Functional Characterization of MHC Class II-Restricted CD8^+CD4^- and CD8^-CD4^+ T Cell Responses to Infection in CD4^-/- Mice

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Functional Characterization of MHC Class II-Restricted CD8\(^+\)CD4\(^-\) and CD8\(^-\)CD4\(^-\) T Cell Responses to Infection in CD4\(^{-/-}\) Mice\(^1\)

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Classical CD4\(^+\) and CD8\(^+\) T cells recognize Ag presented by MHC class II (MHCII) and MHC class I (MHCI), respectively. However, our results show that CD4\(^{-/-}\) mice mount a strong, readily detectable CD8\(^+\) T cell response to MHCII-restricted epitopes after a primary bacterial or viral infection. These MHCII-restricted CD8\(^+\)CD4\(^-\) T cells are more similar to classical CD8\(^+\) T cells than to CD4\(^+\) T cells in their expression of effector functions during a primary infection, yet they also differ from MHCI-restricted CD8\(^+\) T cells by their inability to produce high levels of the cytolytic molecule granzyme B. After resolution of a primary infection, epitope-specific MHCII-restricted T cells in CD4\(^{-/-}\) mice persist for a long period of time as memory T cells. Surprisingly, upon reinfecion the secondary MHCII-restricted response in CD4\(^{-/-}\) mice consists mainly of CD8\(^-\)CD4\(^+\) T cells. In contrast to CD8\(^+\) T cells, MHCII-restricted CD8\(^-\)CD4\(^+\) T cells are capable of producing IL-2 in addition to IFN-\(\gamma\) and thus appear to have attributes characteristic of CD4\(^+\) T cells rather than CD8\(^+\) T cells. Therefore, MHCII-restricted T cells in CD4\(^{-/-}\) mice do not share all phenotypic and functional characteristics with MHCI-restricted CD8\(^+\) T cells or with MHCII-restricted CD4\(^+\) T cells, but, rather, adopt attributes from each of these subsets. These results have implications for understanding thymic T cell selection and for elucidating the mechanisms regulating the peripheral immune response and memory differentiation. *The Journal of Immunology, 2004, 173: 2494–2499.*

CD4\(^+\) and CD8\(^+\) T lymphocytes recognize antigenic peptides presented in the context of MHC class II (MHCII)\(^3\) and MHC class I (MHCI) molecules, respectively. This recognition is mediated by TCR binding to the MHC/peptide complex and simultaneous engagement of CD4 coreceptor with MHCI or of CD8 coreceptor with MHCII (1). Upon TCR ligation, CD4\(^+\) and CD8\(^+\) T cells undergo a differentiation process that gives rise to effector cells. CD4\(^+\) Th cells become either Th1 cells, which produce hallmark Th1 cytokines, including IFN-\(\gamma\), and provide help for cell-mediated immunity, or Th2 cells, which support the generation of humoral responses (2). CD8\(^+\) effector T cells also produce IFN-\(\gamma\), but are generally not described as providing help for cell-mediated or humoral immunity (3). Instead, CD8\(^+\) T cells are endowed with cytotoxic abilities that mediate the killing of infected cells and thus play a crucial role in cell-mediated immune responses (3). Although CD4\(^+\) and CD8\(^+\) T cells have varying effector functions and play different roles in the immune response, they are derived from a common precursor through a complex process of thymic selection (4). Precisely how their thymic education specifies their MHC restriction and functionality has yet to be thoroughly defined (4–6). Similarly, it remains to be determined what further regulates the differentiation of peripheral CD4\(^+\) and CD8\(^+\) T cells that allows them to produce such diverse effector functions during an infection (2).

