CD4⁺ T Cell Division in Irradiated Mice Requires Peptides Distinct from Those Responsible for Thymic Selection

By Jeremy Bender, Tom Mitchell, John Kappler, and Philippa Marrack

Summary

We investigated the mechanism by which α/β T cells expand upon transfer to T cell-deficient host mice by injecting carboxyfluorescein diacetate succinimidyl ester-labeled T cells into mice depleted of T cells by sublethal irradiation. We found that CD4⁺ T cells divided when transferred to irradiated hosts and that the division of more than half of these cells required class II expression. However, division of transferred CD4⁺ T cells did not occur in irradiated hosts that expressed class II molecules occupied solely by the peptide responsible for thymic selection, indicating that peptides distinct from those involved in thymic selection cause the division of CD4⁺ T cells in irradiated mice. These data establish that class II–bound peptides control the expansion of CD4⁺ T cells transferred to T cell-deficient hosts and suggest that the same peptides contribute to the maintenance of T cell numbers in normal mice.

Key words: T cell homeostasis • peptides • peripheral selection • T cell receptor–major histocompatibility complex interaction • T cell–deficiency

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Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; CyC, cytochrome c; li, invariant chain; KO, knockout; SEB, staphylococcal enterotoxin B.
direct measure of the extent of division of individual T cells after transfer to each host. T cells were labeled with the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) before transfer to various sublethally irradiated hosts. Due to the approximately twofold decrease in CFSE fluorescence after each cell division, CFSE allowed us to assess the extent of division of individual transferred T cells (25, 26). We found that a significant fraction of CD4+ T cells divided when placed into irradiated normal mice. Most of the CD4+ T cell division that occurred in irradiated normal mice failed to occur in irradiated MHC class II KO (MHCIIKO) mice, confirming that CD4+ T cell division in T cell–deficient mice requires MHC class II expression. When the peptide presented by class II in the irradiated recipient was restricted to the peptide responsible for positive and negative selection of the T cells, division of the transferred CD4+ T cells did not occur. These experiments establish that peptides distinct from those involved in thymic selection control CD4+ T cell expansion in T cell–deficient mice and suggest that these same peptides may influence the T cell pool in normal mice by causing mature CD4+ T cells to divide.

Materials and Methods
Reagents and Mice. B6.PL-Thy1a/Cy (B6.PL) and BALB/cByJ (BALB/c) mice were purchased from The Jackson Laboratory. C57BL/6NTacBR (B6) and C57BL/6TacBR-KO (MHCIIKO) animals were purchased from Taconic Farms. Aβ EpTKO (β2-microglobulin KO, wαTκO, IκO, AαEpKO), TKO (β2-microglobulin KO, wαTκO, IκO), and DO TCR−RAG2KO BALB/c mice were bred under specific pathogen-free conditions in the Biological Resource Center (BRC) at National Jewish Medical and Research Center. CFSE was purchased from Molecular Probes Inc. PE-labeled mAbs against Thy1.1 and CD4, cy-chrome (CyC)-labeled anti-CD8, allophycoerythrin-labeled anti-CD4, and CyC-labeled streptavidin were purchased from PharMingen. Because whole-body irradiation affects many tissues, it is possible that the T cell division observed in irradiated mice did not result from T cell deficiency, but instead from additional effects of irradiation. To be certain that a T cell deficiency results in the increased division of transferred T cells, CFSE-labeled T cells were transferred to mice that lacked T cells due to induced genetic mutations. CFSE-labeled T cells from BALB/c mice were transferred to RAG2KO (αβ-TCR− T cell−, γδ-TCR− T cell−, B cell−) mice, and CFSE-labeled T cells from B6.PL mice were transferred to TCR−βKO (αβ-TCR− T cell−, γδ-TCR− T cell−, B cell+) mice. Both RAG2KO and TCR−βKO mice lack endogenous αβ T cells. 7 d after transfer, CD4+ and CD8+ T cells had divided in both RAG2KO and TCR−βKO recipient mice (Fig. 2). However, the rate of CD4+ T cell division was much faster in the RAG2KO and TCR−βKO hosts than it was in irradiated hosts. Because the rates of T cell division in irradiated and genetically T cell–deficient hosts differed, it is possible that the mechanism responsible for division in each host also differed. However, together these experiments indicate that the rate of αβ-TCR− CD4+ and CD8+ T cell division increased in response to an αβ T cell deficit.

