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### Mitochondrial Potential and Reactive Oxygen Intermediates in Antigen-Specific CD8<sup>+</sup> T Cells During Viral Infection<sup>1</sup> **FREE**

Jason M. Grayson; ... et. al

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# Mitochondrial Potential and Reactive Oxygen Intermediates in Antigen-Specific CD8<sup>+</sup> T Cells During Viral Infection<sup>1</sup>

Jason M. Grayson,<sup>2</sup> Nathan G. Laniewski, J. Gibson Lanier,<sup>3</sup> and Rafi Ahmed<sup>3</sup>

Following many viral infections, there are large expansions of Ag-specific CD8<sup>+</sup> T cells. After viral clearance, mechanisms exist to ensure that the vast majority of effector cells undergo apoptosis. In studies of thymocyte apoptosis, loss of mitochondrial potential ( $\Delta\Psi_m$ ) and excess production of reactive oxygen intermediates have been implicated as key events in cellular apoptosis. The purpose of the experiments presented in this work was to determine these parameters in Ag-specific CD8<sup>+</sup> T cells during a physiological response such as viral infection. Using lymphocytic choriomeningitis virus infection of mice, we found that Ag-specific CD8<sup>+</sup> effector T cells that had undergone recent TCR stimulation had an increased  $\Delta\Psi_m$ . These cells also had increased levels of superoxide. As these cells progressed through the contraction of the immune response, their potential decreased, but superoxide levels remained similar to naive cells. One of the consequences of reduced mitochondrial potential is membrane permeability and subsequent caspase activation. We examined both the enzymatic activity and levels of cleaved caspase 3, an effector caspase, and could only detect increased levels in Ag-specific CD8<sup>+</sup> T cells on day 5 postinfection, a time point in which virus was still present. This contrasts with Ag-specific effector cells examined during the contraction phase that had no detectable caspase activity directly *ex vivo*. These data suggest that the apoptotic program begins earlier than previously expected on day 5, during the expansion phase. *The Journal of Immunology*, 2003, 170: 4745–4751.

It is now established that during viral infections there are large expansions of Ag-specific CD8<sup>+</sup> T cells (1) (2). In the case of lymphocytic choriomeningitis virus (LCMV)<sup>4</sup> infection, 50% of CD8<sup>+</sup> T cells are virus specific at the peak of the effector response (1). The immune system returns to homeostasis through apoptosis of the vast majority of Ag-specific effector cells. Following this contraction phase, the remaining Ag-specific cells differentiate into memory cells that will be maintained at constant numbers and protect against disease for the life of the animal (3). Understanding the mechanisms that control apoptosis is critical not only for understanding antiviral immune responses, but also for graft rejection, autoimmunity, and cancer therapy.

In recent years, a large amount of research has demonstrated there are at least two mechanisms for cell death in mammalian cells: intrinsic and extrinsic signal-derived pathways. Pathways that are controlled by extrinsic signaling are initiated by binding of a ligand to its cognate receptor. One of the best-characterized examples of this type of pathway is the Fas/Fas ligand interaction (4).

Fas is a member of the TNFR superfamily. This large gene family is composed of over 20 family members, including TNFR1, Fas/CD95, death receptor 3, and death receptor 4/TNF-related apoptosis-inducing ligand. Ligation of these receptors can result in death or proliferation, depending on the signals that cells are receiving. Ligation of the Fas molecule culminates in activation of caspase 8/Fas-associated death domain-like IL-1-converting enzyme and downstream target cleavage. These targets include other caspases, lamins, poly(ADP) ribose polymerase, Bid, and DNases that cleave the genome (5).

Internal signals including DNA damage, production of reactive oxygen intermediates (ROI), NO, xenobiotics, and excess intracellular calcium are also capable of initiating apoptosis. One of the best-characterized pathways is DNA damage, which can induce death through the tumor suppressor p53. When DNA damage occurs, p53 becomes phosphorylated and binds DNA. Depending on the amount of DNA damage, cells either arrest in cell cycle or undergo apoptosis through p53 target genes (6). Some of the known molecular targets of p53 include Bax (7), p21, and PUMA (8, 9).

By various mechanisms, both external and internal signals have been shown to converge on the mitochondria. In addition to producing ATP, mitochondria are a key part of the cell's apoptotic machinery. Mitochondrion are organelles that contain two compartments: the matrix and the intermembrane space. The matrix is relatively impermeable, and this property is used to generate the electrochemical gradient ( $\Delta\Psi_m$ ) or potential that drives production of ATP (10). When cells undergo apoptosis, they undergo changes in mitochondrial membrane permeability that are accompanied by release of various proteins from the intermembrane space including cytochrome *c* (11); apoptosis-inducing factor (12); procaspases 2, 3, and 9 (13); and Hrt2 and second mitochondria-derived activator of caspases/Diablo (14). These proteins then initiate and maintain a caspase cascade and chromatin condensation and degradation. When these proteins are released, the inner membrane also becomes permeable and  $\Delta\Psi_m$  dissipates (10). This loss of potential can be assessed through FACS analysis with fluorometric dyes such as JC-1 and DiOC<sub>6</sub> (15). Loss of  $\Delta\Psi_m$  has been shown

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<sup>4</sup> Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; 7-AAD, 7-amino actinomycin D; DiOC<sub>6</sub>(3), 3,3'-dihexyloxycarbocyanide iodide; HE, dihydroethidium; m.f.i., median fluorescence intensity; ROI, reactive oxygen intermediate; Tg, transgenic.

to be a key event in apoptosis of thymocytes exposed to various apoptotic stimuli (16, 17) and in peripheral cells undergoing apoptosis following administration of staphylococcal enterotoxin B superantigen (18). Additionally, the death of thymocytes has been accompanied by the production of ROI as  $\Delta\Psi_m$  dissipates (16).

