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Brief Antigenic Stimulation Generates Effector CD8 T Cells with Low Cytotoxic Activity and High IL-2 Production

David Usharauli2 and Tirumalai Kamala

It is currently believed that a brief antigenic stimulation is sufficient to induce CD8 T cells to complete their differentiation program, become effector T cells, and subsequently generate memory. Because this concept was derived from studies in which only a single effector function was analyzed (either IFN-γ production or target cell lysis), we wondered whether monitoring for multiple effector functions might reveal novel characteristics of effector CD8 T cells elicited by brief or prolonged Ag exposure. Using an in vitro system to generate effector T cells and an in vivo adoptive transfer model to track donor CD8 T cells, we found that the differentiation programs acquired by CD8 T cells after brief or prolonged antigenic stimulation were different. Although the frequencies of IFN-γ and TNF-α producers were comparable for both effector CD8 T cell populations, there were major differences in cytotoxic potential and IL-2 production. Whereas prolonged (>24 h) Ag exposure stimulated effector CD8 T cells with high cytotoxic activity and low IL-2 production, brief (<24 h) stimulation generated effector CD8 T cells with low cytotoxic activity and high IL-2 production. The latter effector T cells rapidly converted into central memory-like CD8 T cells, exhibited long-term survival in adoptively transferred hosts, and gave robust recall responses upon Ag challenge. These data suggest that not all functions of effector CD8 T cells are equally inherited after brief or prolonged antigenic stimulation. *The Journal of Immunology*, 2008, 180: 4507–4513.

Antigenic stimulation in the context of infection or immunization drives naive CD8 T cells to undergo multiple rounds of cell division and to differentiate into effector cells capable of cytotoxic activity and cytokine production (1). For an efficient immune response, this process should be rapid and generate effector CD8 T cells armed with diverse effector functions. Because of their apparent importance for successful immune responses against intracellular pathogens (2) and also against tumors (3), there has been a wide interest in dissecting the molecular mechanisms underlying the initiation (activation), differentiation (effector function), and maintenance (memory) phases of CD8 T cells involved in immune responses. By design, the adaptive immune response (including CD8 T cells) is Ag specific. This implies that Ag itself may have a role in shaping the outcome of an immune response. It was suggested, and then shown experimentally for both CD8 and CD4 T cells (4), that the strength of the signal or duration of Ag presentation can influence the fate of naive T cells and shift the balance between effector vs memory differentiation (5). In its simplest version, this model predicted that the longer that naive T cells are stimulated with specific Ag, the more they divert their differentiation to effector phenotype and lose the memory characteristic and vice versa (6). This model, however, has been challenged by the observations that brief (<20-h) antigenic stimulation in vitro and in vivo are sufficient for CD8 T cells to acquire the full capacity to differentiate into effector cells and later on generate memory (7–9). Since this “autopilot” model of effector CD8 T cells response was primarily derived from studies in which only a single effector function was analyzed (either IFN-γ production or target cell lysis) (10), we wondered whether monitoring for multiple effector functions might reveal novel characteristics of effector CD8 T cells elicited by brief or prolonged Ag exposure (10–13). In this study, we demonstrate for the first time that effector phenotype and possibly memory potential of CD8 T cells is heavily influenced by the duration of initial antigenic stimulation and has at least one predictable outcome: that brief (<20-h) antigenic stimulation generates effector CD8 T cells with minimal cytotoxic activity but is capable of production of high levels of IL-2 and that the opposite is true for effector CD8 T cells generated by prolonged antigenic stimulation. Interestingly, both types of effector CD8 T cells produce comparable amounts of IFN-γ and TNF-α. These data suggest that not all functions of effector CD8 T cells are equally inherited after brief or prolonged antigenic stimulation (14).

Materials and Methods

**Animals**

OT-I/RAG1 knockout (KO), F5/RAG1 KO, and P14 mice were purchased from Taconic Farms and wild-type (wt) C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Mice were housed in the specific pathogen-free facility at the National Institutes of Health. All experiments were conducted according to National Institutes of Health Animal Care and Use Committee guidelines. The National Institutes of Health is an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility.