CD4\(^{-/-}\) mice have been used as a model to study thymocyte development in the absence of the CD4 molecule (7, 8) and peripheral immune responses to infection in the absence of T cell help (9–14). Early work has revealed the existence of MHCII-restricted T cells in CD4-deficient mice that express neither CD4 nor CD8, but are nonetheless able to develop into mature lymphocytes in the absence of a coreceptor to drive maturation (13, 14). This double-negative CD8\(^-\)CD4\(^-\) (DN) (3) T cell population has been shown to be capable of adopting characteristics of Th cells, in that they are able to protect mice from *Leishmania major* infection (14), mediate Ab class switching (13, 14), and support somatic hypermutation and affinity maturation of germinal center B cells (15). In contrast, studies using MHCII-restricted TCR transgenic mice to investigate thymic selection have shown that in the absence of CD4, MHCII-restricted T cells are misdirected into the CD8 lineage (16). Consistent with this, MHCII-restricted CD8\(^+\) CTLs have been observed in secondary bulk cultures of splenocytes from CD4\(^{-/-}\) mice that have been infected with mouse hepatitis virus (MHV) (17). However, in both the *L. major* and MHV systems, the Ag specificities of MHCII-restricted T cells are not defined, and their responses are very low, requiring secondary in vitro expansion for their detection and analysis. This has precluded direct ex vivo analysis of the phenotypes and functions of MHCII-restricted T cells in CD4\(^{-/-}\) mice, thus leaving many questions unanswered. For example, it is not known why the MHCII-restricted response in CD4\(^{-/-}\) mice is mediated by DN T cells in the case of *L. major*, but by CD8\(^+\) T cells in the MHV system. It remains possible that the MHCII-restricted response in CD4\(^{-/-}\) mice is comprised of both DN and CD8\(^+\) T cells, but a biased expansion of either population under differing in vitro culture conditions results in the disparity observed between the *L.*
major and MHV systems. More importantly, it is not known whether MHCI-restricted CD8+ and DN T cells acquire different effector functions and thus serve different roles in the host defense. In addition, whether either of these populations can persist as memory T cells capable of mounting a robust recall response to reinfection has yet to be determined. In this study we examined the phenotypes and effector functions of MHCI-restricted T cells in CD4−/− mice and the ability of these cells to persist as memory cells and mount a secondary response.

We show that CD4−/− mice mounted a strong CD8+ T cell response to MHCI-restricted epitopes after a primary infection by *Listeria monocytogenes* (LM) or lymphocytic choriomeningitis virus (LCMV). These MHCI-restricted, epitope-specific CD8+ T cells are readily detectable at the single-cell level, thus allowing direct ex vivo analysis of their phenotypes and functions in comparison with MHCI-restricted CD4+ and MHCI-restricted CD8−/− T cells. Our results show that the MHCI-restricted CD8+ T cells are more similar to classical CD8+ T cells than to CD4+ T cells in their expression of effector functions, although they also differ from MHCI-restricted CD8+ T cells by their inability to produce high levels of the cytolytic molecule granzyme B. MHCI-restricted CD8+ T cells were not detectable in wild-type (WT) mice even when CD4+ T cells were depleted, suggesting that they probably arise in CD4−/− mice as a result of misdirected selection in the thymus. Epitope-specific MHCI-restricted T cells in CD4−/− mice can persist for an extended period of time after resolution of a primary infection. Surprisingly, the majority of the secondary MHCI-restricted response in CD4−/− mice upon reinfection is mounted by DN T cells. In contrast to CD8+ T cells, MHCI-restricted DN T cells are capable of producing IL-2 and thus appear to have attributes characteristic of CD4+ T cells, rather than CD8+ T cells. These results have implications for understanding CD4/CD8 lineage commitment during thymic selection and the roles of the coreceptor and MHC restriction in dictating effector differentiation of peripheral CD4+ and CD8+ T cells. In addition, the data suggest that the CD4−/− model might not be an ideal system to study the role of CD4+ T cells and their help in mediating protective immunity to various infections.

**Results**

**CD8+ T cells respond to the MHCI-restricted LLO<sub>190−201</sub> peptide in CD4−/− mice after LM infection**

In our studies investigating the role of CD4+ T cell help in generating functional CD8+ T cell memory, we noted that CD4−/− mice mounted a response to an MHCI-restricted epitope after LM infection. The WT mice produced substantial CD4+ T cell responses to the previously described I-A<sup>b</sup>-restricted LLO<sub>190−201</sub> (22), whereas MHCI− T cells did not mount a detectable T cell response to this epitope. Surprisingly, a subset of T cells in CD4−/− mice was able to respond to the LLO<sub>190−201</sub> epitope, which can be detected even 60 days postinfection (Fig. 1A). To further investigate the nature of the MHCI-restricted T cell response in CD4−/− mice, we infected WT and CD4−/− mice with rLM-OVA and examined T cell responses on day 7 postinfection to LLO<sub>190−201</sub> and, as a control, to the H-2K<sup>b</sup>-restricted OVA<sub>257−264</sub> epitope (Fig. 1B). As expected, CD8+ T cells responded to the OVA<sub>257−264</sub> epitope in both WT and CD4−/− mice, and CD4+ T cells were depleted by the LLO<sub>190−201</sub> epitope in WT mice. However, the LLO<sub>190−201</sub>-specific response in CD4−/− mice was primarily mounted by CD8+ T cells, rather than by DN T cells as seen in other systems (13, 14). These data revealed the existence of a population of MHCI-restricted CD8+ T cells in CD4−/− mice capable of responding to LM infection.

