

Dynamic programming of CD8⁺ T lymphocyte responses

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The initial encounter with an antigen-presenting cell (APC) is the primary force behind the expansion, differentiation and survival of naive T cells. Using an APC that permits temporal control of priming, we examined whether the duration of antigenic stimulation can influence the functional development of CD8⁺ cytotoxic T lymphocytes (CTLs) *in vivo*. Whereas CTLs given a 4-h stimulus underwent an abortive clonal expansion with transient surface CD25 expression, those given a 20-h stimulus sustained CD25 up-regulation, proliferated extensively, and efficiently mediated destruction of peripheral target tissues. Our results show that an instructional program preceding the first cell division integrates differences in signal strength into the decision to activate versus tolerate specific CTL clones.

The rapid clonal expansion of CD8⁺ CTLs in response to antigenic challenge is a hallmark of adaptive immunity and a crucial element of host defense^{1,2}. From a limited number of clonal precursors—1 in perhaps 10⁵–10⁶ naive CTL precursors—successive rounds of division can quickly generate an increase in cytotoxic effectors of up to 10⁴-fold^{3–5}. CTL priming *in vivo* is believed to be initiated mainly by specialized APCs such as dendritic cells. Such APCs occupy a unique position in the activation and differentiation pathways of T cells, controlling the nature and abundance of inductive signals that must be decoded by T cells and integrated into the appropriate response⁶. Depending on their state of activation or maturity, APCs can direct various fates for responding T cells, ranging from full activation and memory to clonal tolerance through anergy or deletion^{7,8}. Although much attention has been focused on the role of specific costimulatory and cytokine pathways in this pathway, the duration and intensity with which these are combined with T cell receptor (TCR) signals may also influence CTL fate *in vivo*. Recent studies have shown that the duration of antigenic stimulation can have an important role in the commitment to proliferation as well as cytokine polarization (T_{H1} versus T_{H2}) of CD4⁺ T cells *in vitro*^{9,10}. Whether duration of stimulation can similarly influence CTL expansion *in vivo* has not been examined.

After a short antigenic stimulation, naive CD8⁺ CTLs become committed to autonomous clonal expansion and functional differentiation. This occurs before the first round of division and without the need for further antigenic stimulation of daughter cells^{11–13}. The period required to achieve this is remarkably brief: a single 2-h stimulation of naive transgenic CTLs is sufficient for expansion of effector CTLs *in vitro*, and a longer stimulation does not confer greater proliferative or cytotoxic capacity¹¹. Although these studies established that CTLs can rapidly be 'programmed' by a stimulatory APC¹¹, their physiological relevance was limited by the fact that functional expansion was largely

assessed *in vitro*. Given the probabilistic nature of T cell–APC interactions within secondary lymphoid organs, it would be useful to know whether CTLs that have received different amounts of stimulation can attain different levels of functional development^{14,15}. We have now examined this question using an engineered adherent fibroblast system in which the duration of antigenic stimulation can be precisely controlled^{11,16}. Our results show that the extent and magnitude of clonal expansion *in vivo* is directly related to the amount of antigenic stimulation provided to the naive CTL precursor during its primary activation.

Results

Stimulation duration regulates clonal expansion *in vivo*

We previously described a CTL priming system featuring an adherent fibroblast APC (MEC.B7.SigOVA) engineered to express a specific ovalbumin (OVA)-derived, major histocompatibility complex (MHC) H-2K^b-restricted peptide epitope, OVA(257–264) or SIINFEKL, along with the costimulatory molecule B7.1 at its surface¹¹. Priming in this system is initiated by addition of naive OVA(257–264)-specific transgenic (OT-I¹⁷) CTLs to the adherent APC monolayer and can be terminated by gentle resuspension of the CTLs and removal from the APC monolayer, thereby permitting the duration of antigenic stimulation to be controlled. To examine whether sustained signaling influences CTL expansion, we primed 5,6-carboxysuccinimidylfluorescein ester (CFSE)-labeled OT-I CTLs for either a short or long period (4 or 20 h) on the MEC.B7.SigOVA APCs and then transferred them either to continued *in vitro* culture in new empty wells or to *in vivo* transfer to naive C57BL/6 recipients. At 48 and 72 h after initiation of priming, the clonal expansion of OT-I CTLs in each case was visualized by CFSE dilution. When OT-I CTLs were cultured *in vitro*, they proceeded through the same number of divisions at 48 and 72 h whether they had received a short or long priming stimulus (**Fig. 1a**). In contrast, extensive clonal

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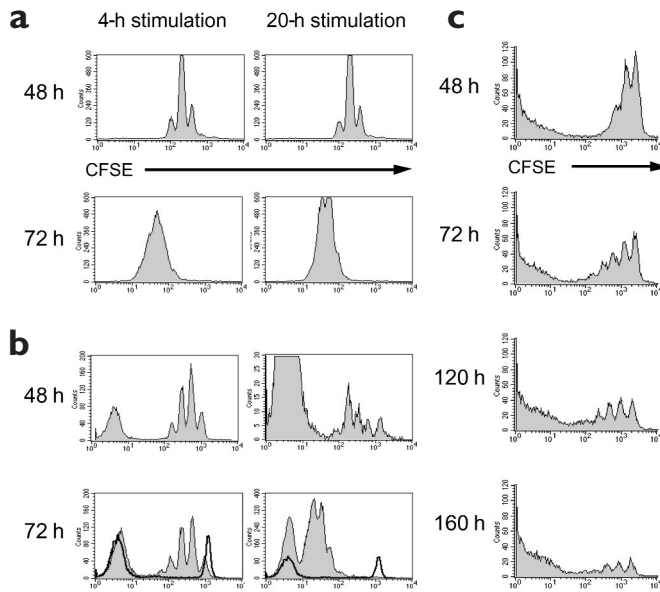