Most CD4+ T Cell Division in Irradiated Mice requires MHC Class II Expression. To determine whether division of T cells that were transferred to irradiated mice required CD8+/CD4+ T Cell Division in Irradiated Mice

Figure 1. CD4+ T cells divide after transfer to irradiated hosts. Purified, CFSE-labeled B6.PL (Thy1.1) T cells were transferred to normal or 450-rad irradiated B6 (Thy1.2) mice. Each host received 1.8 × 10^6 B6.PL CD4+ T cells. 7 d later lymph node and spleen cells were stained with PE anti-Thy1.1, CyC anti-CD8, and allophycoerythrin anti-CD4. Histograms show the intensity of CFSE fluorescence of CD4+Thy1.1+ or CD8+Thy1.1+ cells. T cells from nonirradiated or irradiated hosts were 2.4 × 10^5 and 2.7 × 10^5, respectively.

Results
T Cells Divide When Transferred to T Cell–deficient Mice. The effect of sublethal irradiation of host mice on the rate of division of T cells transferred to the host was determined. T cells were purified from the lymph nodes of B6.PL (Thy1.2+) mice, labeled with CFSE, and injected into B6 (Thy1.2+) recipients that either had or had not been sublethally irradiated. CD4+ and CD8+ T cells that were transferred to normal, nonirradiated B6 mice showed little evidence of division 7 d after transfer (Fig. 1). However, CD4+ and CD8+ T cells that were transferred to irradiated B6 mice divided substantially during the same period. In irradiated B6 hosts, CD8+ T cells divided more quickly than did CD4+ T cells, although a small subpopulation of CD4+ T cells divided so many times that it had almost lost its CFSE fluorescence (Fig. 1, histogram region A). These data showed, as expected from previous studies, that transferred T cells divided more quickly in irradiated host mice than in nonirradiated host mice.
Figure 2. CD4+ T cells divide rapidly after transfer to untreated TCR-βKO (left) or RAG2KO (right) mice. Sublethally irradiated TCR-βKO or RAG2KO mice were injected with 3 × 10^6 CFSE-labeled B6.PL (left) or BALB/c (right) T cells. 7 d later lymph node and spleen cells were removed for analysis. Cells from TCR-βKO recipients were stained with PE anti-Thy1.1, CyC anti-CD8, and allophycoerythrin anti-CD4, and cells from RAG2KO recipients were stained with PE anti-CD4 and CyC anti-TCR-β. The data shown represent CD4+Thy1.1+ lymph node cells from TCR-βKO recipients and CD4+TCR-β+ lymph node cells from RAG2KO recipients.

TCR-MHC interactions, we tested whether CD4+ T cells divided in hosts that did not express MHC class II molecules. Purified B6.PL lymph node T cells were transferred to nonirradiated or irradiated B6 or MHCIIKO mice. Analyses 7 d after transfer showed that transferred CD4+ T cells did not divide in nonirradiated B6 or MHCIIKO hosts (Table I). However, transferred CD4+ T cells did divide in both irradiated B6 and irradiated MHCIIKO hosts, although more division occurred in irradiated B6 hosts than in irradiated MHCIIKO hosts (Fig. 3, summarized in Table I). 55% of transferred CD4+ T cells divided in irradiated B6 hosts, whereas only 22% of transferred CD4+ T cells divided in irradiated MHCIIKO hosts. As shown in Table I, the difference in the percentage of transferred CD4+ T cells that divided in irradiated B6 and irradiated MHCIIKO mice was consistent over many experiments. These data indicate that most of the division that occurred among CD4+ T cells transferred to irradiated B6 mice required class II expression, which confirmed previous studies of CD4+ T cell expansion in T cell-deficient mice.

Although most of the CD4+ T cells that were transferred to irradiated hosts required class II expression to divide, some CD4+ T cells divided in the absence of class II expression. Among the transferred CD4+ T cells that divided in irradiated MHCIIKO mice were two groups of cells. The first group of CD4+ T cells that divided in irradiated MHCIIKO mice went through a single round of cell division. This single round of division may represent a response by the T cells to the cytokine environment generated in the host by irradiation (28–30). The second group of CD4+ T cells divided many times in MHCIIKO mice (see Fig. 1, histogram region A). During and after division these cells expressed high levels of CD44 (data not shown), which suggests that antigen, possibly presented by class I or non-classical MHC molecules, caused these cells to divide. The rapidly dividing cells were excluded from the calculations presented here because they appeared to represent a distinct phenomenon.