To further characterize the phenotype of this unusual subset of MHCI-restricted CD8+ T cells in an epitope-specific manner, we examined the ability of LLO<sub>190−201</sub>-specific T cells to make effector molecules (IFN-γ, IL-2, TNF-α, and granzyme B). Whereas LLO<sub>190−201</sub>-specific CD4+ T cells from WT mice responded strongly by producing IFN-γ, IL-2, and TNF-α (Fig. 1C), the majority of LLO<sub>190−201</sub>-specific CD8+ T cells from infected CD4−/− mice produced only IFN-γ, not IL-2 or TNF-α (Fig. 1C). In addition, neither LLO<sub>190−201</sub>-specific CD4+ T cells from WT mice nor LLO<sub>190−201</sub>-specific CD8+ T cells from CD4−/− mice produced IL-10 or IL-4 in response to LM infection (data not shown). Therefore, during primary LM infection, the cytokine profile of LLO<sub>190−201</sub>-specific CD8+ T cells in CD4−/− mice is more similar to that of OVA<sub>257−264</sub>-specific CD8+ T cells in WT mice (Fig. 1C) than it is to LLO<sub>190−201</sub>-specific CD4+ T cells in WT mice. LLO<sub>190−201</sub>-specific T cells from CD4−/− mice may have a slightly higher level of granzyme B than LLO<sub>190−201</sub>-specific CD4+ T cells from WT mice; however, the granzyme B level in LLO<sub>190−201</sub>-specific T cells is much lower than that in OVA<sub>257−264</sub>-specific CD8+ T cells. Therefore, during primary LM infection in CD4−/− mice, MHCI-restricted CD8+ T cells express some effector properties of CD4+ T cells and some effector properties of traditional MHCI-restricted CD8+ T cells, thus displaying an effector phenotype distinct from that of classical CD4+ or CD8+ T cells.

**CD8+ T cells respond to the MHCI-restricted gp<sub>61−80</sub> peptide in CD4−/− mice after LCMV infection**

To investigate whether the MHCI-restricted CD8+ T cell response in CD4−/− mice is unique to the LLO<sub>190−201</sub> epitope after LM infection or represents a more general phenomenon, we infected WT and CD4−/− mice with the Armstrong strain of LCMV. On day 7 after infection, T cell responses to the H-2K<sup>b</sup>-restricted epitope gp<sub>33−41</sub>, and to the I-A<sup>b</sup>-restricted epitope gp<sub>61−80</sub>, were
zyme B staining are gated on IFN-γ/H9253.

These results indicate that CD8 T cells from CD4 mice are CD8+. Although MHCII-specific T cells can develop in the CD8 lineage when CD4 is absent, an early study indicated that CTLs fail to develop in CD8−/− mice (23). However, this early study was limited by the lack of sensitive assays available at the time for detecting epitope-specific T cells. To rigorously test whether a corresponding MHCII-restricted response might exist in CD8−/− mice, we re-examined this question in our system using intracellular cytokine staining and MHCII/peptide tetramers. WT and CD8−/− mice were infected with rLmOVA, and CD4+ and CD8+ T cell responses to LLO190−201 and OVA257−264 were assessed by intracellular cytokine staining on day 7 postinfection. CD4+ T cells from infected WT and CD8−/− mice mounted a similarly robust response to restimulation with the MHCII-restricted LLO190−201 peptide (Fig. 3). Although CD8+ T cells responded to OVA257−264 in WT mice, we were unable to detect a population of MHCII-restricted, OVA257−264-specific T cells in CD8−/− mice (Fig. 3). We were also unable to detect, by intracellular cytokine staining or staining with MHCII/peptide tetramers, T cells specific

