CD45 can act as a negative regulator for the transition from early to late CD4⁺CD8⁺ thymocytes

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Abstract

The differentiation process from CD4⁻CD8⁻ double-negative (DN) thymocytes to CD4⁺CD8⁺ double-positive (DP) stage is accompanied by vigorous proliferation. The resulting DP cells contain a sizable proportion of large cycling cells, but most DP cells are small resting cells. To explore the molecular mechanisms which regulate cell proliferation of DP thymocytes prior to further development, we used TCR-transgenic (Tg) mice with non-selecting MHC (Tg-Neut), which contain almost exclusively DP thymocytes that are not subject to either positive or negative selection. In Tg-Neut, the thymus contained DP cells of relatively large size, which showed higher extracellular signal-regulated kinase activity and enhanced responsiveness to mitogen compared to small DP cells. This indicates that all the large DP cells in the thymus are not positively selected and that they possess proliferative potential. When Tg-Neut mice were backcrossed with CD45 knockout mice (CD45⁻/⁻ Tg-Neut), the thymus showed an increase of large DP cells and cycling cells, but a decrease of apoptotic cells. Furthermore, Bcl-2 expression and Jun N-terminal kinase activity, which are associated with resistance to apoptosis, were enhanced. These observations suggest that thymocyte proliferation in the DP stage is suppressed by a CD45-related process with regulation of mitogen-activated protein kinase and Bcl-2 unless DP cells receive TCR-mediated signals.

Introduction

Orchestrated regulation of cell proliferation and death generally occurs in ontogenical development (1). In the thymus, the developing process from CD4⁻CD8⁻ double-negative (DN) to CD4⁺CD8⁺ double-positive (DP) cells, which is induced by pre-TCR-mediated signaling, is accompanied by vigorous proliferation (2-4). At the resultant DP stage, however, most DP thymocytes cease proliferation and undergo programmed cell death, whereas only a small proportion of DP cells with low-affinity TCR for self-MHC develop into CD4⁺ or CD8⁺ single-positive (SP) cells. The latter is a process which is referred to as positive selection and the former is referred as default death (5). It is known that DP cells consist of a sizable proportion of large cycling cells, but most are small and non-dividing cells (6,7). Although most small DP cells have been reported to be dead-end cells (8), other studies showed that small DP cells can mature into SP cells when they are intrathymically transferred (9,10). Ernst et al. demonstrated that cell proliferation is not essential for positive selection in an in vitro re-aggregation culture system (6). Furthermore, Huesmann et al. showed that positively selected cells are not dividing cells, using an in vivo DNA-labeling technique (11). From these studies, it can be surmised...
that DP cells stop their proliferation to further development or cell death. However, the regulation mechanism for cell proliferation in the DP stage is not well understood.

Current mutant mice showed that several cytoplasmic molecules such as Lck and Ras are located down-stream of pre-TCR-mediated signals for the DN to DP transition, termed the β-selection process (12–15). Furthermore, Crompton et al. reported in an in vitro system using embryonic thymus culture that activation of extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) superfamily, is also responsible for transition from DN to DP cells (16). However, it is still unclear whether these molecules are involved in regulating cell proliferation of DP thymocytes as they do in the DN to DP transition.

CD45 is a membrane protein whose cytoplasmic region possesses protein tyrosine phosphatase activity (17). Although CD45 is known to activate Lck by dephosphorylating C-terminal tyrosine (18–22), several in vitro studies have shown that Lck activity is greater in CD45-deficient mutant cell lines than in CD45-bearing parent cell lines (19,23,24). The suppressive function of CD45 is also reported for MAPK; purified MAPK can be tyrosine dephosphorylated and inactivated by purified CD45 (25). At the cell level, several studies have shown that MAPK activation via CD2, CD3 or insulin-receptor was suppressed in the presence of CD45 (25). In the absence of negative and positive regulators for signaling, probably depending on cell types or developing stages. Therefore, it is postulated that CD45 could act as a negative modulator for thymocyte development through, for example, regulation of MAPK activities.

In this study, we used TCR-transgenic (Tg) mice without TCR-mediated signals for positive and negative selections in order to determine the molecules regulating cell proliferation of DP thymocytes (29,30). These thymocytes stopped cell proliferation at the DP stage and subsequently died because of the absence of negative and positive selections. Accordingly, these thymocytes are useful for determining the mechanism of regulation of the proliferation at the DP stage. When these mice were backcrossed with CD45 exon 6-deficient mice (31), their thymocytes showed an increase of cycling cells and a decrease of apoptotic cells, with enhanced activity of MAPK, suggesting that CD45 is involved in the cessation of thymocyte proliferation.

