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Involvement of CD1 in Peripheral Deletion of T Lymphocytes Is Independent of NK T Cells

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During peripheral T cell deletion, lymphocytes accumulate in nonlymphoid organs including the liver, a tissue that expresses the nonclassical, MHC-like molecule, CD1. Injection of anti-CD3 Ab results in T cell activation, which in normal mice is followed by peripheral T cell deletion. However, in CD1-deficient mice, the deletion of the activated T cells from the lymph nodes was impaired. This defect in peripheral T cell deletion was accompanied by attenuated accumulation of CD8+ T cells in the liver. In tetraparental bone marrow chimeras, expression of CD1 on the T cells themselves was not required for T cell deletion, suggesting a role for CD1 on other cells with which the T cells interact. We tested whether this role was dependent on the Ag receptor-invariant, CD1-reactive subset of NK T cells using two other mutant mouse lines that lack most NK T cells, due to deletion of the genes encoding either β2-microglobulin or the TCR element Jα281. However, these mice had no abnormality of peripheral T cell deletion. These findings indicate a novel role for CD1 in T cell deletion, and show that CD1 functions in this process through mechanisms that do not involve the major, TCR-invariant set of NK T cells.


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the activated T cells and another cell expressing CD1 promotes the deletion of activated peripheral T cells.

Materials and Methods

**Mice**

C57BL/6 (B6), 1 (B6 × 129)F1, or F2, and β₂-microglobulin-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CD1-deficient mice (on the B6 × 129 background) were created at Beth Israel Deaconess Hospital and kept on ice until staining. and then suspended lymph node cells rinsed in Bruff’s culture medium. Two to 8 days after injection, four LNs (two axillary and two inguinal) were removed by dissection, homogenized, and centrifuged at 1500 × g for 20 min. The cells at the interface were collected, washed with PBS, and counted.

**Results**

**Defective peripheral T cell deletion**

The down-regulation of the TCR is a recognized feature of T cell activation leading to deletion, and has been previously reported in peptide- and superantigen-induced peripheral T cell deletion (2, 10). Similarly, the injection of 100 μg of anti-CD3 resulted in the down-regulation of the TCR. Fig. 1A shows the effect of anti-CD3 injection of CD4⁺ and CD8⁺ T cells of wild-type and CD1-deficient mice at 2 days after injection of the Ab. Lymph node cells of individual, representative wild-type mice are shown in the upper panels of Fig 1A. In PBS-injected controls, 50% of the lymph node cells were CD4⁺, TCRβ⁺ T cells and only 1% were CD4⁺, TCRβ⁺ T cells. In an anti-CD3-injected mouse, there were 23% of CD4⁺, TCRβ⁺ T cells and 18% of CD4⁺, TCRβ⁻ T cells, making 41% of CD4⁺ T cells overall. Thus, at this early time point, the main effect of anti-CD3 treatment was the loss of TCR expression rather than T cell deletion, yet there was also a small loss of CD4⁺ T cells. Similarly, the TCRβ was lost from many of the CD8⁺ cells. The lower panels of Fig. 1A show the effects of anti-CD3 injection on the T cells of individual, representative CD1-deficient mice. The basic pattern was similar, with down-regulation of the TCR on both CD4⁺ and CD8⁺ cells. However, the total of CD4⁺, TCRβ⁺ T cells and CD4⁺, TCRβ⁻ T cells (25 and 26%, respectively) was 51%, against 54% in the PBS-injected controls. Both of these numbers are within the normal range for PBS-injected CD1-deficient mice, in contrast to the loss of 10% of T cells from the anti-CD3-injected normal mice.

This difference was accentuated 2 days later. Fig. 1B shows that at day 4, the TCR-low cells had disappeared from both the CD4⁺ and the CD8⁺ subsets of the anti-CD3-injected wild-type mice. The total number of CD4⁺ cells was reduced from 58% in a representative PBS-injected control to 27% in a representative anti-CD3-injected mouse (Fig. 1B, upper panels). Similar changes of lesser magnitude occurred in the CD8⁺ cells. In CD1-deficient mice the TCR-low cells had also disappeared, but, in contrast, the total CD4⁺ cell frequencies in anti-CD3-injected mice were within the range of the controls. Among the CD8⁺ cells, the modest deletion observed in wild-type mice was not evident in the CD1-deficient mice.

