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Cutting Edge: CD4 and CD8 T Cells Are Intrinsically Different in Their Proliferative Responses[1]

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In this study, we compared the proliferation and differentiation of Ag-specific CD4 and CD8 T cells following *Listeria* infection. Our results show that CD4 T cells responding to infection divide a limited number of times, with progeny exhibiting proliferative arrest in early divisions. Even with increased infectious doses, CD4 T cells display this restricted proliferative pattern and are not driven to undergo extensive clonal expansion. This is in striking contrast to CD8 T cells, which undergo extensive proliferation in response to infection. These differences are also evident when CD4 and CD8 T cells receive uniform anti-CD3 stimulation in vitro. Together, these results suggest that CD4 and CD8 T cells are programmed to undergo limited and extensive proliferation, respectively, to suit their function as regulator and effector cells. ***The Journal of Immunology*****, 2002, 168: 1528–1532.**

Murine listeriosis has been a useful model for investigating T cell responses to infection (reviewed in Ref. 1). The infectious agent, *Listeria monocytogenes* (LM),[3] is a Gram-positive bacterium that invades host cells, escapes from the endosome, and replicates within the host cell cytosol (2). LM proteins are presented by both MHC class I and class II pathways and stimulate strong CD8 and CD4 T cell responses (1, 3). The in vivo dynamics of the CD8 T cell response to infection has been studied extensively through the analysis of specific responses to native LM epitopes as well as foreign epitopes expressed by recombinant LM (3–5). Two recent studies have shown that the extent of CD8 T cell proliferation is not determined by the amount or duration of Ag presentation (6, 7), leading to the hypothesis that CD8 T cells undergo autonomous clonal expansion in an Ag-independent fashion following in vivo priming. Further studies both in vitro and in vivo have demonstrated that stimulation of CD8 T cells triggers a developmental program that instructs daughter cells to continue to divide and differentiate into effector and memory T cells (7–10).

Much less is known about the in vivo dynamics of CD4 T cell responses to infection. While several MHC class II-restricted LM epitopes have been defined through the in vitro analysis of T cell clones (1, 11), the frequencies of CD4 T cells responding to these known epitopes are too low to allow the direct measurement of Ag-specific CD4 T cell responses. This limitation has thus far hindered our ability to assess the in vivo dynamics of CD4 T cell responses in most infections. In this study, we developed a system to quantitate Ag-specific CD4 T cell responses in vivo, using an adoptive transfer model (12) that couples the use of a recombinant LM expressing OVA with OVA-specific transgenic cells. Our results show that the extent of CD4 T cell proliferation and differentiation is strikingly different from that of CD8 T cells.

Materials and Methods

Mice, Abs, and bacterial strains

BALB/c-TgN(DO11.10)10Loh, C57BL/6-P14, OT-I, and OT-II mice were previously described (13–16). OT-I and OT-II mice were bred onto the B6.PL-Thy1.1 background. B6.PL-Thy1.1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and BALB/c-Thy1.1 mice were obtained from C. Surh at The Scripps Institute (La Jolla, CA). All mAbs were from BD PharMingen (San Diego, CA), except the KJ1-26 from Caltag Laboratories (Burlingame, CA). Construction and Western blot analysis of rLM-OVA and rLM-gp33 strains were performed as described (17). Both strains were derived from the wild-type strain 10403s and described previously (18, 19). The LD_{50} of rLM-OVA in BALB/c mice is $\sim 5 \times 10^5$ CFU and the LD_{50} of rLM-OVA and rLM-gp33 in C57BL/6 mice is $\sim 5 \times 10^6$ CFU.

Analysis of T cell proliferation following LM infection in vivo

Splenocytes from DO11.10, P14, OT-I, or OT-II transgenic mice were labeled with CFSE as described (20). A total of 2×10^7 CFSE-labeled splenocytes (2×10^6 specific cells) were transferred into Thy1.1 or Thy1.2 congenic mice (21), which were then infected with the indicated doses of rLM-OVA or rLM-gp33. For the OT-I/OT-II combined transfer, T cells were enriched by depleting splenocytes with B220 and MHC II MicroBeads by MACS (Miltenyi Biotec, Auburn, CA) and CFSE labeled, and 2×10^6 of each cell type were cotransferred per mouse. Proliferation of transferred cells was visualized by FACS analysis of their CFSE profile. Transferred DO11.10 cells were identified by staining with mAb to Thy1.2, CD4, and the KJ1-26 clonotypic mAb, P14 cells were identified by staining with mAb to Thy1.2, CD8, and/or the D^b/gp33 tetramer (22, 23), and OT-I and OT-II cells were identified by staining with mAb to Thy1.1, Vα2, and CD8 or CD4, respectively. Intracellular IFN-γ staining was performed as described following 5 h of in vitro stimulation with 3 μM $OVA_{323–339}$ or 1 μM gp33–41 peptides (23).

