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Differential Requirements of CD45 Protein Tyrosine Phosphatase for Cytolytic Activities and Intrathymic and Extrathymic Development of Intestinal Intraepithelial Lymphocytes

Shinichiro Yada, Kenji Kishihara, Young-Yun Kong, and Kikuo Nomoto

CD45 is a transmembrane protein tyrosine phosphatase essential for Ag receptor-mediated signaling in both T and B cells. In this study we investigated roles of CD45 in development and cytolytic activities of murine intestinal intraepithelial lymphocytes (i-IEL) using CD45 exon 6 knockout (CD45^{−/−}) mice. Interestingly, the total cell number of i-IEL was significantly reduced in CD45^{−/−} mice during aging (10–20 wk of age), whereas the i-IEL number was normally increased in the wild-type littermates. Especially, the number of γδTCR^{+} i-IEL decreased markedly in CD45^{−/−} mice during aging. The i-IEL in CD45^{−/−} mice were more susceptible to in vitro spontaneous apoptosis than the normal i-IEL, implying that CD45 is required for maintenance of the cellularity of i-IEL. Results from in vivo analyses of the extrathymic and intrathymic development of i-IEL suggested that CD45-mediated signaling is required for the intrathymic, but not the extrathymic, development of i-IEL. Moreover, the whole i-IEL from CD45^{−/−} mice showed a significantly reduced cytolytic activity, and the residual cytolytic activity was completely diminished by depleting CD45^{−/−} i-IEL, suggesting that CD45 is indispensable for the TCR-mediated cytolytic activity of i-IEL. Furthermore, we found differential contributions of CD45 and p56^{++} to development and induction of cytolytic activities of i-IEL. The Journal of Immunology, 1998, 161: 2208–2216.

Gut-associated lymphoid tissues represent a prominent part of mucosa-associated lymphoid tissues and include organized lymphoepithelial tissues of the small and large intestines, such as lymphocytes within the epithelial layer, the Peyer’s patches, the appendix, and the solitary lymphoid nodules (1, 2). In mice, intestinal intraepithelial lymphocytes (i-IEL) are classified into two main populations bearing the αβ or γδ heterodimer of TCR, and they are present in significant amounts only in this peculiar localization. The i-IEL are also characterized by surface expression of CD4 and CD8 coreceptors (CD8^{αβ} or CD8^{αα}) (3, 4). In contrast, the bulk of peripheral lymphocytes bear αβTCR and CD4 or CD8^{αβ} molecules but not CD8^{αα}. Both mature αβTCR^{+} and γδTCR^{+} i-IEL (αβ-IEL and γδ-IEL, respectively) contain granules rich in granzymes and perforin and have cytotoxic activity (4–7). Previous studies showed that all i-IEL subsets develop in both intrathymic and extrathymic pathways. Recently, it was suggested that every CD8^{αα} and γδ-IEL does not always develop extrathymically (8–12), and i-IEL development is also regulated by humoral factors, including hormones, growth factors, and cytokines (13–17).

CD45 protein tyrosine phosphatase (PTP) is expressed on all nucleated hemopoietic cells and specifically dephosphorylates negative regulatory tyrosine residues of src family protein tyrosine kinases (PTKs) to activate these kinases (18–20). In T cells, the activation of src family PTKs including p56^{++} (Lck) and p56^{αα} (Fyn) is required for TCR-mediated signaling, and CD45 PTP is essential in this signaling event (19–22). Furthermore, remarkable decreases in mature thymocytes and mature peripheral T cells were previously observed in CD45 exon 6 or exon 9 knockout mice, suggesting that CD45 is important for thymocyte development and maturation (23–25). It was also demonstrated that CD45 is critical for positive/negative selection of T cells in thymus by analyzing CD45 exon 6-deficient, TCR-transgenic mice (26). Notably, the introduction of CD45 isoform transgenes significantly rescued the selection events in thymi in CD45 exon 6-deficient, TCR-transgenic mice, suggesting that CD45 surface expression is crucial for intrathymic T cell maturation (26).

In the case of CD45 exon 6-deficient mice, a small part of mature thymocytes and peripheral mature T cells has a low intensity of CD45 surface expression, although CD45 expression was undetectable on the other leukocytes including B cells, macrophages, mast cells, and so on (23, 25, 27–30). The mechanism of the CD45 surface expression is not clear. CD45 isoforms expressed in the CD45^{−/−} T cells from CD45 exon 6-deficient mice had no use of the CD45RC epitope encoded by the exon 6. The CD45^{−/−} T cells but not the CD45^{−/−} T cells from CD45 exon 6-deficient mice could proliferate in vitro by CD3 cross-linking, suggesting that CD45^{−/−} T cells are not functionally defective except for their low expression level of CD45 (25).

