Phenotypical and functional alterations in the mucosal immune system of CD45 exon 9 KO mice

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Abstract

The protein tyrosine phosphatase CD45 has been shown to be an important regulator of antigen-receptor signaling in both T and B lymphocytes. Lymphocyte populations within the gut mucosa are phenotypically and ontogenetically different from those generally found in secondary lymphoid tissue. In T lymphopenic mice, extrathymic T cell development takes place within the gut. Here we report the characterization of T and B cell populations in the distinct compartments of the gut mucosa and gut-associated lymphoid tissue of CD45-null mice. These data suggest that CD45 is required for the development of the specialized T cell populations within the gut environment as has been previously shown for thymic development. We demonstrate that within the large intestine intraepithelial compartment ab-TCR+ CD4+ T cells are selectively retained by CD45KO mice. T cells and NK cells within the intraepithelium and the gut mucosa associated lymphoid tissue of CD45KO mice frequently possess an activated phenotype, differentiated to produce typical TH1 and TH2 cytokines. These data demonstrate that local environmental differences within the gut can, at least in part, overcome the requirement for CD45 during activation of T cells.

Introduction

CD45 is a transmembrane glycoprotein which is a member of the receptor protein tyrosine phosphatase family (RPTPc); it is expressed by all cells of the hemopoietic lineage and is also known as the leukocyte common antigen (1). One of the interesting features of CD45 is its ability to undergo alternative splicing, resulting in the expression of multiple isoforms that have been associated with different levels of T cell activation in the periphery (2). It has been suggested that CD45 plays an important role in TCR signaling since CD45-deficient T cell lines showed defective signaling through their TCR-CD3 complex (2). In an attempt to further understand CD45 function, a CD45 KO mouse was generated by targeting exon 9, which is common to all isoforms (3). Using this mouse model it was possible to demonstrate that CD45 expression is required at two stages of intrathymic T cell differentiation. Firstly, the efficiency with which immature CD4- CD8- precursors differentiate into the CD4+ CD8- intermediate stage was reduced by 2-fold. Secondly, the ability of double positive thymocytes to become single-positive mature thymocytes was diminished 4- to 5-fold (3). Thus, the number of mature T cells in the periphery was greatly reduced. CD45 is not required for the generation of B cells since the number of B cells in the periphery is increased in CD45KO mice (3). Nevertheless, further analysis indicated that peripheral maturation of B cells to a follicular, recirculating phenotype is impaired in CD45 KO mice (3–5). Therefore, both T and B cells require CD45 for full differentiation and maturation.

Mucosal tissues and skin represent a barrier protecting internal organs from pathogens. Epithelial cells are the first layer and T and B lymphocytes localize along the mucosa, beneath epithelial cells or in between them. T cells in the mucosal associated lymphoid tissues, especially in the gut, can have either a thymic or an extrathymic origin (6,7). In normal mice, a high proportion of gut T cells express the γδ-TCR; such cells are mainly intraepithelial lymphocytes (IELs) (7). In athymic nude mice IELs γδ-TCR+ cells are the dominant population and most ab-TCR+ cells are CD8αα+ or CD4- CD8- (double negative); such cells must develop extrathymically (6,8). However, Guy-Grand and colleagues have recently suggested that in euthymic mice these cells may be derived...
from an atypical, ‘double negative’ thymic differentiation pathway (9,10). In euthymic normal mice αβ-TCR+ cells may express CD8αβ or CD4, the latter more frequently found in the intestinal lamina propria (11); such cells are absent from nude mice and are therefore thought to be exclusively thymically derived. Peyer’s patches (PP) are specialized lymphoid areas within the small intestine where IgM+ B cells differentiate into IgA+ B cells in the presence of T cell derived factors and encounter antigen (12). PP T cells are thymus derived cells. B cells expressing IgA can migrate through lymphatics into the mesenteric lymph nodes (MLN) where they further mature under the influence of T cell derived cytokines (13). Finally, mature B cells exit MLN through lymphatics, reach the blood circulation and populate the intestinal lamina propria and other secretory sites (14).

In this paper we have used the CD45 KO mouse model in an attempt to understand the requirement for CD45 in the differentiation of gut T cells. In addition, we show that the gut NKT cell phenotype and functionality have been altered by CD45 deficiency. Furthermore, we have analyzed Th and B cells in PP and MLN.

Methods

Mice

CD45 KO mice on a C57BL/6 background were described previously (15). They were kept in individually ventilated cages in our breeding unit at the Animal Facility of the Department of Pathology, Cambridge University. Normal C57BL/6 mice were also bred in our facility and used as controls. All studies shown in this paper were performed in mice that were 8–9 weeks old. Both females and males were used in all studies.