Using LCMV infection of mice and MHC class I tetramers, we examined  $\Delta\Psi_m$  and the production of ROI as Ag-specific CD8<sup>+</sup> T cells progressed from naive to effector to memory cells. We found that activated CD8<sup>+</sup> T cells contained increased  $\Delta\Psi_m$  that decreased as the cells underwent apoptosis during the contraction phase. Surprisingly, memory cells, which are more resistant to apoptosis than naive cells (19), also contained a low  $\Delta\Psi_m$  similar to that found in dying effector cells, but were not undergoing apoptosis. When production of ROI and caspase activity was examined, we found that Ag-specific CD8<sup>+</sup> T cells only contained increased amounts of superoxide and active caspase 3 on day 5 postinfection, in the presence of Ag and before the contraction phase. These data suggest that the apoptotic program begins on day 5 with the production of ROI and activation of caspases, but contraction is not observed until day 8, potentially because of lowered Bcl-2 levels and cessation of proliferation.

## Materials and Methods

### Virus infection and mice

Six- to 8-wk-old female BALB/c or C57BL/6 mice were purchased from the National Cancer Institute (Fredericksburg, MD). Perforin knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in our mouse colony. Mice were infected with  $2 \times 10^5$  PFU of LCMV-Armstrong i.p. or  $2 \times 10^6$  PFU LCMV clone 13 i.v. and used at the indicated time points. Virus stocks were grown and quantitated, as described previously (20).

### Priming of transgenic cells

Priming of P14 transgenic (Tg) cells has been described before (21). In these studies,  $10^4$  Tg cells were transferred into naive C57BL/6 mice, challenged with LCMV-Armstrong, and used at the indicated time points.

### Flow cytometry and FACS analysis

The preparation of splenocytes and surface staining was performed, as described previously (1). Samples were acquired on a FACSVantage instrument (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software (BD Immunocytometry Systems, Mountain View, CA).

### Dyes and mitochondrial staining

The 3,3'-dihexyloxycarbocyanide iodide (DiOC<sub>6</sub>(3)) and dihydroethidium (HE) were purchased from Molecular Probes (Eugene, OR). DiOC<sub>6</sub>(3) was dissolved in ethanol at a 10 mM concentration. HE was dissolved in DMSO at a 20 mM concentration. To assess  $\Delta\Psi_m$ , cells were incubated in 40 nM DiOC<sub>6</sub>(3) diluted in 10% FCS + RPMI for 30 min at 37°C. The cells were then washed once in ice-cold FACS buffer and then surface stained with anti-CD8 $\alpha$  Ab and MHC class I tetramers. For assessment of superoxide production, splenocytes were incubated with 5  $\mu$ M HE and stained, as described above.

### Dexamethasone treatment

Freshly explanted thymocytes were incubated in 50  $\mu$ M dexamethasone for 8 h and then used to assess apoptosis, loss of  $\Delta\Psi_m$ , and the production of ROI.

### Caspase substrate cleavage

PhiPhiLuxG<sub>1</sub>D<sub>2</sub> was purchased from OncoImmunin (Gaithersburg, MD). One million splenocytes were resuspended in 50  $\mu$ l of substrate and 5  $\mu$ l FCS for 1 h at 37°C. Cells were then washed once with FACS buffer and then surface stained, as described above, and acquired immediately.

### Detection of active caspase 3

After surface staining with Abs and MHC class I tetramers, as described above, splenocytes were stained intracellularly with PE-conjugated active caspase 3 from BD PharMingen (San Jose, CA), using a CytoFix/CytoPerm kit following the manufacturer's instructions.

### Annexin V and 7-amino actinomycin D (7-AAD) staining

For analysis of direct ex vivo apoptosis, splenocytes were isolated and then surface stained, as described above, and then incubated with annexin V and 7-AAD (BD PharMingen) at room temperature for 15 min and acquired immediately.