As previously reported, γδ T cells were not induced at a site of Listeria monocytogenes infection in CD45 exon 6-deficient mice.
and unresponsiveness of the γδ T cells resulted in a weak protection against the bacterial infection (27). Thus, CD45 is also critical for signaling via the γδTCR/CD3 complex. Interestingly, p56<sup>Lck</sup> (Lck<sup>−/−</sup>) mice showed a more severely impaired thymocyte development but not significantly blocked development of γδ-IEL, suggesting that Lck is not indispensable for γδ-IEL development (31, 32). On the other hand, it was shown that a severely impaired development of γδT cells, which differentiate in the intrathymic pathway, was observed in both CD45<sup>−/−</sup> and Lck<sup>−/−</sup> mice, suggesting that CD45 and Lck are important for the intrathymic pathway of γδ T cell development (31, 33). However, the importance of CD45 and Lck to cytolytic activity and development of i-IEL has not previously been reported in detail.

In this study we investigated the roles of CD45 PTP and Lck PTK in development and cytolytic function of i-IEL. The results of this study showed that CD45 is indispensable for the intrathymic pathway of i-IEL development and induction of cytolytic activities but not for the extrathymic development of i-IEL, while Lck per se is crucial but not essential for i-IEL development and cytolytic function. We also discuss differential contributions of CD45 and Lck to i-IEL development and functions.

Materials and Methods

Mice

CD45 knockout mice lacking CD45 exon 6 (CD45<sup>−/−</sup>) and Lck knockout mice (Lck<sup>−/−</sup>) generated by gene-targeting technology (18, 23) were supplied by Dr. T. W. Mak (Amgen Institute, Toronto, Canada). They were back-crossed more than seven times to C57BL/6 (H-2<sup>b</sup>) mice and maintained under specific pathogen-free conditions at the Kyushu University Animal Care Center. Age-matched littersmates or C57BL/6 mice were used as control CD45<sup>+/+</sup> mice and also maintained under specific pathogen-free conditions. Nude mice (nu/nu) of BALB/c (H-2<sup>d</sup>) background were purchased from Charles River Japan (Yokohama, Japan). (C57BL/6 x C3H/He)F<sub>1</sub> mice, generated by Dr. T. W. Mak (Amgen Institute, Toronto, Canada). They were back-crossed more than seven times to C57BL/6 (H-2<sup>b</sup>) mice and maintained under specific pathogen-free conditions at the Kyushu University Animal Care Center. Age-matched littersmates or C57BL/6 mice were used as control CD45<sup>+/+</sup> mice and also maintained under specific pathogen-free conditions.

Isolation and purification of i-IEL

The i-IEL were obtained as originally described by Cerf-Bensan et al. (20) with minor modification (34). In brief, a small intestine free of the lumen contents and of the Peyer’s patches was turned inside out with the aid of a polyethylene tube and strings. An inverted intestine was cut into two or three segments, and they were transferred to a 50-ml conical tube with 35 ml RPMI 1640 medium (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated FBS, 25 mM NaHCO<sub>3</sub>, 60 mM 2-ME, 100 U/ml penicillin G (potassium salt), 10% heat-inactivated FBS (Intergene, Purchase, NY), 20 mM HEPES, 23 ml RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS at densities of 0.5 x 10<sup>6</sup> to 5 x 10<sup>6</sup> cells/ml in 24-well tissue culture plates and cultured for 37°C in humidified CO<sub>2</sub> incubator at 4°C. At intervals, aliquots of cells were removed, and cell viability was determined by trypan blue dye exclusion, and apoptosis induction was assayed by cell cycle analysis. Flow cytometric detection of apoptosis was performed as described by Fried et al. (36) and Nicolletti et al. (37). Briefly, i-IEL were suspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodine (Sigma, St. Louis, MO) in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)). The cell suspension was placed over-night at 4°C in the dark before flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured by EPICS XL flow cytometer (Coulter, Hialeah, FL).

Production of ATxBMT and FTG mice

ATxBMT (adult thymectomized, lethally irradiated, and bone marrow-transferred) mice were produced according to the method of Tesu Lin et al. (38). In brief, 8-week-old female C57BL/6 mice were anesthetized by an i.p. injection of pentobarbital sodium solution (1.25 mg/mouse), and thymectomy was performed. Two weeks after thymectomy, the mice underwent lethal irradiation of 11 Gy followed by i.v. injection of 1 x 10<sup>6</sup> T cell-depleted bone marrow cells obtained from CD45<sup>−/−</sup>, CD45<sup>+/+</sup>, or Lck<sup>−/−</sup> female mice. T cell depletion from bone marrow cells was achieved by complement lysis using one cycle each of anti-Thy 1.2 mAb (ascites, Meiji Institute of Health Science) followed by Low-Tox rabbit C (diluted 1/10, Cedara Laboratories, Hornby, Canada). It was confirmed that this method depleted virtually all T cells from the bone marrow cells by detecting CD3<sup>+</sup> cells in the treated bone marrow cells (data not shown).

