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Strength in Numbers: Visualizing CTL-Mediated Killing In Vivo

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Cytotoxic CD8$^+$ T lymphocytes (CTLs) have long been believed to be extremely efficient killers. Forster and colleagues (Halle et al., 2016) used in vivo imaging to tell a different story, in which each CTL killed only 2–16 targets a day, and several CTLs per target were needed to get the job done.

Cytotoxic CD8$^+$ T lymphocytes (CTLs) identify their target cells through direct recognition of peptide presented on major histocompatibility complex I (MHC-I). This antigen-specific recognition causes the microtubule-organizing center (MTOC) to position itself just below the plasma membrane where the T cell interacts with the target. Cytotoxic granules containing perforin and granzymes travel along microtubules toward the MTOC and are delivered between the tightly juxtaposed cell membranes into the target cell, triggering apoptosis. In vitro, three peptide-MHC complexes are sufficient to trigger lysis (Purbhoo et al., 2004), and a single activated CTL is capable of sequentially lysing several individual target cells, hence the term "serial killer" (Isaaz et al., 1995). In addition, effector CTLs rapidly kill peptide-pulsed splenocytes injected intravenously (Regoes et al., 2007). These studies suggest that CTL-mediated elimination of antigen-bearing cells is extremely efficient. However, the extent, rate, and efficiency of CTL-mediated killing of cells infected with viruses have not been carefully quantified in vivo.

In this issue of *Immunity*, Halle et al. (2016) used two-photon microscopy to directly assess the efficiency of CTL killing of virus-infected targets in vivo. They generated GFP-expressing, primed ovalbumin-specific OT-I CD8$^+$ T cells in vivo by using a peptide-plus-adjuvant vaccination strategy. Four to six days later, they injected mice subcutaneously with murine cytomegalovirus (MCMV) or modified vaccinia virus Ankara (MVA) expressing the fluorescent protein mCherry to generate target cells that could be directly visualized in the draining lymph node. By using viruses that also expressed (or did not express) ovalbumin, the authors could then accurately quantitate the antigen-specific migratory behavior and killing capacity of recently activated CTLs. Furthermore, elimination of cells infected with viruses could be quantified by loss of mCherry-expressing cells in the draining lymph node. As expected, the killing of target cells infected with either virus required specific antigen recognition, given that activated OT-I CD8$^+$ T cells could only kill cells infected with MCMV or MVA expressing ovalbumin. Cells infected with the wild-type version of MCMV were resistant to CTL-mediated killing, whereas cells infected with an MCMV mutant lacking viral genes that inhibit MHC-I expression (MCMV-ΔRAP) were killed at the same rate as MVA-infected cells (Figure 1). The in vivo imaging provides a beautiful confirmation of previous work (Holtappels et al., 2004; Pinto et al., 2006): seeing is believing. However, it is interesting to note that although expression of the activation marker CD69 also required antigen specificity (ovalbumin expression), it was not affected by the MCMV genes that inhibit MHC-I expression. This suggests that in vivo, more T cell receptor stimulation is required for cytotoxicity than for the expression of at least some activation markers.

In the subcapsular region of the lymph node, which was imaged in this study, MCMV primarily infected non-hematopoetic (CD45$^-$ and CD169$^-$) cells that were located just beneath the lymph node capsule. In contrast, MVA infected macrophages and dendritic cells, enabling the authors to enumerate killing of two different cell types as well as two different viruses. Similar to vaccinia-virus-infected monocytes that were killed in the skin in a previous study (Hickman et al., 2013), the CTL in this study remained motile for approximately 10 min during the killing process. This indicates that stable immunological synapses seem to not be common, or necessary, for killing in vivo.

However, the virus-infected cells were not easily dispatched. Killing usually required multiple CTLs: killed cells had a median of 3.5 CTL contacts and a cumulative median contact time of 50 min. The authors then calculated the "per capita kill rate"—the average number of infected targets that a single CTL could kill in a day—and found that CTLs killed 2–16 (median ~4.5) targets per day (Figure 1). Similar numbers were obtained in experiments of target-cell killing in MVA-infected skin. A second experiment used more physiological polyclonal MCMV-specific CTLs generated in vivo after MCMV infection; these CTLs were then (remarkably) transferred by intralymphatic injection directly into the lymph node. Halle et al. calculated the per capita kill rate with a simple mathematical model after counting the number of infected cells at the beginning and end of an 8 hr window in the presence or absence of CTLs. Again, a similar kill rate was observed.

