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T Cells Undergo Rapid ON/OFF but Not ON/OFF/ON Cycling of Cytokine Production in Response to Antigen

Gail A. Corbin* and John T. Harty2*†

Inflammatory cytokines such as IFN-γ and TNF produced by Ag-stimulated CD4+ and CD8+ T cells are important in defense against microbial infection. However, production of these cytokines must be tightly regulated to prevent immunopathology. Previous studies, conducted with BALB/c mice, have suggested that 1) CD8+ T cells maintain IFN-γ production but transiently produce TNF in the continued presence of Ag and 2) lymphocytic choriomeningitis virus-specific and in vitro-propagated effector CD8+ T cells could rapidly cycle IFN-γ production ON/OFF/ON in response to Ag exposure, removal, and re-exposure. In contrast with CD8+ T cells, our results show that Listeria monocytogenes-specific CD4+ T cells from C57BL/6 mice rapidly initiate (ON cycling) and maintain production of both IFN-γ and TNF in the continued presence of Ag. Upon Ag removal, production of both cytokines rapidly ceases (OFF cycling). However, if the initial stimulation was maximal, Ag-specific CD4+ T cells were unable to reinitiate cytokite production after a second Ag exposure. Furthermore, L. monocytogenes-specific CD8+ T cells in the same mice and lymphocytic choriomeningitis virus-specific CD8+ T cells in BALB/c mice also underwent ON/OFF cycling, but if the initial Ag stimulus was maximal, they could not produce IFN-γ after Ag re-exposure. As the initial Ag dose was reduced, the number of cells producing cytokine in response to the second Ag exposure exhibited a corresponding increase. However, T cells that were marked for IFN-γ secretion during the first stimulation did not contribute cytokite production during the second stimulation.

Thus, T cells are not able to undergo rapid ON/OFF/ON cytokine cycling in vitro in response to Ag. The Journal of Immunology, 2005, 174: 718–726.

Communication between cells of the immune system is conducted through cell-cell contact and release of soluble factors such as chemokines and cytokines (1–4). The latter molecules act through specific receptors on immune cells to activate and mobilize the response to infection (5, 6). However, cytokines are a double-edged sword: essential for the appropriate function of the immune system but also potentially toxic mediators. This scenario would limit cytokine production to sites of active infection and, thus, minimize damage to healthy tissue.

Recent studies support the notion that production of the proinflammatory cytokines IFN-γ and TNF by effector and memory CD8+ T cells is tightly regulated, presumably to limit immunopathology (10–12). In vitro exposure to Ag causes both effector and memory CD8+ T cells to rapidly produce IFN-γ and TNF (10, 13). Whereas IFN-γ production is maintained in the continued presence of Ag, TNF production ceases after an initial burst, even in the continued presence of Ag (11). Thus, Ag-specific CD8+ T cells use different strategies to regulate IFN-γ and TNF production in response to Ag, perhaps as a consequence of the different biological effects controlled by the two cytokines (11, 14, 15). Importantly, IFN-γ production by Ag-stimulated CD8+ T cells rapidly ceases upon removal of Ag (10, 11). Finally, studies with lymphocytic choriomeningitis virus (LCMV)3-specific effector CD8+ T cells (10) and in vitro-propagated CD8+ T cell lines (11), both from BALB/c mice, have suggested that, after Ag removal, CD8+ T cells could rapidly reinitiate IFN-γ production upon subsequent Ag exposure. This ON/OFF/ON cycling (10) of IFN-γ production in response to Ag implies that, in vivo, CD8+ T cells will only make cytokines during their interaction with infected cells. This scenario would limit cytokine production to sites of active infection and, thus, minimize damage to healthy tissue.

Pathogen-specific CD4+ T cells (Th1 type) also produce IFN-γ and TNF in response to Ag stimulation (16), although it is not known whether these cytokines are regulated in the same fashion as observed in CD8+ T cells. In this report, we initially ask how Listeria monocytogenes (LM)-specific CD4+ T cells from infected C57BL/6 mice regulate IFN-γ and TNF production in response to continued Ag stimulation or under conditions of Ag removal and re-exposure. In contrast with previous studies of CD8+ T cells (10, 11), CD4+ T cells maintained both IFN-γ and TNF production in the continued presence of Ag and, after Ag removal and cessation of cytokine production, were unable to reinitiate cytokine production in response to a second exposure to Ag. These results suggested a fundamental difference between CD4+ and CD8+ T cells in the ability to undergo ON/OFF/ON cycling of cytokine production in response to Ag. However, further studies of LM- and LCMV-specific CD8+ T cells also revealed that, after an optimal initial stimulation and Ag removal, cytokine production was not reinitiated in response to subsequent Ag exposure. Thus, at least in vitro, T cells are unable to undergo rapid ON/OFF/ON cycling of cytokine production in response to Ag.

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Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; LM, Listeria monocytogenes; rLM-OVA, recombinant LM expressing secreted OVA protein; LLO, listeriolysin O; NP, nucleoprotein; BFA, brefeldin A; ICS, intracellular cytokine staining.