The results from groups of four mice each are summarized in Fig. 2, which shows the means and SD estimates of the total numbers of CD4⁺ and CD8⁺ T cells in the lymph node, for each experimental group, at 2, 4, 6, and 8 days after a single injection of anti-CD3. We chose to represent absolute CD4⁺ and CD8⁺ cell numbers to show that the effects of CD1 deficiency on the relative depletion of CD4⁺ T cells were not due to the fluctuations in the absolute numbers of other cell types. To obtain these numbers, we multiplied the lymph node cell yield from the pooled superficial (axillary and inguinal) lymph nodes of each individual animal by the percentage of CD4⁺ and CD8⁺ T cells from that animal. In wild-type mice, the absolute number of CD4⁺ T cells was already depleted (from 4.4 × 10⁶ to 1.8 × 10⁶) by day 2, while there was less depletion in the CD1-deficient cells (from 4.6 × 10⁶ to 3.0 × 10⁶). By day 4, there was depletion of CD4⁺ T cells from both wild-type
and CD1-deficient mice, but much less deletion in the CD1-deficient mice. This difference persisted throughout the time-course of the experiment, until the latest time-point evaluated on day 8. We conclude first that, even at early time-points, peripheral T cell deletion was impaired in CD1-deficient mice. Second, the calculation of absolute cell numbers revealed that the deletion of CD8$^+$ cells, as well as CD4$^+$ cells, was impaired in CD1$^{-/-}$ mice, which was not obvious from the study of percentages alone. Thus, at days 4, 6, and 8, respectively, in wild-type mice the number of CD8$^+$ cells was reduced from mean values of 2.2, 2.4, and 2.6 million in PBS-injected control groups to 0.5, 0.2, and 0.1 million in anti-CD3-injected groups of mice. In the CD1-deficient mice, anti-CD3 reduced the mean numbers of CD8$^+$ cells from similar control group values of 2.0, 1.5, and 2.5 million to 0.8, 1.0, and 0.3 million in anti-CD3-injected groups of mice. Thus the deletion of CD8$^+$ T cells was more profound in wild-type mice.

The limited availability of CD1-deficient mice backcrossed to B6 limited our ability to perform a full kinetic analysis on such a uniform background. However, to confirm the difference in T cell deletion, we analyzed the effect of anti-CD3 treatment in groups of fifth-generation B6.CD1$^{-/-}$ backcross mice at 4 days. In this direct comparison, the inguinal plus axillary lymph nodes of PBS-injected wild-type mice contained 5.3 $\pm$ 0.9 $\times$ 10$^6$ CD4$^+$ cells. Injection of four such mice with anti-CD3 caused very substantial deletion of the cells, such that 0.6 $\pm$ 0.2 $\times$ 10$^6$ CD4$^+$ cells remained. In the four congenic CD1$^{-/-}$ mice, the corresponding numbers were 4.0 $\times$ 10$^6$ and 1.03 $\pm$ 0.3 $\times$ 10$^6$. Because the numbers of CD4$^+$ T cells were not identical in the PBS-injected controls, the numbers of cells in each anti-CD3-injected animal was normalized to “the percent of mean PBS control”, and these numbers subjected to a $t$ test. The deletion in the CD1$^{-/-}$ mice was significantly less ($p = 0.0026$).

An identical analysis was applied to the CD8$^+$ T cells in the B6 control vs congenic CD1$^{-/-}$ mice. Lymph nodes of wild-type mice contained 4.4 $\pm$ 0.7 $\times$ 10$^6$ CD8$^+$ cells, depleted to 0.4 $\pm$ 0.1 $\times$ 10$^6$ by anti-CD3. Lymph nodes of CD1$^{-/-}$ mice contained 3.8 $\pm$ 0.2 $\times$ 10$^6$ CD8$^+$ cells, depleted to 1.0 $\pm$ 0.4 $\times$ 10$^6$ with anti-CD3. The difference in T cell deletion was significant ($p = 0.0025$).