### Preparation of MHC class I tetramers

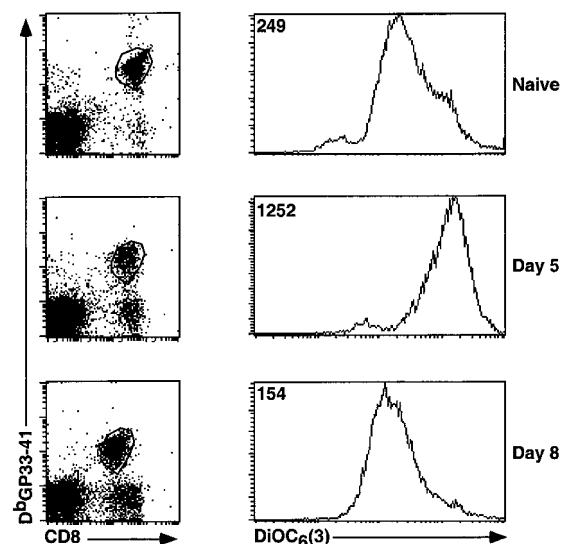
The construction and purification of L<sup>d</sup>NP118–126, D<sup>b</sup>GP33–41, D<sup>b</sup>NP396–404, and D<sup>b</sup>GP276–286 have been described previously (1).

## Results

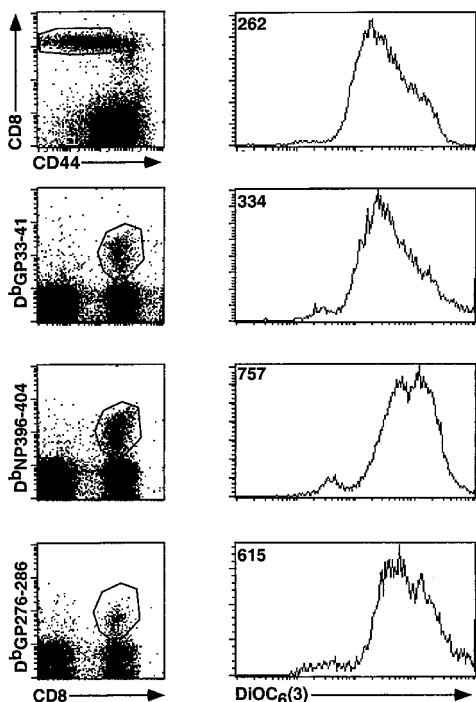
### $\Delta\Psi_m$ increases in response to Ag stimulation, but decreases in apoptotic effector cells

To understand how  $\Delta\Psi_m$  changes with T cell activation, differentiation, and death, we performed flow cytometry on P14 Tg CD8<sup>+</sup> T cells using the potential sensitive dye DiOC<sub>6</sub>(3). These cells express a TCR specific for the LCMV D<sup>b</sup>-restricted epitope GP33–41 and allow a comparison of Ag-specific naive, effector, and memory CD8<sup>+</sup> T cells. Fig. 1 shows that naive T cells had a moderate potential with a median fluorescence intensity (m.f.i.) of 249 that increased to 1252 in Ag-specific effector cells on day 5 postinfection. By day 8 postinfection, virus was cleared and the median fluorescence of the effector cells had decreased to 154. This suggests that increased potential is a consequence of metabolic activation of T cells, and decreasing potential reflects decreased activity and propensity to undergo apoptosis.

In addition to examining how  $\Delta\Psi_m$  changes in a monoclonal response, we also examined how polyclonal Ag-specific effector cells modulate their potential in C57BL/6 mice infected with LCMV (Fig. 2). By day 8 postinfection, there is a sizable response to three D<sup>b</sup>-restricted LCMV epitopes: GP33–41, NP396–404, and GP276–286. Compared with naive (CD8<sup>+</sup>CD44<sup>low</sup>) cells, GP33-specific cells contained a slightly increased fluorescence



**FIGURE 1.**  $\Delta\Psi_m$  in Ag-specific Tg CD8<sup>+</sup> T cells during the effector phase of an immune response. A total of  $10^4$  P14 Tg CD8<sup>+</sup> T cells was transferred into naive C57BL/6 hosts. Four hours after transfer, mice were infected with LCMV-Armstrong and used at the indicated time points. Spleen cells were incubated with DiOC<sub>6</sub>(3) and then double stained with anti-CD8 $\alpha$  and D<sup>b</sup>GP33–41. The DiOC<sub>6</sub>(3) levels of the gated populations are plotted in the histogram format, with the m.f.i. indicated by the value in the upper left-hand corner of the plot. At each time point, six mice were individually examined in two independent experiments. A representative mouse is presented in each histogram.