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[3] Abbreviations used in this paper: LM, *Listeria monocytogenes*; LCMV, lymphocytic choriomeningitis virus.

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Analysis of T cell proliferation following in vitro stimulation

CFSE-labeled splenocytes from BALB/c or C57BL/6 mice were stimulated in vitro as described (24, 25), using soluble anti-CD3 mAb (1 μg/ml; BD PharMingen) in the presence of human rIL-2 (10 U/ml; BD PharMingen). Proliferation of CD4 and CD8 T cells was visualized by FACS analysis and their CFSE profiles were analyzed as described (25).

Results and Discussion

To facilitate the analysis of Ag-specific CD4 T cell responses to infection, we used a recombinant LM strain (rLM-OVA) that expresses a well-defined model Ag, OVA. rLM-OVA contains a chromosomally integrated Ag cassette encoding truncated OVA (aa 134–387). The OVA sequence was fused to a virulence gene (*hly*) promoter and signal sequence that control the expression and secretion of OVA (Fig. 1*A*). Western blot analysis revealed the presence of the OVA fusion protein in the culture supernatant of rLM-OVA but not in that of the parental wild-type strain (wtLM), demonstrating the synthesis and secretion of OVA by rLM-OVA (Fig. 1*B*).

To test whether infection with rLM-OVA induces an OVA-specific CD4 T cell response, we used an adoptive transfer model using DO11.10 transgenic cells labeled with CFSE (20). DO11.10 cells express a TCR that recognizes a MHC class II-restricted OVA peptide ($OVA_{323–339}$) and can be detected with the KJ1-26 clonotypic mAb (22). CFSE-labeled DO11.10 cells were transferred into congenic Thy1.1 BALB/c mice and recipients were infected with rLM-OVA or wtLM. In mice infected with rLM-OVA, KJ1-26$^+$ cells divided numerous times by 8 days postinfection, as seen by incremental loss of CFSE fluorescence intensity (Fig. 1*C*). In contrast, very little proliferation of KJ1-26$^+$ cells occurred in wtLM- or PBS mock-infected recipients. Thus, rLM-OVA specifically induces proliferation of OVA-specific cells in this adoptive transfer model, thereby providing an in vivo system to analyze the response of Ag-specific CD4 T cells in the context of an infection.

Initial examination of the CD4 T cell response to rLM-OVA infection (Fig. 1) revealed a proliferative pattern that is strikingly different from that of CD8 T cells reported recently (7–9). We thus compared proliferation of Ag-specific CD4 and CD8 T cells following LM infection. We used the system described above using rLM-OVA and DO11.10 cells to examine the Ag-specific CD4 T cell response in vivo. To assess the CD8 T cell response, we used a similar adoptive transfer model in which B6.PL-Thy1.1 mice received CFSE-labeled CD8 T cells from P14 TCR-transgenic mice (26), which express a TCR specific to an H-2D^b-restricted epitope (gp33–41) from lymphocytic choriomeningitis virus (LCMV). The recipient mice were then infected with rLM-gp33, which is isogenic to rLM-OVA but expresses the LCMV gp33–41 epitope.

As shown in Fig. 2*A*, the extent of proliferation was strikingly different between CD4 and CD8 T cells. gp33-specific P14 CD8 T cells had already responded with substantial proliferation by day 3 postinfection with rLM-gp33. By day 8, all CD8 T cells recruited into division were CFSE negative, indicating that these cells had divided at least seven times. In contrast, only a few OVA-specific