This calculated kill rate seems inefficient in comparison to previously visualized killing in vitro and to the rapid clearance of peptide-pulsed splenocytes in the widely used in vivo killing assay (Isaaz et al., 1995; Regoes et al., 2007). On the other hand, as Halle et al. point out, killing of tumor cells and malaria-infected hepatocytes is also slow when it is imaged in vivo. We suspect that anti-apoptotic mechanisms that are common to virtually all intracellular pathogens and tumors might make it difficult to kill these cells.

Alternatively, this inefficient killing rate could also be affected by the cytolytic

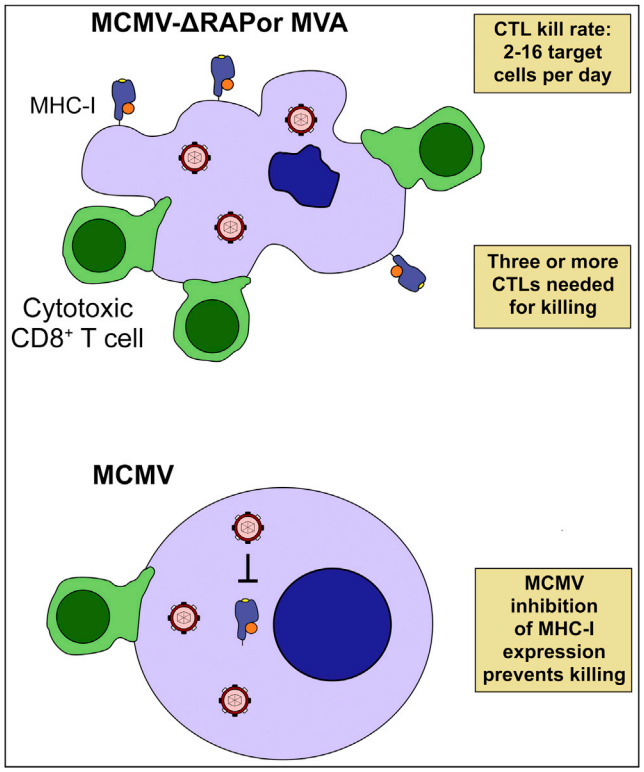

Figure 1. In Vivo Observations of CTL-Mediated Killing of Target Cells Infected with Viruses
(Top) Cells infected with MCMV lacking the genes that inhibit MHC-I expression (MCMV-ΔRAP) or with MVA were killed by CTLs at an approximate rate of 2–16 target cells per CTL per day. Three or more CTLs in contact with the infected cell significantly increased the probability of cell death.
(Bottom) Infecting target cells with wild-type MCMV caused inhibition of MHC-I surface expression and resistance to CTL-mediated killing.

capacity of the killers studied in these experiments. In fact, Halle et al. found that as many as 40% of the CTLs generated after their vaccination protocol were unable to initiate apoptosis of cells infected with MCMV-ΔRAP and that only about 10% were able to initiate apoptosis in three or more targets. Imaging studies are generally subject to some sort of artificial experimental manipulation because of the need to get enough labeled players together at the same time in a location that is amenable to imaging. In this study, activated CTLs were primed in vivo 4–6 days prior to the generation of target cells by a new viral infection. Both the magnitude and timing of inflammatory cytokines such as interleukin-12 and type I interferons (IFNs) are known to directly affect the cytolytic capacity of effector $CD8^+$ T cells (Richer et al., 2013). Thus, the inflammatory environment that is generated during different types of infections could also influence the per capita killing rates of effector $CD8^+$ T cell populations.

Using two-photon microscopy and in vivo analysis, Halle at el. have provided interesting data suggesting that the kill rate of CTLs is not as high as it was previously thought to be. The kill rate is an important factor in trying to understand the actual significance of CTL-mediated killing in antiviral immunity. The ability of CTLs to control a viral infection by cytolysis depends, minimally, on (1) the number of viral progeny released from an infected cell (burst size) per unit of time, (2) the availability of new targets for infection, (3) the number of CTLs present, and (4) the CTL kill rate (Nowak and Bangham, 1996). If one infected cell produces thousands of progeny virions, it would be very difficult for CTLs that can kill only five targets a day to control infection by killing alone. However, direct cell killing is not the only mechanism $CD8^+$ T cells utilize to control viral infections, given that CTLs also produce the cytokines IFN-γ and tumor necrosis factor α. IFN-γ, in particular, is critical for controlling viral infections, given that IFN-$\gamma^{-/-}$ mice fail to clear acute infection with lymphocytic choriomeningitis virus despite enhanced cytolytic activity of the antigen-specific $CD8^+$ T cells (Bartholdy et al., 2000). Thus, future imaging studies might determine whether CTL interactions with virally infected cells also result in local production of IFN-γ and how cytokine production in coordination with CTL-mediated cell killing ultimately protects the host from a variety of different viral infections.

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