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Cutting Edge: OFF Cycling of TNF Production by Antigen-Specific CD8⁺ T Cells Is Antigen Independent

Vladimir P. Badovinac,* Gail A. Corbin,* and John T. Harty²*†

Although they are known for their capacity to kill infected cells, Ag-specific CD8⁺ T cells elaborate other effector mechanisms, including TNF and IFN-γ, that contribute to defense against infection. Ag-specific CD8⁺ T cells rapidly turn ON and turn OFF IFN-γ production in direct response to Ag contact, presumably to minimize the potential immunopathology that could result from inappropriate secretion of this inflammatory mediator. In this study, we show, using in vitro propagated and directly ex vivo-analyzed Ag-specific CD8⁺ T cells, that in contrast to Ag-dependent ON/OFF cycling of IFN-γ production, the cessation of TNF production by the same IFN-γ producing cells is rapid and Ag independent. The Journal of Immunology, 2000, 165: 5387–5391.

The CD8⁺ T lymphocytes are important mediators of adaptive immunity against certain viral, protozoan, and bacterial pathogens. Ag-specific CD8⁺ T cells protect the body against microbial infections by two important mechanisms: lysis of infected cells and production of cytokines such as IFN-γ and TNF (1). To minimize the damage to the host, these effector mechanisms employed by Ag-specific CD8⁺ T cells have to be strictly regulated. Recently, it was shown that ON/OFF cycling of IFN-γ by virus-specific CD8⁺ T cells is rapid and Ag dependent (2, 3). The same virus-specific CD8⁺ T cells produce TNF, and TNF ON cycling is Ag-dependent also. In this study, we address the role of Ag in OFF cycling of TNF in Ag-specific CD8⁺ T cells.

Materials and Methods
Mice, virus, bacteria, and cell lines
BALB/c (H-2b MHC) mice were obtained from the National Cancer Institute (Frederick, MD). Mice were analyzed 8 and 60 days after lymphocytic choriomeningitis virus (LCMV) 1-Arm (5 × 10⁸ PFU i.p., 4)—infection or were immunized with 1 × 10⁹ CFU of the ActA mutant Listeria monocytogenes (LM) strain DP-L1942 (5) and challenged 60 days later with 1 × 10⁹ CFU of the virulent LM strain 10403s as described previously (6). At day 5 after LM challenge, mice were sacrificed and spleens were taken for analysis. BALB/c-derived CD8⁺ T cell lines specific for p60₂₁⁷–₂₂₅ in the context of H-2Kd, P815, and P815-p60 tumor cells were maintained as described previously (7, 8).

Intracellular cytokine staining
Intracellular cytokine staining (ICS) for IFN-γ and TNF was performed using a Cytofix/Cytoperm plus (with GolgiPlug) kit (PharMingen, San Diego, CA) as previously described, except that brefeldin A (BFA) was present for the indicated times (9, 10).

Results and Discussion
Real-time kinetics of Ag-specific TNF and IFN-γ production by CD8⁺ T cell lines
Cycling of IFN-γ production by virus-specific CD8⁺ T cells appears to be strictly controlled by the presence of Ag (2, 3). These cells rapidly produce IFN-γ upon Ag stimulation and also rapidly cease IFN-γ production when Ag contact is broken. Since the same Ag-specific CD8⁺ T cells also produce TNF, we asked whether TNF production is regulated in the same way as IFN-γ in the presence or absence of Ag stimulation.

In vitro propagated CD8⁺ T cell lines with specificity for aa 217–225 of the LM Ag p60 (p60₂₁⁷–₂₂₅) (6, 7, 10) were incubated in the presence of p60₂₁⁷–₂₂₅ peptide (200 nM) with or without P815 MHC class I-expressing tumor cells (Fig. 1A). CD8⁺ T cells were incubated for 1–5 h in the presence of BFA, stained for surface CD8 expression, fixed, and divided before addition of anti-TNF or anti-IFN-γ mAbs (9). Data presented in Fig. 1A show that 1) intracellular staining of Ag-specific CD8⁺ T cells reveals similar frequencies of TNF or IFN-γ-producing cells after incubation with a high peptide concentration (Fig. 1A; Ref. 9); 2) the cytokine production is specific (Fig. 1A, 5-h incubation without peptide) and rapid with the maximal response obtained after 3 h of incubation; and 3) addition of peptide alone is sufficient to drive cytokine production by MHC class I-expressing Ag-specific CD8⁺ T cells.

These results were obtained in the continued presence of the Golgi complex-disrupting compound BFA which prevents secretion of newly synthesized proteins. The presence of BFA from the

*Department of Microbiology and † Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA 52242

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1 This work was supported by National Institutes of Health Grants AI36864 and AI42767.