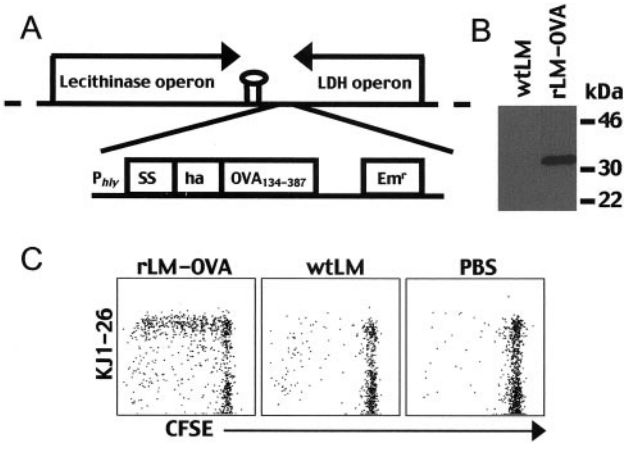

FIGURE 1. rLM-OVA induces an OVA-specific CD4 T cell response. *A*, Schematic diagram of the Ag cassette in rLM-OVA. The cassette is integrated into the genome between two convergent transcriptional units, the lecithinase and lactate dehydrogenase (LDH) operons. It contains truncated OVA (aa 134–387) fused to the ha mAb epitope, the signal sequence (SS) and promoter (P_{hly}) of the *hly* gene, and an erythromycin-resistance gene (Em^r). *B*, Western blot analysis of supernatants from rLM-OVA and wtLM cultures. The OVA fusion protein (35 kDa) was detected using mAb to the ha epitope. *C*, Proliferation of OVA-specific CD4 T cells in rLM-OVA-infected mice. CFSE-labeled splenocytes from DO11.10 mice were adoptively transferred into BALB/c-Thy1.1 recipients, which were then infected with 5×10^4 CFU (0.1 LD_{50}) of rLM-OVA or 5×10^3 CFU (0.1 LD_{50}) of wtLM, or mock-infected with PBS. On day 8 postinfection, splenocytes were harvested and stained with mAb to Thy1.2, CD4, and KJ1-26. DO11.10 cells were identified by gating on Thy1.2$^+$CD4$^+$ cells and analyzed for CFSE fluorescence intensity. Data are representative of three experiments with at least two mice per group.

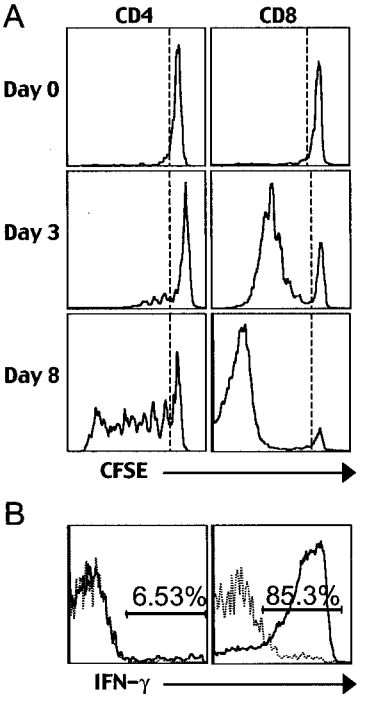

FIGURE 2. CD4 T cells undergo limited division and clonal expansion in comparison to CD8 T cells. CFSE-labeled splenocytes from DO11.10 and P14 mice were adoptively transferred into congenic Thy1.1 recipients, which were then infected with 5×10^4 CFU (0.1 LD_{50}) of rLM-OVA or 5×10^5 CFU (0.1 LD_{50}) of rLM-gp33, respectively. On days 0, 3, and 8 postinfection, splenocytes were harvested and stained with Thy1.2, CD4, and KJ1-26 mAb to identify DO11.10 cells or with Thy1.2, CD8 mAb, and/or D^{b}gp33 tetramer to identify P14 cells. *A*, Proliferation of OVA-specific CD4 T cells and gp33-specific CD8 T cells was analyzed by measuring their CFSE fluorescence intensity (nonrecruited cells are separated from recruited cells by a dashed line). *B*, IFN-γ production by responding OVA-specific CD4 T cells and gp33-specific CD8 T cells on day 8 postinfection was measured by intracellular cytokine staining (dotted lines, nonrecruited cells; solid lines, recruited cells). Data are representative of three experiments with at least two mice per group.

