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Clonal Competition Inhibits the Proliferation and Differentiation of Adoptively Transferred TCR Transgenic CD4 T Cells in Response to Infection

Kathryn E. Fouleds and Hao Shen

CD4 and CD8 T cells have been shown to proliferate and differentiate to different extents following antigenic stimulation. CD4 T cells form a heterogenous pool of effector cells in various stages of division and differentiation, while nearly all responding CD8 T cells divide and differentiate to the same extent. We examined CD4 and CD8 T cell responses during bacterial infection by adoptive transfer of CFSE-labeled monoclonal and polyclonal T cells. Monoclonal and polyclonal CD8 T cells both divided extensively, whereas monoclonal CD4 T cells underwent limited division in comparison with polyclonal CD4 T cells. Titration studies revealed that the limited proliferation of transferred monoclonal CD4 T cells was due to inhibition by a high precursor frequency of clonal T cells. This unusually high precursor frequency of clonal CD4 T cells also inhibited the differentiation of these cells. These results suggest that the adoptive transfer of TCR transgenic CD4 T cells significantly underestimates the extent of proliferation and differentiation of CD4 T cells following infection. The Journal of Immunology, 2006, 176: 3037–3043.
polyclonal CD4 T cells. In addition, the protection provided by these cells increased as the number of transferred cells decreased. Our results suggest that the adoptive transfer of TCR transgenic CD4 T cells may significantly underestimate the extent of proliferation and differentiation of CD4 T cells in response to infection.

Materials and Methods

Mice and bacterial strains

OT-I, OT-II, and BALB/c-TgN(DO11.10)Loh mice were previously described (36–38). OT-I and OT-II mice were bred onto the B6.PL-Thy-1.1 background. C57BL/6 and B6.Ly-5.2/Cr (Ly-5.1) mice were obtained from the National Cancer Institute (Bethesda, MD). B6.PL-Thy-1.1 mice were obtained from The Jackson Laboratory, and BALB/c-Thy-1.1 mice were obtained from C. Suth (The Scripps Institute, La Jolla, CA). Construction of rLM-OVA was described previously (11, 39). The LD₅₀ of rLM-OVA in BALB/c mice is ~5 × 10⁴, and the LD₅₀ of rLM-OVA in C57BL/6 mice is ~5 × 10⁵; experimental mice were infected with 0.1 LD₅₀ of rLM-OVA.

Adoptive transfer

Splenocytes from OT-I, OT-II, DO11.10, or Ly-5.1 mice were transferred with CFSE, as described (40). For OT-I/OT-II combined transfers, T cells were enriched by depleting splenocytes with B220 and MHC class II MicroBeads by MACS (Miltenyi Biotec) and CFSE labeled, and the indicated number of each cell type was cotransferred per mouse. For the Ly-5.1 and individual OT-II and DO11.10 transfers, total splenocytes were labeled with CFSE and the indicated number of splenocytes was transferred. Experimental groups included two to five mice per group, and similar results were observed in at least two independent experiments.

Flow cytometry

Proliferation of transferred cells was visualized by incremental loss of CFSE fluorescence. Transferred OT-I and OT-II cells identified by staining with mAb to Thy-1.1, Vα2, and CD8 or CD4, respectively. DO11.10 mice were identified by staining with mAbs to Thy-1.2, CD4, and KJ1-26. For the OT-II and DO11.10 titration experiment, 3-fold more events were acquired for each dilution of competitors (10⁴, and the LD₅₀ of rLM-OVA in C57BL/6 mice was determined by plating 10-fold serial dilutions of spleen and liver homogenates in 1% Triton X-100PBS on brain heart infusion agar plates (Difco). Bacterial numbers were log transformed before statistical analyses were conducted by t tests (two tailed, 95% confidence intervals) using GraphPad Prism (GraphPad).