**In vitro culture**

For brief antigenic stimulation, 10³–2 × 10⁴ CD8 T cells from lymph nodes of naive OT-I RAG1 KO mice were cultured for a period of 18 h.
with 2–3 × 10^6, irradiated (1500 rad) OVA peptide (1 μg/ml) pulsed wt B6 splenocytes or with 10^7 LPS-matured, SIINFEKL-pulsed BmDCs in advanced DMEM:F-12 medium (Invitrogen Life Technologies) supplemented with 5% FCS, 2 mM glutamine, penicillin/streptomycin/gentamycin, and 50 μM 2-ME (T cell culture medium) and then purified by negative selection using a mixture of biotinylated Abs against non-CD8 T cells (all from BioLegend), followed by streptavidin-coated immunomagnetic beads (Dynal Biotech and Invitrogen Life Technologies). The purity was usually between 93 and 97%. For OT-I-transgenic CD8 T cells, stimulators were prepared by pulsing wt B6 splenocytes with OVA peptide for 2 h and washing the free peptide off before culturing with OT-1 cells. In contrast, F5- and P14-transgenic T cells were cultured with stimulators in the presence of free peptide. After purification, activated CD8 T cells were then recultured for an additional 54 h in the conditioned medium derived from the original coculture (this conditioned medium was fully able to support their expansion (Fig. 1A) and the conditioned medium was sufficient to activate naive OT-I cells (Fig. 1A) and the conditioned medium was derived from the original coculture). No exogenous cytokine was added. The control OT-I cells were left with stimulators for the same length of time. Fold expansion was calculated by dividing the number of recovered viable T cells (at day 3) by the number of initial seeding for each group. Dots represent individual experiments.

Results
Briefly stimulated CD8 T cells show deficiency in cytotoxicity
For CD8 T cells, brief antigenic stimulation is sufficient to drive their differentiation into effectors (7, 8, 10). To this end, we cultured OT-I cells with peptide-pulsed wt B6 splenocytes (as stimulators) for 18 h and then purified them by negative selection. Purified OT-I cells were recultured for an additional 54 h in the conditioned medium derived from the original coculture. No exogenous cytokines were added. The control OT-I cells were left with stimulators for the same length of time. Brief stimulation was sufficient to activate naive OT-I cells (Fig. 1A) and the conditioned medium was fully able to support their expansion (Fig. 1B). All functional experiments were done on day 3 effector OT-I cells. First, we analyzed the cytotoxic activity of briefly stimulated OT-I cells. In contrast to what has been reported previously (7), we found that brief antigenic stimulation consistently generated effector OT-I cells with low/no cytotoxic activity (Fig. 2A).

Two major cytotoxic pathways have been described for effector CD8 T cells: granule exocytosis- and FasL-mediated cytolyis. Deficiencies in either of these pathways could have produced the observed decrease in cytotoxic potential of briefly stimulated OT-I cells. To elucidate the cause for low cytotoxicity, we stained for the lysosomal marker CD107a, which has been shown to be expressed transiently on the surface of the effector CD8 T cells upon degranulation of cytotoxic granules and therefore to mark the effector T cells involved in target cell lysis (17). In this assay, briefly stimulated OT-I cells degranulated equally well compared with control OT-I cells (Fig. 2B). These results suggested that the CD107a-based degranulation assay may not precisely identify effector CD8 T cells involved in cytolyis, a finding that had been reported previously (18). Next, we questioned whether low cytotoxic activity despite normal degranulation could be explained by changes in expression level of grB, or perhaps perforin, in the cytotoxic granules of briefly stimulated OT-I cells. Indeed, intracellular staining for grB showed that briefly stimulated OT-I cells expressed less grB on a per cell basis compared with control OT-I cells (Fig. 2C). In addition, briefly stimulated OT-I cells were also defective in their ability to up-regulate FasL upon Ag recognition on target cells (Fig. 2D). Thus, brief antigenic stimulation generates effector CD8 T cells with deficiencies in both granule- and FasL-mediated cytolyis pathways.
Validation of in vitro model system

