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Cutting Edge: Re-evaluating the In Vivo Cytokine Responses of CD8⁺ T Cells during Primary and Secondary Viral Infections¹

Fei Liu and J. Lindsay Whitton²

Virus-specific CD8⁺ T cells produce IFN- γ after Ag contact and, in the absence of this cytokine, the host often cannot eradicate infection. However, our ability to identify cells that are actively expressing this critical effector function in vivo is limited, because the protein is rapidly secreted. In this study, we describe a simple approach that circumvents the need for ex vivo Ag stimulation and allows the enumeration of CD8⁺ T cells that are actively synthesizing IFN- γ in vivo during primary and secondary virus infections. The proportion of Ag-specific primary CD8⁺ T cells producing IFN- γ peaks at 5 days postinfection, when the T cell population is still expanding exponentially. In vivo IFN- γ synthesis by memory cells is explosive, peaking at ~12 h after secondary infection and terminating hours thereafter. This technique will be useful when evaluating in vivo immune cell activity in many situations, including a variety of noninfectious (e.g., autoimmune) diseases. The Journal of Immunology, 2005, 174: 5936–5940.

CD8⁺ T cells play a key role in protecting against primary and secondary virus infections and, over the past decade, numerous studies have shown that these cells can exert profound antiviral effects by synthesizing and secreting cytokines such as IFN- γ , the production of which helps to control a multitude of viruses (1–5) as well as infections by several intracellular bacteria. Despite the importance of IFN- γ synthesis by T cells, our understanding of its in vivo regulation is surprisingly limited. Conventionally, Ag-specific T cells are enumerated, and their IFN- γ production is evaluated, after ex vivo Ag stimulation. The resulting data tell us little about in vivo cytokine production by these cells over the course of infection, in response to contact with authentic Ag. In this article, we describe a novel approach which allows the identification and enumeration of T cells that are actively expressing cytokines in vivo, thereby illuminating the functional in vivo kinetics of the primary and secondary immune responses to viral infection.

Our ability to detect cells that are actively producing IFN- γ in vivo in response to authentic Ag has been limited by at least two factors. First, IFN- γ synthesis by virus-specific CD8⁺ T cells occurs only when the cell is in contact with Ag and is terminated very soon after Ag contact is broken (6). Consequently, in vivo IFN- γ synthesis by an individual CD8⁺ T cell is probably sporadic and its frequency may be inversely related to Ag load. Second, cytokines are very rapidly secreted, making it difficult to identify the cells of origin. The same two constraints apply to intracellular cytokine staining (ICCS),³ now widely used as a means to enumerate Ag-specific T cells by flow cytometry. However, since ICCS is conducted ex vivo, the two limiting factors are readily circumvented. The sporadic nature of Ag contact is overcome by incubating the cells with cognate Ag (usually a synthetic peptide representing a known T cell epitope), and cytokine secretion is prevented by inclusion of brefeldin A (BFA), an inhibitor of Golgi transport that causes retention of the cytokine(s) within the Golgi apparatus, rendering the accumulated intracellular proteins detectable by Ab staining of permeabilized cells. In this study, we report that the in vivo injection of BFA allows the detection and enumeration of CD8⁺ primary and memory T cells that are actively producing IFN- γ in vivo, in response to authentic viral Ag. This approach, which we term “direct ICCS,” complements the recent development, by other laboratories, of a technique to evaluate the in vivo cytolytic activity of CD8⁺ T cells (7, 8).

Materials and Methods

Mice, viruses, and bacteria

C57BL/6 (H-2^b) mice and congenic knockout mice lacking either perforin or IFN γ were obtained from The Scripps Research Institute breeding facility. Mice received 2×10^5 or 2×10^6 PFU (as specified in the text) of lymphocytic choriomeningitis virus (LCMV), Armstrong strain i.p. *Listeria monocytogenes* encoding the OVA epitope SIINFEKL (Lm-OVA, Ref. 9) was administered i.v. (10^4 CFU in 500 μ l of saline), and recombinant vaccinia viruses VVSC11 and VVNP (10) were administered i.p. (2×10^6 PFU in 500 μ l of saline).

