Bcl-2 and Bcl-xL did not differ significantly between the wild-type and Runt transgenic thymi.

The increased sensitivity of Runt transgenic DP thymocytes to apoptosis is partially due to their up-regulation of Fas expression

We next examined the correlation between the levels of Fas expression and apoptosis. Thymocytes harvested from untreated Runt transgenic and wild-type mice were incubated in vitro with various concentrations of an anti-Fas Ab and then processed for flow cytometry. Fig. 4A shows the percentage of the annexin V-high population in the DP fraction. In the absence of the anti-Fas Ab, apoptosis was low in both genotypes of cells until 3 h of incubation. However, the Runt transgenic DP thymocytes were more sensitive to the anti-Fas Ab treatment and died more readily than the wild-type DP cells. Thus, the up-regulation of Fas levels in the Runt transgenic DP thymocytes is associated with increased sensitivity to anti-Fas Ab-induced apoptosis.

The above result indicates that Fas may be involved in the TCR-mediated apoptosis of DP thymocytes. To test this, we crossed Runt transgenic mice with lpr/lpr mice, which show little Fas expression, injected them with anti-CD3 Ab, and analyzed their thymocytes 7 h later by flow cytometry (Fig. 4B). Only 2.6% of the DP thymocytes of the lpr/lpr mice were annexin V-high but up to 18.5% of the Runt transgenic DP thymocytes reacted to annexin V at high levels. However, in the Runt transgenic/lpr/lpr DP thymocytes, the annexin V-high population had decreased to 8.2%. Therefore, the enhanced apoptosis of the Runt transgenic DP thymocytes upon TCR stimulation appears to be mediated, at least partly, by the up-regulation of Fas expression.
of the annexin V-high population. The numbers of total thymocytes recovered in the different fractions are shown in the lower row. The numbers indicate the percentage of thymocytes that were DP and annexin V-high processed for flow cytometric analysis. The annexin V staining of the DP thymocytes 7 h later. The cells were then stained for CD4, CD8, and annexin V and analyzed. The annexin V staining of Runt transgenic DP thymocytes is plotted as average and SD.

**FIGURE 4.** Effect of anti-Fas Ab or the lpr mutation on the survival of Runt transgenic DP thymocytes. A, Thymocytes were prepared from wild-type and Runt transgenic mice, incubated for 30 min with the indicated concentration of anti-Fas Ab, and processed for flow cytometric analysis of CD4, CD8, and annexin V staining. The percentage constituted by the annexin V-high population in the DP gate is plotted as average and SD. B, Annexin V staining of Runt transgenic DP thymocytes on the lpr/lpr background. Wild-type, Runt transgenic, lpr/lpr, and Runx transgenic:lpr/lpr mice were injected with anti-CD3 Ab and their thymocytes were prepared 7 h later. The cells were then stained for CD4, CD8, and annexin V and processed for flow cytometric analysis. The annexin V staining of the DP fractions are shown in the lower row. The numbers indicate the percentage of the annexin V-high population. The numbers of total thymocytes recovered were as follows: 1.48 × 10^6 for wild-type, 4.28 × 10^6 for Runx transgenic, 5.6 × 10^6 for lpr/lpr, and 5.1 × 10^6 for Runt transgenic:lpr/lpr mice, respectively.

**Effect of overexpression of Runx1 on the fate of DP thymocytes**

Next, we performed a similar analysis using Runx1 transgenic mice in which Runx1 was artificially overexpressed in the T cell lineage (6). The i.p. administration of anti-CD3 Ab into mice did not produce a significant nor constant difference in the extent of apoptosis between the wild-type and Runx1 transgenic cells (data not shown). Therefore, thymocytes were taken from untreated mice and incubated with various concentrations of anti-CD3 Ab in vitro. As shown in Fig. 5A, the Runx1 transgenic DP thymocytes contained a significantly less proportion of annexin V-high cells compared with the wild-type cells. This was evident after the Ab incubation. For the asfor the expression of proapoptotic molecules, that of Fas as detected by flow cytometry in Fig. 5B was significantly downregulated in the Runx1 transgenic DP thymocytes (peak intensities were 330 ± 0; n = 3) compared with the wild-type cells (360 ± 0; n = 3). In contrast, we have encountered a somewhat complicated situation in the case of Bim protein as revealed by immunoblot analysis (Fig. 5C). Before the i.p. injection of anti-CD3 Ab into mice, the level of Bim in the Runx1 transgenic DP thymocytes was similar to or rather higher than that in the wild-type cells. In contrast, 7 h after the Ab administration, Bim expression was substantially down-regulated in the Runx1 transgenic but not the wild-type thymus. A similar pattern of Bim expression was confirmed when the purified DP fraction was processed for immunoblot (data not shown).