Results

Adoptively transferred polyclonal CD4 T cells divide extensively in comparison with monoclonal CD4 T cells

To compare the proliferation of CD4 and CD8 T cells in the context of a natural infection, we developed an adoptive transfer system to visualize primary polyclonal T cell responses. CFSE-labeled CD4 and CD8 T cells from C57BL/6 mice were adoptively transferred into Thy-1.2 or Ly-5.2 congenic mice. For comparison, we also adoptively cotransferred CFSE-labeled monoclonal TCR transgenic CD4 and CD8 T cells from OT-II and OT-I mice (2 × 10⁶ specific cells each), respectively. Recipient mice were subsequently infected with rLM expressing OVA (rLM-OVA). As seen before, OT-II T cells divided to a limited extent with a spectrum of responding cells in each division, while OT-I T cells divided extensively with most of the responding cells becoming CFSE negative, indicating seven or more divisions (Fig. 1A). However,

FIGURE 1. Adoptively transferred polyclonal CD4 T cells undergo extensive division in comparison with monoclonal CD4 T cells. CFSE-labeled OT-II and OT-I cells were transferred into congenic B6.Thy-1.2 mice (A), and CFSE-labeled splenocytes (2 × 10⁵) from B6.Ly-5.1 mice were transferred into congenic B6.Ly-5.2 recipients (B). One day later, the recipients were infected with rLM-OVA, and the response of donor cells was analyzed on days 3 or 5, and 8 postinfection. The cells from the uninfected recipients were analyzed 9 days posttransfer. A. Proliferation of transferred OT-II (Thy-1.1+/Vα2+/CD4+) and OT-I (gated on Thy-1.1+/Vα2+/CD8+) cells as visualized by loss of CFSE. B. Proliferation of polyclonal donor CD4 (CD4+/Ly-5.1+/Ly-5.2-) and CD8 (CD8+/Ly-5.1+/Ly-5.2-) T cells. C. Absolute numbers of CFSE-negative polyclonal donor CD4 and CD8 T cells per spleen on days 5 and 8. D. CD44 expression on CFSE-negative (black line) and CFSE-positive (shaded plots) polyclonal donor CD4 and CD8 T cells on day 8 postinfecction. E. Percentages of host CD4 and CD8 T cells with an activated (CD44high) phenotype on days 0, 5, and 8. Plots in A, B, and D are from a representative mouse, and data in C and E are the mean and SD of each group (five mice per group). Similar results were also obtained with the adoptive transfer of polyclonal T cells using Thy-1.1 donor and Thy-1.2 recipient mice.
much to our surprise, polyclonal CD4 T cells divided to the same extent as polyclonal CD8 T cells with most of the responding cells dividing seven or more times (Fig. 1B). Thus, we observed a difference in the extent of division between adoptively transferred monoclonal CD4 T cells and polyclonal CD4 T cells. Monoclonal CD4 T cells divided a limited number of times in response to infection, while all responding polyclonal CD4 T cells divided extensively. In contrast, both monoclonal and polyclonal CD8 T cells divided extensively in response to infection with the vast majority of responding CD8 T cells becoming CFSE negative (Fig. 1, A and B).

Although the division profiles of polyclonal CD4 and CD8 T cells are similar at the peak of the response on day 8, CFSE-negative CD8 T cells accumulated more rapidly than CFSE-negative CD4 T cells (Fig. 1B). On day 5 postinfection, 1.6 × 10⁵ CFSE-negative CD4 T cells were present compared with 4.1 × 10⁵ CFSE-negative CD8 T cells (Fig. 1C). However, at the peak of the response on day 8, the total numbers of CFSE-negative CD4 and CD8 T cells were nearly the same, 7.4 × 10⁵ and 6.2 × 10⁵ cells, respectively. These results indicate that the CD8 T cell response is greater than the CD4 T cell response early on, yet the magnitude of total CD4 and CD8 T cell responses reaches comparable levels at the peak of the response.