BFA and synthetic peptides

BFA (catalogue no. B6542) was purchased from Sigma-Aldrich and a 20-mg/ml stock was prepared in DMSO. Further dilution to 0.5 mg/ml was made in PBS, and 500 μ l was injected i.v. For standard ICCS assays (below), peptides

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³ Abbreviations used in this paper: ICCS, intracellular cytokine staining; BFA, brefeldin A; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; MF1, mean fluorescent intensity; Lm-OVA, *Listeria monocytogenes* OVA.

GP₃₃ (KAVYNFATC) and NP₃₉₆ (FQPQNGQFI) were purchased from Alpha Diagnostic International.

Direct and standard ICCS

For direct ICCS, splenocytes were harvested, and rapidly processed on ice. Briefly, 2×10^6 cells/well were stained with $0.25 \mu\text{g/ml}$ rat anti-mouse CD8a (Ly-2) Ab (Caltag Laboratories) at 4°C , either for 1 h or overnight (the resulting data were indistinguishable). After washing, cells were fixed in 2% formaldehyde for 5 min and then permeabilized in 0.1% saponin, 0.1% sodium azide, and 1% FBS in PBS (Perm Wash) before being stained with $0.5 \mu\text{g/ml}$ rat anti-mouse IFN- γ (XMG1.2) for 30 min at 4°C . Cells were washed, first with Perm Wash, then with 5% FBS-PBS before being acquired on a FACScan flow cytometer. Analyses were done using CellQuest software (BD Biosciences). For standard ICCS, cells were incubated for 6 h at 37°C with a mix of GP₃₃ and NP₃₉₆ peptides (each at 10^{-6} M) in the presence of BFA, then processed as described above.

Evaluation of viral RNA content by RT-PCR

RNA was purified from the spleens of the infected mice using TRIzol reagent (Invitrogen), and cDNA was generated with the Superscript First Strand Synthesis System (Invitrogen). PCR amplification (30 rounds) was conducted using Taq polymerase (Invitrogen) at an annealing temperature of 60°C and an extension temperature of 72°C .

Results and Discussion

BFA inoculation enhances detection of CD8⁺ T cells that are actively producing IFN- γ in vivo during virus infection

The effects of BFA administration are shown in Fig. 1. Naive C57BL/6 mice or C57BL/6 mice that had been infected 5, 7, or 12 days previously with LCMV (2×10^5 PFU) were inoculated with $250 \mu\text{g}$ BFA or with diluent alone. Six hours later, the mice were sacrificed and analyzed by direct ICCS. No IFN- γ ⁺ cells were identified in the spleens of naive mice regardless of whether or not they had received BFA. Thus, the in vivo administration of this transport inhibitor does not generate a false positive signal. However, strong positive signals were present at the three selected time points postinfection (p.i.) and, in all cases, the percentage of IFN- γ ⁺ cells was higher in animals that had received BFA 6 h before sacrifice. The increase in frequency of CD8⁺ T cells detected in BFA-injected mice varied, from 6- to 19-fold. The findings that 1) some signal is detected even without BFA at earlier time points and 2) the largest benefit of BFA is seen at the later time point can be attributed to changing Ag load. Early in infection, Ag load in the spleen is high, increasing the probability that, at the moment of harvest, a number of T cells will be in contact with Ag and actively producing

IFN- γ ; these cells may be detected by direct ICCS, even in the absence of BFA. However, by 12 days p.i., encounters between T cells and cognate Ag will be infrequent, thereby maximizing the effects of BFA. The effects of BFA also are demonstrated by differences in the quantities of IFN- γ present in T cells, as judged by mean fluorescence intensity (MFI). At day 12 p.i., the few cells that were identified as IFN- γ ⁺ in mice that had not received BFA had very low MFIs, whereas IFN- γ ⁺ cells from mice that had received BFA had much higher MFIs. We conclude that the in vivo inoculation of BFA markedly facilitates the detection of splenic CD8⁺ T cells that are actively producing IFN- γ in vivo during virus infection. BFA inoculation also allows the detection of CD8⁻ cells that are making IFN- γ in vivo (most obviously at the day 7 time point in Fig. 1); at least some of these cells are CD4⁺ T cells (data not shown).