After the transfer of T cells to irradiated mice, the total number of transferred T cells recovered from the lymph nodes and spleen of an irradiated recipient was typically 10–20% of the total number of cells that were initially

Table I. Unfamiliar Class II–bound Peptides Are Involved in CD4+ T Cell Division in Irradiated Hosts

<table>
<thead>
<tr>
<th>Source of donor T cells</th>
<th>Recipient</th>
<th>Recipient irradiated</th>
<th>No. of independent experiments</th>
<th>Percentage of transferred CD4+ T cells that have undergone a given number of divisions</th>
<th>Average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6.PL</td>
<td>B6</td>
<td>no</td>
<td>5</td>
<td>97.0 ± 0.5</td>
<td>2.8 ± 0.4</td>
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<tr>
<td></td>
<td>AβKO</td>
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<td>4</td>
<td>96.8 ± 0.5</td>
<td>3.3 ± 0.3</td>
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<tr>
<td></td>
<td>B6</td>
<td>yes</td>
<td>5</td>
<td>57.7 ± 2.9</td>
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<tr>
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<td>4</td>
<td>79.2 ± 2.3</td>
<td>19.3 ± 2.2</td>
</tr>
<tr>
<td>AβEpTKO</td>
<td>AβEpTKO</td>
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<td>4</td>
<td>91.8 ± 4.6</td>
<td>7.5 ± 4.0</td>
</tr>
<tr>
<td></td>
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<td>85.7 ± 9.8</td>
<td>13.3 ± 9.7</td>
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<tr>
<td>AβEpTKO*</td>
<td>AβEpTKO</td>
<td>yes</td>
<td>1</td>
<td>96.6 ± 2.7*</td>
<td>3.4 ± 2.6*</td>
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<tr>
<td></td>
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<td>1</td>
<td>90.2 ± 2.2*</td>
<td>7.9 ± 2.1*</td>
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<tr>
<td>DO TCR + RAG2KO</td>
<td>BALB/c</td>
<td>yes</td>
<td>3</td>
<td>89.6 ± 2.1</td>
<td>6.5 ± 1.0</td>
</tr>
</tbody>
</table>

Donor T cells were purified and labeled with CFSE before transfer to untreated or irradiated recipients. 7 d after transfer, recipients were analyzed and the percentage of transferred CD4+ T cells that had not divided, divided once, or divided twice was determined using histogram markers as indicated in Fig. 3. In the experiment in which AβEpTKO donor mice were injected with SEB and LPS before transfer, the data shown indicate the percentage of Vβ8+CD4+ (Vβ8+ T cells are activated by SEB) cells that went through a given number of divisions.

1 Data from CD4+Vβ8+ cells.

2 Injected 1 wk before transfer with 150 μg SEB and 7 μg LPS.
transferred. To exclude the possibility that transferred T cells trafficked to and divided within tissues other than spleen and lymph node, lymphocytes were prepared from the liver, lung, and small intestine of irradiated B6 or irradiated MHCIIKO mice that had been injected with CFSE-labeled B6.PL T cells 7 d earlier. The total number of transferred T cells obtained from these tissues represented ~4% of the number initially transferred, but the T cells obtained from these tissues had divided less than the T cells in the lymph node and spleen from the same mice (data not shown). Thus, trafficking of transferred CD4+ T cells to tissues other than the spleen and lymph node did not influence the division of CD4+ T cells in irradiated B6 or MHCIIKO mice. This finding provided additional support for the conclusion that most CD4+ T cells required class II expression to divide in irradiated hosts.