The CFSE-negative polyclonal CD4 and CD8 T cells had increased expression of the activation marker CD44 (Fig. 1D), CD25, and CD132 (data not shown) in comparison with the non-responding CFSE-positive cells, consistent with them having responded to antigenic stimulation. We also compared the activation of host CD4 and CD8 T cells over the course of infection (Fig. 1E). Naive mice contained nearly equal percentages of activated/memory (CD44<sup>high</sup>) CD4 and CD8 T cells, 30 and 34%, respectively. By day 5 postinfection, the CD8 T cell subset had more activated cells (~65% CD44<sup>high</sup>) than the CD4 T cell subset (~45% CD44<sup>high</sup>). However, by the peak of the response on day 8, both CD4 and CD8 T cells contained ~70% activated cells. These results are consistent with the above CFSE data showing that more donor CD8 T cells had accumulated by day 5, yet similar numbers of CFSE-negative CD4 and CD8 T cells were present by day 8 (Fig. 1C). Together, these results strongly indicate that, while more activated CD8 T cells are present early during the course of the immune response, the total CD4 T cell response is as robust as the CD8 T cell response.

In summary, the adoptive transfer of CFSE-labeled splenocytes enabled the simultaneous visualization of polyclonal CD4 and CD8 T cell responses during a primary LM infection. Our results show that polyclonal CD4 T cells, like CD8 T cells, divide extensively in response to LM infection. These results also reveal a discrepancy between the proliferative responses of adoptively transferred monoclonal and polyclonal CD4 T cells.

**Adoptively transferred polyclonal T cells become activated and differentiate to the same extent as host T cells**

We reasoned that the response of adoptively transferred polyclonal T cells was more likely to mimic the endogenous response of host T cells than the response of adoptively transferred monoclonal T cells. However, it is still possible that adoptively transferred T cells may become activated during ex vivo manipulation or may not engraft properly. We therefore examined whether the response of the adoptively transferred polyclonal T cells accurately reflected the response of the endogenous host T cells by comparing the activation and differentiation of the donor and host polyclonal T cells. Donor CD4 and CD8 T cells were activated to the same extent as recipient CD4 and CD8 T cells in naive mice and on days 5 and 8 postinfection, as measured by increased surface expression of CD44 (CD44<sup>high</sup>) (Fig. 2A). For example, at the peak of the response on day 8 postinfection, 73.4 and 70.0% of donor and host CD4 T cells were activated, respectively, and 72.1 and 70.4% of donor and host CD8 T cells were activated, respectively. No statistical differences occurred between donor and host T cells at any time point examined. These results demonstrate that the adoptively transferred T cells followed the same activation kinetics as endogenous T cells.

We also examined the Ag-specific responses of donor and recipient CD4 and CD8 T cells by intracellular IFN-γ staining on days 5 and 8 postinfection (Fig. 2B). CD4 T cells mount a broad response to many different LM epitopes; however, we examined the response to LLO<sub>190–201</sub> because it is clearly immunodominant (41). The donor and host CD4 T cell responses to LLO<sub>190–201</sub> were nearly the same for both time points. Approximately 2% of both donor and host CD4 T cells were LLO<sub>190</sub> specific on day 5. This increased to 9% at the peak of the response on day 8. Likewise, both the donor and host CD8 T cell responses to OVA<sub>257–264</sub> were nearly identical. Approximately 1% of both donor and host CD8 T cells were OVA<sub>257–264</sub> specific on day 5, increasing to ~4% on day 8. No statistical differences were observed between donor and recipient T cells for either T cell subset on the two time points examined. Thus, the same levels of Ag-specific CD4 and CD8 T cells were generated from the donor and host T cell populations.

The activation and Ag-specific response data show that adoptively transferred polyclonal T cells behave like the endogenous T cells in response to LM infection. Thus, CD4 T cells responding to infection most likely undergo extensive proliferation, as seen with adoptively transferred polyclonal CD4 T cells, rather than limited proliferation, as seen with TCR transgenic CD4 T cells.
Reducing the precursor frequency of monoclonal CD4 T cells results in a proliferative profile similar to polyclonal CD4 T cells