Fetal thymus-grafted (FTG) mice were produced by implanting two thymic lobes of fetuses on gestation days 15 to 18 from CD45<sup>−/−</sup> or CD45<sup>+/+</sup> mice under the kidney capsule of 8-week-old BALB/c nu/nu female mice as previously described (34).

Redirectional assay of cytolitic activities of i-IEL

Cytolytic activities of i-IEL were measured by 51Cr release assay using hybridomas producing anti-CD3 mAb (2C11-145, provided by Dr. J. A. Bluestone, University of Chicago, Chicago, IL), anti-αβTCR (H57-597, by Dr. R. T. Kubo, Cytel Co., San Diego, CA), anti-γδTCR mAb (UC7-13D5, by Dr. J. A. Bluestone), and control hamster IgG (UCH-4B3, by Dr. J. A. Bluestone) as target cells according to Gug-Grand et al. (5). Cells were labeled with 51Cr by the conventional method. The i-IEL were incubated with 1 x 10<sup>6</sup> of the 51Cr-labeled target cells at 37°C in 5% CO<sub>2</sub> for 4.5 h at various E/T cell ratios ranging from 1:1.5 to 1:50 in a total volume of 200 μl/well. The cytolytic assay was performed in a 96-well round-bottom plate. After incubation, 100 μl of supernatant was removed from each well, and the radioactivity was determined using a gamma counter (Shimazu, Kyoto, Japan). The cytotoxicity

Abs and flow cytometric analysis

mAbs used for staining were FITC-conjugated anti-CD3ε (2C11-145, Pharmingen); FITC- and phycoerythrin (PE)-conjugated anti-CD4 (Life Technologies, Gaithersburg, MD); FITC-, PE-, allophycocyanin- and biotin-conjugated anti-CD8α (PharMingen); biotin-conjugated anti-CD8β (Meiji Institute of Health Science, Tokyo, Japan); FITC- and PE-conjugated anti-αβTCR (H57-597; Pharmingen); FITC- and biotin-conjugated anti-γδTCR (GL-3; Pharmingen); FITC-, PE-, and biotin-conjugated anti-pan-CD45 (30F11.1; Pharmingen); FITC-conjugated anti-H-2K<sup>d</sup> (Meiji Institute of Health Science, Tokyo, Japan) and streptavidin-RED670 (Life Technologies). Cells were incubated with nonconjugated anti-FcγRII/III mAb (anti-CD32/CD16, 2.4G2) for 15 min at 4°C to block nonspecific Ab binding via FcγR, followed by staining for flow cytometry. The purified anti-FcγRII/III mAb was prepared from the culture supernatant of 2.4G2 hybridoma cells grown in a serum-free medium (101, Nissui Pharmaceutical, Tokyo, Japan). Freshly isolated i-IEL were stained with an appropriate combination of dye-conjugated mAbs or streptavidin as described above for 20 min at 4°C following by loading them on a flow cytometer, FACSCalibur (Becton Dickinson, Sunnyvale, CA). The data obtained by three- or four-color flow cytometry were analyzed using CellQuest analysis software (Becton Dickinson).

Induction of in vitro spontaneous apoptosis of i-IEL

Spontaneous in vitro apoptosis of i-IEL was induced according to the method of Viney et al. (35). Briefly, isolated i-IEL were cultured in complete RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS at densities of 0.5 x 10<sup>6</sup> to 5 x 10<sup>6</sup> cells/ml in 24-well tissue culture plates and cultured for 37°C in humidified CO<sub>2</sub> incubator at 4°C. At intervals, aliquots of cells were removed, and cell viability was determined by trypan blue dye exclusion, and apoptosis induction was assayed by cell cycle analysis. Flow cytometric detection of apoptosis was performed as described by Fried et al. (36) and Nicolletti et al. (37). Briefly, i-IEL were suspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodine (Sigma, St. Louis, MO) in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)). The cell suspension was placed overnight at 4°C in the dark before flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured by EPICS XL flow cytometer (Coulter, Hialeah, FL).
was defined as the percentage of specific $^{51}$Cr release calculated according to the following formula: % cytotoxicity = \[ \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximal release (cpm)} - \text{spontaneous release (cpm)}} \times 100. \]

### Results

#### Development of i-IEL in CD45$^{-/-}$ and Lck$^{-/-}$ mice

Small intestines of CD45$^{-/-}$ and Lck$^{-/-}$ mice were macroscopically normal. Three- or four-color flow cytometric analysis was performed using freshly isolated i-IEL from 10- and 20-wk-old CD45$^{+/+}$, CD45$^{-/-}$, and Lck$^{-/-}$ mice. The data obtained from cytometric analyses were summarized in Figure 1, and representative profiles were shown in Figure 2. As shown in Figure 1, the i-IEL numbers of the both mutant mice at 10 wk of age were approximately 1.5 to 2 times more than those of the control wild-type (CD45$^{+/+}$) mice. Unexpectedly, the i-IEL numbers of CD45$^{-/-}$ and Lck$^{-/-}$ mice significantly decreased during aging (10–20 wk of age), although the i-IEL number of the CD45$^{+/+}$ mice increased during aging (Fig. 1). No significant difference in the cell number or the composition of i-IEL subsets was observed between CD45$^{+/+}$ and heterozygously mutated (CD45$^{1/2}$) littermates (data not shown).