The Peptide-MHC Ligand Responsible for Thymic Selection Does Not Cause Division of CD4+ T Cells in Irradiated Mice. To assess whether peptides influenced MHC class II–dependent CD4+ T cell division, we exploited mice that express a single class II–peptide combination, Aβ covalently linked to a protein and no invariant chain (Ii) (AbEpTKO). A recent study found that although naive transgenic CD8+ T cells required antigen to divide in irradiated hosts, memory transgenic CD8+ T cells divided in irradiated mice in the absence of specific antigen (12). To address whether the peptide–MHC ligand responsible for thymic selection was capable of causing the division of memory CD4+ T cells, Vβ8+ T cells from AβEpTKO mice were activated before transfer by injecting the mice with 150 μg
staphylococcal enterotoxin B (SEB) and 7 μg LPS. Injection of SEB and LPS resulted in the activation of Vβ8+ T cells, as assessed by the increased number of Vβ8+ cells observed 2 d after SEB injection (data not shown). 1 wk after the SEB injection, T cells from these AαEpTKO mice were purified, labeled with CFSE, and transferred to irradiated AαEpTKO, TKO, and wtAbβ-DKO recipients. 1 wk later, the amount of division that had occurred among transferred Vβ8+ CD4+ T cells was determined (Table I). The data indicate that little division of previously activated Vβ8+ CD4+ T cells occurred in irradiated mice that lacked MHC expression or expressed only the selecting MHC–peptide ligand. As expected, the transferred Vβ8+ CD4+ cells divided rapidly in wtAbβ-DKO mice, indicating that the lack of division of these cells in AαEpTKO and TKO mice was not due to anergy induced by SEB priming (data not shown). Thus, unlike memory CD8+ T cells, memory CD4+ T cells do not appear to divide in irradiated mice that express only the selecting MHC molecule.

Transgenic DO T Cells Do Not Divide in Irradiated Hosts That Support Positive Selection. To provide additional evidence that the peptide–MHC ligand responsible for thymic selection could not induce division of CD4+ T cells in irradiated mice, CFSE-labeled transgenic CD4+ DO T cells, which bear TCRs specific for OVA peptide in the context of Aα and are positively selected in BALB/c mice (33), were transferred to irradiated BALB/c mice. 7 d after transfer, analysis showed that few DO T cells divided in irradiated BALB/c mice and not due to anergy induced by SEB priming (data not shown). Thus, unlike memory CD8+ T cells, memory CD4+ T cells do not appear to divide in irradiated mice that express only the selecting MHC molecule.

To demonstrate that DO T cells were able to divide in response to antigen, irradiated BALB/c mice that had previously received CFSE-labeled DO T cells were injected with OVA peptide, for which DO T cells are specific. 7 d after the injection of 0.1, 1.0, or 10 μg of OVA peptide, the extent of division among DO T cells was determined (Fig. 5). Analysis showed that the amount of division of DO T cells reflected the amount of antigen administered to the irradiated BALB/c recipient. Although injection of 0.1 μg OVA peptide into an irradiated BALB/c host induced little division, injection of 1.0 or 10 μg OVA peptide caused a progressive increase in the extent of division of DO T cells. This result confirmed the previous finding that transgenic CD4+ T cells required antigen to divide when transferred to T cell–depleted mice (23) and indicated that the extent of division among DO T cells in irradiated BALB/c mice reflected the amount of peptide antigen administered. These findings suggest that the one or two rounds of division seen among normal B6.PL CD4+ T cells that were transferred to irradiated B6 hosts may have

![Figure 4](image-url)  
**Figure 4.** The selecting ligand does not drive CD4+ T cell division in irradiated hosts. 3.8 × 10^5 CFSE-labeled CD4+ AαEpTKO T cells were transferred to 450-rad sublethally irradiated hosts. Hosts were: TKO (β2M KO, wtAβKO, IiKO); AαEpTKO (β2M KO, wtAβKO, IiKO, AαEp+); wtAbβ-DKO (β2M KO, wtAbβ+IiKO). 7 d after transfer were stained with PE anti-CD4 and CyC anti-TCR-β and analyzed. Histograms represent the data from five CD4+ TCR+ lymph node cells.

![Figure 5](image-url)  
**Figure 5.** Transgenic DO T cells do not divide in irradiated mice that support thymic selection of the DO TCR. DO TCR RAGKO BALB/c, CFSE-labeled cells were transferred to sublethally irradiated BALB/c mice. 24 h later, mice were injected intravenously with soluble OVA peptide as indicated on the figure. 7 d later, cells were stained with PE anti-CD4 and CyC KJ1 (anti-DO idiotype) and analyzed. Data shown are CD4+KJ1+ lymph node cells.
occurred in response to TCR stimulation by relatively low levels of peptides.