We considered the possibility that the limited division of the monoclonal CD4 T cells was due to clonal competition imposed by a high precursor frequency of cells with the same specificity. Therefore, we transferred 3-fold serial dilutions of OT-II TCR transgenic CD4 T cells into recipient mice and examined the extent of division of these cells in response to infection with rLM-OVA. Graded levels of transferred cells were present in the spleens of uninfected control mice, as expected. In mice infected with rLM-OVA, we found that the transfer of decreasing amounts of TCR transgenic CD4 T cells resulted in more extensive division of these cells (Fig. 3A). As seen before, the OT-II cells were nearly equally distributed in divisions 1 through 7 in mice that received the largest number of transferred cells (4.5 x 10⁶ OVA-specific CD4 T cells) on day 8 postinfection. This trend was apparent as early as day 3 postinfection. In contrast, the majority of OT-II cells responding to infection in the mice that received the lowest number of transferred cells (1.5 x 10⁵ OVA-specific CD4 T cells) were CFSE negative on day 8 postinfection, resembling the proliferative pattern of the polyclonal CD4 T cells seen in Fig. 1B. Similar results were also seen with DO11.10 cells, which are on the BALB/c background and are specific for the same CD4 T cell epitope of OVA as OT-II cells (Fig. 3). We have shown previously that the DO11.10 cells that proliferate in response to rLM-OVA infection do so in an Ag-specific manner because there is no proliferation of the TCR transgenic cells in response to infection with wild-type LM (11). Furthermore, use of the KJ1-26 clonotypic Ab in this model ensures that the CFSE-negative cells are indeed Ag specific.

The above experimental design required the FACS acquisition of increasingly more cells as the number of transferred cells decreased to have sufficient donor cells for analysis. To exclude the potential biases this may introduce, we next cotransferred CFSE-labeled OT-II and OT-I TCR transgenic T cells (2 x 10⁵ specific cells each) with increasing numbers of an unlabeled mixture of purified OT-II and OT-I splenocytes, and then infected the recipients with rLM-OVA. This allowed us to follow the proliferation of the same number of labeled TCR transgenic CD4 and CD8 T cells in the face of increasing numbers of clonal T cells in the same mouse. TCR transgenic CD4 T cells underwent fewer rounds of division with increasing numbers of unlabeled OT-II competitors (Fig. 3B), as seen when large numbers of labeled OT-II cells were transferred (Fig. 3A). In contrast, OT-I TCR transgenic CD8 T cells underwent at least seven rounds of division even in the presence of high numbers of unlabeled OT-I competitors (Fig. 3B). A direct comparison between CD4 and CD8 T cells is difficult to make because the number of class I and class II OVA epitopes presented during infection with rLM-OVA is not likely the same, and the affinity of the OT-II TCR is weaker than the OT-I TCR. Nevertheless, these results demonstrate that the extent of proliferation of CD4 T cells is greatly inhibited by high precursor frequencies and suggest that the differences between the proliferation of adoptively transferred monoclonal and polyclonal CD4 T cells are due to the different precursor frequencies of Ag-specific clonal T cells in the two systems. In addition, these results indicate that the presence of CD4 T cells in various stages of division (Fig. 1A) is the result of an abnormally high precursor frequency and may not occur under physiological conditions.

High precursor frequencies of TCR transgenic CD4 T cells do not rapidly clear bacteria from the spleens and livers of mice

The limited proliferation of transferred monoclonal CD4 T cells could also be the result of less antigenic stimulation due to the rapid clearance of bacteria by the large number of Ag-specific
FIGURE 5. Clonal competition limits the differentiation of CD4, but not CD8, T cells. The indicated number of OT-II (A) and OT-I (B) cells was transferred into congenic Thy-1.2 mice. The following day, the recipients were infected with rLM-OVA. On day 8 postinfection, proliferation of OT-II (Thy-1.1°Vα2°CD4°) and OT-I (Thy-1.1°Vα2°CD8°) cells was visualized by loss of CFSE, and differentiation of these cells was analyzed by intracellular IFN-γ staining. Numbers indicate the percentage of responding OT-II or OT-I cells that secrete IFN-γ.