CD4 T cells had divided by day 3 postinfection with rLM-OVA. Even by day 8, the responding CD4 T cells had divided only a limited number of times and very few cells were present in the peak representing seven or more rounds of division. This difference in proliferation was reflected in the expansion of Ag-specific CD4 and CD8 T cell populations. At the peak of T cell responses on day 8, the OVA-specific CD4 T cell population increased 10-fold from 3.9×10^4 cells/spleen before infection to 3.4×10^5 cells/spleen, while the gp33-specific CD8 T cell population expanded >100-fold from 1.2×10^5 cells/spleen to 1.6×10^7 cells/spleen. Thus, CD8 T cells undergo extensive proliferation and massive clonal expansion while CD4 T cells undergo limited division and restricted clonal expansion.

We also compared CD4 and CD8 T cell differentiation in response to LM infection. Upon activation, CD4 T cells differentiate into polarized Th1 or Th2 cells, which produce distinct sets of cytokines (27). Previous studies have demonstrated that LM infection induces a strong Th1 response and LM-specific CD4 T cells in bulk culture secrete substantial amounts of IFN-γ (11), a hallmark cytokine of the Th1 response. Surprisingly, our results show that only a small fraction (<7%) of CD4 T cells that were recruited into division produced IFN-γ (Fig. 2*B*), indicating that most responding CD4 T cells did not fully differentiate into IFN-γ-producing Th1 cells. In sharp contrast, most (>85%) of the recruited CD8 T cells differentiated into IFN-γ-producing effector cells (Fig. 2*B*). These results are consistent with the recent reports that differentiation and polarization of CD4 T cells are remarkably inefficient (28, 29) and that CD8 T cells become committed to differentiating fully into effector cells upon stimulation (8, 9).

We considered the possibility that the CD4 T cells present in the early divisions could continue to divide many rounds over time, resulting in a pattern of proliferation and extent of clonal expansion comparable to those of CD8 T cells. Thus, we analyzed the CFSE profile of OVA-specific CD4 T cells at later time points after rLM-OVA infection. Surprisingly, a large number of responding CD4 T cells on days 14 and 21 postinfection were still present in early divisions and their proliferative patterns remained different from those of CD8 T cells (Fig. 3*A*). These results indicate that responding CD4 T cells did not continue to divide and instead exhibited proliferative arrest at early divisions. To further confirm the resting state of these CD4 T cells, we examined their forward and side scatters. On day 3 postinfection, recruited CD4 T cells had greater forward and side scatter compared with naive cells, indicating that they were actively dividing. By day 8, responding CD4 T cells displayed a phenotype of forward scatter similar to that of naive cells, suggesting that they had returned to a resting state and remained so on days 14 and 21 (Fig. 3*B* and data not shown). While it remains to be determined whether these cells are capable of mounting a recall response, these results clearly show that CD4 T cells exhibit proliferative arrest in the early divisions and persist for at least 21 days.

The differences in proliferative patterns and clonal expansion of CD4 and CD8 T cells may result from disparity in the efficiency of Ag presentation by the MHC class I and II pathways in the context of LM infection. We tested this possibility by examining CD4 and CD8 T cell responses to increasing Ag doses. Specifically, we asked whether limiting Ag dose in our in vivo system would result in proliferative arrest of CD8 T cells in early divisions and, more importantly, whether increasing Ag dose could stimulate CD4 T cells to overcome proliferative arrest and undergo extensive division. Increasing the dose of rLM-gp33 resulted in higher percentages of naive P14 CD8 T cells recruited into division but had little effect on the proliferative pattern of the recruited P14 cells. Even at low doses, recruited CD8 T cells still divided more than seven times, and relatively few cells were detected in early divisions (Fig. 4). Thus, limiting antigenic stimulation did not lead to proliferative arrest of CD8 T cells in early divisions, consistent with results from previous studies (6, 9). Similarly, increasing the dose of rLM-OVA resulted in recruitment of more naive KJ1-26$^+$ cells and generation of higher numbers of total OVA-specific KJ1-26$^+$ cells. However, varying Ag dose did not alter the basic proliferative patterns of these responding cells (Fig. 4). Even at high doses, most CD4 T cells were arrested in the early divisions and did not proceed beyond seven rounds of division. Thus, increasing antigenic stimulation within the limits of sublethal LM infection does not prevent proliferative arrest of CD4 T cells in early divisions. However, this does not mean that CD4 T cells cannot be driven to further divide. Indeed, it is estimated that Ag-specific CD4 T cells proceed through approximately nine cell divisions following LCMV infection. Although CD4 T cells undergo more divisions following LCMV infection than LM infection, CD4 T cells still divide less than CD8 T cells, which undergo 15 divisions on average after LCMV infection (30, 31).