**Normal activation of CD1$^{-/-}$ T cells**

One possible explanation for these findings could be that CD1-deficient T cells simply failed to become activated. Therefore, we verified that the lack of CD1 expression did not compromise T cell activation. In a 48-h in vitro proliferative response to anti-CD3 Ab, in which the T cell response was measured using a [$^3$H]thymidine incorporation assay, the activation of CD1$^{-/-}$ T cells was identical with the activation of wild-type T cells (the data were: means of 260, 534 cpm in wild-type T cells and 264, 279 cpm in CD1$^{-/-}$ T cells, with backgrounds of less than 2,000 cpm in both cases). In vivo injection of 100 $\mu$g of purified 145-2C11 anti-CD3 Ab resulted in a burst of T cell proliferation, which was very similar between CD1-deficient and wild-type mice based on the percent-age of T cells that incorporated 2-bromo-deoxyuridine (20.4% in wild type, and 24.2% in CD1$^{-/-}$).

**Defective accumulation of T cells in liver in CD1-deficient mice**

The activation and deletion of T cells is associated with their accumulation in the liver. Our previous studies have shown that the accumulation of T cells in the liver during peripheral T cell deletion is independent of Fas function (29). To test whether such
accumulation was dependent on CD1, we isolated IHL from the livers of wild-type control mice and CD1-deficient mice during anti-CD3-induced peripheral T cell deletion. In normal mice, anti-CD3 induced a transient increase in the IHL count, with a peak at $5 \times 10^6$ at day 4 after anti-CD3 injection (Fig. 3, left, dark shading). Control mice injected with PBS showed an IHL count of around $1 \times 10^6$ throughout the experiment, which is in the normal range for unmanipulated mice. Mice lacking CD1 had a normal number of IHL in the PBS-injected controls. However, in anti-CD3-injected CD1-deficient mice, the increase in IHL numbers was smaller, with a peak of $3 \times 10^6$, and the peak was delayed until day 6. Therefore, the normal process of liver accumulation of T cells during peripheral T cell deletion was defective in CD1-deficient mice.

To validate the conclusions, we normalized the mean number of liver lymphocytes in PBS-injected control mice on day 4 to 100%. Day 4 was chosen because it was the peak of the liver lymphocytosis in normal mice. In wild-type mice, the IHL count in anti-CD3-injected mice on day 4 was $4.6 \pm 0.6 \times 10^6$, compared with $1.0 \times 10^6$ in the PBS-injected controls. In CD1$^{-/-}$ mice, the IHL count in anti-CD3-injected mice on day 4 was $2.7 \pm 0.4 \times 10^6$, compared with $0.91 \pm 0.3 \times 10^6$ in the PBS controls. The difference between wild-type and CD1$^{-/-}$ mice was significant, using a $t$ test ($p = 0.0008$).

The isolated IHL were stained for CD4 and CD8 expression (Fig. 3, center left and center right panels). As expected, the frequency of CD4$^+$ cells was unchanged among the IHL, and fluctuations were within the limits of normal. However, a dramatic effect was evident in the CD8$^+$ cells. In wild-type mice, anti-CD3 injection caused a 4-fold increase in the percentage of CD8$^+$ IHL at day 2, followed by a return to normal on day 4. In contrast, in CD1-deficient mice, the percentage of CD8$^+$ IHL showed a much smaller increase on day 2, but remained elevated by 2- to 3-fold on days 4 and 6 (Fig. 3, inner left and right panels). Thus in terms of both cell accumulation and phenotypic change, the liver phase of peripheral T cell deletion was attenuated and delayed in CD1-deficient mice. The most striking difference between IHL subsets of wild-type and CD1-deficient mice was in the percentage of CD8$^+$ cells, observed in anti-CD3-injected mice on day 2. This difference was statistically significant ($p = 0.002$).

It is noteworthy that in anti-CD3-injected normal mice, the peak in the percentage of CD8$^+$ cells occurred at 2 days, while the peak in total IHL numbers occurred at 4 days. This could have been because CD8$^+$ T cells, trapped in the liver, undergo loss of recognition molecules resulting in CD4$^+$, CD8$^+$ T cells, as we have reported before (10, 30). Alternatively, it could have been because of expansion of the liver NK T cells, induced by the anti-CD3. The right panels of Fig. 3 show that anti-CD3 treatment did not change the percentage of NK-1.1$^+$ cells in the liver, either in wild-type of in CD1$^{-/-}$ mice.