Isolation of iIELs and LPLs

Small intestines were collected on cold modified HBSS (calcium and magnesium free; Sigma-Aldrich, St Louis, MO) after removing all PP, blood vessels, adherent fat and mesentery. Intestines were opened longitudinally and washed thoroughly with ice-cold HBSS. Then, intestines were cut in 0.5 cm pieces and placed individually in 50 ml tubes with HBSS supplemented with 1 mM DTT (HBSS/DTT) and were shaken at 37°C, 210 r.p.m. for 20 min. The isolation procedure was repeated twice using modified HBSS (for ~30 min each time) or until the supernatant was clear. After each incubation period, supernatants were resuspended in their wells and transferred to a 96-well V-bottom plate to perform the staining. Plates were spun down, vortexed and 100 μl of PBS/2% BSA/0.01% Na azide, supplemented with Brefeldin A, were added to each well. Cells were fixed with 100 μl of 4% paraformaldehyde (pH 7.4) per well and the plate was incubated on ice for 20 min. Plates were spun down and cells were resuspended in PBS/2% BSA/0.5% saponin/0.01% Na azide, and incubated in the cold for 20 min, to facilitate pore formation. Then, fluorochrome-conjugated antibodies that recognize mouse cytokines were added. Plates were incubated in the cold room with shaking for 30 min. Then, they were spun down, washed twice with PBS/2% BSA/0.5% saponin, and once with PBS/2% BSA. Antibodies recognizing specific surface markers were added, and plates were incubated with shaking in the cold room for 30 min. Afterwards, plates were spun down and the cells were washed with PBS/2% BSA. Finally, cells were resuspended in 2% paraformaldehyde, stored in darkness at 4°C, until analyzed in a FACSscan (BD Biosciences). Samples were always run within 24 h after the staining was finished.

Antibodies used in flow cytometric staining

The following directly conjugated anti-mouse antibodies were used: FITC-DX5, FITC-anti-αβ-TCR, FITC-anti-Fas, Chromo-anti-CD3, FITC-anti-CD90.2, FITC-anti-CD62L, Chromo-anti-CD5, FITC-anti-CD44, PE-anti-IFN-γ, from BD Pharmingen (San Diego, CA); PE- and TC-anti-CD4, PE- and TC-anti-αβ-TCR, FITC-, PE- and TC-anti-γδ-TCR, FITC-, PE- and TC-anti-CD8α, PE-anti-CD8β, PE-anti-C-kit receptor (CD117), PE-anti-NK1.1, PE-anti-CD25, FITC-anti-IL-10, PE-anti-IL-2, PE-anti-IL-4, PE-anti-TNF-α, PE-anti-IL-5 from Caltag ( Burlingame, CA); FITC-anti-CD2 from Serotec (Oxford,
Frozen tissue processing and staining

Small intestines were dissected, washed with HBSS to eliminate feces, and frozen in isopentane cooled in acetone/dry ice. Tissues were stored wrapped in aluminum foil at −80°C until cut. Frozen tissues were cut and sections were left drying overnight. Sections were fixed in cold acetic acid for 10 min, air dried and wrapped up back-to-back in aluminum foil before being frozen at −80°C. Sections were defrosted and rehydrated in PBS for 5 min. Tissue sections were covered with 50 µl of PBS/2% BSA (blocking reagent) and incubated for 10 min, at room temperature. Anti-mouse-αβ-TCR or γδ-TCR antibodies (Caltag) were added in the presence of either anti-mouse-CD8a (clone YB2/34 supernatant) or CD4 (clone 145/2C11, kind gift of Dr Jenny Phillips, Cambridge University) antibodies, and sections were incubated for 1 h. Then two washes with PBS/0.01% Tween and two washes with PBS were performed. Then, FITC-conjugated goat-anti-hamster and Texas Red-conjugated donkey-anti-rat (Jackson ImmunoResearch, West Grove, PA) were added and the sections were incubated for another 30 min. Afterwards, washes were performed as described above. Then, biotinylated-mouse anti-human-cytokeratin 8/18 (Novoceastra, Newcastle, UK) was added for 30 min, followed by washes as described above, AMCA-conjugated streptavidin (Jackson ImmunoResearch) for further 30 min and the final washes as before. Then, sections were mounted in 10% PBS/90% glycerol and covered with a coverslip sealed with nail polish. Sections were kept in cold and darkness until they were analyzed using an Axioshot microscope (Zeiss, Jena, Germany). Photographs were taken using slide film (Fuji Provia 1600) exposed at 800 ASA.

Immunomicroscopy of Peyer’s patch and mesenteric lymph node cells

Cytospins were prepared using PP and MLN cells in a Shandon cytocentrifuge (Thermo Shandon, Pittsburgh, PA). They were air-dried, fixed in methanol, air-dried and stored at −80°C until stained. Staining was performed following a procedure similar as described above for frozen sections. Cytospins were incubated for 1 h in the presence of biotin-anti-mouse IgM (Serotec) and FITC-anti-mouse IgA (Southern Biotechnology). Cytospins were washed and sequentially incubated with streptavidin, followed by biotinylated-goat anti-streptavidin (both Vector, Burlingame, CA) and Texas Red streptavidin (Jackson ImmunoResearch), all incubations lasted for 30 min and washes were performed in between incubations. Slides were mounted and analyzed as mentioned above.