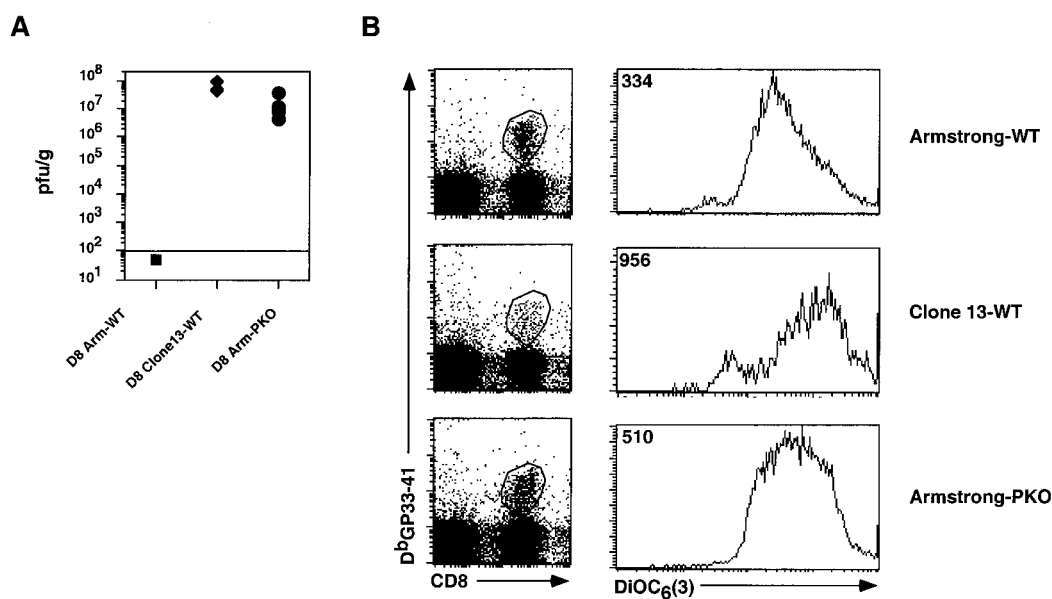
**FIGURE 2.** Analysis of  $\Delta\Psi_m$  in Ag-specific CD8<sup>+</sup> T cells of different Ag specificities by MHC class I tetramers. Spleen cells from naive and LCMV-infected mice (8 days postinfection) were incubated with DiOC<sub>6</sub>(3) and then double stained with CD8 $\alpha$  and CD44 Ab (naive) or D<sup>b</sup> MHC class I tetramers (GP33–41 or NP396–404 or GP276–286). The DiOC<sub>6</sub>(3) levels of the gated populations are plotted in the histogram format, with the m.f.i. indicated by the value in the upper left-hand corner of the plot. At each time point, six mice were individually examined in two independent experiments. A representative mouse is presented in each histogram.

(334 vs 262). When NP396- and GP276-specific cells were examined, they also had an increased potential (757 and 615 vs 262). Because it is unlikely that all three viral epitopes are expressed at

or cleared at the same level, the differences observed between the various populations of Ag-specific CD8<sup>+</sup> T cells could be due to more recent Ag stimulation.

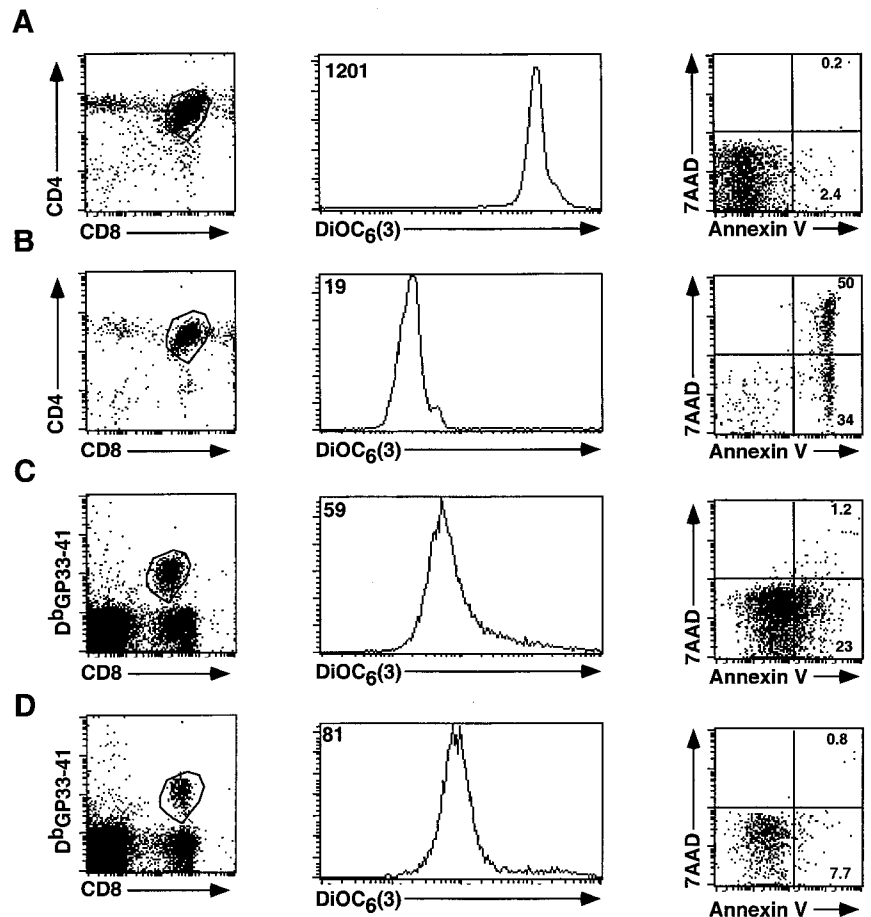
To formally test this possibility, we used situations in which viral Ag continued to persist and examined  $\Delta\Psi_m$ . Fig. 3A shows that when C57BL/6 mice were infected with the Armstrong strain of LCMV, 8 days postinfection the amount of virus in the spleen was below the limit of detection by plaque assay ( $\leq 50$  PFU/g). This contrasts with infection by the Clone 13 strain of LCMV, which induces a chronic infection that takes 90–120 days to be brought under control. Eight days postinfection, there was a large amount of virus in the spleen (average =  $5 \times 10^7$  PFU/g). To test the possibility that viral milieu causes differences in  $\Delta\Psi_m$ , we infected perforin knockout mice with the Armstrong strain of LCMV. These mice cannot clear the virus, and by 8 days postinfection, there was a large amount of virus in the spleen (average =  $8 \times 10^6$  PFU/g). When  $\Delta\Psi_m$  was examined in GP33-specific cells (B), we found that during Clone 13 infection, the Ag-specific cells contained a higher  $\Delta\Psi_m$  than during Armstrong infection (956 vs 334). This effect was not restricted to Clone 13 infection, as perforin knockout mice infected with the Armstrong strain of LCMV also had an increased potential (510 vs 334). Thus, increased TCR stimulation correlates with increased  $\Delta\Psi_m$ .

