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An In Vivo Cytotoxicity Threshold for Influenza A Virus-Specific Effector and Memory CD8⁺ T Cells

John Stambas, Peter C. Doherty, and Stephen J. Turner

Influenza A virus infection of C57BL/6 (B6) mice is characterized by prominent CD8⁺ T cell responses to H2Db complexed with peptides from the viral nucleoprotein (NP366, ASNENMETM) and acid polymerase (PA224, SSLENFRAYV). An in vivo cytotoxicity assay that depends on the adoptive transfer of peptide-pulsed, syngeneic targets was used in this study to quantitate the cytotoxic potential of DⁿNP₃₆₆⁻ and DⁿPA₂₂₄⁻specific acute and memory CD8⁺ T cells following primary or secondary virus challenge. Both T cell populations displayed equivalent levels of in vivo effector function when comparable numbers were transferred into naive B6 hosts. Cytotoxic activity following primary infection clearly correlated with the frequency of tetramer-stained CD8⁺ T cells. This relationship looked, however, to be less direct following secondary exposure, partly because the numbers of CD8⁺DⁿNP₃₆₆⁺ T cells were greatly in excess. However, calculating the in vivo E:T ratios indicated that in vivo lysis, like many other biological functions, is threshold dependent. Furthermore, the capacity to eliminate peptide-pulsed targets was independent of the differentiation state (i.e., primary or secondary effectors) and was comparable for the two T cell specificities that were analyzed. These experiments provide insights that may be of value for adoptive immunotherapy, where careful consideration of both the activation state and the number of effector cells is required.

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A sensitive in vivo cytotoxicity assay has been developed and used to assess the cytotoxic potential of multiple effector and memory CD8⁺ T cell populations following systemic lymphocytic choriomeningitis virus (LCMV) and mouse polyoma virus infection. The capacity of this assay to detect what is presumed to

Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; i.n., intranasal; NP, viral nucleoprotein; PA, acid polymerase; NA, neuraminidase; PI, propidium iodide; FSC, forward scatter; SSC, side scatter.

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be phagocytic removal of killed targets rapidly after recall (18, 19) adds a dimension that is not measured by in vitro $^{51}$Cr release. The present study uses this in vivo CTL assay to quantitate effector function mediated by CD8$^+$ D$^b$NP$^{566}$- and CD8$^+$ D$^b$PA$^{224}$ T cell populations in the influenza model of localized virus infection, where (unlike the situation for LCMV) virus replication is constrained to the respiratory epithelium, due to restriction of the serine protease (20) required for cleavage of the viral hemagglutinin molecule. The analysis covers the effector and memory phases following both primary and secondary challenge and suggests a threshold model for target cell elimination.

Materials and Methods

Mice and infection
The B6 (H2$^b$) mice used throughout this study were bred and housed under specific pathogen-free conditions at the Microbiology and Immunology Department Animal Facility (University of Melbourne, Parkville, Melbourne, Australia) and the study was reviewed and approved by the animal ethics committee. Naive 6- to 8-wk-old mice were infected intranasally (i.n.) with 1 $\times$ 10$^3$ PFU of the HKx31 virus and spleens were sampled at various time points: (A) primary day 10 acute, (B) secondary day 7 acute, (C) primary day 181 memory, or (D) secondary day 153 memory. The CD8$^+$ D$^b$NP$^{566}$ and CD8$^+$ D$^b$PA$^{224}$ cell numbers were equalized after tetramer staining and titrated before addition of $^{51}$Cr-labeled NP$^{566}$ or PA$^{224}$ peptide-pulsed EL-4 target cells for 4 h in round-bottom 96-well plates. Direct ex vivo cytoxicity was then determined as specific $^{51}$Cr release and the results were expressed as means ± SD.

Adoptive transfer experiments
To generate T cell populations that were enriched for either the CD8$^+$ D$^b$NP$^{566}$ or CD8$^+$ D$^b$PA$^{224}$ sets while lacking the other population, B6 mice were primed with rHKx31 viruses that had been engineered to disrupt the epitope-specific cells were equalized and separately titrated in 96-well plates. Direct ex vivo cytotoxicity was then determined as described below.