OT-I CD8 T cells express a high-affinity transgenic TCR specific to the H2-Kb-SIINFEKL complex (19). To exclude the possibility that the observed effector phenotype of briefly stimulated CD8 T cells was a peculiar characteristic of OT-I cells (20), we did similar studies with F5 and P14-transgenic CD8 T cells expressing TCRs specific for peptides derived from influenza virus and lymphocytic choriomeningitis virus, respectively. In both cases, briefly stimulated F5 and P14 effector CD8 T cells showed a deficiency in cytotoxicity (Fig. 3, A and B). In addition, to exclude the possibility that the negative selection protocol used to purify briefly stimulated OT-I cells could be depleting precursors of highly cytotoxic effector T cells, we used positive selection instead and obtained similar results (Fig. 3C). To further validate these observations, we used LPS-matured, OVA peptide-pulsed BmDCs to stimulate naive OT-I cells. Again, similar to results obtained with stimulatory splenocytes, effector OT-I cells generated by brief exposure to BmDCs plus OVA showed minimal cytotoxic activity (Fig. 4A). Finally, to exclude the possibility that briefly stimulated OT-I cells might have used a killing pathway other than the one that causes DNA fragmentation in target cells detected by the JAM test (15), we did flow cytometry-based killing and target detachment assays. In both cases, briefly stimulated OT-I showed defective cytotoxicity (Fig. 4B and data not shown).

Briefly stimulated CD8 T cells produce high levels of IL-2

These data prompted us to question whether briefly stimulated OT-I cells are impaired in effector functions in general or whether they have a selective deficiency in cytotoxic activity. We went on to investigate briefly stimulated OT-I cells for various other effector functions. In agreement with previously published observations (10, 21), upon restimulation, briefly stimulated OT-I cells produced IFN-γ/H9253 and TNF-α/H9251 at levels equivalent to those produced by the control OT-I cells, as detected by both intracellular staining and ELISA (Fig. 5). However, there was a major difference in IL-2 production. Briefly stimulated OT-I cells produced 10–100 times more IL-2 compared with control OT-I cells in the restimulation assay (Fig. 5). These data implied that briefly stimulated OT-I cells develop a distinct effector phenotype characterized by low cytotoxic activity and high IL-2 production.
Briefly stimulated CD8 T cells develop a central memory-like phenotype