Evaluating cytokine production by primary CD8⁺ T cells over the course of virus infection

Next, the above experiment was expanded in two ways. First, more time points p.i. were analyzed by direct ICCS of splenocytes from BFA-treated mice to identify the proportion of the Ag-specific cells producing IFN- γ over the course of infection. Second, the splenocytes also were analyzed by standard ICCS; they were incubated with a mix of GP₃₃ and NP₃₉₆ peptides in vitro for 6 h in the presence of BFA. These two peptides represent the two dominant epitopes in C57BL/6 mice, so the resulting standard ICCS data approximate (but somewhat underestimate) the total LCMV-specific response. The data for standard ICCS and direct ICCS at eight time points are shown in Fig. 2A. The differences between the two methods are dramatic and are presented graphically in Fig. 2B. The standard ICCS (patterned circles) shows the expected increase in CD8⁺ T cell numbers between days 4 and 8, after which the number of virus-specific cells very rapidly declines. However, the proportion of these virus-specific cells that are actively producing cytokines in vivo (gray squares) peaks much earlier in infection, at 5 days p.i., and drops very rapidly thereafter. Furthermore, it is clear that, after ~ 7 days p.i., the vast majority of splenic CD8⁺ T cells appear to be inactive in vivo, at least in terms of IFN- γ production.

These findings reveal a disconnect between T cell abundance and the expression of T cell effector functions, and dovetail nicely with previous observations that Ag stimulation triggers naive CD8⁺ T cells to begin a program of expansion that continues even in the absence of further Ag contact; thus, T cell abundance is preprogrammed, and does not require repeated Ag encounters, but the expression of T cell effector functions in vivo is exquisitely sensitive to Ag contact. Furthermore, we have shown that the functional avidity of virus-specific CD8⁺ T cells (their ability to respond to low levels of Ag) increases early in infection and is complete by days 6–8 (11), the time at which the proportion of T cells that are actively producing IFN- γ declines (Fig. 2B). We propose that functional avidity maturation may be driven by repeated contact with viral Ag and, perhaps, by the resultant production of IFN- γ .

BFA injection reveals an explosive in vivo activation of LCMV-specific CD8⁺ memory T cells following secondary virus challenge

We have recently reported that the rapidity with which primary CD8⁺ T cells initiate IFN- γ synthesis is heterogeneous, with

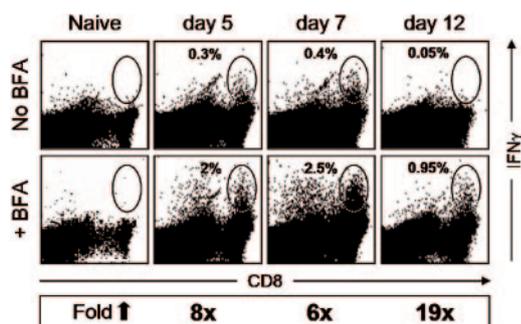


FIGURE 1. BFA inoculation enhances detection of CD8⁺ T cells that are producing IFN- γ in vivo. Naive mice or mice infected 5, 7, or 12 days previously were injected with BFA or with diluent, and 6 h later their splenocytes were recovered and were analyzed by direct ICCS (defined in the text). The percentages of CD8⁺ T cells that were actively producing IFN- γ are indicated, and the fold increases in signal in mice that received BFA are shown. These data are representative of four separate experiments.