Flow cytometric analysis of CD8$^+$ T cells
The kinetics, magnitude, and phenotype of primary and secondary virus-specific CD8$^+$ T cell responses were measured by flow cytometry. The D$^b$NP$^{566}$-specific and D$^b$PA$^{224}$-specific CD8$^+$ T cell populations were characterized using tetramers (24) generated by complexing H2D$^d$, conjugated to streptavidin-PE (Molecular Probes), with NP$^{566}$ or PA$^{224}$ peptide. Spleenocytes were incubated with tetrameric complexes for 60 min at room temperature, washed, and stained with rat anti-mouse CD8α-PerCP Cy5.5 (BD Pharmingen) and rat anti-mouse CD62L-allophycocyanin (BD Pharmingen) for 30 min on ice. Following further washing, cells were resuspended in PBS containing 1% BSA/0.02% NaN$_3$ and data were acquired on a FACSCalibur (BD Biosciences). CellQuest software (BD Biosciences) was used for analysis.

Ex vivo $^{51}$Cr release cytotoxicity assay
Target H2$^b$ EL-4 cells were pulsed with 1 $\mu$M NP$^{566}$ or PA$^{224}$ and 750 $\mu$Cl $^{51}$Cr for 1 h at 37°C. Tetramer staining was used to determine the number of effector CD8$^+$ D$^b$NP$^{566}$ and CD8$^+$ D$^b$PA$^{224}$ T cells after primary and secondary influenza A virus infection. The numbers of both epitope-specific cells were equalized and separately titrated in 96-well round-bottom microtiter plates before the addition of 1 $\times$ 10$^5$ $^{51}$Cr-labeled targets for direct analysis of ex vivo cytoxicity. Supernatants (50 $\mu$l) were harvested after 4 h of culture at 37°C, 5% CO$_2$, and transferred to Lumina plates (Packard Instrument), where they were left to dry overnight. Gamma irradiation was then measured on a Topcount NXT microplate scintillation and luminescence counter (Packard Instrument). The percentage of specific $^{51}$Cr release was then calculated as ($($experimental-spontaneous release)/($maximum-spontaneous release$)) × 100, where spontaneous release of $^{51}$Cr was measured from culture of target cells in the absence of effectors and the maximum
release was measured by the addition of 1% Triton X-100 to \(^{51}\)Cr-labeled targets.

**In vivo cytotoxicity assay**

Target cell populations were prepared from naive B6 mice using RBC-lysed splenocytes. The residual white cells (1.5 × 10^6 cells/ml PBS) were first labeled with 5 \(\mu\)M Vybrant DiD (Molecular Probes) for 25 min at 37°C, before quenching with 1% BSA/PBS. They were then separated into three populations and costained with 0.001, 0.05, or 0.5 \(\mu\)M CFSE in PBS. CFSE labeling was subsequently quenched with 1% BSA/PBS and targets pulsed with NP\(_{366}\) or PA\(_{224}\) peptides, or left unpulsed (0.5 \(\mu\)M target population) as an internal control. Five million cells from each population were then mixed together in equal proportions and injected i.v. into naive or infected B6 mice. Spleens were removed 4 h later and single-cell suspensions were generated before acquisition on a FACS Calibur (BD Biosciences). DiD\(^+\) donor target splenocytes were differentiated from host cells and the percentage of target cell killing was determined as: 100 − (percentage of peptide-pulsed targets in infected recipients/percentage of unpulsed targets in infected recipients)/(percentage of peptide-pulsed targets in naive recipients/unpulsed targets in naive recipients) × 100.

**Flow cytometric analysis of residual peptide-pulsed targets cells**

Splenocytes recovered from day 7 secondary infected mice following the in vivo cytotoxicity assay were stained with Annexin V\(^{PE}\) Ab and propidium iodide (PI) for 15 min at room temperature to determine the level of apoptosis and ability of DiD\(^+\)CFSE\(^+\) target cells to exclude PI. Data was acquired on a FACS Calibur (BD Biosciences) and CellQuest software (BD Biosciences) used for analysis.