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Materials and Methods

Mice, virus, bacteria, peptides, and tetracycles

C57BL/6 (B6) and BALB/c mice were obtained from the National Cancer Institute. Recombinant LM expressing secreted OVA protein (rLM-OVA) (17) was kindly provided by Dr. H. Shen (Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA). B6 mice were analyzed 7 days after primary LM-OVA infection (5 × 10^6 CFU i.v.; BALB/c mice were analyzed 8 or 30 days after LCMV-Armstrong (5 × 10^5 PFU i.p.) infection. Synthetic peptides listed below OVA257–264, and nucleoprotein (NP) were purchased from Biosynth International. Allophycocyanin-conjugated L^2-116–123 tetramers were synthesized as described (19).

Cell lines and cell culture media

P815 and E.G7 cell lines were obtained from the American Type Culture Collection. P815 is a DBA/2-derived mastocytoma (H-2^d MHC) (ATCC TIB-66). E.G7 is the EL-4 (C57BL/6-derived thymoma, H-2^b MHC) cell line stably transfected with a plasmid construct expressing the OVA Ag and G418 resistance (ATCC CRL-11213) (20). CHB3 is a B10.129(2M)-derived B cell lymphoma (H-2^b/L^v-2M MHC) (21), kindly provided by Dr. G. Bishop (Department of Microbiology, University of Iowa, Iowa City, IA). P815 and CHB3 cell lines were maintained in RP10 (RPML 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 10 mM HEPES, 2 mM L-glutamine, and 50 μg/ml 2-ME). EG7 cells were maintained in RPMI supplemented with 400 μg/ml G418. T cell medium is RPMI supplemented with 5% supernatant from Con A-stimulated rat spleen cells and 50 mM α-methylmannoside.

Intracellular cytokine staining

Splenocytes were cultured directly ex vivo in the presence of peptide-coated biotinylated APCs, with brefeldin A (BFA; 2 μg/ml final concentration) added at the initiation or at the last hour of incubation to facilitate intracellular cytokine accumulation. Intracellular cytokine staining (ICS) for IFN-γ and TNF was performed using a Cytofix/Cytoperm plus (BD Pharmingen) kit as previously described (22). The following mAbs were also obtained from BD Pharmingen: PE anti-IFN-γ, PE anti-TNF-α, FITC anti-TNF-α, PerCP anti-CD4, and PerCP anti-CD8.

APC biotinylation and depletion

T cell-APC contact was disrupted at the indicated time points as previously described (10). Briefly, CHB3 or P815 cells were resuspended at 10^6 per ml in PBS supplemented with 1 mM MgCl_2, 0.1 mM CaCl_2, and 1.0 mg/ml washable biotinylated human serum (Calbiochem) for 20 min at 4°C. Biotinylation was quenched by adding RP10, and cells were washed twice before resuspension in medium containing peptide (10 μM LLO190–201, 100 nM OVA257–264, or 100 nM LCMV NP118–126). Cells were incubated at 37°C for 1 h and washed five times in 50 ml of RP10 to remove unbound peptide. Spleen cells (1 × 10^6) from LM-OVA or LCMV-infected mice were incubated with biotinylated APCs coated with peptide. After 5 h of incubation, APC-T cell contact was disrupted by vigorous pipetting. Streptavidin-coated magnetic beads (MagPrep Streptavidin Beads; Novagen) were added and mixed by pipetting. To deplete APCs, a magnet (Magnetically Separation Stand; Novagen) was used to remove the magnetic beads and associated cells. More than 95% of the target cells were removed by the depletion procedure as detected by flow cytometry. The recovered T cells were transferred to tubes containing 1 × 10^6 uncoated APCs. After 2 h of incubation with APCs in the absence of peptide, some samples were re-exposed to saturating amounts of peptide (10 μM LLO190–201, 100 nM OVA257–264 or 100 nM LCMV NP118–126). After Ag re-exposure, samples were incubated for the indicated periods of time. At each time point, cells were placed at 4°C overnight in PBS containing 1% FBS. Intracellular cytokine production was detected as described above.

IFN-γ secretion capture assay

Cellular Affinity Matrix Technology (MACS Cytokine Secretion Assay: Miltenyi Biotec) was used as previously described (23). Splenocytes obtained from LCMV-infected mice were stimulated with NP118–126-coated APCs for 5 h as indicated. After stimulation, cells were harvested and reacted for 5 min at 4°C with IFN-γ-specific high-affinity capture matrix, i.e., a bispecific Ab-Ab conjugate consisting of the anti-CD45 mAb coupled to the anti-IFN-γ mAb (Miltenyi Biotec). The cells were then transferred into 37°C medium at 2 × 10^6 cells/ml and permitted to secrete cytokines at 37°C for 45 min. Cells were then washed, and captured IFN-γ was detected by staining with a second anti-IFN-γ mAb conjugated to PE (Miltenyi Biotec) for 10 min at 4°C. APCs were then depleted from the cultures. T cells were then restimulated with peptide and fresh APCs for 5 h before ICS for TNF. For staining, cells were washed and incubated with PerCP anti-CD8 (BD Pharmingen) at 4°C for 30 min. For intracellular staining, cells were treated with Cytofix/Cytoperm plus (BD Pharmingen) and incubated with FITC-labeled anti-TNF Abs (BD Pharmingen).