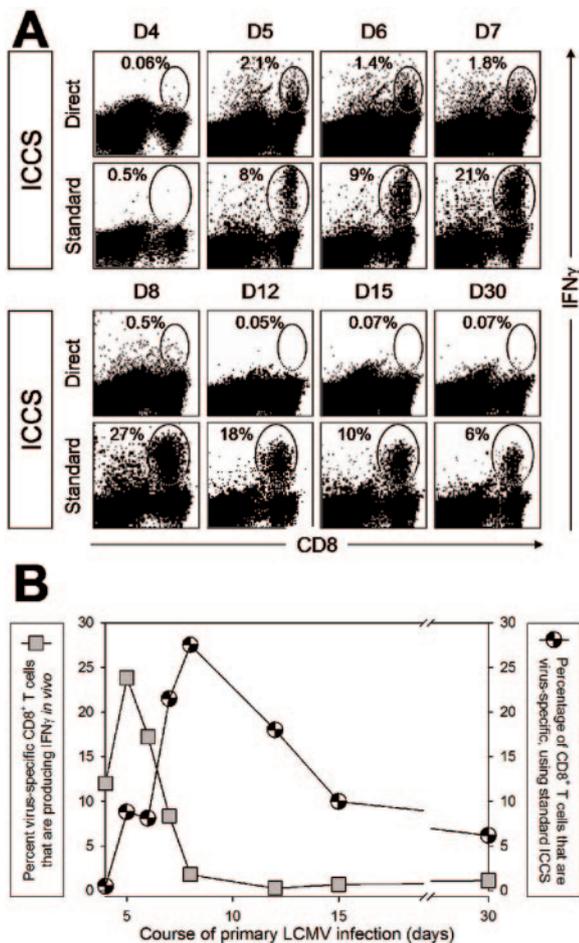


FIGURE 2. Mapping the in vivo activity of CD8⁺ T cells over the course of virus infection. C57BL/6 mice were infected with LCMV and inoculated with BFA on the indicated days p.i. Six hours later, the mice were sacrificed and their splenocytes either were immediately stained for CD8 and IFN- γ (direct ICCS); or analyzed by standard ICCS. *A*, Dot plots from each time point p.i. are shown along with the percentages of CD8⁺ T cells that were IFN- γ ⁺. *B*, The results of the standard ICCS assay, the conventional way to evaluate virus-specific T cells during virus infection, are presented graphically (patterned circles). At each time point, the proportion of the virus-specific cells that was actively producing cytokines was calculated [(percent direct) \times 100/(percent standard)], and these values are plotted (gray squares).

some cells responding rapidly (within \sim 1 h of contact) and others responding more slowly (within \sim 5 h); but, in contrast, all memory CD8⁺ T cells are fast responders (12). However, those studies were conducted *ex vivo* using standard ICCS. Thus, we applied the in vivo BFA inoculation approach to investigate the rapidity of memory CD8⁺ T cell responses in vivo. To this end, long-term LCMV-immune mice, who had recovered from LCMV infection at least 6 wk previously, were reinfected with 2×10^6 PFU of virus. The mice were inoculated with BFA at four time points following secondary infection, indicated diagrammatically in Fig. 3*A*, and were sacrificed 6 h later. Their cells were analyzed immediately after sacrifice, using direct ICCS, and also were evaluated using standard ICCS. Standard ICCS showed that, before secondary infection, 6.4% of an immune mouse's CD8⁺ T cells responded to in vitro Ag stimulation (Fig. 3*A*), a number consistent with previous reports from several laboratories. In the 4 days following secondary infection, this percentage increased by \sim 2-fold (Fig. 3*A*) and, as a result of