The i-IEL from CD45$^{-/-}$ mice were slightly larger in cell size than those from CD45$^{+/+}$ mice, although the blast-like CD45$^{-}$ i-IEL showed no up-regulated expression of activation markers.

![Flow cytometric analysis of i-IEL from CD45$^{+/+}$ and CD45$^{-/-}$ mice](image_url)
including CD25, CD40L, CD69, and Ia (data not shown). However, the morphology of i-IEL from Lck\(^{-/-}\) mice was not different from that from CD45\(^{+/+}\) mice (data not shown). Unexpectedly, CD4\(^+\) CD8\(^{+}\) αβ-IEL at 10 wk of age were markedly higher in percentage and number in CD45\(^{-/-}\) mice (25%) than in CD45\(^{+/+}\) mice (5%) as shown in Figures 1 and 2A. Therefore, the absolute number of CD4\(^+\) αβ-IEL (7.5 \(\times\) 10\(^3\)) in CD45\(^{-/-}\) mice was nearly 7 times more than that in CD45\(^{+/+}\) mice (1.1 \(\times\) 10\(^3\)) at 10 wk of age. Moreover, the percentage of CD4\(^+\) CD8\(^{+}\) αβ-IEL at 10 wk of age was higher in CD45\(^{-/-}\) mice (10%) than in the CD45\(^{+/+}\) mice (5%) (Fig. 1). Interestingly, the percentages of γδ-IEL from CD45\(^{-/-}\) mice (25 and 15% in 10- and 20-wk-old mice, respectively) were remarkably lower than those of the CD45\(^{+/+}\) littermates (55 and 52%, respectively) as shown in Figures 1 and 2A. The significantly decreased number of the γδ-IEL in CD45\(^{-/-}\) mice at 20 wk of age (0.33 \(\times\) 10\(^6\)) was observed compared with that in CD45\(^{+/+}\) mice (2.3 \(\times\) 10\(^6\)). Moreover, the distribution of i-IEL subsets in CD45\(^{+/+}\) mice showed no significant change during aging, but the γδ-IEL subset decreased evidently in the CD45\(^{-/-}\) mice during aging (0.85 \(\times\) 10\(^6\) at 10 wk of age, 0.33 \(\times\) 10\(^6\) at 20 wk of age). Furthermore, the expression level of CD8α was higher, but the γδTCR expression was lower in the CD8α\(^+\) γδ-IEL subset of CD45\(^{-/-}\) mice compared with their expression levels in those of CD45\(^{+/+}\) mice (Fig. 2B). On the other hand, the i-IEL number of Lck\(^{-/-}\) mice was similar to that of CD45\(^{-/-}\) mice at 10 wk of age, and the number of γδ-IEL decreased markedly also in Lck\(^{-/-}\) mice during aging as observed in CD45\(^{-/-}\) mice (Fig. 1).