2 Address correspondence and reprint requests to Dr. John T. Harty, Department of Microbiology, University of Iowa, 3-512 Bowen Science Building, 51 Newton Road, Iowa City, IA 52242. E-mail address: john-harty@uiowa.edu

3 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; LM, Listeria monocytogenes; ICS, intracellular cytokine staining; BFA, brefeldin A; SSC, side light scatter; FSC, forward light scatter.

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beginning of incubation permits the determination of the cumulative frequency of the CD8$^+$ T cells that responded with Ag-specific TNF and IFN-γ production but not the real-time kinetics of cytokine synthesis.

Using an unlimited Ag concentration (either with high concentrations of p60$_{217-225}$ peptide alone or with P815 cells) p60-specific CD8$^+$ T cells were stimulated in the presence of BfA only during the last hour of incubation (Fig. 1B). Our results confirm those of Slifka et al. (2, 3), showing that even after 23 h of incubation all of the CD8$^+$ T cells produce IFN-γ (Fig. 1B). In contrast, TNF production by the same cells was dramatically different. Although, ON cycling for both cytokines was similar, TNF OFF cycling starts after 4 h and TNF production is not detectable at 24 h, a time the same CD8$^+$ T cells still produce IFN-γ.

In the same experiment, >95% of p60-specific CD8$^+$ T cells were TNF and IFN-γ positive after a 6-h incubation in the presence of BfA added at $t = 0$ (data not shown). The observed pattern of cycling of TNF and IFN-γ production was also detected with H-2b NP$_{396-404}$-specific C57BL/6 and allospecific (H-2$d$ anti-H-2$b$) BALB/c CD8$^+$ T cell lines (data not shown).

**FIGURE 1.** Real-time kinetics of Ag-specific cytokine production by CD8$^+$ T cells. A, The p60$_{217-225}$-specific CD8$^+$ T cells were incubated for the indicated period of time either with or without P815 cells (E:T ratio = 1) in the presence or absence of p60$_{217-225}$ peptide (200 nM) and BfA (added at $t = 0$). After incubation, cells were stained for surface CD8 expression, fixed, and divided before staining for intracellular TNF or IFN-γ. Data are presented as frequencies of cytokine-producing CD8$^+$ T cells detected at different time points. B, p60-specific CD8$^+$ T cells were incubated with P815 cells and p60$_{217-225}$ (200 nM) peptide, and BfA was added only during the last hour of incubation. Data were obtained and presented as described above. Data are representative of at least three independent experiments.

**OFF cycling of TNF production by Ag-specific CD8$^+$ T cells in the presence of Ag**

Detection of cytokine production by Ag-specific CD8$^+$ T cell is dependent on Ag dose, incubation time, and the presence of Golgi-disrupting compounds (Fig. 1; Ref. 10). p60-specific CD8$^+$ T cells were incubated for 4 h with P815 expressing the LM p60 Ag (P815-p60; Ref. 7) in the continued presence of BfA (Fig. 2). By keeping the incubation time constant and varying the amount of P815-p60 cells present during the incubation, we were able to show that the frequency of p60-specific CD8$^+$ T cells that produce TNF and IFN-γ depends on the dose of Ag. Moreover, the killing capacity of the p60-specific CD8$^+$ T cells measured in the $^{51}$Cr release assays inversely correlated with the magnitude of cytokine production (data not shown and Ref. 11), suggesting that after the P815-p60 cells are destroyed (limited Ag concentration) cytokine production is OFF.

Using limited Ag concentrations and different incubation times in the presence or absence of BfA, we asked whether 1) we have a system in which we can correlate the disappearance of Ag with OFF cycling of IFN-γ production and 2) to confirm data presented...
in Fig. 1A that OFF cycling of TNF is Ag independent (Fig. 3). At an E:T ratio of 10, ~20% of CD8+ T cells make IFN-γ after a 3- and 5-h incubation in the presence of BfA added at t = 0. When BfA was added during the last 2 h of a 5-h incubation, the frequency of IFN-γ producing CD8+ T cells was ~6% (Fig. 3A), suggesting that Ag-dependent OFF cycling had occurred. In contrast, at an E:T ratio of 1, all of the cells still produce IFN-γ after 5 h of incubation (Fig. 3A). Also, we were able to follow the survival of P815-p60 cells (side light scatter (SSC) vs forward light scatter (FSC) panels) and show that real-time kinetics of cytokine production by p60-specific CD8+ T cells correlates with the frequencies of remaining target cells after different incubation times. The disappearance of P815-p60 cells is not complete (E:T = 1, 5-h incubation) and it is possible that some cells lost the expression of the p60 molecule and therefore cannot be recognized by p60-specific CD8+ T cells (maximal lysis of P815-p60 targets is ~80%; data not shown). Despite continued production of IFN-γ (E:T ratio, 1), the same cells begin to down-regulate TNF production after 3 h of incubation (Fig. 3B, 5-h incubation).