increased splenic cellularity combined with an increased relative content of CD8⁺ T cells, the overall abundance of peptide-specific CD8⁺ T cells in the spleen increased \sim 8-fold (data not shown). Strikingly, direct ICCS revealed that the in vivo functional response of memory CD8⁺ to secondary virus challenge was explosive in nature, as shown graphically in Fig. 3*B*, where the total percentages of CD8⁺ T cells that are peptide specific are plotted (patterned circles), along with the proportions of virus-specific cells that are actively producing cytokines in vivo (gray squares). Essentially, none of the virus-specific memory cells were actively producing IFN- γ in the absence of secondary infection, as expected, because no viral Ag should be present. However, IFN- γ ⁺ cells were already abundant as early as 6 h after infection and, at 12 h p.i., fully 4% of all splenic CD8⁺ T cells (i.e., \sim 50% of all splenic virus-specific CD8⁺ T cells) were actively producing the cytokine in vivo. Thus, the rapid in vitro responses to synthetic peptides previously reported for all memory T cells (12) are paralleled by extremely rapid in vivo responses to secondary virus infection. Perhaps surprisingly, IFN- γ production in vivo appears to be very short-lived; by 24 h after secondary challenge, IFN- γ ⁺ cells were almost undetectable in vivo. To ensure that the responding cells were LCMV specific, rather than bystanders, two experiments were conducted (Fig. 3*C*). First, mice were infected with Lm-OVA (see *Materials and Methods*) and 6 wk later, these mice were infected with LCMV. The mice received BFA 6 h later and were sacrificed after another 6 h. As shown in Fig. 3*C* (left column), even though a substantial population of OVA-specific memory cells was detectable by standard ICCS using the SIINFEKL peptide, almost no IFN- γ ⁺ cells were detected by direct ICCS. Thus, LCMV infection does not lead to extensive IFN- γ production by non-LCMV-specific CD8⁺ memory T cells. To obtain positive evidence for the LCMV specificity of responding cells, a second experimental approach was taken. LCMV-immune mice were infected either with VVSC11 (a recombinant vaccinia virus that contains no LCMV sequences) or with VVNP (identical to VVSC11 except that it expresses the LCMV NP). The mice received BFA 6 h later and were sacrificed after an additional 6 h. As expected, both groups showed good responses by standard ICCS (after stimulation with LCMV NP₃₉₆ peptide). However, IFN- γ ⁺ cells were detected by direct ICCS only in the VVNP-infected mice (Fig. 3*C*, top right dot plot). Thus, BFA inoculation of LCMV-immune mice allows the detection of cells that are responding in vivo to authentic Ag contact in the spleen, both when this Ag is delivered by LCMV and by recombinant vaccinia virus (Fig. 3, *A* and *C*, respectively).

CD8⁺ memory T cells play a key role in protecting against LCMV infection. Is the transient pulse of IFN- γ by CD8⁺ memory T cells (Fig. 3*B*) related to their protective effects? PCR was used to determine the quantity of LCMV RNA in the spleen at various times following infection with LCMV (2×10^6 PFU), comparing naive mice to mice that had been DNA immunized using a plasmid encoding the LCMV nucleoprotein, which we (13) and others (14, 15) have shown induces CD8⁺ memory T cell-dependent immunity. In naive mice, the quantity of viral RNA rose over the course of several days (Fig. 3*D*, top panel). In contrast, DNA-immunized C57BL/6 mice controlled the infection much more rapidly; compared with naive mice, viral RNA was much reduced at 1 day postinfection, indicating that the DNA vaccine-induced CD8⁺ memory T