In addition to examining  $\Delta\Psi_m$  during the effector phase of a CD8<sup>+</sup> T cell response, we were interested in assessing how it changed during the contraction phase. For a point of comparison, we examined CD4<sup>+</sup>CD8<sup>+</sup> thymocytes as they underwent apoptosis after treatment with dexamethasone. Previous studies have shown that dexamethasone is a potent inducer of thymocyte apoptosis. In Fig. 4, A and B, thymocytes before addition to dexamethasone had a median fluorescence of 1201 that decreased to 19 after treatment. Examination of P14 Tg Ag-specific CD8<sup>+</sup> T cells during the contraction phase (day 14, C) revealed that the  $\Delta\Psi_m$  was decreased compared with either naive or day 8 effector cells (59 (D14) vs 154 (Fig. 1, D8) vs 249 (Fig. 1, naive)). When memory cells were examined (D), their  $\Delta\Psi_m$  had only increased



**FIGURE 3.**  $\Delta\Psi_m$  in Ag-specific CD8<sup>+</sup> T cells is related to Ag exposure. C57BL/6 or perforin knockout (PKO) mice were infected with either LCMV-Armstrong or Clone 13. A, Viral titer was assessed 8 days postinfection in the spleen by plaque assay. B,  $\Delta\Psi_m$  was assessed by incubation in DiOC<sub>6</sub>(3), followed by staining with anti-CD8 $\alpha$  and D<sup>b</sup>GP33–41. The DiOC<sub>6</sub>(3) levels of the gated populations are plotted in the histogram format, with the m.f.i. indicated by the value in the upper left-hand corner of the plot. At each time point, six mice were individually examined in two independent experiments. A representative mouse is presented in each histogram.





**FIGURE 4.** Apoptosis of Ag-specific CD8<sup>+</sup> T cells is characterized by loss of  $\Delta\Psi_m$ .  $\Delta\Psi_m$  of thymocytes directly ex vivo (A) and after 8-h incubation in 50  $\mu$ m dexamethasone (B) or Tg CD8<sup>+</sup> T cells 14 (C) or 300 (D) days postinfection was examined by staining with DiOC<sub>6</sub>(3), as previously described. Thymocytes were surface stained with anti-CD8 $\alpha$  and anti-CD4 Abs, and splenocytes with anti-CD8 $\alpha$  and D<sup>b</sup>GP33–41. Apoptosis was assessed by a brief incubation with annexin V and 7-AAD. The samples were acquired immediately, and the value in the dot plot indicates the percentage of each gated region that falls into the quadrant. At each time point, six mice were individually examined in two independent experiments.

slightly (m.f.i. 81). Previous studies have shown that loss of mitochondrial potential is associated with apoptosis. Cells that are in the early stages of apoptosis are annexin V<sup>+</sup>, while those that are dead or in the late stages are annexin V<sup>+</sup> 7-AAD<sup>+</sup>. Using these reagents, we found that before treatment CD4<sup>+</sup>CD8<sup>+</sup> thymocytes had very few cells in either region (2.4% early, 0.2% late). After dexamethasone treatment, almost all the cells were in the early stages of apoptosis or dead (34% early, 50% late). Examination of cells from Tg mice on day 14 postinfection revealed an increase in the number of cells in the early stages of apoptosis (23% early, 1.2% late). In this situation, increased apoptosis is observed in a population that has a reduced  $\Delta\Psi_m$ . Ag-specific memory cells had few cells in either stage of apoptosis and were similar to naive cells (7.7% early, 0.8% late). In this situation,  $\Delta\Psi_m$  and resistance to apoptosis are uncoupled. Memory cells are more resistant to apoptosis than naive cells (19), yet they contain a reduced  $\Delta\Psi_m$ .