CD8−/− mice do not mount a response to the MHCII-restricted OVA257-264 peptide

Although MHCII-specific T cells can develop in the CD8 lineage when CD4 is absent, an early study indicated that CTLs fail to

FIGURE 1. CD8+ T cells respond to the MHCII-restricted LLO190–201 epitope in CD4−/− mice after LM infection. A, C57BL/6 WT and CD4−/− mice, but not MHCII−/− mice mount an LLO190–210-specific response. B, LLO190–201-specific T cells in CD4−/− mice are CD8+. C, MHCII-restricted CD8+ T cells produce IFN-γ, but little TNF-α, IL-2, and granzyme B. Splenocytes from rLmOVA-infected mice (A, >day 60 postinfection; B and C, day 7 postinfection) were stimulated by MHCII-restricted LLO190–201- or MHCII-restricted OVA257–264 peptides, followed by intracellular staining for cytokines or granzyme B. The numbers indicate the percentage of total splenic T cells (Thy1.2+ gated). Histograms for granzyme B staining are gated on IFN-γ+ cells responding to LLO190–210 or OVA257–264 stimulation.

FIGURE 2. CD8+ T cells respond to the MHCII-restricted gp61–80 epitope in CD4−/− mice after LCMV infection. Splenocytes from LCMV-infected mice (day 7 postinfection) were stimulated with MHCII-restricted gp61–80, or MHCII-restricted gp33–41 peptides, followed by intracellular IFN-γ staining. The numbers indicate the percentage of total splenic T cells (Thy1.2+ gated).

FIGURE 3. CD8−/− mice do not respond to the MHCII-restricted OVA257–264 epitope after LM infection. Splenocytes from rLmOVA-infected WT and CD8−/− mice (day 7 postinfection) were stimulated with MHCII-restricted OVA257–264 or MHCII-restricted LLO190–201, followed by intracellular IFN-γ staining. The numbers indicate the percentage of splenic T cells (Thy1.2+ gated).
to MHC-I-restricted gp34-41 and nuclear protein 2497 epitopes after LCMV infection (data not shown). Thus, unlike CD4–/– mice that are able to respond to MHCII-presented Ag, CD8–/– mice do not mount MHC-I-restricted responses after LM infection.

CD8+ T cells responding to the MHCII-restricted LLO190–201 peptide are absent in CD4-depleted WT mice

It is possible that MHCII-restricted CD8+ T cells exist in WT mice, but they are difficult to detect because of the presence of MHCII-restricted CD4+ T cells, which are capable of out-competing MHCII-restricted CD8+ T cells due to their larger numbers or greater binding affinity for MHCII ligand. To investigate this possibility, we used anti-CD4 mAb to deplete WT mice of CD4+ T cells before infection with LM and assayed T cell responses to LLO190–201 and OVA257–264 7 days postinfection by intracellular IFN-γ staining. As anticipated, the control mice mounted robust CD4+ and CD8+ T cell responses to LLO190–201 and OVA257–264 peptides, respectively (Fig. 4). The CD8+ T cells in CD4-depleted mice responded strongly to the OVA257–264 epitope, but there was no response against the LLO190–201 epitope in CD4-depleted mice. These data suggest that there is no endogenous population of MHCII-restricted CD8+ T cells in WT mice and that the MHCII-restricted CD8+ T cells in CD4–/– mice are probably a result of aberrant selection during thymic development.

MHCII-restricted CD8+ T cells persist as memory cells in CD4–/– mice and mount effective recall responses

Although MHCII-restricted T cells develop in the CD8 lineage in CD4–/– mice and are capable of mounting a response to primary infection, it is possible that due to the absence of the predicted coreceptor match with the presenting MHCII, these cells would fail to develop into a stable memory population and/or respond normally during a challenge infection. To examine the ability of MHCII-restricted CD8+ T cells to become memory cells, we infected WT and CD4–/– mice with rLmOVA and >60 days postinfection assayed for the presence of LLO190–201-specific T cells. MHCII-restricted LLO190–201-specific CD8+ T cells were detectable in CD4–/– mice at levels similar to those of MHCII-restricted CD4+ T cells in WT mice (Fig. 5A, top panels). These results indicate that LLO190–201-specific CD8+ T cells in CD4–/– mice can persist as long-lasting memory lymphocytes.