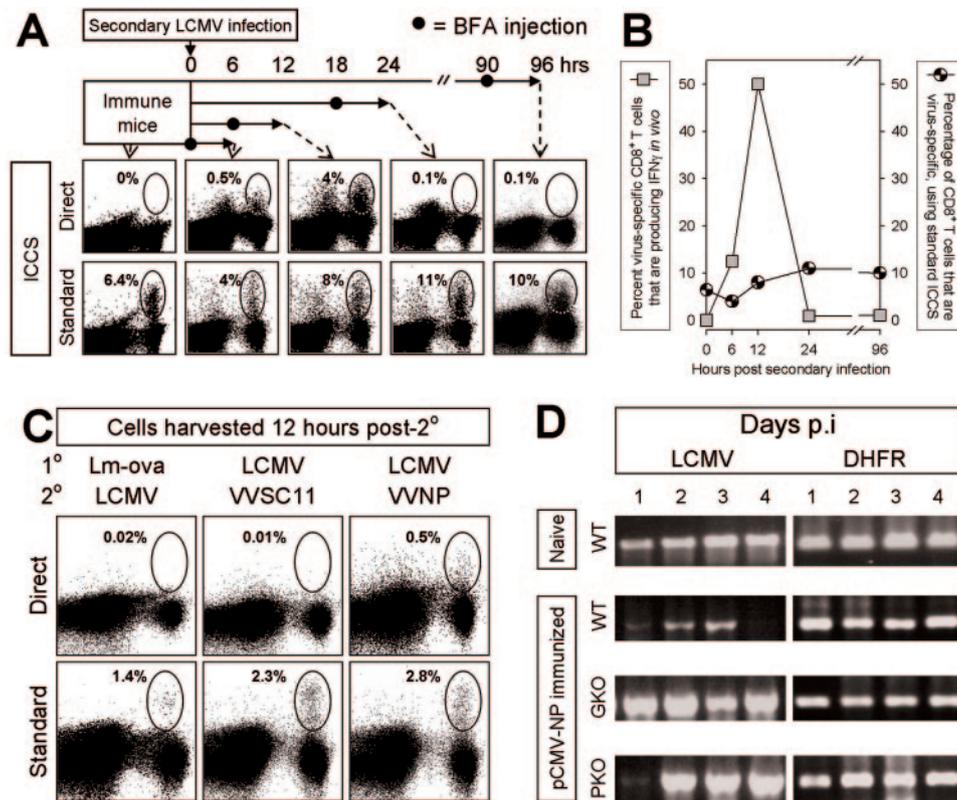


FIGURE 3. Secondary LCMV infection leads to rapid but transient IFN- γ production by LCMV-specific CD8⁺ memory T cells in vivo. *A*, LCMV-immune C57BL/6 mice were infected with LCMV, and at the times (hours) indicated by solid circles were inoculated with BFA. Six hours after receiving BFA, the mice were sacrificed and their splenocytes were evaluated by both direct and standard ICCS procedures. The percentages of CD8⁺ T cells that were IFN- γ ⁺ are shown. The left-hand pair of dot plots show the results for immune mice that had received BFA 6 h before sacrifice, but were not secondarily infected. *B*, Plots equivalent to those described in detail for Fig. 2*B* are shown; patterned circles show the total percentages of Ag-specific T cells at each time point (standard ICCS) and gray squares show the proportion of these cells that are actively expressing IFN- γ in vivo. *C*, Mice received primary (1°) infection with Lm-OVA, or with LCMV, and ≥ 6 wk later were given a second (2°) infection with LCMV, VVSC11, or VVNP. Six hours later, the mice received BFA and the spleens were harvested 6 h thereafter. For standard ICCS, cells from 1° Lm-OVA mice were incubated with SIINFEKL peptide, while cells from 1° LCMV mice were incubated with the NP-GP mix. The percentages of CD8⁺ T cells that were IFN- γ ⁺ are shown. *D*, C57BL/6 mice, or congenic equivalents lacking either IFN- γ (GKO) or perforin (PKO), were immunized with 100 μ g of a plasmid encoding LCMV NP, which is known to induce CD8⁺ T cell-dependent immunity. These mice and naive C57BL/6 mice were infected with 2×10^6 PFU of LCMV and were sacrificed at the indicated days p.i. Splenic LCMV RNA was detected using RT-PCR. The RNA control for each sample was the gene dihydrofolate reductase (DHFR). WT, wild type.

cells exercised very rapid and effective, antiviral functions. To determine the contribution of IFN- γ to this early antiviral effect, mice that were deficient either in IFN- γ (GKO mice) or perforin (PKO mice) were DNA immunized and then challenged with virus. Vaccinated GKO mice were completely unable to control the infection but, in dramatic contrast, vaccinated PKO mice showed highly effective early control of infection, but later “escape” of the virus. These data suggest that the two major antiviral effector functions of CD8⁺ memory T cells, IFN- γ production and perforin-mediated cytotoxicity, exert their effects in waves, with IFN- γ acting immediately, but transiently, and perforin taking over at a later stage. Therefore, the transient pulse of IFN- γ production revealed in Fig. 3*B* correlates well with the antiviral effects of IFN- γ observed in Fig. 3*D*.

In conclusion, we have demonstrated the validity of BFA injection for detecting CD8⁺ cells that are actively synthesizing IFN- γ in vivo during primary and secondary virus infections. We also have found the technique useful for enhancing the detection of IFN- γ -producing CD4⁺ T cells, and we suggest that it might be used to permit the in vivo enumeration of many different cell types that produce secreted molecules such as cy-

tokines and chemokines. Furthermore, the benefits may not be limited to models of infectious disease; BFA injection also may allow the analysis of, for example, autoreactive cells which are known to be present in a wide variety of autoimmune disease models, but whose in vivo activity has, to date, been a matter of conjecture.

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Disclosures

The authors have no financial conflict of interest.

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