A part (10–20%) of whole i-IEL from CD45\(^{-/-}\) mice had CD45 surface expression (Fig. 2C). Their intensity of the CD45 surface expression of whole i-IEL from CD45\(^{-/-}\) mice was lower than that of i-IEL from CD45\(^{+/+}\) mice as shown in Figure 2B. A low intensity of CD45 surface Ag expression was also observed previously in a part of peripheral T cells of splenocytes and lymph node cells from CD45\(^{-/-}\) mice compared with that in cells from CD45\(^{+/+}\) mice (23, 25). The percentage of CD45\(^+\) i-IEL in CD45\(^{-/-}\) mice was significantly lower in the CD8α\(^+\) γδ-IEL subset (1–3%) than in other i-IEL subsets (25–50%; Fig. 2C). Some reports strongly suggested that the CD8α\(^+\) γδ-IEL subset developed predominantly in the extrathymic pathway (4, 6, 39). Therefore, it can be considered that the CD8α\(^+\) γδ-IEL subset developed extrathymically through an irrelevant pathway of CD45. To further analyze the CD45\(^+\) i-IEL population, i-IEL from CD45\(^{-/-}\) mice (10 wk of age; \(n = 5\)) were examined by four-color flow cytometry. The CD45\(^{-/-}\) mice used for this analysis were distinct from the mice used previously (in Figure 1). In the results of the analysis, the distribution of i-IEL subsets in CD45\(^{-/-}\) mice was comparable to that in the wild-type CD45\(^{+/+}\) mice, except for lower percentages of CD4\(^+\) CD8\(^+\) γδTCR\(^+\) and CD4\(^+\) αβCD8\(^+\) αβTCR\(^+\) i-IEL subsets. The composition of each i-IEL subset in CD3\(^+\) CD45\(^{-/-}\) i-IEL from CD45\(^{-/-}\) mice was as follows: CD4\(^+\) αβCD8\(^+\) γδ-IEL, 51.7 \(\pm\) 3.7%; CD4\(^+\) CD8\(^+\) γδ-IEL, 1.4 \(\pm\) 0.8%; CD4\(^+\) αβCD8\(^+\) αβ-IEL, 2.2 \(\pm\) 1.6%; CD4\(^-\) αβCD8\(^+\) αβ-IEL, 36.2 \(\pm\) 4.0%; CD4\(^+\) αβCD8\(^+\) αβ-IEL, 1.6 \(\pm\) 0.3%; CD4\(^-\) CD8\(^+\) αβ-IEL, 6.9 \(\pm\) 1.2%. On the other hand, the numbers of CD45\(^+\) i-IEL in CD45\(^{-/-}\) mice showed differences between individuals, and the CD45\(^+\) i-IEL contained a high percentage of CD4\(^+\) subset (data not shown). Therefore, the compositions of the i-IEL subsets in CD3\(^+\) and CD45\(^+\) i-IEL populations were remarkably different from each other in CD45\(^{-/-}\) mice. Similar results were obtained from the old CD45\(^{-/-}\) mice (20 wk of age), but the incidence of CD45\(^+\) i-IEL (20–35%) was higher than that in the younger mice (10–20%; 10 wk of age; data not shown).

### Extrathymic and intrathymic development of i-IEL

To confirm whether CD45 and Lck are critical for the extrathymic pathway of i-IEL development, ATxBMT mice were produced by reconstitution of B6C3F\(_1\) mice (H-2\(^b\)) with T-depleted bone marrow cells from wild-type (CD45\(^{+/+}\)), CD45\(^{-/-}\), or Lck\(^{-/-}\) mice (H-2\(^b\)), which were designated CD45\(^{+/+}\)ATxBMT, CD45\(^{-/-}\)ATxBMT, or Lck\(^{-/-}\)ATxBMT mice, respectively, as shown in Table I. Donor-derived (H-2\(^b\)-negative) i-IEL were first detected 4 wk after the bone marrow cell transfer, and cell number increased later (data not shown). The i-IEL in the ATxBMT mice were isolated and analyzed 6 wk after the bone marrow transfer. The donor-derived i-IEL numbers in the CD45\(^{-/-}\) ATxBMT and Lck\(^{-/-}\) ATxBMT mice (0.78 \(\times\) 10\(^6\)/mouse and 0.87 \(\times\) 10\(^6\)/mouse, respectively) were approximately half that in the CD45\(^{-/-}\) ATxBMT mice (1.56 \(\times\) 10\(^6\)/mouse) as shown in Table II. The composition of donor-derived i-IEL subsets in the CD45\(^{-/-}\) ATxBMT mice was similar to that in the CD45\(^{+/+}\) ATxBMT mice, while immature i-IEL without TCR expression were considerably accumulated in the Lck\(^{-/-}\) ATxBMT mice, since the total percentage of αβ-IEL and γδ-IEL in the Lck\(^{-/-}\) ATxBMT mice (24.3% \(\pm\) 5.0) was about half that in the CD45\(^{+/+}\) ATxBMT or CD45\(^{-/-}\) ATxBMT mice (50.6 \(\pm\) 17.8% or 51.6 \(\pm\) 10.1%, respectively) as calculated from the data shown in Table II, implying that Lck is partly involved in the extrathymic differentiation of i-IEL. In the CD45\(^{-/-}\) ATxBMT mice, the percentage of the donor-derived CD4\(^+\) i-IEL population (4.4 \(\pm\) 2.2%; Table II) was obviously lower than that of CD4\(^+\) IEL in the nontreated CD45\(^{-/-}\) mice (24.0 \(\pm\) 4.2% at 10 wk of age; Fig. 1). Furthermore, each i-IEL subset generated from the CD45\(^{-/-}\) bone marrow cells contained a significantly lower percentage of CD45\(^+\) cells compared
with the corresponding i-IEL subset in the nontreated CD45<sup>−/−</sup> mice (Fig. 3).

