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

Fei Liu and J. Lindsay Whitton

J Immunol 2005; 174:5936-5940; doi: 10.4049/jimmunol.174.10.5936

http://www.jimmunol.org/content/174/10/5936

References
This article cites 15 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/174/10/5936.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Re-evaluating the In Vivo Cytokine Responses of CD8+ T Cells during Primary and Secondary Viral Infections

Fei Liu and J. Lindsay Whitton

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.

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), 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-2b) mice and congenic knockout mice lacking either perforin or IFNγ were obtained from The Scripps Research Institute breeding facility. Mice received 2 × 104 or 2 × 105 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. 7) was administered i.v. (106 CFU in 500 μl of saline), and recombinant vaccinia viruses VVSC11 and VVNP (10) were administered i.p. (2 × 106 PFU in 500 μl 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 μl was injected i.v. For standard ICCS assays (below), peptides encoding the OVA epitope SIINFEKL were synthesized and used as control stimuli.

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037

Received for publication January 5, 2005. Accepted for publication March 15, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health R-01 Awards AI-27028 and AI-52351.

2 Address correspondence and reprint requests to Dr. J. Lindsay Whitton, Department of Neuropharmacology, CVN-9, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: lwhitton@scripps.edu

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

**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 × 106 PFU) were inoculated with 250 μg 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).

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...
spleenocytes either were immediately stained for CD8 and IFN-γ or virus infection. C57BL/6 mice were infected with LCMV and inoculated with 2×10^6 PFU of virus. The mice were inoculated with BFA at 6 h later. As shown in Fig. 3C (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 another 6 h. As expected, both groups showed good responses by standard ICCS (after stimulation with LCMV NP), but no detectable in vivo. To ensure that the responding cells were LCMV specific, rather than bystanders, two experiments were conducted (Fig. 3C). 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. 3C (top panel), 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] × 100/[percent standard]), and these values are plotted (gray squares).

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. 3B) 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. 3D, 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
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 cytolysis, 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. 3B correlates well with the antiviral effects of IFN-γ observed in Fig. 3D.

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 cytokines 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.

Acknowledgments
We are grateful to Annette Lord for excellent secretarial support. This is manuscript number 16498-NP from the Scripps Research Institute.

Disclosures
The authors have no financial conflict of interest.

References