Methods

Mice

I-A<sup>d</sup> + ovalbumin (OVA)<sub>323-339</sub>-specific TCR-Tg mice, OVA23-3, were produced as previously reported (29). These mice were backcrossed with BALB/c and C57BL/6. Their founders were designated Tg-Posi and Tg-Neut respectively. Tg-Posi and Tg-Neut were backcrossed with Rag2-deficient and CD45 exon 6-deficient mice (31).

Flow cytometry analysis and cell sorting

Phycocyanin (PE)-anti-mouse CD4 and streptavidin–Red670 were purchased from Caltag (San Francisco, CA) and Life Technologies (Gaithersburg, MD) respectively. PE-anti-mouse CD25 and biotin-anti-mouse CD69 were purchased from PharMingen (San Diego, CA). Biotin-anti-Ly1(CD5) was purchased from Becton Dickinson (Mountain View, CA). Surface-stained thymocytes were analyzed by a FACScan (Becton Dickinson) or sorted on a FACStar Plus flow cytometer.

Proliferation assay

Thymocytes were cultured in 96-well plate for 48 h in the presence of indicated concentrations of phorbol myristate acetate (PMA) and ionomycin. Either 1 × 10<sup>5</sup> or 5 × 10<sup>5</sup> cells/well were cultured in round-bottomed or flat-bottomed 96-well plates respectively. Then 1 μCi/well [3H]thymidine was added during the final 18 h and incorporated radioactivities were counted.

Cell cycle analysis

Mice were i.p. injected with the thymidine analogue BrdU. At 2 h after injection, the thymus was removed and fixed with 70% ethanol. Fixed thymocytes were treated with 2 N HCl plus 0.5% Triton X-100 and neutralized with 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>. After washing with PBS, FITC–anti-BrdU (Becton Dickinson) was added. Following incubation with 5 μg/ml propidium iodine, stained cells were analyzed on a FACScan by the CellFIT program.

In vitro kinase assay

Between 5 and 50 × 10<sup>5</sup> cells were lysed with 0.5 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM EGTA, 1 mM DTT, 0.1 M NaF, 1% Triton X-100, 1 mM PMSF, 20 μg/ml aprotinin and 20 ng/ml leupeptin). After incubation on ice for 30 min, the sample was centrifuged (10,000 r.p.m., 4°C) and the supernatant was collected. The lysate was pre-cleared with Protein G–Sepharose and immunoprecipitated with anti-ERK-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Jun N-terminal kinase (JNK)-1 (Santa Cruz)-Protein G–Sepharose. After washing with lysis buffer 3 times, the immunoprecipitate was rinsed with kinase buffer (50 mM Tris–HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 2 mM EGTA and 1 mM DTT). Kinase reaction was performed at room temperature for 10 min (ERK) or at 30°C for 30 min (JNK) in 25 μl of kinase buffer containing 1 μg/ml protein kinase inhibitor (Sigma), 2 μM ATP, 1 μCi [γ<sup>32</sup>P]ATP and myelin basic protein (MBP; Sigma) or c-jun-GST (Santa Cruz Biotechnology) as substrate for ERK and JNK respectively. When the sample was small scale (5 × 10<sup>5</sup> cells/sample), cold ATP was not added. After 25 μl of sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol and 0.2 g/ml glycerol) was added and boiled for 5 min, the sample was separated by SDS-PAGE. The dried gel was exposed to Scientific Imaging Film (Kodak, Rochester, NY).

Immunoblotting

Between 2.5 and 20 × 10<sup>5</sup> cells were lysed with sample buffer and boiled for 5 min. The lysates were separated by SDS-PAGE and transferred onto Clear-blot Membrane (Atto, Tokyo, Japan). After blocking with BSA, the membrane was incubated with anti-ERK-1, anti-JNK-1, anti-p27<sup>KIP1</sup> (Santa Cruz) or anti-Bcl-2 (PharMingen, San Diego, CA). Detection was performed
using ECL Western blotting reagent (Amersham, Amersham, UK) and Hyperfilm ECL (Amersham).