To further examine LLO190–201-specific memory in CD4–/– mice, we assayed the ability of these animals to mount a recall response to the LLO190–201 epitope. WT and CD4–/– mice that resolved a primary rLmOVA infection (>60 days) were challenged with rLmOVA, and the LLO190–201-specific response was measured on day 7 after challenge. CD4–/– mice were able to mount an LLO190–201-specific recall response similar in magnitude to that of WT mice (Fig. 5A, bottom panels). Interestingly, the IFN-γ-producing cells responding to challenge infection in the CD4–/– animals consisted of DN and CD8+ T cells, with the majority of the population being DN. Furthermore, the DN population in the challenge-infected CD4–/– mice was also capable of producing IL-2 in response to LLO190–201 (Fig. 5B), whereas the CD8+ LLO190–201-specific cells in these animals did not make this cytokine (Fig. 5). Thus, LLO190–201-specific T cells can persist in CD4–/– mice as memory cells and mount a strong recall response that consists primarily of DN T cells. This is in striking contrast to the primary response in CD4–/– mice, where the LLO190–201-specific response is comprised mainly of CD8+ T cells.

Discussion

Previous studies have shown the existence of MHCII-restricted DN and CD8+ T cells after L. major and MHV infection of CD4–/– mice, respectively (14, 17). However, the Ag specificities of these MHCII-restricted T cells have not been defined, and their responses are very low, requiring secondary in vitro expansion for their detection and analysis. In this study we show that CD4–/– mice mounted a strong CD8+ T cell response to well-defined, MHCII-restricted epitopes after a primary bacterial or viral infection. These MHCII-restricted, epitope-specific CD8+ T cells in
CD4+/− mice are readily detectable and reach levels close to those of MHCI-restricted CD4+ and MHCII-restricted CD8+ T cells, suggesting that they make up a substantial number of peripheral T cells in CD4+/− mice. These results are consistent with a recent study demonstrating that the CD8+ T cell pool in CD4+/− mice is heavily contaminated with MHCI-restricted T cells (24). Our results further show that MHCI-restricted CD8+ T cells do not share all phenotypic characteristics with MHCII-restricted CD8+ T cells or with MHCI-restricted CD4+ T cells, but, rather, adopt attributes from each of these subsets. MHCI-restricted CD8+ T cells are more similar to classical CD8+ T cells than to CD4+ T cells in that they produce IFN-γ, but little TNF-α and IL-2, during a primary infection. However, they differ from MHCI-restricted CD8+ T cells by their inability to produce high levels of the cytolytic molecule granzyme B, consistent with the finding that these cells are less cytotoxic (24). These results are surprising when considered with previous studies showing that MHCI-restricted responses in CD4+/− are mediated by DN T cells, and that these T cells function like classical CD8+ T cells, providing help for B cells and Th-dependent protection against L. major (13–15).

In light of a strong MHCI-restricted response in CD4+/− mice, we considered the possibility that MHCI-restricted CD8+ T cells might also exist in WT mice at a very low frequency and that their response might be inhibited by competition from large numbers of CD4+ T cells, which are likely to have higher binding affinity for MHCI. Indeed, a small population of CD8+ T cells in infected mice did produce IFN-γ upon in vitro stimulation with the MHCI-restricted peptides (Figs. 1B and 2). However, depleting CD4+ T cells in WT mice before infection did not result in the outgrowth of an MHCI-restricted CD8+ T cell response (Fig. 4). Instead, the slight CD8+ cell response to LLO190-201 observed in WT mice was greatly decreased after CD4+ T cell depletion, suggesting that the WT CD8+ LLO190-201 response is most likely due to bystander activation from activated CD4+ T cells in the same in vitro culture. Thus, the lack of MHCI-restricted CD8+ T cell responses in WT mice is not due to competition or active suppression by endogenous MHCI-restricted CD4+ T cells. We interpret these data as evidence that MHCI-restricted CD8+ T cells in CD4+/− mice arise as a result of aberrant positive selection during thymic development when the CD4 coreceptor is absent.