To examine whether CD45 is important for the intrathymic pathway of i-IEL development, FTG mice were produced by transplantation of fetal thymic lobes from CD45<sup>+/+</sup> or CD45<sup>−/−</sup> mice under the kidney capsule of BALB/c <i>nu/nu</i> mice, which were designated CD45<sup>+/+</sup>/FTG or CD45<sup>−/−</sup>/FTG mice, respectively, as shown in Table I. Donor-derived (H-2<sup>b</sup>-positive) i-IEL were first detected 4 wk after grafting the fetal thymus, and cell number increased later (data not shown). The i-IEL were isolated and analyzed 6 to 8 wk after the thymus graft. As shown in Table III, the donor-derived i-IEL number in the CD45<sup>−/−</sup>/FTG mice was much lower than that in the CD45<sup>+/+</sup>/FTG mice (2.73 × 10<sup>6</sup>/mouse) but there was no remarkable difference in the distribution of i-IEL subsets. Importantly, the donor-derived i-IEL in CD45<sup>−/−</sup>/FTG mice showed that >90% of them were CD45 positive as shown in Figure 3, suggesting that CD45 is indispensable for the intrathymic development of i-IEL. Conclusively, the results in the ATxBMT and FTG mice demonstrate that Lck and CD45 are not essential for the extrathymic development of i-IEL, but are crucial for their intrathymic development.

### An enhanced spontaneous apoptosis in i-IEL from CD45<sup>−/−</sup> mice

It has been reported that apoptosis was induced spontaneously in the in vitro culture of i-IEL without any stimuli (35). As shown in Figure 4, i-IEL from the both CD45<sup>+/+</sup> control and CD45<sup>−/−</sup> mice showed decreases in viable cell number during the cell culture at 37°C in the complete medium but not at 4°C. However, the cell death rates were significantly higher in i-IEL from CD45<sup>−/−</sup> mice compared with those from CD45<sup>+/+</sup> mice (Fig. 4 A and C). Cell death was characterized as apoptosis by detecting apoptotic cells containing small fragmented nuclei in the cell cycle analysis as shown in Figure 4A and by DNA fragmentation (data not shown). Cell death was induced in both αβ- and γδ-IEL subsets as demonstrated by flow cytometric analysis (data not shown).

The susceptibilities of the i-IEL against spontaneous apoptosis were estimated as susceptibility indexes calculated by ratios of γδTCR<sup>+</sup> cells/αβTCR<sup>+</sup> cells in the viable i-IEL populations after 6-h culture vs those at 0 h of culture (n = 10 for each experimental group). The susceptibility indexes were 1.29 ± 0.81 and 0.81 ± 0.10 in the i-IEL from CD45<sup>+/+</sup> and CD45<sup>−/−</sup> mice, respectively (data not shown). Thus, the γδ-IEL from CD45<sup>−/−</sup> mice were more susceptible to the induction of the spontaneous cell death than the αβ-IEL. The susceptibilities may be responsible for the markedly decreased number of γδ-IEL in CD45<sup>−/−</sup> mice during aging, although other possibilities cannot be excluded. These data indicate that one of the main causes of the decreased number of i-IEL in CD45<sup>−/−</sup> mice during aging is the defect of a signal that regulates CD45 such as TCR/CD3-mediated signaling. Together these results imply that an adequate CD45-mediated signal

### Table II. Analysis of i-IEL generated extrathymically in the ATxBMT mice at 6 wk after bone marrow transfer<sup>a</sup>

<table>
<thead>
<tr>
<th>Mice</th>
<th>CD45&lt;sup&gt;+/+&lt;/sup&gt; ATxBMT</th>
<th>CD45&lt;sup&gt;−/−&lt;/sup&gt; ATxBMT</th>
<th>Lck&lt;sup&gt;−/−&lt;/sup&gt; ATxBMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number (&lt;i&gt;×10&lt;sup&gt;6&lt;/sup&gt;&lt;/i&gt;)</td>
<td>1.56 ± 0.66</td>
<td>0.78 ± 0.28</td>
<td>0.87 ± 0.18</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>2.2 ± 0.6</td>
<td>4.4 ± 2.2</td>
<td>6.0 ± 5.5</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>60.0 ± 20.1</td>
<td>58.4 ± 11.1</td>
<td>28.3 ± 5.5</td>
</tr>
<tr>
<td>α&lt;sup&gt;β&lt;/sup&gt; IEL (%)</td>
<td>4.4 ± 1.2</td>
<td>11.0 ± 3.4</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>γδ&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>46.2 ± 18.3</td>
<td>40.6 ± 11.9</td>
<td>17.3 ± 6.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> ATxBMT mice were prepared by reconstitution of adult thymectomized B6C3F<sub>1</sub> (H-2<sup>b</sup>/k) with T-depleted bone marrow cells from CD45<sup>+/+</sup>, CD45<sup>−/−</sup>, and Lck<sup>−/−</sup> mice. The donor-derived i-IEL were isolated from ATxBMT mice and analyzed by flow cytometry in a gate on H-2<sup>b</sup>-negative cells.

The data were obtained from eight individual mice of each experimental group.