Results

Rationale for use of the rLM-OVA infection model

Initially, we wished to study regulation of IFN-γ and TNF production and cytokine cycling by LM-specific CD4^+ T cells in response to Ag exposure in vitro. We also wished to compare the CD4^+ T cell results with CD8^+ T cells from the same infected mice. Previous studies analyzed the regulation of cytokine production in LM and LCMV-specific CD8^+ T cells (direct ex vivo effector cells or in vitro-propagated lines) from BALB/c mice (10, 11). Unfortunately, no strong CD4^+ T cell epitopes are defined from LM- or LCMV-infected BALB/c mice; thus, we could not use the BALB/c model to study CD4^+ T cells and CD8^+ T cells from the same host. In contrast, LM infection of C57BL/6 (B6) mice stimulates a very strong, I-A^b-restricted CD4^+ T cell response to amino acids 190–201 of the LLO protein (LLO190–201) (18). However, no strong MHC class Ia-restricted CD8^+ T cell epitopes are identified in LM-infected B6 mice (17). To circumvent this problem, we infected B6 mice with a recently described recombinant LM strain expressing hen OVA (rLM-OVA) as a secreted protein (17). Infection of B6 mice with rLM-OVA stimulates a vigorous expansion and the generation of memory CD4^+ T cells specific for LLO190–201 as well as a strong H-2K^d-restricted CD8^+ T cell response to the well-characterized OVA257–264 epitope (Fig. 1). This system allowed us to directly compare the regulation of cytokine production in CD4^+ T cells and CD8^+ T cells from the same host, stimulated by the same infection.

CD4^+ and CD8^+ T cells generated by rLM-OVA infection differentially regulate TNF production in response to Ag stimulation

Upon addition of defined peptide epitopes in vitro, Ag-specific CD8^+ T cells from LM- or LCMV-infected BALB/c mice rapidly initiate production of IFN-γ and TNF, which is detectable by ICS

FIGURE 1. Ag-specific CD4^+ and CD8^+ T cell responses after rLM-OVA infection. C57BL/6 mice were infected with 1 × 10^7 CFU LM-OVA. Splenocytes obtained at 7 or 30 days postinfection were cultured directly ex vivo in the presence of 10 μM LLO190–201 peptide (■) or 100 nM OVA257–264 peptide ( Enumeration of the LLO190–201-specific CD4^+ T cells or OVA257–264-specific CD8^+ T cells per spleen was determined after ICS for IFN-γ. Data are representative of three independent experiments.
specific T cells that still produced cytokine during the later stages of incubation (Fig. 2, A and C, right panels).

As previously observed (16), when BfA was added at the beginning of culture, the same frequency of LLO_{190-201}-specific CD4^+ T cells was detected by intracellular staining for either IFN-γ or TNF (Fig. 2A, left panels). Costaining for both IFN-γ and TNF demonstrated that both cytokines were produced by the same cells (data not shown). In contrast, not all CD8^+ T cells stimulated to produce IFN-γ in response to OVA_{257-264} also produce TNF (Fig. 2C, left panels), a previously described characteristic of effector CD8^+ T cells (13, 16). Interestingly, similar frequencies of CD4^+ T cells producing IFN-γ (Fig. 2, A and B, top panels) and TNF (Fig. 2, A and B, bottom panels) in response to the LLO_{190-201} epitope were detected in culture incubated with BfA for the entire period or only the last hour. Thus, CD4^+ T cells maintain production of both IFN-γ and TNF for at least 6 h in the continued presence of Ag. In contrast, although the majority of OVA_{257-264}-specific CD8^+ T cells continued to make IFN-γ (Fig. 2, C and D, top panels), TNF production was significantly (p < 0.05) reduced when BfA was added for only the last hour of incubation (Fig. 2, C and D, bottom panels). Similar results were obtained after analysis of cytokine production by memory CD4^+ and CD8^+ T cells from rLM-OVA-infected B6 mice (data not shown). Together, these data demonstrate that, in contrast with CD8^+ T cells that exhibit a rapid but transient burst of TNF production in response to Ag, CD4^+ T cells maintain TNF production for at least 6 h in the presence of Ag.