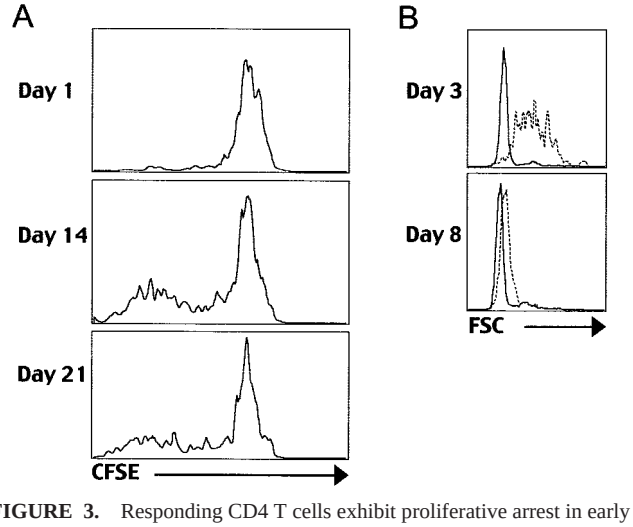

FIGURE 3. Responding CD4 T cells exhibit proliferative arrest in early divisions. *A*, CFSE profiles of OVA-specific cells following rLM-OVA infection. Experiments were performed as described in Fig. 1. *A*, On days 1, 14, and 21 postinfection, CFSE profiles of DO11.10 cells were analyzed. *B*, On days 3 and 8 postinfection, the forward scatters of DO11.10 cells were analyzed (dotted lines, recruited cells; solid lines, naive cells).

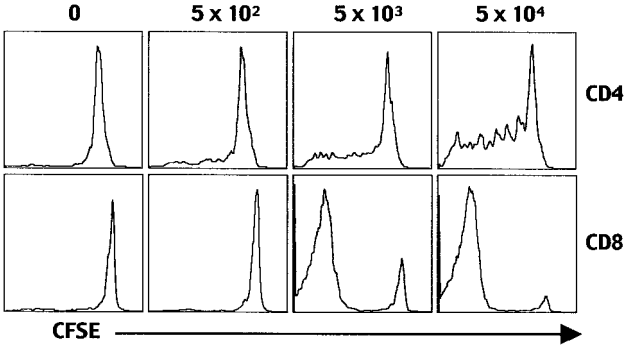

FIGURE 4. Infectious dose has little effect on the proliferative patterns of CD4 and CD8 T cells. Experiments were conducted as described in Fig. 2. CFSE profiles of DO11.10 and P14 cells were analyzed on day 8 postinfection with 0, 5×10^2, 5×10^3, or 5×10^4 CFU of rLM-OVA or rLM-gp33.

To support our data using other transgenic mice, we compared the proliferation of OT-II and OT-I transgenic cells (recently available to us), which recognize H-2^b MHC class II- and MHC class I-restricted epitopes of OVA, respectively. Following adoptive transfer of these cells and infection with rLM-OVA, we observed the same basic proliferative differences between CD4 and CD8 T cells as with DO11.10 and P14 cells (Fig. 5*A*). Furthermore, similar results were seen when OT-II and OT-I cells were cotransferred into same recipient (Fig. 5*B*). These results show that the differences between CD4 and CD8 T cell proliferation hold true with different transgenic cells and are not due to different mouse backgrounds or different strains of rLM.

The proliferative differences between CD4 and CD8 T cells we observed may be unique to LM infection. However, early proliferative arrest of responding CD4 T cells is also evident after infection with recombinant vaccinia virus expressing OVA (data not shown), immunization with OVA peptide in CFA (32), and in vitro stimulation with peptide or anti-CD3 (21, 24). Because T cell proliferation in vivo can be influenced by many factors, we compared the proliferation of CD4 and CD8 T cells following in vitro stimulation with soluble anti-CD3 mAb in the presence of IL-2. This system delivers uniform stimulatory signals to both CD4 and CD8 T cells and allows us to visualize the proliferation of polyclonal T cell populations rather than clonal transgenic populations. In agreement with our in vivo findings, in vitro stimulated CD8 T cells divided more extensively than CD4 T cells. By day 4, most CD8 T cells had undergone more than seven rounds of division while responding CD4 T cells were mostly found in early (1–4) divisions (Fig. 5*C*). Similar differences in the proliferative patterns of CD4 and CD8 T cells were seen when anti-CD28 mAb was included (data not shown). Together, our results suggest that the distinct proliferative patterns of CD4 and CD8 T cells are not due to external signals but are likely controlled by intrinsic regulatory mechanisms. A previous study indicates that CD4 and CD8 T cells have different requirements for activation and clonal expansion (33). Two recent studies show that proliferation of CD4 T cells is negatively regulated by DR6 (34) and is tightly controlled by a cell cycle-coupled regulatory mechanism (24). These inherent differences may provide an explanation for why the magnitude of CD8 T cell responses vastly exceeds that of CD4 T cells in most infections.