The occurrence of MHCI-restricted CD8+ T cells in CD4+/− mice may be explained on the basis of the strength of signal model of CD4/CD8 lineage commitment during thymic positive selection (4–6, 25). This model postulates that commitment to the CD4 vs CD8 lineage depends on the intensity or duration of signaling from the TCR. Strong and prolonged signals lead to the CD4 pathway, whereas weak and short duration signals promote CD8 lineage choice. Thus, it is possible that some MHCI-restricted TCR, which normally have a strong affinity and direct differentiation to the CD4 pathway when the CD4 coreceptor is engaged, would provide a weaker signal in CD4+/− mice, leading to differentiation into the CD8 lineage. This model would also predict that it is unlikely for MHCI-restricted CD4+ T cells to develop in CD8+/− mice because lack of the CD8 coreceptor would only result in weaker binding affinity and thus be even less conducive to CD4 lineage commitment. Consistent with this prediction, our results show that unlike in CD4+/− mice, there is not a corresponding population of MHCI-restricted CD4+ T cells in CD8+/− mice capable of responding to LM or LCMV. Furthermore, our data show that the MHCI-restricted CD4+ T cell response in CD8+/− mice is not reduced (Fig. 3). This is in contrast to the decreased MHCI-restricted CD8+ T cell response evident in CD4+/− mice, which is thought to be due to contamination of the CD8+ population by MHCI-restricted T cells (24). Our findings of no MHCI-restricted response and normal CD4+ T cell response in CD8+/− mice suggest that the CD4+ T cell pool in these mice is not contaminated with MHCI-restricted T cells, in contrast to the CD8+ T cell pool in CD4+/− mice.

Our data show that MHCI-restricted T cells in CD4+/− mice are capable of persisting as long-lasting memory cells and of mounting an effective recall response to LM reinfection. Surprisingly, however, the secondary response to MHCI-restricted epitopes in CD4+/− mice consists of both CD8+ T cells and DN T cells, but it is the DN population that responds most strongly after reinfection. This is in striking contrast to the primary MHCI-restricted response in CD4+/− mice, which is comprised mostly of CD8+ T cells. At present it is not known why different populations dominate the primary and secondary responses. This may simply reflect the fact that the precursor frequency of MHCI-restricted DN T cells is much lower than that of MHCI-restricted CD8+ T cells in naive CD4+/− mice. In contrast, memory pools may contain more MHCI-restricted DN T cells due to preferential differentiation of these cells into long-lasting memory cells or conversion of CD8+ T cells into DN T cells during memory differentiation. In this regard, it is interesting to note that there were DN T cells detectable in the memory population (Fig. 5A, top panels). Recent studies have shown that memory CD8+ T cells generated in CD4+/− mice are defective in their ability to expand upon restimulation in vitro and in vivo (11, 12, 26, 27). It is thus possible that MHCI-restricted CD8+ T cells might be less capable of mounting a robust recall response than MHCI-restricted DN memory T cells. If true, this raises the possibility that MHCI-restricted responses to L. major in CD4+/− mice may also consist of both CD8+ and DN T cells, but the DN T cell population is preferentially expanded upon in vitro restimulation, which is required for detecting MHCI-restricted responses in this system. Alternatively, the MHCI-restricted response to L. major infection in CD4+/− mice may consist of only DN T cells. Unlike LM and LCMV, L. major does not grow in the host cell cytoplasm, and this difference may explain the apparent lack of an MHCI-restricted CD8+ T cell response. However, this is unlikely, because MHC II-restricted Ags should not require access to the cytoplasm for their presentation. Furthermore, CD8+ T cells are known to respond to MHCI-restricted Ags from L. major and other pathogens that do not replicate in the cytoplasm, such as Salmonella and Mycobacterium (28–30). Nevertheless, it would be interesting to determine whether CD4+/− mice mount an MHCI-restricted CD8+ T cell response to an LM mutant that is unable to escape into the cytoplasm.

In addition to being a major component of the recall response in CD4+/− mice, the MHCI-restricted DN T cells produce IL-2 upon ex vivo stimulation, indicating that they have attributes characteristic of CD4+ T cells rather than CD8+ T cells. In contrast to DN T cells, CD8+ T cells specific to the same MHCI-restricted epitope did not produce IL-2. These data suggest a possible role for the CD8 coreceptor in down-regulating the production of IL-2 in CD8+ T cells. Together, our results have implications for understanding thymic T cell selection, regulation of peripheral immune responses and memory differentiation, and the limitations of CD4+/− mice as a model for studying the role of Th cells in immunity.

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References