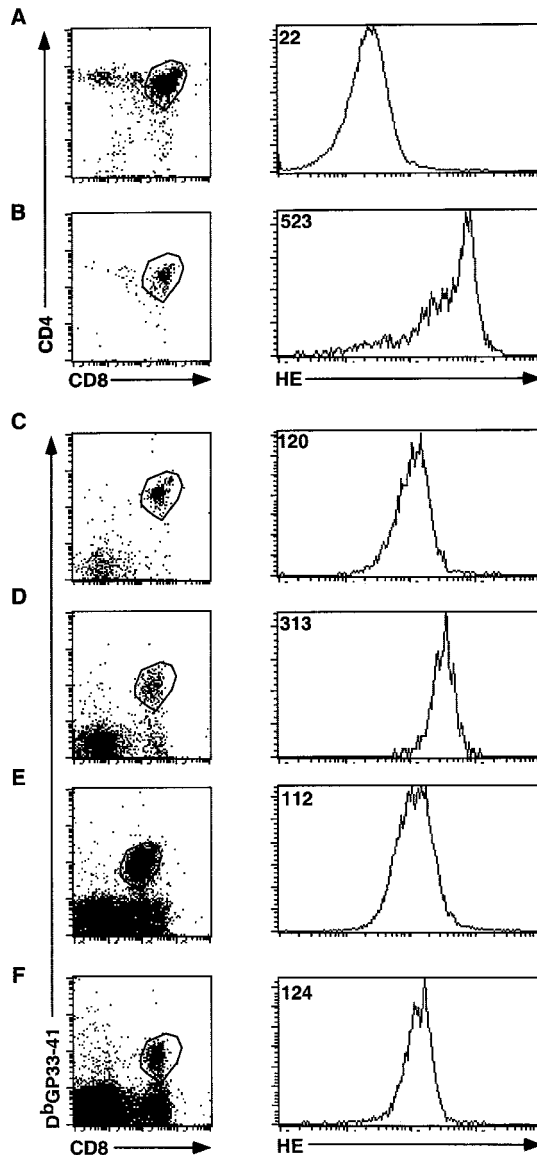
#### *Ag-specific CD8<sup>+</sup> T cells produce increased ROI early, but not late in an immune response*

In addition to decreases in  $\Delta\Psi_m$ , apoptosis in T cells has been accompanied by increases in production of ROI. We assessed production of superoxide in Ag-specific CD8<sup>+</sup> T cells using the fluorescent dye HE. We used CD4<sup>+</sup>CD8<sup>+</sup> thymocytes treated with dexamethasone as a positive control. In Fig. 5, A and B, the median fluorescence increased from 22 for thymocytes analyzed directly ex vivo to 523 after treatment with dexamethasone. Ag-specific naive Tg CD8<sup>+</sup> T cells had higher levels of superoxide compared with thymocytes (C, 120 vs 22) that increased in effector cells 5 days postinfection (D, 313). After the virus was cleared and the effector cells began to undergo apoptosis on day 8 (E), the levels

of superoxide actually decreased and remained at lower levels, as memory was established (F). These data are consistent with production of ROI early, a period of increased metabolic activity due to rapid proliferation and cytokine production.

#### *Increased levels of caspase activity are observed when Ag-specific CD8<sup>+</sup> T cells are exposed to virus*

One of the more critical steps in apoptosis is activation of caspases. These enzymes exist as inactive zymogens that undergo cleavage to become functional. After its release from the mitochondria, cytochrome *c* associates with APAF-1, dATP, and procaspase 9. In this complex, caspase 9 becomes active and cleaves downstream targets including procaspase 3. Using both a fluorescent labeled substrate for caspase 3 and an Ab that detects the cleaved active form of the enzyme, we followed caspase activation as cells progressed from naive to effector to memory cells (Fig. 6). We used BALB/c mice, which direct a majority (95%) of their Ag-specific CD8<sup>+</sup> T cell response to the L<sup>d</sup>-restricted LCMV CTL epitope NP118–126. Naive (CD8<sup>+</sup>LFA-1<sup>low</sup>) cells contained little detectable caspase enzymatic activity or active form of the enzyme (m.f.i. 9 (substrate) and 8 (active form)). On day 5 postinfection (B), an increase in the amount of enzymatic activity and active form of the enzyme was observed (m.f.i. 26 and 21, respectively). From days 5 to 8 (C), the amount of enzymatic activity and active form of caspase 3 returned to levels comparable to those found in naive cells. These levels were maintained throughout the contraction phase on day 15 (D) and into the memory phase on day 80 (E). These data show that caspase 3 activation occurs during the expansion phase and not during the contraction phase.



**FIGURE 5.** Superoxide production in Ag-specific CD8<sup>+</sup> T cells during an immune response. Thymocytes were isolated from naive C57BL/6 mice (A) and incubated in 50  $\mu$ M dexamethasone for 8 h (B) and serve as a reference point for increased superoxide production during T cell apoptosis. A total of  $10^4$  P14 Tg CD8<sup>+</sup> T cells was transferred into naive C57BL/6 hosts. Four hours after transfer, mice were infected with LCMV-Armstrong and used at 0 (C), 5 (D), 8 (E), and 300 days (F) postinfection. Spleen cells were incubated with HE and then double stained with anti-CD8 $\alpha$  and D<sup>b</sup>GP33–41. Thymocytes were treated in a similar manner, except the cells were stained with anti-CD8 $\alpha$  and anti-CD4 Abs. The HE levels are plotted in the histogram format, with the m.f.i. indicated by the value in the upper left-hand corner of the plot. At each time point, six mice were individually examined in two independent experiments. A representative mouse is presented in each histogram.