Clonal competition limits the differentiation of CD4, but not CD8, T cells. The indicated number of OT-II (A) and OT-I (B) cells was transferred into congenic Thy-1.2 mice. The following day, the recipients were infected with rLM-OVA. On day 8 postinfection, proliferation of OT-II (Thy-1.1°Vα2°CD4°) and OT-I (Thy-1.1°Vα2°CD8°) cells was visualized by loss of CFSE, and differentiation of these cells was analyzed by intracellular IFN-γ staining. Numbers indicate the percentage of responding OT-II or OT-I cells that secrete IFN-γ.

High precursor frequencies result in less differentiation of TCR transgenic CD4 T cells

Because the differentiation of naive T cells into cytokine-producing effectors has been linked to the process through multiple rounds of division (13–15), and our results have shown that CD4 T cell division can be greatly limited by unusually high precursor frequencies, we examined whether high precursor frequencies limit the extent of CD4 T cell differentiation. We transferred 1.5 × 10⁶ and 1.5 × 10⁷ CFSE-labeled OVA-specific cells from OT-II or OT-I mice into congenic recipients and infected them with rLM-OVA. On day 8 postinfection, we analyzed the proliferation and IFN-γ production of the TCR transgenic cells. We have seen previously that no IL-4° or IL-2 single+ (IL-2°IFN-γ− double-positive cells can be detected) CD4 T cells exist in the spleen following i.v. infection with LM (our unpublished results). In mice that received 1.5 × 10⁵ OT-II cells, 26.8% of the responding cells (those that have divided at least once) differentiated into IFN-γ-producing cells compared with only 10.7% in mice that received 1.5 × 10⁶ OT-II cells (Fig. 5A). As expected, 98.1 and 97.2% of responding OT-I cells produced IFN-γ in mice that received 1.5 × 10⁵ or 1.5 × 10⁶ OT-I cells, respectively (Fig. 5B). Therefore, nearly all of the CD8 T cells that were recruited into division differentiated into cytokine-producing effectors regardless of initial precursor frequency. The absolute number of Ag-specific donor CD4 T cells capable of secreting IFN-γ was ~4.1 × 10⁴ and 2.1 × 10⁵ in mice that received 1.5 × 10⁵ and 1.5 × 10⁶ OT-II cells, respectively. Thus, twice as many differentiated CD4 T cells were generated when the initial precursor frequency was 10 times lower. In contrast, the absolute number of Ag-specific donor CD8 T cells capable of secreting IFN-γ was 2-fold less (2.2 × 10⁴ vs 4.4 × 10⁵) when the initial precursor frequency was 10-fold lower. These data show that the extent of differentiation of CD4 T cells in this system is inhibited by abnormally high precursor frequencies, and that the differentiation of CD4 T cells is inversely related to the initial precursor frequency.

Discussion

The magnitude of the CD4 T cell response is generally believed to be lower than that of the CD8 T cell response (7, 22, 43, 44). These conclusions stem from studies that quantified Ag-specific T cells using techniques such as intracellular staining for IFN-γ, tetramer staining, or tracking T cell clones (45). However, these techniques may underestimate the CD4 T cell response because the percentage of responding CD4 T cells that differentiate into cytokine-producing effectors may be lower than that of responding CD8 T cells (13, 16), the availability of MHC class II tetramers is limited, and the response to a few isolated epitopes may not reflect the CD4 T cell response as a whole. For example, for many years, the Ag-specific CD4 T cell response to LM infection was considered to be very weak because the frequency of CD4 T cells responding to all known LM epitopes was almost too low to study. However, a recently identified CD4 T cell epitope, LLO(305-313), reacts with more T cells than any LM-specific CD8 T cell epitope identified to date (41). In this study, we adoptively transferred naive polyclonal T cells and compared the CD4 and CD8 T cell responses during a primary LM infection. We found that both CD4 and CD8 T cells divide extensively following infection, with nearly all responding cells in both subsets becoming CFSE negative (dividing seven or more times). In addition, the absolute number of responding CD4 and CD8 T cells was similar at the peak of response on day 8 after infection. Combined, these data indicate that the CD4 T cell response to primary LM infection is as robust as the CD8 T cell response in terms of the total number of responding cells generated.