The capacity to produce high levels of IL-2 upon Ag recognition is a feature of central memory T cells (22). The surface staining for activation/differentiation markers on day 3 effector OT-I cells revealed the following characteristics: briefly stimulated OT-I cells expressed low levels of CD25 (IL-2Rα), IL-7Rα, PD1, and CTLA-4 and high levels of CD62L and CXCR3 (Fig. 6). There was no difference in CD43 expression compared with control OT-I cells, as detected by the mAb 1B11, which has been shown to mark effector CD8 T cells with high cytotoxic activity (23). Thus, confirming the conclusion from the results with CD107a, changes in O-glycosylation of CD43 do not differentiate between effector OT-I cells with high and low cytotoxic activity in this model. In addition, the killer cell lectin-like receptor G1 expression was undetectable on both effector CD8 T cell populations (data not shown), consistent with a previous report (21) that it is up-regulated on CD8 T cells from day 5 after antigenic stimulation in vivo. Of note, on day 3, briefly stimulated OT-I cells expressed a low level of IL-7Rα compared with control OT-I cells (Fig. 6); however, on day 4, the reverse was true: briefly stimulated OT-I cells up-regulated IL-7Rα and control OT-I cells down-regulated it (data not shown). At this time, it is unclear as to what is the reason for this delay in IL-7Rα up-regulation on briefly stimulated OT-I cells (24, 25). Overall, this staining pattern suggested that briefly stimulated OT-I cells were primarily developing into central memory-like CD8 T cells (26). Previous studies have supported the idea that central memory CD8 T cells, with a capacity to secrete large amounts of IL-2 upon Ag recognition that lead to accelerated secondary responses, play a decisive role in host protection against reinfection. To test whether the central memory-like phenotype and function acquired by OT-I cells after brief antigenic stimulation were restricted only to in vitro models or could also be maintained in vivo, we adoptively transferred briefly stimulated OT-I cells.
cells into wt B6 mice, rested them for 30–100 days, and challenged them with LPS-activated BmDCs pulsed with specific Ag. Briefly stimulated OT-I cells showed robust responses to antigenic challenge (Fig. 7A). These data suggest that briefly stimulated CD8 T cells can survive in vivo long term after adoptive transfer and, more importantly, can robustly respond to secondary antigenic challenge (27). On day 4 of BmDCs plus OVA challenge, staining for grB on ex vivo-isolated OT-I cells showed that the progeny of briefly stimulated OT-I cells expressed similar levels of grB compared with the progeny of control OT-I cells (Fig. 7B), implying that epigenetic modifications, if any, in briefly stimulated OT-I are not fixed or transmitted into daughter cells, thus permitting development of an array of effector functions, including cytotoxicity (28). Further study is required to examine in more detail the in vivo implications of brief antigenic stimulation on CD8 T cell effector functions.

Discussion

The fate of naive CD8 T cells can be influenced by multiple factors, like strength of signal, state of APCs, frequency of responders, etc. It has been shown in several studies that naive CD8 T cells require only a brief antigenic stimulation to develop full effector function and transit into memory. However, the validity of this concept has been questioned (29, 30). More recently, two groups (21, 31) have reported the phenotypic and functional difference between CD8 T cells responding to short-lived vs prolonged infection. The data in our study are the first to demonstrate that observed functional differences of CD8 T cells are already imprinted on them after the initial 20-h antigenic stimulation and have at least one predictable outcome: that brief (20-h) antigenic stimulation generates effector CD8 T cells with minimal cytotoxic activity but is capable of production of high levels of IL-2 and that the reverse is true for effector CD8 T cells generated by prolonged antigenic stimulation, while both types of effector CD8 T cells produce comparable amounts of IFN-γ and TNF-α. In our experiments, day 3 was chosen for analysis of CD8 T cell effector functions for the following reasons. First, on day 3, control CD8 T cells displayed maximal cytotoxic activity. Second, our main experiments were done with T cell cultures that were at no time replenished with fresh medium supplemented with exogenous cytokines. In these settings, the control CD8 T cell culture medium becomes acidic (yellow) by day 5. In addition, an analysis on day 4 showed similar differences in cytotoxic activity and IL-2 production between two types of effector T cells. Thus, day 3 appeared to be an optimal time for effector function analysis.

How can we reconcile these results with those already published? One study used expression of IFN-γ as the only readout of effector function in briefly stimulated CD8 T cells (10) and our own data are in agreement with this. Two other studies (8, 9) tested cytotoxic activity in addition to IFN-γ production. However, in these studies, briefly stimulated CD8 T cells were generated either by low-dose infection or, alternatively, infection was artificially curbed by antibiotic treatment at 24 h of initiation (9). In both cases, the exact timing of antigenic stimulation for the Ag-specific

FIGURE 6. Surface staining for activation/differentiation markers on day 3 effector OT-I cells. A, Histograms show representative staining. Top numbers indicate percent positive cells and bottom numbers are mean fluorescence intensity (MFI) for each marker. B, As in A, except only percent is shown. Dots represent individual experiments.