A difference in the apoptotic sensitivity that was seen between the Runx1 transgenic and wild-type cells was not as dramatic as in the case of Runt transgenic versus wild-type cells. This might be partly due to a complex pattern of Fas and Bim expression in the Runx1 transgenic cells.

**Runx1:HY-TCR-double transgenic DP thymocytes are more resistant to HY Ag-specific TCR engagement**

So far, we have evaluated the effect of the artificial cross-linking of the TCR on the fate of DP thymocytes. To examine whether endogenous TCR signaling also affects the survival or death of DP thymocytes, we crossed Runt transgenic with HY-TCR transgenic mice. Runx1 transgenic mice were also crossed with HY-TCR transgenic mice. The HY-TCR recognizes the male-specific HY Ag (13). The thymocytes were prepared from male mice and processed for flow cytometry (Fig. 6). The cells were first gated for the T3.70high population that expresses the HY-TCR, and the CD4 and CD8 expression profiles of this population were then analyzed. The proportion of the DP cells did not differ significantly between the HY-TCR-single and the Runx:HY-TCR-double transgenic thymus (Fig. 6A). This is probably because the signal was so strong that no effect was observed (since the number of 3% as the DP fraction was already very low, a possible further reduction to, for example, 2% or 1%, in the percentage of DP fraction by the Runx transgene would not be reasonably evaluated). However, the proportion of thymocytes that were DP was significantly greater in the Runx1:HY-TCR-double transgenic thymus (16.3%) compared with that in the HY-TCR-single transgenic thymus (1.6%) (Fig. 6B). Therefore, overexpression of Runx1 can render DP thymocytes resistant to the apoptosis induced by endogenous TCR signaling due to HY Ag presentation.

**Discussion**

In this study, we have demonstrated that the DP thymocytes in Runx1 transgenic mice are more susceptible to apoptosis than those in wild-type mice. We also showed that the enhanced apoptotic sensitivity of Runx1 transgenic DP cells is mediated at least partly by their up-regulation of Fas expression because this sensitivity was abrogated to a significant extent by the lpr/lpr background. Furthermore, modulation of Runx1 activity affected the fate of DP thymocytes not only in the context of artificial cross-linking of the TCR but also in the context of endogenous TCR stimulation via the HY Ag. Thus, we propose that Runx1 functions to suppress the apoptotic sensitivity of DP thymocytes in the context of TCR signaling.

There have been many reports to date that have examined the role that Fas plays in the negative selection of thymocytes. A generally accepted consensus is that Fas may not be essential for negative selection to occur because the clonal deletion of thymocytes that is observed in TCR transgenic mice after the administration of the Ag peptide can still be observed in the TCR transgenic:lpr/lpr mice (20). However, it is possible that...
multiple and redundant pathways are involved in negative selection and that some of these are involved in the cell death of Fas-deficient thymocytes. It is also possible that the dose of Ag given influences the degree to which the Fas-dependent death signal is mobilized. Indeed, more detailed analysis suggests that Fas does appear to play some roles in the negative selection of thymocytes. For example, the administration of a high dose of Ag peptide-specific for the transgenic TCR (21, 22) or a high dose of superantigen (23) eliminates the DP or immature SP thymocytes from the thymus of wild-type but not lpr/lpr mice. Furthermore, blockade of endogenous Fas-Fas ligand interaction in the wild-type thymus renders the DP thymocytes less sensitive to apoptosis (21), whereas overexpression of Fas ligand as a transgene induces massive apoptosis of DP thymocytes (24). Therefore, it may not be unreasonable to speculate that the apoptotic sensitivity (or the negative selection) of DP thymocytes is enhanced if their endogenous expression of Fas is somehow elevated, as it is in the Runx1 transgenic thymus whose Runx1 transcription factor activity is reduced.