The extent of polyclonal CD4 T cell division reported in this study differed from previous findings that adoptively transferred TCR transgenic CD4 T cells undergo significantly limited division in the spleen (11, 12, 46) in comparison with adoptively transferred TCR transgenic CD8 T cells in response to infection (1–3,
11. What accounts for the difference in the extent of monoclonal and polyclonal CD4 T cell division? Our results show that the proliferation of monoclonal CD4 T cells is limited due to an unusually high precursor frequency. We titrated the number of adoptively transferred TCR transgenic CD4 T cells and found that the extent of proliferation increased as the precursor number decreased. Our results are consistent with the findings that recruitment of CD4 T cells into division and burst size are inversely related to precursor frequency (47). Furthermore, we demonstrated that when the number of precursors was decreased to ~1 × 10^6 cells (2 × 10^5 transferred cells with ~5% engraftment), which is still 100 times greater than the estimated precursor frequency for naive Ag-specific T cells (48), the majority of cells divided seven or more times in response to infection. Combined with the results of our polyclonal T cell experiments, we believe that this extensive division of CD4 T cells reflects the natural response of CD4 T cells to LM infection. Although we don’t know whether all of the CFSE-negative cells are Ag specific, several lines of evidence suggest that they are. First, we have shown previously that the DO11.10 cells that proliferate in response to rLM-OVA infection do so in an Ag-specific manner because there is no proliferation of the TCR transgenic cells in response to infection with wild-type LM, thus ruling out bystander proliferation. Furthermore, OT-1 cells and P14 TCR transgenic CD8 T cells don’t proliferate in response to infection with Listeria strains that don’t contain the peptides for which the transgenic T cells are specific (49).

Both CD4 and CD8 T cells have been shown previously to compete for access to Ag on APCs in several different experimental systems (47, 50–60). In this study, we examined the effects of high precursor frequencies on the proliferation and differentiation of CD4 T cells. When the precursor frequency of Ag-specific CD4 T cells is high, fewer CD4 T cells undergo extensive division and a smaller percentage of responding CD4 T cells produce IFN-γ than when the precursor frequency of these cells is low. A previous report demonstrated that clonal competition serves as a mechanism to control the overall size of the CD4 T cell response so that the same number of Ag-specific CD4 T cells is generated despite large differences in initial precursor frequencies (47). However, we show that clonal competition due to high precursor frequencies causes fewer differentiated CD4 T cells to be generated. Thus, while the same number of responding CD4 T cells may be generated in the face of clonal competition, fewer of these cells will be differentiated effectors. A number of reports have proposed that procession through multiple rounds of division is necessary for differentiation (13, 14, 61). However, a study by Laour and Crispe (62) suggests that the relationship between proliferation and differentiation is correlative and not mechanistically linked. Our results show that during an infection, most, if not all, of the IFN-γ production comes from the population of CD4 T cells that have divided multiple times. Moreover, we see a greater percentage of differentiated CD4 T cells when these cells progress through more divisions. CD4 T cells at a high clonal precursor frequency have been shown to undergo more rounds of division in the presence of more Ag (47), and numerous reports have suggested that T cell competition occurs at the level of Ag-APC (47, 51, 52). Thus, we propose that adoptively transferred TCR transgenic CD4 T cells have less access to Ag, and therefore have inhibited proliferation and differentiation, due to clonal competition.

In summary, we showed that the natural proliferative response of CD4 T cells to infection is more extensive than previously described, with the majority of responding CD4 T cells dividing seven or more times. In addition, we demonstrated that high precursor frequencies inhibit the proliferation and differentiation of CD4 T cells due to clonal competition. Thus, the adoptive transfer of TCR transgenic CD4 T cells to study the response of CD4 T cells may significantly underestimate the extent of division and differentiation of the natural response to the same Ag. The effects of clonal competition on CD4 T cell proliferation and differentiation may need to be considered in designing rational vaccine strategies, particularly ones that incorporate multiple boosts in which pools of memory cells may compete with each other.

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Disclosures
The authors have no financial conflict of interest.

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