FIGURE 7. Memory CD8 T cells derived from briefly stimulated effector OT-I cells show robust responses to secondary antigenic challenge in vivo, and their progeny can develop full cytotoxic activity. A, Briefly stimulated and control effector OT-I cells were adoptively transferred into wt B6 mice, rested for 30–100 days, immunized with LPS-stimulated BmDCs pulsed with OVA peptide, and donor T cell expansion in the spleen 3–5 days later was analyzed by flow cytometry. Histograms are representative of three independent experiments. B, grB was detected by intracellular staining on ex vivo-isolated donor T cells day 4 after BmDC + OVA immunization. Percent grB-positive progeny of control and briefly stimulated OT-I cells are shown in dark and gray lines, respectively. Shaded histogram is gated on recipient CD8 T cells and used to define the background staining.
CD8 T cells cannot be controlled accurately, a limitation the authors themselves acknowledged. We have also observed that antigenic stimulation for >24 h resulted in significant increases in cytotoxic activity of effector OT-I cells (data not shown). Lastly, in two studies (7, 32), briefly stimulated OT-I cells showed high cytotoxic activity. Although the exact nature of this discrepancy is not clear, we think, cells genetically modified for Ag presentation (7) or exogenous IL-2 added to the culture of briefly stimulated OT-I cells (32) were likely to have contributed to this effect in their studies. Our own data suggest that adding exogenous IL-2 to the culture of briefly stimulated OT-I cells greatly improves their cytotoxic activity. Although the exact nature of this discrepancy is not clear, we think, cells genetically modified for Ag presentation (7) or exogenous IL-2 added to the culture of briefly stimulated OT-I cells (32) were likely to have contributed to this effect in their studies. Our own data suggest that adding exogenous IL-2 to the culture of briefly stimulated OT-I cells greatly improves their cytotoxic activity (Fig. 8A). In addition to IL-2, IL-12 (18) and IL-21 (33), but not IFN-γ (34), can partially rescue the development of cytotoxic activity in briefly stimulated OT-I cells (Fig. 8B–D). In addition, exogenous IL-12 and IL-21 highly up-regulated IL-10 production in briefly stimulated effector T cells (Fig. 8E). Of note, in contrast to control OT-I cells, IL-21 did not decrease IFN-γ production by briefly stimulated OT-I cells (Fig. 8F) (33).

What is the biological relevance of brief antigenic stimulation in vivo? What kind of role, if any, does or can it play? The stochastic nature of interaction between immune cells during immune responses can predict the generation of effector CD8 T cells with phenotypes similar to the one described in this manuscript (31, 35). This will be especially true for the secondary response where rapidly reactivated Ag-specific memory CD8 T cells would quickly deplete Ag-carrying APCs, creating a condition permissive for brief stimulation of naive CD8 T cells (36). This scenario would allow naive CD8 T cells involved in an immune response to bypass full effector differentiation and transit directly into the memory stage (37), thus preventing excessive generation of full-blown effector CD8 T cells while preserving efficient recruitment into the memory pool. Further studies are necessary to deepen our understanding as to how Ag affects the balance between effector vs memory generation (21, 31, 38–40), knowledge that will give new tools in designing vaccines.

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Disclosures
The authors have no financial conflict of interest.

References

**FIGURE 8.** IL-2, IL-12, and IL-21, but not IFN-α, can partially rescue the development of cytotoxic activity of briefly stimulated OT-I cells. Briefly stimulated OT-I cells were cultured without or with A) 50 U/ml IL-2 (A), 100 U/ml IL-12 (B), 100 ng/ml IL-21(C), and 1000 U/ml IFN-α (D). Error bars indicate SD and n is number of experiments. E, Day 3 effector OT-I cells generated in the presence of exogenous cytokines were restimulated with OVA peptide-pulsed EL-4 target cells at an E:T ratio of 2:1 for 4 h in the presence of 2 μg/ml brefeldin A, and expression of IL-10 was detected by intracellular staining. Background gate was set based on the staining of duplicate cultures for each effector CD8 T cell in the absence of peptide. Staining is representative of two independent experiments. F, As in E, except staining was done to detect IFN-γ expression in effector OT-I cells generated in the absence or presence of exogenously added IL-21.