We showed here too that Bim protein levels were increased in the Runx1 transgenic thymocytes compared with the wild-type cells. As in the case of Fas, Bim is involved in the apoptosis of peripheral activated T lymphocytes (25, 26). With regard to its role in thymocyte differentiation, Bim-deficient thymocytes are resistant to TCR-mediated apoptosis (27). Homozygous targeting of Bim remarkably abrogates both the elimination of endogenous self-reactive clones and anti-CD3 Ab-induced apoptosis. It remains to be seen whether the increased expression of Bim in Runx1 transgenic cells contributes to their higher sensitivity to apoptosis.

Two mechanisms have been proposed that explain how Bim, which only contains a Bcl-2 homology domain 3 (BH3), induces apoptosis. In one, Bim binds to the BH1–4-containing anti-apoptotic molecules Bcl-2 and/or Bcl-xL, thereby inactivating them (19). In the other mechanism, Bim is activated by an apoptotic signal and released from microtubules and then binds to the BH1–3-containing proapoptotic molecule Bax (28). This activates Bax, which alters its conformation and builds a pore in the mitochondrial membrane. Analogous to the latter mechanism is that Bid, which like Bim only bears a BH3, interacts with the BH1–3-containing proapoptotic molecule Bak. Importantly, a death signal of Fas can be transmitted to Bid and then to Bak via the recruitment of Fas-associated death domain protein and caspase 8 (29). It is not known whether Bim is located in the Fas-signaling pathway. However, it must be noted that Fas signaling can induce apoptosis in a mitochondria-dependent or independent manner (30, 31). It is striking, therefore, to see that the expression of both the proapoptotic Fas and Bim molecules, which is induced by TCR stimulation, is enhanced by reducing the endogenous Runx1 activity.

Some nuclear orphan receptors have been reported to regulate the survival or death of thymocytes. RORγt (−/−) mice show a profound decrease in DP thymocyte numbers and an increased rate of DP thymocyte apoptosis (32, 33). Bcl-xL transcript and protein levels are very low in these mice. Transgenic mice overexpressing Nur77 also exhibit massive depletion of thymocytes (34, 35). Overexpression of Bcl-2 or lack of Fas ligand do not block the Nur77-mediated apoptosis of thymocytes, which excludes Bcl-2 or Fas ligand as the target genes of Nur77 (36, 37). Thus, different proapoptotic and/or anti-apoptotic molecules appear to be involved in the death of Runx1 transgenic, RORγt (−/−), and Nur77 transgenic thymocytes. The elucidation of the molecular mechanism by which Runx1 regulates, directly or indirectly, the expression of Fas and Bim in the context of TCR signaling will be a fascinating subject of future studies. A fact that the consensus sequence for Runx1 binding
FIGURE 6. Flow cytometric analysis of thymocytes prepared from male Runt:HY-TCR- or Runx1:HY-TCR-double transgenic mice. Thymocytes were prepared from HY-TCR transgenic, Runt:HY-TCR-double transgenic, and Runx1:HY-TCR-double transgenic mice. CD4, CD8, and T3.70 and processed for flow cytometric analysis. The thymocytes were first gated for the T3.70high populations and the CD4, CD8, and T3.70 expression profiles in these populations were then analyzed. The numbers in each quadrant represent the percentages of cells in each gate. The numbers of total thymocytes recovered from littermate mice were 1.36 × 10^6 for Runt:HY-TCR-transgenic and 5.8 × 10^6 for Runx1:HY-TCR-transgenic mouse (A) and 9.9 × 10^6 for HY-TCR-transgenic and 9.4 × 10^6 for Runx1:HY-TCR-double transgenic mouse (B), respectively.

(PuACCPuCA) was detected in the promoter regions of murine Fas (38) and Bim (39) (M. Satake, unpublished observation) might favor for a direct involvement of Runx1 in the regulation of these genes’ expression.

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Disclosures

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