Statistical analysis

Data from CD45 KO and normal mice were compared using the two-tailed Student's t-test included in Excel software.

Results

It has been previously shown that exon 9 CD45 KO mice possess a complete CD45 null phenotype in thymus, spleen and lymph nodes (3). We have confirmed, using both flow cytometry (Fig. 1A) and tissue staining (Fig. 1B and C) that CD45 is absent from intestinal intraepithelium and lamina propria lymphocytes. In addition, tissue section staining indicated the paucity of mature IgA+ B cells in the small intestine lamina propria of CD45 KO mice (compare Fig. 1D and E). In the intestinal intraepithelium there are also conspicuously fewer cells which express CD8α and lack αβ-TCR (i.e. stained red not yellow in Fig. 1F and G), such cells are presumably CD8αγδ-TCR+ T cells.

Small intestine intraepithelial lymphocytes

Small intestines were recovered from CD45 KO and normal mice and studied individually. Cell numbers recovered from CD45 KO mice were significantly lower than from normal mice (Table 1). Several T and NK cell markers were used to analyze iIEL subsets; data are presented as the percentage and absolute number of positive cells, in Table 1. The percentage and absolute number of CD3+ T cells was significantly decreased in CD45 KO mice (Table 1). The CD3+ cells in mouse small intestine comprise three major subpopulations. For two of these the percentage of iIELs recovered differed significantly between CD45KO and wild-type mice; the frequency of αβ-TCR+ CD8α–CD8β+ cells is reduced by approximately three-quarters and the γδ-TCR+ cells by about one-half (Table 1 and Fig. 2). However, there is little observable difference in the percentage of αβ-TCR+ CD8α+ CD8β+ cells between CD45KO and wild-type mice (this percentage can be obtained by subtracting the percentage of αβ-TCR+ CD8α+ CD8β+ from the percentage of αβ-TCR+ CD8β+ cells) (Table 1 and Fig 2). On the contrary, NK cells were found at increased frequency (Fig. 3A). The results concerning the thymus-derived αβ-TCR+ CD8α+ CD8β+ cells and NK cells are similar to those previously reported in spleen, albeit that the reduction in αβ-TCR+ CD8α+ CD8β+ cells is more dramatic in spleen (3).

Further analysis combining either two or three different markers showed more subtle differences. Previous studies have shown that CD8α+ was expressed at higher levels in the CD4+CD8+ double positive thymocytes, and that this pattern was associated with the inability of CD8+ cells to terminally differentiate in the absence of CD45 (3,16). Figure 2 shows similar results for CD8α+ iIELs (MFI 663.6 ± 75.6 for CD45KO and 237.8 ± 10.9 for wild-type; P < 0.005) and this effect is observable on both αβ-TCR+ and γδ-TCR+ cells (Fig. 2). Interestingly, only a modest reduction in the percentage of αβ-TCR+ CD4+ cells was observed (Table 1). The proportion of αβ-TCR+ cells expressing CD90+ was not altered by the lack of CD45, but a highly significant decrease in the proportion of γδ-TCR+ cells expressing CD90+ was found (Fig. 3A). Interestingly, the percentage and the absolute number of CD90+
CD3^+ cells increased in CD45KO mice (Table 1). This population could represent immature T cells that did not acquire CD3 due to the lack of CD45 expression.

Two different antibodies were utilized to analyze NK cell marker expression: DX5 and NK1.1. The percentage of NK1.1^+ or DX5^+ cells expressing T cell markers was diminished in CD45 KO mice (Fig. 3B and C). Notably, while the proportion of DX5^+ cells expressing NK1.1 was not altered by the CD45 mutation, the proportion of NK1.1^+ cells expressing DX5 was lower in the mutated mice (Fig. 3B and C). Recent data indicated that DX5 recognizes CD49b and that its expression is associated with higher cytotoxic capacity in NK cells (17). Analysis of the NK1.1^+ CD3^- population indicated an increase in the percentage and absolute number of cells in CD45KO mice (Table 1).

**Large intestine intraepithelial and lamina propria lymphocytes**

In order to study T cell subsets in the large intestine, we pooled cells from 15 mice of the same sex. In each experiment one pool of normal and one pool of CD45 KO large intestine IELs were obtained. A total of four experiments were performed, two with females and two with males. After comparing pools, we were able to rule out sex-induced variations in cell subset distribution, therefore we performed statistical analysis using all pools. No statistically significant difference was observed comparing the numbers of large intestine IELs and lamina propria lymphocytes isolated from CD45 KO and normal mice, although there was a tendency to lower yields among CD45 KO mice (Table 1).