Detection of apoptotic cells

Formalin-fixed sections of thymuses at day 2 after birth were treated with proteinase K (final 20 µg/ml) at room temperature for 15 min. After washing, tissue sections were incubated with 2% H2O2 diluted in methanol for 5 min to neutralize endogenous peroxidases. After washing with H2O 4 times, sections were incubated with 5 U TdT (Boehringer Mannheim, Mannheim, Germany) and 0.5 nmol biotin–dUTP (Boehringer Mannheim) in 30 µl of TdT buffer (Boehringer Mannheim) at 37°C for 60 min. Sections were incubated with TB buffer (300 mM NaCl/30 mM citrate), 2% BSA in PBS and then with horseradish peroxidase (HRP)–streptavidin. Bound HRP was detected with substrate DBA and sections were lightly counterstained with methylgreen or haematoxylin.

A computer-assisted image analysis system with the Interactive Build Analysis System (Carl Zeiss, Zena, Germany) was used to evaluate apoptotic cells. The input image resolution was 512×512 pixels, 8 bits, 256 gray scales. The stained nuclei and DAB reaction products were detected using interference bandpass filter IF 436 and 600 (Olympus, Tokyo, Japan) respectively. The binary images of hematoxylin and DAB were overlaid and segmented images were counted.

Results

DP large cells in TCR-Tg mice with non-selecting MHC showed increased MAPK activity and enhanced responsiveness to mitogen

The thymus of TCR-Tg mice, OVA23-3 (29,30), with selecting MHC (I-Aα, Tg-Posi) contains large numbers of CD4 SP cells (~20 %, data not shown) and DP cells. On the other hand, the thymus of TCR-Tg mice with non-selecting MHC (I-Aβ, Tg-Neut) does not contain SP cells. DP cells of both TCR-Tg mice include a significant number of cells of relatively large cell size. Large DP cells in Tg-Neut did not show characteristics of positive selection, such as high expression of CD69 and CD5 (32–34) (Fig. 1). Such large DP cells with low CD69 and CD5 expression on large and small DP cells. DP and small DP cells are shown as solid lines. Dotted lines are non-stained controls.

Another function of CD45 on DP thymocytes development

In our previous study using Tg-Neut mice, we demonstrated that TCR expression is high at the early DP stage even though the cells are not positively selected and the expression is gradually down-regulated thereafter (30). We also reported that the down-regulation of TCR is inhibited in CD45-deficient mice. Then, we examined the role of CD45 in thymocyte proliferation in the absence of TCR-mediated signals. In Tg-Neut whose thymocytes are not positively selected by appropriate MHC, the thymus is composed of mostly DP cells but not SP cells in both CD45-bearing (CD45+/+ Tg-Neut) and CD45-deficient (CD45−/− Tg-Neut) lines. Interestingly, an increased number of DP cells of large size was found in CD45−/− Tg-Neut thymocytes (Fig. 4a). In addition, enhancement of proliferation and aggregation was observed in CD45−/− Tg-Neut when they were cultured with PMA plus ionomycin (Fig. 4b and c). At the same time, ERK activity was greater in CD45−/− Tg-Neut than in CD45+/+ Tg-Neut, although the
Another function of CD45 on DP thymocytes development

Fig. 2. Large DP cells of TCR-Tg mice with non-selecting MHC (Tg-Neut) showed enhanced aggregation and proliferation responding to PMA plus ionomycin. (a) Thymocytes of Tg-Neut were stained with FITC–anti-CD8 and PE–anti-CD4. Small and large DP cells were fractionated by a FACStar. (b) Phase contrast micrographs of culture after 1 day with PMA (0.2 ng/ml) and ionomycin (1.2 µg/ml). (c) Small and large DP cells were cultured with 0.2 ng/ml PMA and 1.2 µg/ml ionomycin for 48 h in round-bottomed 96-well plates (1x10⁵ cells/well). [³H]Thymidine incorporations (± SD) during the final 18 h are indicated.

amounts of ERK-1 and -2 protein were not different between the two types of mice (Fig. 4d).

These features observed in CD45⁻/⁻ Tg-Neut thymocytes are similar to those of large DP cells in CD45⁺/⁺ Tg-Neut (Figs 2 and 3). Because there is no ligand for TCR in either of the Tg-Neut thymuses, these changes are considered to result from CD45 deficiency independently of TCR-mediated signals. This is the first evidence to show the suppressive effect of CD45 on cell proliferation of non-selected DP cells in the thymus, although CD45 has been reported to be important in both positive and negative selections (18,31,42). CD45 seems to be involved in the negative regulation of DP cell proliferation, as is discussed later.