### Ag-dependent cycling of IFN-γ production by CD4^+ and CD8^+ T cells from rLM-OVA-infected mice

Ag-stimulated, virus-specific CD8^+ T cells and Ag-specific CD8^+ T cell lines from BALB/c mice rapidly cease production of IFN-γ upon Ag removal (10, 11), and it has been suggested that these cells are capable of rapidly reinitiating cytokine production upon subsequent Ag exposure (10, 11). Thus, CD8^+ T cells may exhibit Ag-dependent ON/OFF/ON cycling of cytokine production to focus the immune response to the area of infected cells to limit collateral damage and immunopathology (10). To determine whether CD4^+ T cells cycle cytokine production in response to Ag in vitro, splenocytes obtained from mice 7 days post-rLM-OVA infection were cultured for 4 h in the presence of biotinylated CHB3 cells (1-A^b+T, APC) coated with LLO_{190-201} peptide (Fig. 3A). If the cultures were not manipulated further, the percentage of CD4^+ T cells making IFN-γ (Fig. 3A) and TNF (data not shown) was maintained for at least 6 h. If T cell-APC contact was disrupted and peptide-coated APCs were removed by incubation with avidin-coated magnetic beads at t = 4 h, both IFN-γ and TNF production rapidly ceased. To determine whether CD4^+ T cells could reinitiate cytokine production, a saturating amount of LLO_{190-201} peptide and fresh APCs were added back to one group of the APC-depleted cultures at t = 6 h. In contrast with previous results with CD8^+ T cells (10, 11), Ag-specific CD4^+ T cells did not re-express IFN-γ (Fig. 3A) or TNF (data not shown) in response to Ag re-exposure. Similar results were obtained for memory (more than day 30 post-rLM-OVA infection) CD4^+ T cells (data not shown). Thus, CD4^+ T cells were able to undergo rapid ON/OFF cycling of cytokine production in response to Ag; however, they were not able to reinitiate cytokine production in response to Ag re-exposure.

Because this result for CD4^+ T cells differs from previous reports describing rapid ON/OFF/ON cycling of cytokine production by CD8^+ T cells from BALB/c mice (10, 11), we performed the same type of experiment to determine whether rLM-OVA-specific CD8^+ T cells from B6 mice could rapidly cycle cytokine production in response to Ag. OVA_{257-264}-specific CD8^+ T cells continued to make IFN-γ in the presence of Ag, whereas removal of Ag caused a rapid down-regulation of cytokine production (Fig. 3B).
However, and in contrast with previous results, subsequent addition of a saturating amount of OVA257–264 peptide and fresh APCs stimulated only a small fraction of Ag-specific CD8+ T cells to initiate IFN-γ (Fig. 3B) and TNF (data not shown) production. Similar results were obtained for memory (more than 30 day post-rLM-OVA infection) CD8+ T cells (data not shown). These results suggest that most of the Ag-specific CD8+ T cells from B6 mice were unable to reinitiate cytokine production in response to a second Ag stimulation in vitro.

**Ag dose and APC numbers modulate the frequency of LM-specific T cells producing IFN-γ in response to the first and second exposure to Ag**

CD4+ T cells cultured with LLO190–201-coated APCs received an optimal initial stimulation with ~3.5% of CD4+ T cells producing IFN-γ, similar to the maximum percentage of CD4+ T cells producing IFN-γ detected after culture with a saturating amount of free LLO190–201 peptide (Fig. 3A, dotted line). In contrast, CD8+ T cells cultured with OVA257–264-coated APCs received a suboptimal initial stimulation with ~5% of CD8+ T cells producing IFN-γ, compared with ~6.5% IFN-γ producing CD8+ T cells after culture with a saturating amount of free OVA257–264 peptide (Fig. 3B, dotted lines). We noted that the difference between the maximum and suboptimal responses (~1.5%) of OVA257–264-specific CD8+ T cells was similar to the percentage of T cells stimulated to make IFN-γ during the second Ag exposure (~1.2%; Fig. 3B). This result suggested the hypothesis that the T cells producing cytokines in response to the second Ag exposure may not have been stimulated to produce cytokines during the first Ag exposure. This hypothesis predicts that decreasing the fraction of Ag-specific CD8+ T cells stimulated to produce cytokines by initial Ag encounter will cause a corresponding increase in the fraction of cells producing cytokines in response to Ag re-exposure. To address this prediction, we varied the level of initial stimulation by titrating the ratio of APCs to CD4+ T cells (Fig. 4, A–C) or by coating a fixed number of APCs with different amounts of peptide (Fig. 4, D–G), and then we determined the percentage of LLO190–201-specific CD4+ T cells that produced IFN-γ in response to Ag re-exposure. Initial stimulation with a high number of peptide-coated APCs (Fig. 4A) or with APCs coated with a high Ag dose (Fig. 4D) resulted in a maximal response compared with a saturating dose of free LLO190–201 peptide (dotted line) and, after Ag removal, no response to Ag re-exposure (Fig. 4, A and D). Decreasing the number of APCs (Fig. 4, B and C) or amount of coating peptide (Fig. 4, E–G) resulted in a reduced initial response and a corresponding increase in the percentage of CD4+ T cells that made IFN-γ in response to Ag re-exposure. After normalizing to the maximum possible response, we plotted the percentage of Ag-specific CD4+ T cells making IFN-γ after initial Ag stimulation vs the percentage making IFN-γ in response to Ag re-exposure from several experiments. These data revealed an inverse relationship (slope ~1.004, r² = 0.992) between the percentages of cells making IFN-γ in response to first and second exposure to Ag (Fig. 4H).