Large intestine IELs showed a similar pattern of changes in the percentage of T cell subsets as reported for small intestines (Table 1) but with some significant changes in detail. A sharp decrease in the percentage of cells expressing CD8^αβ^+ was again observed, but in the case of the large intestine a significant reduction in CD8^α^+ CD8^β^^-TCR^+ was also seen (calculated as described above for small intestines) (Table 1). The percentage of αβ^- and γδ^-TCR^+ CD4^+ T cells was significantly increased in CD45KO mice in the large intestine (Table 1) and this represents a genuine increase in numbers of CD4^+ cells as well as frequency. The same observations can be made about the increase in NK cells (Fig. 4 and Table 1). Moreover, large intestine NK cells also lost the expression of T cell markers (Fig. 4). The proportion of both αβ^- and γδ^-TCR^+ cells expressing CD25 was increased (Fig. 4).

Large intestine lamina propria cells presented a similar pattern characterized by decreased percentage of T cells associated with an increase in NK cells (data not shown).

**Cytokine secretion profile of small and large intestine lymphocytes**

The cytokine secretion profile in CD45 KO mice was studied using combined staining for surface markers and cytoplasmic cytokines after 5 h incubation in the presence of PMA, ionomycin and Brefeldin A as described previously (18). Data are presented as percentage of gated cells secreting a specific cytokine, and have not been transformed into absolute number of cells secreting cytokines. As described above, CD45 KO small intestines yielded ~43% as many cells

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Fig. 1. Exon 9 CD45KO mice show a null phenotype in the gut as well as lack of mature T and B cells. Small intestine IELs from CD45 KO and normal mice were stained with rat anti-mouse CD45 (clone YBM 42.2) followed by a FITC-conjugated mouse anti-rat IgG2a showing a completely null phenotype (A). (B–G) Small intestine cryostat sections from wild-type C57BL/6 mice (B, D and F) and CD45 KO mice (C, E and G); (B and C) are stained with anti CD45 (clone YBM 42.2) followed by TR-conjugated donkey anti rat IgG; (D and E) are stained for IgA^+ cells (FITC, green) and anti-cytokeratin (AMCA, blue); (F and G) are stained with FITC-αβ-TCR (green), TR-CD8α^- (red) and anti-cytokeratin (AMCA, blue); (H and I) show cells from PP cytospun, fixed and stained with anti-IgA (FITC, green) and anti-IgM (TR, red). (B, C, F and G) 40x objective; (D, E, H and I) 100x objective.
Table 1. The proportion of T cell subsets among small and large intestine intraepithelial lymphocytes

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Small intestine</th>
<th>Normal</th>
<th>P</th>
<th>Absolute Number of Cells</th>
<th>CD45 KO</th>
<th>Normal</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number $\times 10^5$</td>
<td>CD45 KO</td>
<td>Normal</td>
<td>P</td>
<td>CD45 KO</td>
<td>Normal</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>CD45 KO</td>
<td>42.1 $\pm$ 13.1</td>
<td>96.2 $\pm$ 2.1</td>
<td>0.025</td>
<td>6.5 $\times 10^{-5}$</td>
<td>5.2 $\times 10^4$</td>
<td>29.3 $\pm$ 6.5</td>
<td>4.5 $\times 10^{-7}$</td>
</tr>
<tr>
<td>NK1.1+ CD3-</td>
<td>14.4 $\pm$ 3.5</td>
<td>0.7 $\pm$ 0.2</td>
<td>0.0005</td>
<td>1.8 $\pm$ 0.5</td>
<td>0.2 $\pm$ 0.05</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>CD90+ CD3-</td>
<td>32.4 $\pm$ 7.0</td>
<td>7.1 $\pm$ 1.2</td>
<td>6.5 $\times 10^{-6}$</td>
<td>4.2 $\pm$ 0.8</td>
<td>2.2 $\pm$ 0.6</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-TCR</td>
<td>37.7 $\pm$ 5.6</td>
<td>46.0 $\pm$ 4.5</td>
<td>0.03</td>
<td>4.9 $\pm$ 1.3</td>
<td>13.9 $\pm$ 2.4</td>
<td>3.1 $\times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-TCR</td>
<td>23.9 $\pm$ 4.7</td>
<td>49.5 $\pm$ 2.0</td>
<td>2.1 $\times 10^{-6}$</td>
<td>3.1 $\pm$ 0.7</td>
<td>15.2 $\pm$ 3.4</td>
<td>5.0 $\times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-TCR CD4</td>
<td>6.5 $\pm$ 2.8</td>
<td>8.4 $\pm$ 1.3</td>
<td>N.S.</td>
<td>0.8 $\pm$ 0.3</td>
<td>2.5 $\pm$ 0.5</td>
<td>0.00022</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-TCR CD8 $\alpha$</td>
<td>4.6 $\pm$ 3.5</td>
<td>21.1 $\pm$ 4.6</td>
<td>0.0001</td>
<td>0.6 $\pm$ 0.4</td>
<td>6.4 $\pm$ 1.7</td>
<td>3.2 $\times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-TCR CD8 $\alpha$</td>
<td>14.2 $\pm$ 1.4</td>
<td>33.1 $\pm$ 1.9</td>
<td>9.4 $\times 10^{-6}$</td>
<td>1.8 $\pm$ 0.2</td>
<td>10.2 $\pm$ 3.2</td>
<td>0.0045</td>
<td></td>
</tr>
</tbody>
</table>

The values given are the mean and SD of the percentage of the indicated subset as a fraction of all lymphocytes (as defined by light scattering) recovered from the stated compartment. For small intestine, the values are those for individual animals ($n > 25$) and for large intestine, the means of four pools (each of 15 individuals). The exact level of significance using the two-tailed Student’s t-test is shown for each marker.