Cycling cells increase in CD45⁻/⁻ Tg-Neut in vivo

To ascertain whether large DP cells with proliferative potential substantially increase in the thymus of CD45⁻/⁻ Tg-Neut, we injected the thymidine analogue BrdU i.p. into CD45⁻/⁻ and CD45⁺/⁺ Tg-Neut mice. Two hours after the injection, the thymocytes were fixed and stained with FITC–anti-BrdU and propidium iodine for analysis by flow cytometry. The number of thymocytes in the S phase of the cell cycle was 2-fold greater in CD45⁻/⁻ Tg-Neut mice than in CD45⁺/⁺ Tg-Neut (Fig. 5). A concomitant decrease of the cell number in G₀/G₁ phase was observed in the CD45⁻/⁻ Tg-Neut thymus. These findings indicate that CD45 deficiency results in augmentation of thymocyte proliferation in vivo, which is consistent with the increased number of large DP cells in CD45⁻/⁻ Tg-Neut.

Apoptotic cells decrease in CD45⁻/⁻ Tg Neut thymus

In addition to the increase of cycling cells, the decrease of small DP cells by apoptosis may also cause the high frequency of large DP cells in CD45⁻/⁻ Tg-Neut. Then, we examined apoptotic cells in the fresh thymus of CD45⁻/⁻ and CD45⁺/⁺ Tg-Neut by the TUNEL staining method. In the thymus sections, the frequency of TUNEL-positive cells in total cells was lower in CD45⁻/⁻ Tg-Neut than in CD45⁺/⁺ Tg-Neut (Fig. 6). Moreover, both the frequency of DP cells and the total cell number were larger in CD45⁻/⁻ Tg-Neut than in CD45⁺/⁺ Tg-Neut (data not shown). The correlation of the decrease of apoptotic cells with the increase of cycling cells is consistent with the previous report showing that apoptotic
Another function of CD45 on DP thymocytes development

Nishina et al. reported that the number of DP thymocytes is decreased in mutant mice lacking the JNK kinase, Sek-1, and that Sek-1-deficient DP cells are more susceptible to Fas- or CD3-mediated apoptosis (43). This raises the possibility that JNK plays a role in the survival of DP thymocytes. Thus, we examined the JNK activity in thymocytes of CD45−/− Tg-Neut. As shown in Fig. 7(a), the JNK activity was higher in CD45−/− Tg-Neut than that in CD45+/+ Tg-Neut thymocytes. Moreover, the expression of the anti-apoptotic gene, bcl-2, was higher in DP cells of CD45−/− Tg-Neut than of CD45+/+ Tg-Neut (Fig. 7b). These findings are consistent with the decrease of apoptosis in CD45−/− Tg-Neut.

When compared with Tg-Neut, JNK activity was much higher in thymocytes of TCR-Tg mice with selecting MHC (Tg-Posi) where all DP cells are considered to have escaped from apoptosis and to develop into SP cells (Sato, unpublished data). Taken together, it is possible that JNK as well as Bcl-2 are down-regulated at the DP stage to make cells prone to die, presumably by CD45-mediated mechanisms, which may be cancelled when DP cells are positively selected via TCR-mediated signals.

**Discussion**

We demonstrated that DP thymocytes of relatively large size are present in TCR-Tg mice with non-selecting MHC (Tg-Neut), and that these DP cells do not express cell surface molecules characteristic for positively selected cells such as CD69 and CD5 (32–34) (Fig. 1). However, they showed the increased activity of MAPK, ERK-1 and -2, compared with small DP cells (Fig. 3). This indicated that all the large DP cells in the thymus are not positively selected cells and that such large DP cells without TCR-mediated signals possess potential for proliferation because MAPK was reported to be associated with cell proliferation (37,38). In fact, they proliferated in response to PMA plus ionomycin (Fig. 2).
The expression level of ERK in large DP cells was higher than in small cells (data not shown), but the kinase activity per ERK protein could not be estimated clearly from the intensity of blotting bands of the activity and amount of ERK. It cannot be excluded that the increased ERK activity in large DP cells may reflect higher expression of ERK, but it is absolutely clear that in CD45-deficient Tg-Neut thymocytes, the kinase activity of ERK is increased but its expression is not changed (Fig. 4). Furthermore, CD45^{-/-} Tg-Neut thymocytes showed enhanced JNK activity and Bcl-2 expression (Fig. 8), both of which are thought to be involved in resistance to apoptosis (43–45).