Similarly, decreasing the number of APCs coated with OVA257–264 (Fig. 5, A–C) for initial stimulation of CD8+ T cell responses led to a corresponding increase in the percentage of CD8+ T cells making cytokine after Ag re-exposure. Again, plotting the normalized results revealed a strong inverse correlation (slope ~0.929, r² = 0.953) between the fraction of Ag-specific CD8+ T cells producing cytokine in response to the initial and subsequent Ag exposures (Fig. 5D). Together, these results provide evidence that, after optimal initial Ag stimulation and Ag removal, neither CD4+ nor CD8+ T cells from rLM-OVA-infected B6 mice are able to rapidly reinitiate cytokine production in response to Ag re-exposure.

**Cycling of cytokine production by virus-specific effector CD8+ T cells**

Previous studies suggesting ON/OFF/ON cycling of IFN-γ production in response to Ag were conducted with in vitro-propagated CD8+ T cell lines (11) or LCMV (NP118–126)-specific effector CD8+ T cells from BALB/c mice (10). Thus, both the mouse strain and pathogen differ from our experiments with T cells from rLM-OVA-infected B6 mice, which could account for the differences in results. To address this possibility, we infected BALB/c mice with LCMV and performed cytokine cycling experiments with effector CD8+ T cells obtained at day 8 postinfection (Fig. 6). At this time point, ~52% of CD8+ T cells scored as Ag specific for IFN-γ production after incubation with free NP118–126 peptide (Fig. 6C, dotted line), consistent with previous results in the LCMV system (10). Incubation of day 8 spleen cells with relatively high, intermediate, and low numbers of NP118–126-coated, biotinylated P815 (H-2d MHC) APCs for the initial Ag exposure resulted in decreasing percentages of IFN-γ-positive CD8+ T cells (Fig. 6, A and C) and, in all cases, a return to background levels after Ag removal (Fig. 6C). As observed with both CD4+ and CD8+ T cells from
Regulation of cytokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells

CD<sup>+</sup> T cells

A 10<sup>6</sup> APCs

B 3 x 10<sup>4</sup> APCs

C no Ag

APC maintained

\[ \bullet \text{peptide removed at } t = 5h \]

\[ \bullet \text{peptide and APC restored at } t = 7h \]

Hours of incubation (BA last hour)

% expressing IFN-γ

D 3 μM

E 30 nM

F 3 nM

G no Ag

H slope = -1.004x

\[ \chi^2 = 0.982 \]

IFN-γ, second time

IFN-γ, first time

FIGURE 4. Regulation of IFN-γ production by rLM-OVA-specific CD4<sup>+</sup> T cells after Ag exposure, Ag removal, and Ag re-exposure. C57BL/6 mice were infected with 1 x 10<sup>5</sup> CFU rLM-OVA. A total of 1 x 10<sup>6</sup> splenocytes obtained at 7 days postinfection were cultured directly ex vivo for 5 h in the presence of 1 x 10<sup>5</sup> (A) or 3 x 10<sup>4</sup> (B) biotinylated CHB3 cells coated with 10 μM LLO<sub>190-201</sub> peptide, 1 x 10<sup>5</sup> biotinylated CHB3 cells not coated with peptide (C and D), or 1 x 10<sup>6</sup> biotinylated CHB3 cells coated with 3 μM (D), 30 nM (E), or 3 nM (F) LLO<sub>190-201</sub> peptide. At t = 5 h, cells were either unmanipulated (○) or separated from APC contact (□). The recovered effector cells were immediately transferred to wells containing 1 x 10<sup>6</sup> APCs and then costained for L<sub>d</sub>-NP<sub>118-126</sub> tetramer and intracellular IFN-γ. The percent IFN-γ<sup>+</sup> after the second Ag stimulation was plotted against the percent IFN-γ<sup>+</sup> after the second Ag stimulation (H). Data are representative of three independent experiments.

rLM-OVA-infected B6 mice, the percentage of NP<sub>118-126</sub>-specific CD8<sup>+</sup> T cells that made IFN-γ in response to Ag re-exposure was low if the initial stimulation was high (Fig. 6, B and C), but it increased as the percentage of Ag-specific CD8<sup>+</sup> T cells responding to the initial stimulation decreased (Fig. 6, B and C). As seen in Fig. 6, B and C, it was possible to observe conditions where the percentages of CD8<sup>+</sup> T cells producing IFN-γ after the first and second Ag exposures was very similar, which may account for the previous findings that suggested ON/OFF/ON cycling of NP<sub>118-126</sub>-specific CD8<sup>+</sup> T cells in this model (10). Taken together, our data do not support the notion that, after an initial stimulation and Ag removal, T cells rapidly reinitiate cytokine production in response to Ag re-exposure in vitro.