**Results**

**Ex vivo \(^{51}\)Cr release by D\(^0\)NP\(_{366}\) and D\(^0\)PA\(_{224}\)-specific CTL**

Short-term \(^{51}\)Cr release from virus-infected or peptide-pulsed targets incubated with freshly isolated T cells (16), T cell clones (8), or ex vivo lymphocyte populations stimulated in vitro with peptide-pulsed syngeneic feeders (9) has been the standard measure of influenza virus-specific CD\(^8\) CTL activity. We thus, for comparison, present (Fig. 1) some limited \(^{51}\)Cr release data for the stages of the response that we analyze here in more detail by the in vivo cytotoxic assay (Figs. 2–4).

Splenic CD\(^8\) T cells from the peak of the acute primary (day 10), acute secondary (day 7), and the memory (primary day 181; secondary day 153) phases following influenza A virus infection were stained with tetramer complexes to determine the number of D\(^0\)NP\(_{366}\)- and D\(^0\)PA\(_{224}\)-specific CD\(^8\) T cells so that equivalent E:T ratios could be calculated before adding peptide-pulsed targets. The primary day 10 response (Fig. 1A) shows low-level, but detectable, lysis of NP\(_{366}\) and PA\(_{224}\)-pulsed EL4 targets (10 ± 2 and 2 ± 4\%, respectively). Analysis of the acute, secondary response demonstrated that freshly isolated CD\(^8\)D\(^0\)NP\(_{366}\)- and CD\(^8\)D\(^0\)PA\(_{224}\)-specific effectors eliminated their respective peptide-pulsed targets equivalently (51 ± 7 and 44 ± 5\% at 3:1) (Fig. 1B). No such ex vivo \(^{51}\)Cr release CTL activity was found for the “resting,” memory T cell populations that persist after primary (Fig. 1C) or secondary (Fig. 1D) challenge.

**Phenotype of the primary response and in vivo CTL activity**

Quantitative, phenotypic, and functional data for primary CD\(^8\)D\(^0\)NP\(_{366}\) and CD\(^8\)D\(^0\)PA\(_{224}\)-specific T cell responses is presented here as dot plots and histograms for the peak of the primary response (day 10) (Fig. 2) or as part of a detailed time course (Fig. 3). Analysis by tetramer staining showed the expansion, then contraction, of virus-specific CD\(^8\) T cell populations that we would expect for these influenza-specific responses (Fig. 3A), together with the rapid acquisition and slower loss of the “activated” CD62L\(^{low}\) phenotype (Fig. 3B). These mice were, of course, injected with the peptide-pulsed targets that we use for the in vivo CTL assay, so it is worth noting that the profiles for CD62L expression (Fig. 3B) are essentially equivalent to those seen previously in a kinetic analysis from virus-infected, but otherwise unmanipulated, mice (25).

Equivalent numbers of DiD\(^+\)CFSE\(^+\) NP\(_{366}\), PA\(_{224}\) peptide-pulsed and DiD\(^+\)CFSE\(^+\) unpulsed target cells were adoptively transferred into HKx31-infected mice to measure CD8\(^+\)D\(^0\)NP\(_{366}\) and CD8\(^+\)D\(^0\)PA\(_{224}\)-specific CTL activity. In general, the measure of in vivo cytotoxicity (Fig. 3C) correlated with virus-specific T cell frequencies (Fig. 3A). The significantly smaller numbers of CD8\(^+\)PA\(_{224}\) T cells transiting into memory on day 15 (Fig. 3A) were reflected in lower CTL activity at the same time point (Fig. 3C). One, perhaps minor, divergence from this theme that T cell counts and in vivo effector function are tightly linked was that the “memory” T cells detected on day 276 seemed more potent effectors than the initial (day 5) responders (CD8\(^+\)D\(^0\)NP\(_{366}\); \(p < 0.04\), CD8\(^+\)D\(^0\)PA\(_{224}\); \(p < 0.002\); Fig. 3C), although there was no comparable difference in the prevalence of tetramer\(^+\) T cells or in the CD62L phenotype (Fig. 3, A and B). Clearly, there is an effect of differentiation state in this in vivo CTL assay that goes beyond a simple correlation with T cell prevalence.