### Table III. Analysis of i-IEL generated intrathymically in the FTG mice at 6 to 8 wk after the fetal thymus graft<sup>a</sup>

<table>
<thead>
<tr>
<th>Mice</th>
<th>CD45&lt;sup&gt;+/+&lt;/sup&gt; FTG</th>
<th>CD45&lt;sup&gt;−/−&lt;/sup&gt; FTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number (&lt;i&gt;×10&lt;sup&gt;6&lt;/sup&gt;&lt;/i&gt;)</td>
<td>2.73 ± 0.30</td>
<td>0.05 ± 0.18</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>39.7 ± 11.7</td>
<td>22.3 ± 8.0</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>67.1 ± 12.3</td>
<td>84.4 ± 17.3</td>
</tr>
<tr>
<td>α&lt;sup&gt;β&lt;/sup&gt; IEL (%)</td>
<td>93.8 ± 5.1</td>
<td>78.0 ± 13.7</td>
</tr>
<tr>
<td>γδ&lt;sup&gt;+&lt;/sup&gt; IEL (%)</td>
<td>5.5 ± 1.6</td>
<td>30.9 ± 11.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> FTG mice were prepared by grafting thymic lobes from CD45<sup>+/+</sup> and CD45<sup>−/−</sup> fetal mice under the kidney capsule of BALB/c <i>nu/nu</i> (H-2<sup>b</sup>). The donor-derived i-IEL were isolated from FTG mice and analyzed by flow cytometry in a gate on H-2<sup>b</sup>-positive cells.

The data were obtained from eight individual mice of each experimental group.
Repeated five more times, and similar results were obtained in each experiment.

Thus, the other PTK(s), e.g., Fyn, could compensate a part of the cytolytic activities in i-IEL but is not essential for the function.

Expectedly, the slightly remaining cytolytic activities of i-IEL from CD45 knockout mice disappeared completely. CD45 PTP, a major surface Ag of lymphocytes, influences numerous biologic functions, and its most prominent role is to activate src family PTKs including Lck and Fyn in signaling pathways of lymphocyte activation (19–22). In this study we demonstrated the importance of CD45 PTP for i-IEL development and cytolytic activities and differential requirements of CD45 and Lck for such immunologic events. The i-IEL development occurs through two main differentiation pathways in thymus-dependent (intrathymic) and thymus-independent (extrathymic) manners. It has been considered that CD45 is absolutely required for inducing cytolytic activities in i-IEL. On the other hand, the cytolytic activities of i-IEL from Lck knockout mice was slightly reduced, but a considerable level of cytotoxicity was detected as shown in Figure 6, suggesting that Lck is involved in inducing cytolytic activities in i-IEL but is not essential for the function. Thus, the other PTK(s), e.g., Fyn, could compensate a part of the Lck function to induce cytolytic activities in i-IEL.

Discussion

The i-IEL population from CD45 exon 6-deficient (CD45^−/−) mice contained CD45^+ cells as observed in the thymus and the peripheral lymphoid organs of CD45^−/− mice. It is still not definite how CD45^+ cells can generate in CD45^−/− mice. In the knockout mice, a genomic sequence including exon 6 was replaced by the neomycin-resistance gene cassette, which was inserted at the antisense direction (23). The inserted gene cassette may have influenced not only a precise splicing but also the transcription and/or mRNA stability in mature T cells from CD45^−/− mice, since more CD45^+ T cells should have generated in CD45^−/− mice if the precise splicing occurred, and CD45 isoforms except the CD45RC epitope were normally synthesized in T cells in CD45^−/− mice. In this study, the CD45^+ T cells (i-IEL) are a good indicator to examine a requirement of CD45 for development and function of i-IEL.

CD45 PTP, a major surface Ag of lymphocytes, influences numerous biologic functions, and its most prominent role is to activate src family PTKs including Lck and Fyn in signaling pathways of lymphocyte activation (19–22). In this study we demonstrated the importance of CD45 PTP for i-IEL development and cytolytic activities and differential requirements of CD45 and Lck for such immunologic events. The i-IEL development occurs through two main differentiation pathways in thymus-dependent (intrathymic) and thymus-independent (extrathymic) manners. It has been considered that CD45^+ αβ-IEL developed intrathymically and CD8αγδ-IEL developed in the extrathymic pathway (38–40). However, several reports showed that the idea is controversial (9, 11, 12, 41). The i-IEL development from CD45^−/− BMT mice was considerably blocked, and almost all of the developed i-IEL were CD45 positive. The adult and fetal thymocyte development was also blocked in CD45^−/− mice (23, 42, 43). On the other hand, the extrathymic pathway of i-IEL development was not significantly influenced in the absence of CD45 surface expression in the CD45^−/− ATxBMT mice because the i-IEL was reconstituted...
from the bone marrow cells of CD45−/− mice without a remarkable defect, and they were predominantly CD45 negative. Thus, the CD45− i-IEL subset in CD45−/− mice was comparable to that in CD45+/− mice. These results suggest that the CD45 PTP-mediated signaling is indispensable for the intrathymic pathway of i-IEL development, but is not essential for their extrathymic development. Moreover, they suggest that CD45− i-IEL do not influence the development of CD45+ i-IEL, since no increased percentage of CD45+ i-IEL was observed in the CD45−/− ATxBMT mice, which included many host-derived CD45+ cells as the background.