CD45 is also reported to activate Lck and Fyn by dephosphorylation of their C-terminal tyrosine (18–22). However, several reports have shown the reverse function of CD45; Lck activity is increased in CD45-deficient mutant cell lines compared to that in CD45-bearing parent cell lines (19,23,24). D’Oro et al. found that CD45 can dephosphorylate Tyr394 of Lck, in addition to C-terminal Tyr505, and that the absence of CD45 drives the hyperphosphorylation of Tyr394 and a resultant increase of the kinase activity of Lck though Tyr505 of Lck was not dephosphorylated (49). Similarly, CD45 can act as a negative regulator for some other kinases (25,50). It is reported that ERK can be tyrosine dephosphorylated and inactivated directly by purified CD45 in vitro (25). Furthermore, several studies have shown that ERK activation via CD2, CD3 or insulin-receptor was suppressed in the presence of CD45 (26–28). Cross-linking of CD3 on thymocytes of RAG-deficient mice induced transient up-regulation of ERK activity (16), which was more increased in CD45^{-/-} RAG-deficient mice (T. Sato, unpublished data). Taken together, it is suggested that CD45 may negatively regulate phosphorylation and activation of ERK in DP thymocytes without TCR signals. Similarly, JNK, which is also a member of the MAPK superfamily, could be inactivated by CD45. It is reported that positively selected DP cells increase their TCR expression (51,52). However, all of the TCR^{hi} DP cells were not always post-selected cells. We and Swat et al. found that DP cells with TCR^{hi} exist in TCR-Tg mice with non-selecting MHC, in which DP thymocytes are not positively selected (30,36). We have also reported...
that TCR expression on DP cells in Tg-Neut at gestation days 16 to 17, when DP cells begin to appear, is at the same high level as that of positively selected DP cells, but that the expression decreases during the further course of gestation (30). This observation suggests that ‘early DP cells’, which recently transited from the DN stage, can express TCR at a higher level. Moreover, the decrease of TCR expression did not occur in the absence of CD45 (30), indicating that there exist some CD45-mediated mechanisms which down-regulate the TCR expression when DP cells do not receive TCR-mediated positive signals. This finding seems to be consistent with the previous reports that Lck regulates TCR surface expression and lysosomal degradation (53,54), because CD45 can positively regulate the kinase activity of Lck.

We found that large DP cells in TCR-Tg mice with non-selecting MHC contained many high TCR-expressing cells, whereas small DP cells did not contain any at all (data not shown). Considering the present findings together with the previous ones of TCR expression, it is strongly suggested that large DP thymocytes without TCR-mediated signals belong to DP cells at the early stage and that in the absence of TCR-mediated signals CD45 is involved in the transition from early to late DP cells by negatively regulating the proliferation and presumably by promoting apoptosis (Fig. 6), as well as in TCR down-regulation. This hypothesis is depicted in Fig. 8. Early DP cells have greater activity of both ERK and JNK, and express Bcl-2 at a higher level. Then, they are down-regulated and early DP cells become small DP cells at the late stage. CD45 may mediate this transition. When DP cells are positively selected by TCR-mediated signals, JNK activity and Bcl-2 expression are again up-regulated. Subsequently, selected cells acquire a long lifespan, whereas non-selected cells die.

It is believed that the lifespan of DP thymocytes is 3–4 days (11,55,56). However, it is not known what molecules set the clock. It is attractive to postulate that CD45 can transduce signals to repress thymocyte proliferation or to induce apoptosis in non-selected DP cells. This hypothesis may be consistent with a recent report that CD45 ligation can induce apoptosis of DP cells (57). On the other hand, Novak et al. proposed the possibility that the shorter isoform, CD45RO, can diffuse more freely in the plane of cell membrane and...
Another function of CD45 on DP thymocytes development

can interact with substrates more readily than the longer isoform (CD45RABC) does (58). It is reported that the switch from the shorter to longer isoform occurs when DP cells are positively selected (59). Therefore, it can be speculated that the shorter isoforms on non-selected DP cells transduce signals to effectively suppress the cell cycle or to induce apoptosis and that the switch to longer forms prevents this effect when they are positively selected.

On the other hand, Chen et al. reported that the inducibility of the DNA binding activity of AP-1 (38), which is one of the targets of ERK and JNK pathways, is extinguished at most cortical thymocytes, whereas it is found at earlier and full mature stages (60,61). It is believed that the alteration of AP-1 inducibility is associated with mitogen responsiveness of thymocytes. We found that CD45<sup>−/−</sup> Tg-Neut thymocytes showed enhanced cell division in response to PMA plus ionomycin. It will be of interest to examine whether the DNA binding activity of these transcription factors can be induced at a greater level in CD45<sup>−/−</sup> Tg-Neut than CD45<sup>+/+</sup> thymus.

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Abbreviations

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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>DN</td>
<td>double negative</td>
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<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>JNK</td>
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<td>Tg</td>
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References

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