As normal mice did, moreover the percentage of CD3$^+$ cells decreased from $\sim$90% to $\sim$40% in CD45 KO small intestines. Therefore, differences in the percentage of cells secreting a given cytokine do not necessarily reflect differences in the absolute number of cells secreting such cytokine, but differences in activation levels between cells expressing the same phenotype in KO and normal mice.

Furthermore, although we have focused on T cells and NK cells in order to study their cytokine expression, other cells might also contribute to the production of cytokines. Only data showing statistical differences were presented in the figures. The percentage of $\alpha$-TCR$^+$ CD8$^+$ and DX5$^+$ cells producing IL-2 or TNF-$\alpha$ decreased in CD45 KO mice (Fig. 5A). The proportion of $\alpha$-TCR$^+$, CD8$\alpha^+$ and $\gamma$-TCR$^+$ cells producing IL-4 significantly increased in CD45 KO mice (Fig. 5A). The percentage of CD8$\alpha^+$ cells producing IL-5 also increased in CD45 KO mice. Nevertheless, the percentage of $\alpha$-TCR$^+$ cells producing IL-10 decreased in CD45 KO mice (Fig. 5A).

Among large intestine IELs the percentage of $\alpha$-TCR$^+$ CD4$^+$ cells producing IFN-$\gamma$ significantly increased (Fig. 5B). Similar results were obtained when the percentage of $\alpha$-TCR$^+$ CD8$\alpha^+$ cells producing IL-4 was examined. On the contrary, the percentage of DX5$^+$ cells producing IL-2 and IL-4 decreased (Fig. 5B) as observed in the small intestine (Fig. 5A). Many large intestine lamina propria lymphocytes seemed to be activated since the percentages of different T cell subsets producing cytokines were increased to encompass a large proportion of cells (Fig. 5C). There was an increase in the percentage of CD3$^+$ cells producing IFN-$\gamma$ and of $\alpha$-TCR$^+$ cells synthesizing IL-10 (data not shown).

As expected, the proportion of cells producing IL-2 and IL-4 was lower in CD45 KO mice than in normal mice (Fig. 5C). The percentage of $\alpha$-TCR$^+$ CD8$\alpha^+$ cells synthesizing IL-2 and of IL-5 increased at much greater frequency (Fig. 5C).

Peyer’s patches and mesenteric lymph nodes

Cells recovered from CD45 KO PP and MLN were larger than cells from normal mice (Fig. 1H and I). The total number of cells recovered from CD45 KO PP was nearly twice as many as the cells recovered from normal PP (4.36 $\times 10^6$ vs 2.73 $\times 10^6$ (n = 23)) vs 2.73 $\times 10^6$ ± 1.57 (n = 25); P < 0.0025). Meanwhile, the total number of CD45 KO MLN cells recovered was nearly three times the number of cells recovered from normal mice (20.06 $\times 10^6$ ± 9.21 (n = 24) vs 6.86 $\times 10^6$ ± 3.26 (n = 21); P < 10$^{-5}$). Nevertheless, the proportion of T cells decreased so dramatically in both lymphoid compartments, the absolute numbers for T cell subsets are greatly diminished. T cells that populate PP and MLN are thymus derived cells and, in agreement with previous data, their presence was greatly reduced in CD45KO PP [4.36 $\times 10^6$ (n = 24); P = 0.0025]. Meanwhile, the total number of CD45 KO MLN cells recovered was nearly three times the number of cells recovered from normal mice MLN [20.06 $\times 10^6$ ± 9.21 (n = 24) vs 6.86 $\times 10^6$ ± 3.26 (n = 21); P < 10$^{-5}$]. Nevertheless, the proportion of T cells decreased so dramatically in both lymphoid compartments, the absolute numbers for T cell subsets are greatly diminished. T cells that populate PP and MLN are thymus derived cells and, in agreement with previous data, their presence was greatly reduced in CD45KO PP (3). Flow cytometric analysis of PP T cells indicated that the proportion of $\alpha$-TCR$^+$ cells expressing either CD4 or CD8$\alpha^+$ was highly diminished (Fig. 6A). Interestingly, the expression of CD62L and CD44 was increased for both CD4$^+$ and CD8$\alpha^+$ cells.