**The secondary response**

The same analysis for the secondary response showed the enormous disparity (seven times on day 7) in CD8\(^+\)D\(^0\)NP\(_{366}\) and CD8\(^+\)D\(^0\)PA\(_{224}\) T cell numbers (Fig. 4A) that we have described previously (9, 13, 14). Again, secondary challenge was associated
with loss of the CD62L marker from the majority of the T cells (Fig. 4B), and the relative prevalence of the CD8+ D\textsuperscript{NP\textsubscript{366}} and CD8+ D\textsuperscript{PA\textsubscript{224}} CD62L\textsubscript{low} sets was comparable through to very long-term memory (days 129 and 482; Fig. 4B). The significantly lower level of activation achieved for the CD8+ PA\textsubscript{224} set during the acute phase (days 7 and 60; Fig. 4B) may reflect the lower Ag dose for D\textsuperscript{PA\textsubscript{224}} (23).

Despite this substantial difference in numbers for the two epitope-specific T cell populations (Fig. 4A), the correlation between tetramer\textsuperscript{+} lymphocyte counts and in vivo CTL activity that we saw for the primary response (Fig. 3, A and C) was, especially in the memory phase (from day 60), much less apparent following secondary challenge (Fig. 4, A and C). This could, of course, reflect that, even for the smaller secondary CD8+ D\textsuperscript{PA\textsubscript{224}} response, the memory T cells persist at a level (26) above that at which we might expect to see a titration of the in vivo CTL effect. An alternative possibility is that the secondary CD8+ D\textsuperscript{PA\textsubscript{224}}-specific T cells are, on a cell-for-cell basis, more efficient killers than the CD8+ D\textsuperscript{NP\textsubscript{366}} set.

**Adjusted T cell numbers and in vivo CTL efficacy**

The possibility that individual CD8+ D\textsuperscript{PA\textsubscript{224}} memory T cells generated after secondary challenge may be more effective than the
CD8⁺DbNP366⁺ set at mediating in vivo CTL activity was addressed by transferring the same number of each (6.9 x 10⁵) into normal mice (Table I). Administration of peptide-pulsed targets showed equivalent killing by both T cell populations (CD8⁺DbNP366⁺, 21 ± 3% and CD8⁺DbPA224⁺, 19 ± 3%). Because the DanP366⁺ or DbPA224⁺-specific CD8⁺ T cell counts (determined by tetramer staining) in spleen (4.40 ± 0.05 log₁₀ and 4.52 ± 0.15 log₁₀) were also comparable, there was clearly no difference in CTL efficacy for the CD8⁺DbNP366⁺ and CD8⁺DbPA224⁺ memory T cells.

The threshold effect

Because we transferred a set number of targets, and we knew (from tetramer staining) the numbers of epitope-specific T cells in spleen, it was possible to calculate in vivo E:T ratios for every time point following primary or secondary virus challenge. The E:T ratio for CD8⁺DbNP366⁺ CTL detected in spleen at the peak of the primary response on day 10 (80% lysis; Fig. 3C) was calculated as 6.90 ± 3.30 (Fig. 5A). These values (80% kill at ~7:1) were then used as the point of reference for the secondary analysis (Fig. 5B). All E:T ratios for CD8⁺DbNP366⁺ CTL remained above the 7:1 level from the acute secondary response through to long-term memory (day 482) and the level of CTL activity was consistently >80% (Fig. 5B). Only one calculated E:T ratio (day 7, 10.1 ± 4.24) was found to be above the 7:1 threshold (>80% kill) for the secondary CD8⁺DbPA224⁺ response (Fig. 5B). Otherwise, though, the E:T ratios for secondary CD8⁺DbPA224⁺ T cells that were uniformly >0.7:1 showed at least 40% lysis (Figs. 4C and 5B); levels below 0.1:1 in the primary response were associated with much lower levels of killing (Figs. 3C and 5A). It thus seems that any effector or memory T cell population that achieves this 7:1 ratio will show evidence of maximal in vivo CTL activity (80%), with the level still remaining at something above 40% at 0.7:1, then falling as the T cells become less prevalent.