TCR down-modulation after initial Ag stimulation prevents the response to Ag re-encounter

T cells down-modulate their TCRs in response to Ag stimulation in vitro (24, 25). To determine whether the failure to respond to a second Ag stimulation correlates with TCR down-regulation, LCMV-specific CD8<sup>+</sup> T cells were stimulated for 5 h with varying numbers of NP<sub>118-126</sub>-coated APCs and were separated from APC contact. The percentage of NP<sub>118-126</sub>-specific CD8<sup>+</sup> T cells that had not down-modulated their TCRs was then determined by staining with the L<sup>4</sup>-NP<sub>118-126</sub> tetramer (Fig. 6D). Strikingly, the fraction of CD8<sup>+</sup> T cells that were capable of binding the L<sup>4</sup>-NP<sub>118-126</sub> tetramer after Ag removal in the different groups (Fig. 6D) correlated very well with the percentage of CD8<sup>+</sup> T cells that made IFN-γ in response to re-exposure to NP<sub>118-126</sub>-specific peptide (Fig. 6E). To confirm that TCR down-regulation correlated directly with IFN-γ production and did not result in death of the initially stimulated T cells, CD8<sup>+</sup> T cells were stimulated with various numbers of APCs and then costained for L<sup>4</sup>-NP<sub>118-126</sub> tetramer and intracellular IFN-γ (Fig. 6F). This result shows that CD8<sup>+</sup> T cells that produced cytokine in response to initial Ag stimulation in vitro remain in the culture; however, these stimulated cells down-modulate surface TCR expression and are not detectable by tetramer staining. In addition, the fraction of Ag-specific T cells that remain detectable by tetramer staining correlates with the fraction of Ag-specific CD8<sup>+</sup> T cells able to produce cytokines in response to a subsequent Ag stimulation.

Coordinate regulation of IFN-γ and TNF production by NP<sub>118-126</sub>-specific memory CD8<sup>+</sup> T cells

In contrast with effector CD8<sup>+</sup> T cells, which contain populations that produce IFN-γ alone as well as both IFN-γ and TNF, most memory CD8<sup>+</sup> T cells produce both cytokines after in vitro stimulation (11, 26, 27) (Fig. 7). As also shown in Fig. 7, decreasing the initial Ag dose also decreased the percentage of NP<sub>118-126</sub>-specific memory CD8<sup>+</sup> T cells making IFN-γ and TNF in response to primary Ag exposure and allowed a corresponding increase in the percentage of CD8<sup>+</sup> T cells making these cytokines in response to Ag re-exposure. These results show that memory CD8<sup>+</sup>
T cells regulate IFN-γ and TNF similarly in response to Ag cycling in vitro. Thus, detection of IFN-γ and TNF can be used interchangeably to study cytokine regulation in memory CD8+ T cells, allowing us to examine cytokine cycling on a per cell basis (see below).

**Cytokine cycling by NP118–126-specific memory CD8+ T cells on a per cell basis**

Together, the results do not support the ability of T cells to rapidly reinitiate cytokine production in response to Ag re-exposure; however, this conclusion is based on analysis of Ag-specific T cell populations, not on single cell analysis. To address this issue, we made use of the fact that memory CD8+ T cells coordinate regulation of IFN-γ and TNF production (Fig. 7) to evaluate on a per cell basis whether CD8+ T cells that made cytokines during the initial Ag stimulation were capable of re-expressing cytokines during Ag re-exposure. NP118–126-specific memory CD8+ T cells that produced IFN-γ during the initial Ag stimulation were identified using the MACS Cytokine Secretion Assay (Miltenyi Biotec), and TNF production was measured after subsequent Ag exposure by ICS. This was possible because, as opposed to ICS, the MACS Cytokine Secretion Assay maintains viability in the responding cells. IFN-γ production could not be used to detect cells making cytokine in response to the second Ag exposure because not all captured IFN-γ from the first stimulation was blocked with anti-IFN-γ detection reagent (data not shown). To determine the frequency of Ag-specific CD8+ T cells in the population, splenocytes obtained 30 days post-LCMV infection were cultured with BFA in the absence (Fig. 8A) or presence (Fig. 8B) of free NP118–126 peptide for 5 h and then were stained intracellularly for IFN-γ and TNF. We found that 19% of the memory CD8+ T cells produced both IFN-γ and TNF. For determination of cytokine expression/re-expression, LCMV-specific CD8+ T cells received no Ag stimulation (Fig. 8C), which resulted in no cytokine production, or suboptimal initial stimulation in the presence of an intermediate number of APCs coated with NP118–126 peptide, which caused a fraction of the Ag-specific CD8+ T cells to produce IFN-γ; these cells were marked by the MACS Cytokine Secretion Assay (Fig. 8E). APCs were depleted, resulting in the down-regulation of both IFN-γ and TNF production (data not shown). After 2 h of rest, fresh APCs and NP118–126 peptide were restored to the cultures (Fig. 8, D and F). After incubation for 5 h, CD8+ T cells were stained for intracellular TNF to identify those cells stimulated to produce cytokine during the second Ag exposure. In the group that received no initial stimulation (Fig. 8D), the majority (18% of 19% maximum) of Ag-specific CD8+ T cells produced TNF during the second stimulation. In the group that received a suboptimal initial stimulation (Fig. 8F), the second Ag exposure stimulated a substantial percentage of the CD8+ T cells to secrete TNF (11.4%). Importantly, the vast majority of these cells (10.3%, or >90%) are not marked as having produced IFN-γ in response to the initial Ag exposure, demonstrating that the majority of CD8+ T cells responding to Ag re-exposure are those that did not respond to the initial Ag stimulation. Similar results were obtained with LM-specific memory CD4+ T cells (data not shown). Ld-NP118–126 tetramer staining of CD8+ T cells marked for IFN-γ production in the MACS cytokine secretion assay revealed that these cells have down-regulated TCR expression (data not shown). Thus, on a per cell basis, the majority of T cells that made cytokine in response to a second Ag stimulation did not produce cytokine in response to the initial Ag exposure.