Within the cells recovered from mesenteric lymph nodes, CD45 KO mice presented a significant decrease in the percentage of CD3$^+$ and $\alpha$-TCR$^+$ cells (Fig. 7A). The percentage of $\alpha$-TCR$^+$ cells expressing CD8$\alpha^+$ significantly decreased. More than 80% of the residual $\alpha$-TCR$^+$ cells recovered from CD45KO PP were CD4$^+$ (Fig. 7A).

It is well known that PP are the inductive site for IgA responses and that immunoglobulin switching from synthesizing IgM to IgA takes place in PP (12). The proportion of CD19$^+$ and of IgM expressing cells is highly increased in the PP and MLN of CD45 KO mice (Figs 6B and 7B). The increase in the percentage and number of B cells in PP and MLN was in
agreement with previous findings in CD45 KO spleens (3). Nevertheless, the proportion of total IgA+ and of CD19+ CD5− cells expressing IgA in the PP of CD45 KO mice decreased (Fig. 6B). A clear paucity of CD19+ cells lacking sIgM, which we presume to be class-switched B cells, is also observable (data not shown). These findings may be ascribed to the diminished number of T cells in PP, since the switching step is believed to be T cell dependent (12). Moreover, the percentage of CD5+ IgA+ and CD5− IgA+ cells expressing CD23 was higher in CD45 KO mice PP (Fig. 7B). Indeed, CD23-negative IgA+ cells are largely absent from CD45KO PP. A similar increase in the percentage of IgA+ CD5− cells expressing CD23 was observed in the MLN of CD45 KO mice (Fig. 7B). The percentage of IgM+ CD5− cells expressing CD23 was also increased in the MLN of CD45 KO mice (Fig. 7B).

Cytokine secretion profile of Peyer’s patches and mesenteric lymph node T cells

Data from PP pointed to an increase in the total percentage of IL-5+ in the PP of CD45 KO mice. The proportion of αβ-TCR+ CD8α+ and DX5+CD3− cells producing IL-5 increased in the PP of CD45 KO mice (Fig. 8A). Moreover, the percentage of CD4+ cells producing IL-4 also increased (Fig. 8A), which may be particularly significant since CD4+ cells are the major source of IL-4 within PP (data not shown). The proportion of αβ-TCR+ cells producing IL-2, αβ-TCR+ CD4+ cells producing IFN-γ and the percentage of αβ-TCR+ CD4+ cells producing TNF-α increased in the PP of CD45 KO mice (Fig. 8A). These data suggested that many T cells in the PP of CD45 KO mice were activated to produce cytokines.

The analysis of cytokine producing cells in the MLN of CD45 KO mice suggested both a TH1 type and TH2 type activation. The proportion of αβ-TCR+ CD4+ cells secreting IL-4 decreased while the percentage of CD8α+ cells producing IL-4 increased as well as the percentage of αβ-TCR+, CD4+ and CD8α+ cells producing IL-5 (Fig. 8B). Furthermore, the proportion of αβ-TCR+ producing IL-2, of αβ-TCR+ CD4+, αβ-TCR+ CD8α+ producing IFN-γ, and of αβ-TCR+ and CD3+ cells producing TNF-α, increased in the MLN of CD45 KO mice (Fig. 8B). Interestingly, the proportion of NK1.1+ and NK1.1+ CD3+ cells producing IL-10 decreased in the MLN of CD45 KO mice (data not shown ). Moreover, the percentage of DX5+ cells
producing IL-2 and IL-5 decreased in these MLN, suggesting once more that changes in NK cell phenotype were associated with a different capacity to secrete cytokines (data not shown).