Characteristics of the residual targets

Though we have not analyzed the effect in any detail for these in vivo experiments (Figs. 3 and 4), we noticed an odd, differential effect on the characteristics of the residual target cells that were recovered from mice. At, or near to, the peak of the primary response (days 7 and 10, Fig. 3) the NP366±pulsed targets recovered at 4 h after transfer were larger (forward scatter (FSC), Fig. 6A) and more granular (side scatter (SSC), Fig. 6B) than the comparable PA224⁺ set. This was not an effect of the peptide per se as where there were much lower levels of killing in memory (days 15–276; Fig. 3C), the NP366⁺ and PA224⁺ populations were similar.

This difference in the FSC and SSC phenotypes for residual NP366⁻ and PA224⁻pulsed targets at the acute phase of the response is also shown in Figure 5. The B6 mice were infected as described in the legend to Fig. 1. E:T ratios were calculated for CD8⁺DbNP366⁺ and CD8⁺DbPA224⁺ responses during (A) primary and (B) secondary influenza A virus infection. The broken line represents the threshold for maximal target cell elimination.

Table I. Comparison of in vivo cytotoxicity following adoptive transfer of equal numbers of secondary memory D²NP₃₆₆ and D²PA₂₂₄ CD₈⁺ T cells

| In vivo cytotoxicity | D²NP₃₆₆(log₁₀) | D²PA₂₂₄(log₁₀) |
|----------------------|---------------|---------------|
| % killing of peptide-pulsed targets | NP₃₆₆ pulsed | 21 ± 3⁶ | PA₂₂₄ pulsed | 19 ± 3 |
| E:T ratio | D²NP₃₆₆-NP₃₆₆ pulsed | 0.09 | D²PA₂₂₄-PA₂₂₄ pulsed | 0.13 |
| FSC or SSC ratio (Infected/naive) | FSC | 1.40 | SSC | 1.14 |
| NP₃₆₆-pulsed targets | 1.04 | 1.14 |
| PA₂₂₄-pulsed targets | 0.99 | 0.95 |
| Unpulsed targets | 1.00 | 0.96 |

⁶ Equal numbers (6.93 x 10⁵) of day 53 secondary memory D²NP₃₆₆ and D²PA₂₂₄ effectors, generated as described in Materials and Methods, were adoptively transferred i.v. into naive B6 hosts and then rested for 2 days. D²CFSE⁺ peptide-pulsed target cells were then transferred i.v. to determine the in vivo cytotoxicity of both CD8⁺ T cell populations using the previously described 4-h assay. Splenocyte populations were also stained with tetramer to confirm the transfer of equivalent numbers of day 53 secondary memory cells. Data represent means ± SD. FSC and SSC means of the D²CFSE⁺ target cell populations were used to calculate infected/naive ratios.
primary response (Fig. 6, A and B) was also found at all time points for the “secondary memory” populations (Fig. 6, C and D). One possibility is that more of the NP366-pulsed target cells are in the process of dying, reflecting the larger numbers of CD8\(^+\)/H11001/\(^{\text{DbNP366}}\)/H11001 T cells at the acute phase of the primary response (Fig. 3A) and throughout memory following secondary challenge (Fig. 4A). Before cells die by the standard cell death mechanisms, they can be shown to swell (27–29). We did not see much difference when we transferred equivalent numbers of effector T cells (see Adjusted T cell numbers and in vivo efficacy above and Table I) where the ratios for FSC (NP, 1.04; PA, 0.99) and SSC (NP, 1.14; PA, 0.95) were essentially similar.

Clearly, this observation merits further analysis as it suggests that targets damaged by effector CTL can, at least for a time, survive in the in vivo situation. Is recovery possible, or are such cells on an irreversible pathway to final elimination?

Cell death can also be characterized using Abs and dyes that detect apoptosis (annexin V) and membrane integrity (PI) (Fig. 7) Costaining of splenocytes following in vivo cytotoxicity (secondary day 7 time point) showed that most of the residual DiD\(^+\)/CFSE\(^-\) NP\(_{366}\)-pulsed target cells had undergone complete cell death (Annexin V\(^+\)/PI\(^-\)). Interestingly, at the equivalent 4 h time point, the majority of residual DiD\(^+\)/CFSE\(^-\) PA\(_{224}\)-pulsed target cells were only in the early stages of apoptosis (Annexin V\(^+\)/PI\(^+\)).