## Discussion

In this study, we have examined how mitochondrial potential, superoxide production, and activation of caspases change in Ag-specific CD8<sup>+</sup> T cells during viral infection. We found that effector cells, in the presence of virus, had increased  $\Delta\Psi_m$  compared with naive cells, but that this potential decreased as the immune response contracted. Surprisingly, we found that memory cells also had a reduced  $\Delta\Psi_m$  compared with naive cells. Examination of the production of superoxide revealed that Ag-specific cells only make

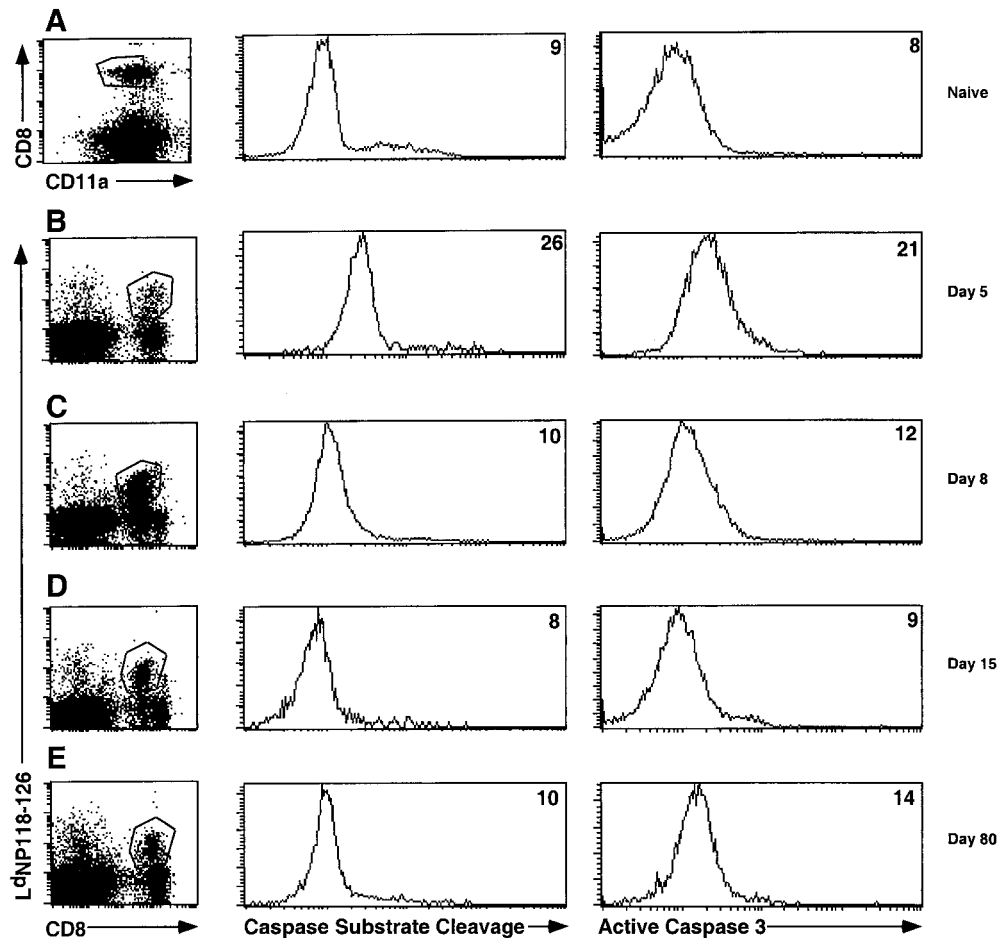
increased levels of ROI in the presence of virus during a period of high metabolic activity. Additionally, we detected maximal caspase activity on day 5 postinfection before the contraction of the immune response. These data are consistent with a model in which cellular damage from ROI induces the apoptotic program to begin during the expansion. Actual reduction in cell numbers does not occur until proliferation and antiapoptotic survival signals cease.

What are the implications of our observed changes in  $\Delta\Psi_m$ ? In many studies, loss of  $\Delta\Psi_m$  has been observed as cells undergo apoptosis (22). This decrease in potential is accompanied by changes in membrane permeability and release of apoptogenic factors such as cytochrome *c* and apoptosis-inducing factor. To date, these studies examined either thymocytes treated with chemical agents and irradiation or peripheral T cells of unknown specificity (23). In our study, we examined how mitochondrial potential changed as cells progressed from naive to effector to memory cells after activation by a physiological stimulus, viral infection. Dramatically increased  $\Delta\Psi_m$  is only observed in effector cells in the presence of virus. A recent pair of studies by Gergely et al. (24, 25) also observed mitochondrial hyperpolarization in lymphocytes isolated from systemic lupus erythematosus patients. Increased mitochondrial potential may be due to TCR stimulation *in vivo*, which may be due to an increased need for oxidative metabolism to make cytokines and perforin granules, and to sustain the rapid replication that these cells undergo. When virus is cleared *in vivo*, the demand for effector function also decreases. For example, *in vitro* studies of cytokine production have shown that removal of Ag from effector cells almost immediately terminates cytokine production (26). After virus is cleared, cells proceed through a brief Ag-independent replication phase (27) (28). These divisions may not be as energetically demanding for the cells, so mitochondrial potential may drop.