Discussion

Previous studies using CD45-deficient mouse models have shown that CD45 expression is a requirement for successful differentiation of T cell precursors in the thymus (3,19,20). This study investigated the requirement for CD45 during the development, maintenance and differentiation of T lymphocytes in gut and gut-associated lymphoid tissues. It has been thought that T cells within the IEL were derived in part from thymic emigrants and in part from an extrathymic source. However, very recent data have cast doubt on whether extrathymic differentiation makes any significant contribution to the IEL populations in euthymic mice (10). What is clear, however, is that in athymic nude mice the $\gamma\delta$-TCR$^+$ and $\alpha\beta$-TCR$^+$ CD8$^+$ IEL populations can still develop (10). This extrathymic T lymphopoiesis also seems to take place in mice which are genetically or experimentally T lymphopenic (10). CD45-null mice are profoundly deficient in T cells in spleen and peripheral lymph nodes (3). This observation is reinforced by our analysis of mesenteric lymph nodes, in which we found CD3$^+$ cells at approximately one-fifteenth the normal frequency (Fig. 7). Thus, we expect that extrathymic T lymphopoiesis will play a role in the development of IEL T cells in CD45-null mice. If CD45 is necessary for extrathymic differentiation of gut T cells, those populations which can be extrathymically derived should be deficient in CD45KO mice, and reduced to an extent comparable to that for those T cell populations believed to have an obligate thymic origin. Whereas, if CD45 is not required for extrathymic differentiation of gut T cells, the $\gamma\delta$-TCR$^+$ and $\alpha\beta$-TCR$^+$ CD8$^+$ IEL populations should be less reduced than the $\alpha\beta$-TCR$^+$ CD4$^+$ and $\alpha\beta$-TCR$^+$ CD8$^+$ populations. However, neither of these outcomes were observed. $\alpha\beta$-TCR$^+$ CD8$^+$ cells, which are believed to be thymus dependent, do behave as predicted and are significantly reduced in both frequency and absolute numbers in IELs recovered from both the small and large intestine (Table 1). However the $\alpha\beta$-TCR$^+$ CD4$^+$ population was found at increased frequency and numbers in large intestine IELs and at equal frequency, albeit reduced numbers, in small intestine IELs. Furthermore, our results clearly show a 2–3-fold decrease in the proportion of $\gamma\delta$-TCR$^+$ cells in the small and large intestine, while the proportion of $\alpha\beta$-TCR$^+$ CD8$^+$ cells decreased only 29% in the small intestine.
Thus, both among those T cells which are thymus dependent and those which may be extrathymic, we observed a dichotomy of effects not explicable simply by differential requirements for CD45 in thymic versus extrathymic development. In a previous study, Martin et al. (21) compared the IEL populations of wild-type and CD45KO mice. Their study differed in detail since they did not separately examine large intestine IELs, nor did they combine αβ-TCR with CD4, CD8α and CD8β staining. By contrast, they reported a reduction in CD4+ cells in CD45-null KO mice. However, their data would appear to have been based on only three mice, whereas we analyzed 25 individual mice for small intestine IELs and four pools, each of 15 mice, for large intestine IELs. Nevertheless, their results for γδ-TCR+ cells are similar to our findings (21). Whether these γδ T cells are derived from the thymus or extrathymically, our results strongly suggest that CD45 is required for their efficient maturation. Kawai et al. have reported that CD45 is also required for the thymic maturation of the atypical Vβ3+ dendritic epidermal T cells found in the skin (22). Thus, all T cell development so far investigated appears CD45 regulated.

All the available evidence is consistent with a greatly reduced thymic output of ‘conventional’ T cells in CD45KO animals, so the difference in the effect of CD45 deficiency on αβ-TCR+ CD8αβ+ versus αβ-TCR+ CD4+ cells cannot simply be explained by the requirement for CD45 during thymic development. One possible explanation might be that the requirement for CD45 is much more stringent in the development of CD8 T cells than CD4 T cells. There is some evidence to suggest that this is not the case. The reduction in CD4+ and CD8+ single positive thymocytes is similar in CD45KO mice (3,20) and the selection of TCR transgenic thymocytes is impaired for both class I and class II MHC (20). An alternative explanation is that there is differential survival and/or expansion of CD4+ and CD8+ T cells in the periphery of CD45KO mice. This hypothesis is supported by the observations that CD4+ cells are consistently less severely reduced than CD8αβ+ cells in all compartments examined in this study, including the mesenteric lymph node and PP (see Figs 6 and 7). Seddon and Zamoyska (23) have recently shown that the half-life of CD4 T cells is longer than that of CD8 T cells in mice in which TCR signaling is impaired by lack of src-kinases. TCR signaling is highly impaired in CD45KO mice (24).

Furthermore, homeostatic proliferation of T cells can be TCR independent and in such circumstances is dependent on cytokines, particularly IL-7 and IL-15 (23,25–27). It is noteworthy that CD45KO cells are more responsive than normal cells to certain cytokines, which should include IL-7.
CD45 is reversed and γδ-TCR+ CD8αα+ cells are reduced by ~6-fold compared to wild-type, whereas the γδ-TCR+ population is ~3-fold rarer. Moreover, fewer γδ-TCR+ cells in CD45 KO mice expressed CD90 and CD2 (Fig. 3A and data not shown). Other studies using germ-free and conventionalized mice have shown that CD90 expression increases with conventionalization and that CD90 is required for γδ-TCR+ cells to mediate cytotoxic responses (31). Our data may therefore suggest that γδ-TCR+ cells cannot acquire full functionality in the absence of CD45 expression. These observations imply that CD45 KO mice might be more susceptible to gut infections. In fact, we have observed a high morbidity in CD45 KO mice after pinworm infection, whereas CD45-sufficient mice cleared the parasite infection (M.C. López and N. Holmes, unpublished observations). Interestingly, although the expression of CD90 decreased on γδ-TCR+ cells, the total percentage and absolute numbers of CD90+ CD3− cells increased in the small intestine (Table 1). It has been described that extrathymic T cell precursors found in the cryptopatches along the small intestine express c-kit, Thy 1 and IL-7R (32). Nonetheless, the increase in c-kit+ cells observed in one experiment (data not shown) does not account for the increase in CD90+ cells observed, thus suggesting that these CD90+ cells could be immature T cells at an intermediate stage of differentiation. Taking into account the elegant experiments described by B. Rocha’s group (33), gut T cells differentiate from Thy 1+ cells in the cryptopatches, slowly acquiring the expression of CD3, as they become either γδ- or γβ-TCR+ and B220+, before losing CD90 expression (33). The presence of an increased frequency of intermediate precursors of gut T cells is consistent with our proposition that extrathymic hemopoiesis is taking place in the gut of CD45 KO mice and that this differentiation is significantly impaired by the absence of CD45.