Discussion

These experiments set out to investigate the in vivo cytotoxic potential of two prominent H2D\(^+\)-restricted CTL populations using the in vivo cytotoxicity assay. The results clearly indicate

![FIGURE 6. Size and granularity of DiD\(^+\) adoptively transferred targets. (A and B) FSC and (C and D) SSC means were obtained for all DiD\(^+\) target cell populations from infected and naive mice. Infected:naive DiD\(^+\) 0.01 \(\mu\)M CFSE\(^-\), DiD\(^+\) 0.05 \(\mu\)M CFSE\(^-\), and DiD\(^+\) 0.5 \(\mu\)M CFSE\(^-\) ratios were calculated following (A and C) primary and (B and D) secondary infection.](image)

![FIGURE 7. Apoptosis and PI staining of DiD\(^-\) residual target cells. The percentage of annexin V- and PI-positive cells was determined for each peptide-pulsed population at the secondary day 7 time point using unpulsed target cells as a control. Data represents the mean for three mice.](image)
that in vivo target cell lysis, like many other biological functions, is threshold dependent. Careful analysis of the immunological synapse has led to the proposition of threshold models for T cell activation (CD4+ and CD8+ T cell) (30), cytotoxicity, and cytokine production (31, 32). The present analysis indicates that maximum CTL lysis of 5 × 106 adaptively transferred targets occurs when the calculated E:T cell ratio in vivo reaches a value of ~7 using our experimental conditions. The model is robust and changes in the E:T cell ratio foretell variations in cytotoxicity. Additionally, the influence of E:T cell ratios on CTL elimination in vivo seems to be independent of the differentiation state (i.e., primary or secondary effectors) and specificity of these immune T cells. It is important to note that we saw no background killing of unpulsed targets that would indicate a role for nonspecific killing in either the primary or secondary response (our unpublished data).

This is, to our knowledge, the first description directly relating in vivo cytotoxicity to the frequency of Ag-specific CD8+ T cells following a respiratory virus infection. McGhee and colleagues (33) have previously highlighted a possible correlation in vitro, by measuring cytotoxicity and the total number of CD8+ IFN-γ+ producing cells by ELISPOT from human peripheral blood mononuclear populations cultured in the presence of influenza virus-infected autologous stimulators. The results suggested a general correlation between CD8+ T cell numbers and cytotoxicity, but did not analyze the significance for different T cell specificities.

Previous studies of the influenza virus-specific CTL response have focused largely on the nature of the effectors and the character of T cell memory following primary virus challenge (18, 19). The present experiments provide the additional insight that secondary memory T cells, like their primary memory counterparts, rapidly recall cytotoxic responses in vivo. Recent studies using transgenic T cells and LCMV infection (34) demonstrated that the CD8+ secondary memory sets maintain their effector phenotype for longer and are more protective than primary memory populations. Our data, however, suggest that such apparent differences are simply related to lymphocyte numbers. When E:T ratios are compared, the in vivo efficacy of primary and secondary DNP366 and DPAPA224 CD8+ memory T cells may be essentially comparable. Furthermore, despite a well-defined divergence in TCR avidity profiles and peptide-induced cytokine production characteristics (13, 14), there is no discernable difference between the capacities of DNP366- and DPAPA224-specific CD8+ T cells to mediate in vivo CTL effector function.

An unexpected observation was the discovery of differences in mean FSC, SSC, and Annexin V/PI staining characteristics for the residual DNP366 or DPAPA224-pulsed target cells. A tentative explanation could be that CD8+ DNP366 and CD8+ DPAPA224 T cells kill their target cells by different mechanisms and/or different kinetics. However, a much more detailed analysis would be required both to illustrate the possible difference in mechanism and to exclude other potential explanations.

In conclusion, these studies have implications for adoptive cellular immunotherapy where, for example, the transfer of tumor-infiltrating lymphocytes or CTL clones is used for the treatment of malignancy (35). Similar protocols have been used in attempts to diminish viremia and to promote virus clearance in HIV and hepatitis C virus infections. Possible threshold effects are an important consideration when gauging the appropriate T cell dose for such experimental therapies.

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Disclosures
The authors have no financial conflict of interest.

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