Fig. 4 shows examples of the changes in TCR$\alpha\beta$, CD4, and CD8 expression in liver lymphocytes during anti-CD3 induced T cell deletion. All data are taken from day 4. In wild-type mice, anti-CD3 treatment caused down-regulation of the TCR$\alpha\beta$, both on T cells and on NK T cells (Fig. 4, upper left panels). In CD1$^{-/-}$ mice, there was less down-modulation of the TCR$\alpha\beta$, and very few NK T cells, as expected (Fig. 4, lower left panels). Within the population of TCR$\alpha\beta^+$ cells, anti-CD3 treatment caused a decrease in the percentages of CD4$^+$ cells, with a compensatory increase in the percentage of both DN and CD8$^+$ cells (Fig. 4, lower right panels).
CD1 FUNCTION INDEPENDENT OF NK T CELLS

Changes in subsets of liver lymphocytes on day 4. Injection of anti-CD3 into wild-type mice caused reduced expression of the TCRαβ on both T cells and NK T cells (two upper left panels). In CD1-deficient mice, there was no such anti-CD3-induced loss of TCR staining, and NK T cells were very rare, as expected (two lower left panels). Among TCRαβ⁺ liver lymphocytes of wild-type mice, anti-CD3 caused relative loss of CD4⁺ cells, with compensating increases in both CD8⁺ and DN cells (two upper right panels). Among CD1-deficient liver lymphocytes, DN T cells were rare, due in part to the lack of NK T cells. Anti-CD3 injection caused some loss of CD4⁺ cells, but the magnitude of the loss was smaller than in wild-type mice.

The role of CD1 is non-T cell autonomous

CD1 molecules are expressed on lymphoid cells, as well as on nonlymphoid bone marrow-derived cells and on extrahepatic tissues, particularly the liver and the intestine. To determine whether the CD1 on the activated T cells themselves was a target of the T cell deletion mechanism, tetraparental bone marrow chimera was constructed in which 50% of the bone marrow was CD1-intact, and 50% was CD1-deficient. The radioresistant host tissues in these chimera were also CD1-intact. Four weeks after irradiation and reconstitution we obtained stable, balanced chimeras. These chimeras were injected with anti-CD3 Ab, and the effect on the CD1-intact and the CD1-deficient T cells in each chimera was determined. Fig. 5A shows two chimeras. One has been injected with PBS as a control; this chimera contained 47% of CD1⁺ lymphocytes, most of which were T cells. The other chimeras were injected with anti-CD3 Ab, and the effect on the CD1-intact and the CD1-deficient T cells in each chimera was tested. These data suggested that CD1 is an active player in the trapping of CD8⁺ T cells in liver during T cell deletion, but the nature of its involvement is not yet clear. Cell interactions that involve CD1 may change the adhesion or recirculation properties of activated CD8⁺ T cells in the circulating pool, increasing the chance that they home to liver. Alternatively, CD1 expressed in the liver may have a direct role in the trapping of CD8⁺ T cells.

Abnormal T cell deletion was not due to lack of Va14, Ja281 NK T cells

The impairment of peripheral T cell deletion in CD1-deficient mice might be accounted for by various mechanisms. These mice lack TCR-invariant NK T cells, and a mechanism could be envisaged in which such NK T cells engage CD1 ligands on activated T cells, and deliver a death signal. In support of this concept, we have shown that liver NK T cells are cytotoxic by both Fas ligand-dependent and perforin-dependent mechanisms (25). Therefore, we tested this hypothesis by repeating the anti-CD3 deletion experiment in young β₂-microglobulin-deficient mice. These mice contain very few NK T cells, although the defect is not absolute and the cells increase in number as the mice age (24). They also lack CD8⁺ T cells, but we were able to evaluate the peripheral deletion of CD4⁺ T cells. Fig. 6A shows that anti-CD3-induced peripheral CD4⁺ T cell deletion was normal in lymph nodes of β₂-microglobulin-deficient mice, with the disappearance of around half of all the CD4⁺ T cells at 4 days (compare with Fig. 1B). However, β₂-microglobulin-deficient mice could potentially be abnormal in ways that would confound the interpretation of the experiment, for example due to the lack of CD8⁺ T cells. To overcome this problem, we addressed the same issue using an alternative mutant mouse line that lacks the same population of NK T cells, but contains CD8⁺ cells. Because most NK T cells use a highly specific TCRα-chain in their Ag receptors, namely Va14Ja281, it is possible to test the involvement of these NK T cells in mice unable to make this specific TCR due to lack of the J region. In Ja281-deficient mice, the deletion of peripheral lymph node CD4⁺ T cells by anti-CD3 was intact (Fig. 6B). These two experiments, taken together, argue strongly against a role for both, if not all NK T cells in the CD1-dependent component of peripheral T cell deletion.