**Discussion**

Proinflammatory cytokines such as IFN-γ and TNF are necessary for the proper function of the immune response to infection but are also potential mediators of immunopathology (10). Pathogen-specific CD8+ T cells and Th1-type CD4+ T cells produce IFN-γ and TNF in response to Ag stimulation, and several recent studies have analyzed how BALB/c-derived CD8+ T cells regulate IFN-γ and TNF production in response to Ag in vitro (10, 11). Here, we show that Ag-stimulated IFN-γ and TNF production by CD4+ T cells from LM-infected B6 mice exhibit both similarities and differences to CD8+ T cells from the same infected mice. Both populations of
Ag-specific T cells rapidly produce cytokines in response to Ag exposure in vitro (ON cycling). In contrast with Ag-specific effector CD8^+ T cells, which contain populations producing both IFN-γ and TNF or only IFN-γ (13), most effector CD4^+ T cells produce both cytokines, confirming a recent report in the same system (16). The significance of the effector CD8^+ T cell population that only produces IFN-γ is currently unknown, although a similar population of cells in mice with chronic LCMV infection has been described as partially exhausted (28). In the presence of Ag, both CD4^+ and CD8^+ T cells continue to produce IFN-γ, and CD4^+ T cells also maintain TNF production. However, Ag-stimulated CD8^+ T cells exhibit a burst of TNF, which ceases within 3–4 h (11) despite the continued presence of Ag. Thus, CD4^+ T cells and CD8^+ T cells differentially regulate TNF production in response to Ag stimulation.

Ag-stimulated CD4^+ T cells and CD8^+ T cells rapidly extinguish cytokine production upon Ag removal (ON/OFF cycling). However, if the initial Ag stimulation was maximal, neither population of T cells is able to rapidly re-express cytokines after Ag removal and subsequent Ag re-exposure. Similar results were obtained in analyses of effector T cells and memory T cells, from LM- and LCMV-infected B6 and BALB/c mice. These data do not support the notion that T cells can undergo rapid ON/OFF/ON cycling of cytokine production (10, 11), at least in vitro.

The ability of CD4^+ T cells to maintain TNF production under conditions where CD8^+ T cells exhibited a transient burst of TNF and then ceased production was the major difference in cytokine regulation that we observed in our studies. Given the complex biology controlled by TNF, ranging from cellular activation to cell death (15), this difference in TNF regulation by Ag-specific CD4^+ T cells and CD8^+ T cells likely reflects the different roles these cell populations play in the immune response to infection. For example, effector and effector memory CD8^+ T cells migrate to tissues (29, 30), where due to the expression of MHC class I on most nucleated cells, they can respond by cytokine production to any cell type expressing the appropriate peptide-MHC class I complex. In contrast, MHC class II molecules are usually expressed by most nucleated cells, they can respond by cytokine production to any cell type expressing the appropriate peptide-MHC class II complex. In contrast, MHC class II molecules are usually expressed by monocytes, macrophages, dendritic cells, and thus, activated CD4^+ T cells have many fewer target cells that can stimulate cytokine production. In concert with the finding that
CD8+ T cell responses to infection tend to be larger than CD4+ T cell responses (31, 32), the potential damage caused by prolonged TNF production by CD8+ T cells may be more likely to lead to immunopathology than prolonged TNF production by CD4+ T cells.

Alternatively, prolonged TNF production in response to Ag may serve an important regulatory function as CD4+ T cells interact with dendritic cells and B cells to orchestrate immune responses in secondary lymphoid organs (33). Under this scenario, anatomical containment to secondary lymphoid organs or the importance of cellular activation by CD4+ T cell-derived TNF may outweigh the potential that prolonged TNF production by CD4+ T cells in the periphery may lead to immunopathology. Resolution of these issues will require comparison between wild-type and TNF-deficient CD4+ T cells under various conditions of immune stimulation.