After this short period of Ag-independent replication, the immune response begins to contract. At these time points (days 8 and 14), we observed decreased  $\Delta\Psi_m$  and increased numbers of cells in the early stages of apoptosis. It is important to note that while  $\Delta\Psi_m$  does decrease compared with naive cells, it doesn't decrease to the same level as thymocytes treated with dexamethasone. If Tg cells are irradiated, their potential does drop to similar levels (J. Grayson, data not shown), showing that it is possible to decrease  $\Delta\Psi_m$  further. Previous studies have demonstrated that low  $\Delta\Psi_m$  is accompanied by changes in mitochondrial permeability with release of cytochrome *c* and apoptosis-inducing factor. Whether these death inducers are released in Ag-specific cells remains to be determined.

Although we observe low  $\Delta\Psi_m$  and high levels of annexin V binding in the dying effector population, this contrasts with memory cells, which also have a low  $\Delta\Psi_m$ , but very few cells are apoptotic. One key difference between memory cells and effector cells in the contraction phase is that memory cells have very high levels of Bcl-2, whereas the effector cells have effectively shut off Bcl-2 expression (29). Even with a lower mitochondrial potential, memory cells are more resistant to apoptotic stimuli than naive cells (19).

In addition to decreases in  $\Delta\Psi_m$ , apoptosis in T cells is often accompanied by bursts of ROI. Ag-specific CD8<sup>+</sup> T cells in the contraction phase have a lowered  $\Delta\Psi_m$ , but the levels of superoxide are equivalent to that of a naive CD8<sup>+</sup> T cell. The only situation in which increased levels of superoxide are observed is when Ag-specific effector cells have undergone recent TCR stimulation. There are two potential interpretations of these results. First, the levels of superoxide are less than those observed in thymocytes treated with dexamethasone, and it is likely that it may be



**FIGURE 6.** Maximal caspase activity occurs before the initiation of the contraction phase. Spleen cells from naive (A) and LCMV-infected mice on days 5 (B), 8 (C), 15 (D), and 80 (E) postinfection were incubated with PhiPhiLuxG<sub>1</sub>D<sub>2</sub> and then surface stained with CD8 $\alpha$  and CD11a Ab (naive) or L<sup>d</sup>NP118–126–126 tetramer. The active form of caspase 3 was detected by surface-staining cells, followed by intracellular staining performed with the active caspase 3 Ab. The level of cleaved substrate or active fragment of the gated populations is plotted in the histogram format, with the m.f.i. indicated by the value in the upper right-hand corner. At each time point, six mice were individually examined in two independent experiments. A representative mouse is presented in each histogram.

functioning as a signaling intermediate. Previous studies have shown that at low levels superoxide can enhance proliferation in fibroblasts, and that T cells treated with antioxidants, which should decrease superoxide, undergo less proliferation (30–33). The second interpretation is that the increased superoxide generated at this time is damaging macromolecules, and apoptosis follows when cells cannot repair the damage. These two interpretations do not have to be mutually exclusive; superoxide could induce proliferation and induce macromolecule damage at the same time.

Activation of caspases is a critical step in apoptosis, leading to cleavage of proteins and activation of nucleases that accelerate the destruction of the cell. These enzymes can be divided into two groups: initiator/upstream caspases such as caspases 2, 8, and 9, and downstream/effector caspases such as caspases 3, 6, and 7 (5). These downstream caspases are thought to be the main executioners of the apoptotic program. Recent studies have shown that caspases 3, 6, 7 (34), and 8 (35) are activated by TCR stimulation in cells that do not appear to be undergoing apoptosis. The downstream targets of activated caspases in these cells have not been identified. We only observe activation of caspase 3 on day 5 postinfection. Coupled with our observations that these cells are producing large amounts of ROI, these data suggest that the apoptotic program of contraction may begin earlier than previously

thought, but the number of Ag-specific CD8<sup>+</sup> T cells do not decrease due to proliferative and survival signals. Previously, we demonstrated that effector cells on day 5 postinfection contain moderate levels of Bcl-2 (29), while effectors on day 8 contain dramatically reduced Bcl-2. One function of caspase activation on day 5 may be to degrade antiapoptotic signals such as those conveyed by Bcl-2 or Akt kinase. As the infection is cleared, cytokine signaling will decrease, leading to further decreases in prosurvival molecules and increased apoptosis. In conclusion, we have shown that  $\Delta\Psi_m$  is modulated as Ag-specific CD8<sup>+</sup> T cells become activated, expand, and die. We found that Ag-specific CD8<sup>+</sup> T cells increase  $\Delta\Psi_m$  in the presence of Ag, while dying effector cells have a reduced  $\Delta\Psi_m$  and normal levels of superoxide. Ag-specific memory CD8<sup>+</sup> T cells also contain a reduced  $\Delta\Psi_m$ , yet they are resistant to apoptosis. Additionally, we found that caspase activation occurred earlier than predicted during the expansion phase, suggesting that the apoptotic program may be initiated earlier than previously thought.

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