Discussion

Previous reports have demonstrated that depletion of T cells in mice creates an environment in which T cells divide when transferred into these mice (19–23). Past experiments have also shown that the T cell division in such mice depends on TCR contact with MHC. For example, division of CD4+ T cells transferred to T cell-deficient mice depends on the expression of class II molecules (22, 23). The findings presented here confirm that CD4+ T cell division in T cell-deficient mice requires class II expression and establish that class II-bound peptides control this division.

As expected, a smaller percentage of CD4+ T cells from normal mice divided when transferred to irradiated MHCIIKO mice than divided when transferred to irradiated B6 mice. To determine whether the peptides presented by class II were involved in this class II-dependent division, CD4+ T cells from mice expressing only one class II-peptide combination were transferred to irradiated hosts. AαEpTKO CD4+ T cells, which were selected in the thymus exclusively by Ep bound to Aα, did not divide when transferred to irradiated AαEpTKO hosts, in which all Aβ molecules were occupied by the selecting Ep peptide. Because AαEpTKO hosts expressed class II molecules, the lack of division among transferred AαEpTKO T cells indicated that division of CD4+ T cells in irradiated mice depends on peptide presentation by class II molecules and not on other effects class II expression may have on irradiated hosts. Therefore, the class II-dependent division of naturally occurring B6.PL CD4+ T cells after transfer to irradiated B6 hosts was caused by peptides presented by class II molecules.

The lack of AαEpTKO T cell division in irradiated AαEpTKO hosts also established that the peptides that controlled division of CD4+ T cells in irradiated mice were distinct from those responsible for thymic selection. These peptides have not been specifically identified, but we refer to them as unfamiliar because they are distinct from those involved in thymic selection. These unfamiliar peptides were expressed extrathymically after irradiation and may be derived either from intestinal flora or from the mouse itself.

The requirement for unfamiliar peptides suggests that TCR specificity determined whether a particular CD4+ T cell divided when transferred to an irradiated host. This suggestion is supported by findings described here and in a previous report (23) that transgenic CD4+ T cells did not divide in T cell-deficient hosts in the absence of antigen. Thus, CD4+ T cells divided when transferred to irradiated mice in response to specific interactions between TCRs and unfamiliar peptides presented by class II molecules. The reason these specific interactions occurred in irradiated mice but did not occur in nonirradiated mice is unknown. There are at least two explanations for the observation that CD4+ T cells experienced greater exposure to unfamiliar peptides when transferred to irradiated mice than when transferred to normal mice. First, irradiation may have changed the distribution of peptides presented by class II molecules by altering the representation of specific self- or foreign peptides. This could have resulted from the phagocytosis of large numbers of apoptotic mouse cells by APCs or from transient infection of irradiated mice by normal flora due to the breakdown of mucosal barriers. Second, by causing apoptosis of lymphocytes, irradiation could have decreased the competition among T cells for interaction with peptides normally presented by class II molecules. The explanation that decreased competition led to increased exposure of CD4+ T cells to peptides is particularly appealing because it could also explain the rapid rate of division of T cells that were transferred to TCR-βKO and RAG2KO hosts, both of which lack endogenous T cells to compete with transferred CD4+ T cells for interactions with APCs.

Further work will be required to determine whether increased contact between transferred CD4+ T cells and unfamiliar peptides in irradiated mice occurred because of decreased competition among T cells or due to a change in the distribution of peptides presented by class II molecules in the host.

The data presented here demonstrate that CD4+ T cells from normal mice divide in response to interactions with unfamiliar class II-bound peptides when transferred to irradiated hosts. Because the unfamiliar peptides that induce division in irradiated mice may also be presented, perhaps at lower levels, by class II molecules in normal mice, it is possible that the same peptides that cause division of CD4+ T cells in irradiated mice are also responsible for causing extrathymic expansion (13) of T cells in normal mice. Thus, the 20% of the CD4+ T cell repertoire that required class II expression to divide in irradiated hosts may have been created in donor mice by extrathymic expansion in response to the same peptides that induced division in irradiated mice. If so, then the peptides presented by class II molecules in a normal, healthy mouse may significantly influence the T cell repertoire, as has been suggested in an earlier study of peripheral T cell selection (24). Additional work will have to be done to determine whether unfamiliar peptides influence the T cell repertoire in normal mice by causing CD4+ T cell division and, if so, whether this division occurs only in the context of an active immune response.

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References