In CD45−/− mice, a markedly increased number of CD4+ αβ-IEL was observed, but it could not be observed in the CD45−/− ATxBMT mice. It is reasonable that the CD4+ αβ-IEL were mainly generated intrathymically, since they included a high percentage of CD45− cells, and the CD4− i-IEL in CD45−/− mice showed an only slightly higher percentage of CD4+ cells compared with i-IEL from CD45+/− mice. As reported previously, the intrathymic development of CD8+ T cells was more severely blocked than that of CD4+ T cells, and the ratio of CD4+ T cells/CD8+ T cells in the peripheral lymphoid tissues including lymph nodes and spleen of CD45−/− mice was extremely high (23). Therefore, CD4+ i-IEL with CD45 expression may have accumulated in the gut epithelia of CD45−/− mice more than CD8+ i-IEL, although few i-IEL were generated in the FTG mice as shown in Table III. However, it cannot be excluded that the absence of thymus in the CD45−/− ATxBMT mice might have influenced the development of CD4+ i-IEL, as it was reported that the presence of thymus per se (a certain thymus-derived factor) influences the extrathymic development of i-IEL (10–12). In this study, the Lck−/− ATxBMT mice showed a considerable accumulation of immature αβTCR−γδTCR+ i-IEL as shown in Table II, and Lck−/− mice also showed a markedly decreased i-IEL number during aging (Fig. 1). Therefore, Lck seems to be more critically involved in both intra- and extrathymic differentiation of i-IEL than CD45.

The cause of the age-dependently decreased number of i-IEL in CD45−/− mice is not clear. It was, however, found that i-IEL from CD45+/− mice were more susceptible to in vitro spontaneous apoptosis than those from CD45+/− mice at least in vitro. Thus, i-IEL from CD45+/− mice could undergo cell death more rapidly than those from CD45−/− mice. In the previous report the in vitro apoptosis was much more rapidly induced in γδ-i-IEL than in αβ-i-IEL (35). In this study such an extremely biased induction of apoptosis in the i-IEL subsets from CD45+/− mice was not observed.
but the cell death was more readily induced in γδ-IEL than in αβ-IEL in the absence of CD45, while the result was reverse in the case of i-IEL from CD45−/− mice. As recently reported, i-IEL express high Bcl-2 and Bcl-x levels, but it has not been confirmed that the gene products are involved in protecting i-IEL from apoptosis (44). Actually, the both gene and surface expression were equally detected in i-IEL from CD45−/− and CD45+/− mice by RT-PCR (data not shown). In recent studies it was suggested that CD45 is involved in apoptosis of thymocytes under positive/negative selection events (26, 45). The present results imply that CD45 PTP regulates the programed cell death and the turnover of i-IEL. Furthermore, an enhancement of apoptosis induction by CD3 stimulation was detected in i-IEL from CD45−/− mice more than in those from CD45+/− mice (data not shown). It is consistent with the previous results that TCR/CD3-mediated signaling is defective in CD45− T cells, T lymphoma clones, and thymoma clones (18–20).

The difference of i-IEL development between CD45−/− mice and Lck−/− mice suggests a differential contribution of such signaling molecules to i-IEL development. Recently, Lck/Fyn double knockout mice were produced and analyzed (44–48). A severely impaired development of i-IEL was observed in the double knockout mice, but not in the each single knockout mouse, suggesting that Lck and Fyn can compensate each function for i-IEL development. In the present study CD45−/− mice did not show such a severe block in extrathymic i-IEL development, although CD45 regulates the activation of Lck and Fyn, implying that the CD45-independent signaling pathway is involved in the extrathymic development of i-IEL or that the CD45-regulated TCR/CD3-mediated signaling is not necessary for extrathymic i-IEL development.

The cytotolytic activity of i-IEL was severely suppressed in CD45−/− mice, and CD45-depleted i-IEL showed virtually no cytoltyc activity, suggesting that CD45 is indispensable for the induction of cytotoxicities in i-IEL as well as TCR-mediated activation in T cells as reported previously (23, 25). On the other hand, cytotolytic activity was only partially blocked in i-IEL from Lck−/− mice, indicating that Lck is involved in the induction of cytotoxicities but is not essential for the cytotolytic function. Therefore, the other PTK(s), e.g., Fyn or Lyn, may compensate the Lck function to induce cytoltytic activities in i-IEL, although Lck expression is the highest and specific in T cells.

Conclusively, in this study, differential contributions of CD45 and Lck to i-IEL development and cytoltytic activities were observed. It can be emphasized that CD45 is exclusively important for the induction of cytoltytic activities and the intrinsic development of i-IEL, but not for the extrathymic development of i-IEL.

Acknowledgments

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References