We also found that NK1.1+ cells were present at increased frequency and numbers in IELs from both small and large intestine (Figs 3 and 4) as well as a more limited increase in the frequency of CD3+ NK1.1+ T cells. Martin et al. reported similar results (21). Our extensive phenotypic analysis combining multiple surface markers helped us in our attempt to elucidate the complexity of the changes observed in the NK and NKT cell populations. As previously described by Guy-Grand and colleagues, the NK cells found in wild-type gut IELs are mainly CD3− cells that also express DX5 (31). The presence of an increased frequency of immature NK1.1+ cells is consistent with our proposition that extrathymic hemopoiesis is taking place in the gut of CD45 KO mice and that this differentiation is significantly impaired by the absence of CD45.

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synthesize cytokines ex vivo. This should reflect the degree to which the various cell populations are fully differentiated and activated in situ. With a few exceptions, the cells recovered from CD45KO mice had an increased ability to synthesize cytokines. The main exceptions to the general observation were from IELs. The ability to secrete TNF-α or IL-2 was reduced amongst both CD8α+ T cells and DX5+ NK cells from small intestine and in large intestinal IELs, DX5+ cells were less likely to make IL-2 or IL-4. Particularly striking was the increase in the tendency of γδ-TCR+ CD4+ cells from large intestine IELs to make IFN-γ, especially as such CD4+ cells are more frequent in CD45KO mice. The greater degree of activation in CD45KO T cells extended to PP- and MLN-derived cells as well as IELs and LPL. This activated phenotype may also be the cause of the increase in CD25 expression among large CD4+ T cells and extrathymically. Given that it is established that the development of CD4+ T cells is CD45 dependent pathway and extrathymically. The ability of IELs to secrete IL-4 after short-term anti-CD3 stimulation and the ability of LAK (3 day, IL-2 cultured NK) cells to secrete IFN-γ after anti-NK1.1 stimulation. By measuring the ability of cells to accumulate intracellular cytokine after short-term, non-specific (PMCA and ionomycin) stimulation, we are assaying only for the existence of cells differentiated in vivo to produce the relevant cytokine mRNA. We make no assumptions about the receptors which might trigger cytokine release. Nevertheless, our data for small intestine IELs, which should form the majority of the cells examined by Martin et al., show an increased frequency of cells making IL-4 overall and in every T cell subpopulation (Fig. 5A and data not shown).

Our data are also informative about the differentiation of B cells within the PP and mesenteric lymph nodes. Associated with the lack of T cells, there was an increase in the percentage of B cells in PP and MLN in CD45 KO mice as previously described for the spleen (3). Within the CD19+ B cell fraction, there was a decrease in the percentage of CD19+ cells which lack surface IgM, which we assume to be class-switched, memory B cells expressing other isotypes (IgA, IgG etc.). Class-switching is recognized to be T cell-dependent, thus the reduction in such cells is presumably accounted for by the paucity, and probably also the dysfunction (3,25) of CD45-negative T cells in PP and MLN. The reduced number of IgA plasma cells within the lamina propria in sections of small intestine (Fig. 1D and E) is further evidence for impaired class-switching to IgA within the gut-associated lymphoid tissues of CD45KO mice.

In summary, our results demonstrate that γδ-TCR+ cells within the intestinal IEL compartment are severely reduced in CD45KO mice whereas γδ-TCR+ CD4+ cells are not. These data suggest that the development of γδIELs is CD45 dependent, probably both via the thymic ‘double negative’ pathway and extrathymically. Given that it is established that the thymic development of CD4+ T cells is CD45 dependent (3,19,20), the selective retention of CD4+ cells within the IEL populations argues for a CD45-independent survival or expansion mechanism operating to a greater degree in the gut epithelial environment than within secondary lymphoid tissue. Previous results suggest that this is unlikely to be via stimulation of the T cell antigen receptor, for which CD45 has been shown to be required (24). It has been suggested that expansion and maintenance of T cells in lymphopenic mice may be TCR, MHC and Ick independent (23) and can be driven by cytokines alone (25,26). There is evidence that such pathways are active, possibly enhanced, in the absence of CD45 (28).

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Abbreviations

| AMCA | 7-amino-4-methylcoumarin-3-acetic acid |
| IELs | intraepithelial lymphocytes |
| LPL | lamina propria lymphocytes |
| MLN | mesenteric lymph node |
| PP | Peyer’s patch |
| TR | Texas red |

References