Finally, overnight incubation of p60-specific CD8+ T cells with P815-p60 cells at an E:T ratio of 2 (limited Ag concentration) in the continued presence of BfA or during the last hour of incubation demonstrates that the same cells that have produced both cytokines down-regulate IFN-γ and TNF production (Fig. 3C). Addition of fresh P815-p60 cells elicits rapid ON cycling of both IFN-γ and TNF production. Again, the cells responding to the new Ag exhibit relatively rapid OFF cycling of TNF production at times when continued IFN-γ production is observed.

Taken together, data obtained using in vitro propagated Ag-specific CD8+ T cells demonstrate that the same Ag-specific CD8+ T cells differentially regulate OFF cycling of cytokine production in an Ag-dependent (IFN-γ) and Ag-independent (TNF) fashion. Despite this difference, production of both cytokines can be reinitiated by reencounter with Ag.

**OFF cycling of TNF production by ex vivo-analyzed Ag-specific CD8+ T cells derived from LCMV and LM-infected mice**

LCMV-derived NP118–126-specific CD8+ T cells analyzed directly ex vivo exhibit Ag-dependent ON/OFF cycling of IFN-γ (2, 3). We used the same model to determine whether the Ag-independent OFF cycling of TNF production occurs in Ag-specific effector and memory CD8+ T cells analyzed directly ex vivo.

Splenocytes derived from mice at 8 days (effectors) or 60 days (memory) after LCMV-Arm infection were cultured directly ex vivo with NP118–125 peptide (200 nM) in the presence of BfA from t = 0 or during the last hour of incubation. As reported previously and shown here, ICS of Ag-specific CD8+ T cells at the peak of primary response to LCMV infection detects different frequencies of CD8+ T cells that make TNF and IFN-γ in the presence of BfA from t = 0, but this difference is much smaller with memory cells (Refs. 2, 9, and 12; Fig. 4, A and C). Using peptide (unlimited Ag) stimulation, we detected similar frequencies of NP-specific CD8+ T cells that make IFN-γ whether the BfA was present all the time or only during the last hour of incubation (Fig. 4, A and C). The maximal frequency of NP118–126-specific CD8+ T cells detected by intracellular IFN-γ staining is similar to the frequency of Ag-specific CD8+ T cells detected by staining with tetrameric L4(NP118–126) complexes (Fig. 4B). In contrast, the same NP118–125-specific CD8+ T cells that produce IFN-γ start to down-regulate TNF production after 2 h (effector cells) or 4 h...
(memory cells). After 7 h, all of the effector NP\textsubscript{118–126}-specific CD8\textsuperscript{+} T cells are still IFN-\(\gamma\) whereas only a small fraction of NP\textsubscript{118–126}-specific CD8\textsuperscript{+} T cells are producing detectable TNF (Fig. 4, A and C). A similar pattern is observed with memory cells, although the decay of TNF production appears to be protracted compared with effector cells, a finding that may result from the higher levels of TNF detected after Ag stimulation of memory vs effector cells (our unpublished observation and Ref. 3).

Finally, analyzing the real-time kinetics of Ag-specific CD8\textsuperscript{+} T cell response after secondary challenge with LM reveals the same pattern of cytokine production by Ag-specific CD8\textsuperscript{+} T cells (Fig. 5A). Addition of LLO\textsubscript{91–99} peptide to splenocytes derived from LM-immune BALB/c mice resulted in sustained IFN-\(\gamma\) production by CD8\textsuperscript{+} T cells up to 22 h of incubation. Again, the same cells completely turned OFF TNF production by 22 h (Fig. 5B).

In this study, we show that Ag-stimulated IFN-\(\gamma\) and TNF production by CD8\textsuperscript{+} T cells is differentially regulated in the presence of Ag. Both of the cytokines analyzed here can mediate a wide variety of biological responses in the host, and overproduction of cytokines, at least in some instances, can lead to immune-mediated...
pathology (reviewed in Refs, 13 and 14). In contrast, IFN-γ and TNF are clearly important as CD8⁺ T cell effector mechanisms in the fight against infection (1). Rapid synthesis of IFN-γ and TNF in direct contact with Ag limits the production of cytokines to the site of infection (1, 13, 14). When Ag contact is broken, Ag-specific CD8⁺ T cells immediately cease IFN-γ production, presumably until they encounter the next infected cell. In contrast, TNF production ceases after a short period even when Ag contact is sustained. Although the mechanism(s) for the differential regulation of TNF and IFN-γ are unknown, the data suggest that OFF cycling to prevent unwanted immunopathology. Thus, CD8⁺ T cells are used to control Ag-independent control of expression of effector molecules that are employed in response to infection.

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