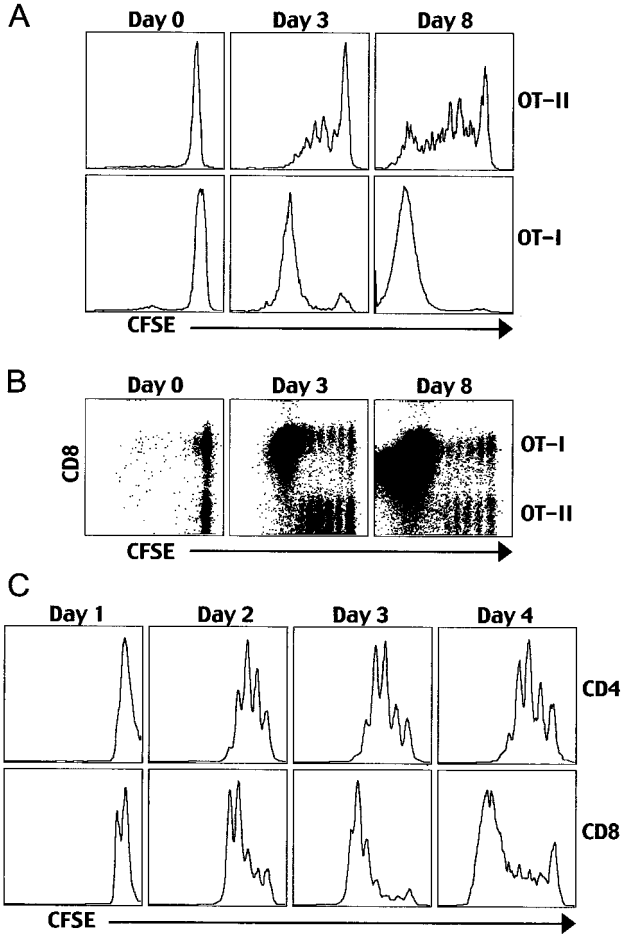

FIGURE 5. CD4 and CD8 T cells are inherently different in their proliferative responses. *A*, CFSE-labeled splenocytes from OT-II and OT-I mice were adoptively transferred into congenic Thy1.2 recipients, which were then infected with 5×10^5 CFU (0.1 LD_{50}) of rLM-OVA. On days 0, 3, and 8 postinfection, proliferation of OVA-specific CD4 and CD8 T cells was analyzed by measuring their CFSE fluorescence intensity. *B*, Same as in *A*, except that enriched T cells from OT-II and OT-I mice were pooled, CFSE-labeled, and adoptively transferred into the same congenic Thy1.2 recipients. OT-I and OT-II cells were identified by first gating on the Thy1.1$^+$Vα2$^+$ subset and then separated by CD8$^+$ staining. *C*, CFSE-labeled splenocytes from C57BL/6 mice were stimulated with anti-CD3 mAb and CFSE profiles of CD4 and CD8 T cells were analyzed at different time points to measure their proliferation. Similar results were obtained when splenocytes from BALB/c mice were used.

CD4 and CD8 T cells are known to recognize different classes of Ags and execute distinct immune functions. In this study, we have demonstrated another fundamental difference between CD4 and CD8 T cells: they are programmed to undergo limited and extensive proliferation, respectively, in response to antigenic stimulation. While the molecular basis for this difference remains to be elucidated, intrinsic control mechanisms likely exist to regulate the proliferative response of CD4 and CD8 T cells to suit their respective roles as regulators and effectors (24, 34). Generation of appropriate helpers and rapid deployment of numerous cytotoxic effectors are essential to the development of an effective adaptive immune response. Understanding their differences will help to develop vaccine strategies tailored to induce optimal CD4 and CD8 T cell responses and thus protective immunity.

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