Discussion

The highly polymorphic, peptide-binding “classical” molecules of the MHC are clustered on chromosome 17 in the mouse, and on chromosome 6 in the human. In addition to this gene complex,
both humans and mice express structurally related but less polymorphic “nonclassical” MHC like molecules encoded elsewhere. In the human, these are termed CD1a, b, c, d, and e. In mice, there is only a tandem duplication of a homologue of CD1d (31). The CD1 molecules, like their classical MHC counterparts, contain a pocket on the apical surface. However, this cavity is deeper and its inner recesses are more hydrophobic that is the case for classical MHC molecules, and differs between species. In the mouse, CD1 expression has been reported on thymocytes and peripheral T lymphocytes, B cells, dendritic cells, and macrophages (34), on hepatocytes in the liver, and on intestinal epithelial cells where it appears to act as a T cell ligand (26, 35). The reported CD1 expression in the livers of mice, and also of rats (27), is provocative because this tissue site has been implicated in the trapping of activated T cells (10, 11, 13). Furthermore, the liver is a site in which NK T cells are unusually abundant (36, 37), raising the possibility that the NK T cells are involved in the liver component of peripheral T cell deletion. The availability of mice deficient in CD1 allowed us to examine these issues. In the present study, we have induced peripheral T cell activation leading to T cell deletion in normal vs CD1-deficient mice.

We tested peripheral T cell deletion using a single injection of anti-CD3, which was chosen in preference to a peptide Ag because it allowed us to induce the activation and deletion of both CD4+ and CD8+ T cells in the same experimental animals. This technique suffers from the disadvantage that the TCR is ligated at an unusual point (i.e., on the extracellular domain of the CD3 e-chain), but offers the compensating advantage that general conclusions may be drawn without the risk of artifacts due to the vagaries of an individual transgenic TCR. After the injection of anti-CD3, lymph node cells and IHL were isolated at 2, 4, 6, and 8 days. As previously reported by others (4, 5, 38), this treatment caused peripheral T cell activation followed by deletion in wild-type mice.

We used this model to show that CD1d was required for the early phase of peripheral T cell deletion in mice. This role was independent of TCR-invariant, CD1-reactive NK T cells, because neither β2-microglobulin-deficient mice nor Jα281-deficient mice reproduced the abnormality found in CD1-deficient mice. The defect appears to control the homing of activated T cells to the liver. Chimera experiments have shown that the expression of CD1d on the T cells is not important for its role in T cell deletion. To explain these data, we propose that activated T cells interact with CD1d expressed on non-T cells, and that this interaction predisposes them to undergo deletion from the periphery. At present, we do not know whether the relevant CD1d is expressed on specialized, bone marrow-derived APCs such as macrophages, B cells, or dendritic cells (39, 40), or whether it is on tissue cells such as intestinal epithelium or hepatocytes (41, 42).
Liver shows a variety of unusual immunological properties, apart from the expression of CD1 and the presence of populations of lymphocytes distinct from those found in secondary lymphoid organs or in the general circulation. It has been proposed as a site of extrathyminic T cell development (43–45), and is a site at which systemic tolerance may be induced. This tolerance is manifest as failure to reject liver allografts (46), as failure to reject pancreatic islet allografts when they are introduced into the liver via the portal vein (47), and as the induction of tolerance in T cells specific for allogeneic MHC Ags expressed on hepatocytes through transgenesis (48, 49). Liver is also a site at which an important pathogenic virus, Hepatitis C virus, establishes a persistent infection (50). The present study suggests an important role for CD1 in T cell accumulation in the liver, and thus raises the possibility that its presence at that site may be linked to liver tolerance.

This study documents the importance of CD1-based mechanisms in the peripheral deletion of T cells induced by anti-CD3 Ab. Presumably these mechanisms also have a role in the immune response to Ag. It is clear from our data that CD1 is important in the earliest phase of the deletion process, because when it is missing, abnormalities are evident as early as 2 days after anti-CD3 injection. In a normal immune response to specific peptide Ag, this is the time of the clonal expansion phase, while T cell deletion does not start until day 5 or later (51–53). On this basis, we speculate that the CD1-based mechanism is involved in removing cells that did not receive a full activation signal, i.e., in the induction of tolerance rather than in the termination of a full-blown immune response.

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