Both CD4+ T cells and CD8+ T cells rapidly ceased cytokine production upon Ag removal in vitro. These data are consistent with the idea that limiting the production of proinflammatory cytokines to times of direct recognition of infected cells or professional APCs is important to the proper function of the immune response as well as to limit potential immunopathology (10). However, in contrast with previous reports (10, 11), we did not find evidence to support the suggestion that T cells could rapidly re-express cytokines in response to a second Ag exposure. This was true for effector and memory CD4+ and CD8+ T cells from LM-infected B6 mice as well as for effector and memory CD8+ T cells from LCMV-infected BALB/c mice. For each of these populations we showed that stimulation of all of the Ag-specific T cells to produce cytokines in the initial culture prevented a detectable response to Ag re-exposure. Decreasing the fraction of Ag-specific T cells stimulated by the initial exposure to Ag allowed a reciprocal increase in the percentage of cytokine-producing cells in response to Ag re-exposure. Importantly, it was possible to find conditions where the percentages of T cells responding to the first and second Ag encounters were similar, which may explain the difference between our conclusion and the conclusion reached in previous studies (10, 11). In the one system we tested (NP118-126-specific effector CD8+ T cells), the fraction of Ag-specific T cells expressing detectable TCR decreased as the amount of amount of initial Ag stimulation increased. This is consistent with the finding that Ag stimulation in vitro leads to TCR internalization (24, 25). Importantly, the fraction of Ag-specific CD8+ T cells that remained detectable by MHC class I tetramer staining after initial Ag stimulation correlated with the fraction of cells that produced cytokine in response to Ag re-exposure. Thus, the inability of T cells to rapidly cycle cytokine production in vitro may be due to TCR down-regulation (24, 25). Finally, we showed that, on a per cell basis, the majority of cells producing cytokine in response to a second Ag exposure had not produced cytokine in response to the initial Ag exposure. Thus, at least in vitro, T cells exhibit Ag-dependent rapid ON/OFF cycling, but most cells do not rapidly turn cytokine production back on in response to Ag re-exposure. Although cytokine production by T cells is vital for controlling infection in vivo, our data suggest
that T cells may be refractory to cytokine production immediately after Ag stimulation. This finding is important in light of recent studies examining CD8$^+$ T cell exhaustion during chronic LCMV infection (28). In these mice, CD8$^+$ T cells exhibit a hierarchical loss of the capability to produce IL-1, TNF, and finally IFN-γ, in conditions of increasing Ag load (28).

In our system, T cells did not regain the ability to respond to Ag after in vitro culture for up to 24 h after initial stimulation (data not shown). However, several important potential differences between the in vivo and in vitro settings must be considered, including the quality of Ag exposure delivered by the APCs and the effect of this Ag exposure on the T cell long term. It is possible that after APC stimulation in vivo, TCR down-regulation is not complete, as we have observed after in vitro stimulation of CD8$^+$ T cells. It is also possible that TCR re-expression in vivo may occur at a time point several days removed from the initial Ag encounter, perhaps after proliferation has occurred. Others have shown that after in vitro stimulation, TCR re-expression occurs after 3 days of in vitro culture (34). Although it has been suggested that IL-2 promotes TCR re-expression (34), we found that incubating CD4$^+$ or CD8$^+$ T cells in medium containing IL-2 for 24 h after the initial Ag stimulation did not restore Ag responsiveness (data not shown). However, the consistent ability to detect peptide-induced cytokine production by Ag-specific effector and memory CD4$^+$ and CD8$^+$ T cells at various times after infection provides indirect evidence that permanent Ag nonresponsiveness does not occur in vivo. Thus, our results do not rule out the possibility that T cells undergo ON/OFF cycling of cytokine production in vivo; however, they suggest that the ability to reinitiate cytokine production may require a relatively long period of rest from the initial Ag exposure.

In contrast, CTL activity is recycled, allowing effector CD8$^+$ T cells to kill target cells in sequence (35). However, we show that Ag-regulated cytokine secretion by effector CD4$^+$ and CD8$^+$ T cells does not occur in response to sequential target cell encounters. Clearly, cytolyis is focused on the infected cell, whereas cytokines may act on other cells in the vicinity. Thus, it is possible that the inability of T cells to reinitiate cytokine production may limit local tissue destruction associated with cytokine release from the large effector population, or it may allow T cells to differentiate between different target cells or tissues.

In summary, our data show that IFN-γ and TNF production by CD4$^+$ and CD8$^+$ T cells is differentially regulated by the presence of Ag and that both of these cytokines are rapidly down-regulated after Ag removal. We also show that after Ag stimulation in vitro, T cells are not responsive to subsequent Ag stimulation. Translated to the in vivo setting, this phenomenon may serve to limit immunopathology associated with cytokine production from the large T cell effector pool and/or expanding memory T cell population.

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