Figure 1. *In vitro* and *in vivo* expansion of OT-I CTLs. Naive CD8⁺ OT-I cells were isolated from spleen and lymph nodes of C57BL/6 OT-I RAG-1-deficient mice by antibody-directed complement lysis and labeled with CFSE. **(a,b)** OT-I cells were then added to culture wells that had been seeded with a monolayer of MEC.B7.SigOVA cells as described previously¹¹. After a 4- or 20-h coculture, OT-I cells were harvested by gentle washing and transferred to empty wells **(a)**. In parallel, 5×10^6 cells stimulated for 4 h or 2×10^6 cells stimulated for 20 h were injected i.v. into naive C57BL/6 mice. **(b)** At 48 h and 72 h after initiation of stimulation, OT-I cells from the *in vitro* cultures and splenocytes from recipient mice were harvested and proliferation of OT-I cells was visualized by CFSE dilution (filled histograms). CFSE fluorescence intensity of unstimulated OT-I cells 3 d after transfer into naive C57BL/6 mice is shown as unfilled histogram in the lower panels ($t = 72$ h). **(c)** CFSE-labeled, naive OT-I cells were stimulated *in vitro* for 4 h with MEC.B7.SigOVA cells and then transferred (5×10^6) i.v. to naive C57BL/6 mice. *In vivo* proliferation of OT-I cells was analyzed 2, 3, 5 and 7 days later by FACS analysis of splenocytes from recipient mice. All histograms represent gating on live CD8⁺, V α 2⁺ cells. Similar results were obtained in seven separate experiments.

expansion *in vivo* occurred only in transferred CTLs that had received a 20-h stimulation (**Fig. 1b**). OT-I CTLs given 4 h of stimulation proliferated more slowly *in vivo* by 48 h after the initiation of priming, and by 72 h they seemed to have become arrested in their proliferation. Expansion of the briefly stimulated CTLs was truly abortive and not simply retarded, as examination of this population at 2, 3, 5 and 7 d after transfer showed that they failed to proceed through additional rounds of division at each time point (**Fig. 1c**). Thus, although unimpaired in their capacity to undergo clonal expansion under *in vitro* culture conditions, CTLs given a brief stimulation proceed through only a limited number of divisions *in vivo* and subsequently disappear.

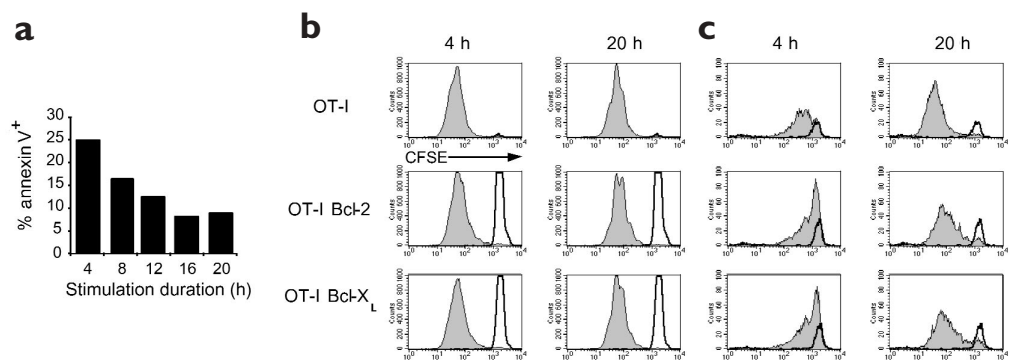
Stimulation duration and CTL survival

The extent and magnitude of clonal expansion in T cells is directly related to their capacity to survive after primary activation¹⁸. We therefore investigated whether CTLs that receive a brief stimulation undergo more apoptosis than CTLs receiving a longer stimulus. We measured the frequency of apoptotic (annexin V⁺) cells at 72 h after initiation of priming in OT-I CTLs that had received 4, 8, 12, 16 or 20 h of antigenic stimulation *in vitro* (**Fig. 2a**). There was a progressive decrease in the frequency

of apoptotic cells that was inversely proportional to the stimulation period provided during priming, with OT-I cells given the longest (20-h) stimulation containing more than 60% fewer apoptotic cells than those given a 4-h stimulation. These data support the interpretation that the limited *in vivo* expansion shown by OT-I cells stimulated for 4 h is related to insufficient survival of CTLs in later rounds of division, perhaps mediated through passive death pathways. To assess this directly, we investigated whether transgenic expression of the anti-apoptotic proteins Bcl-2 or Bcl-x_L^{19,20} could overcome the abortive expansion observed under conditions of brief stimulation. OT-I Bcl-2 and OT-I Bcl-x_L CTLs stimulated for 4 h underwent the same abortive expansion as wild-type OT-I CTLs *in vivo*, even though *in vitro* expansion was unaffected (**Fig. 2b,c**). When stimulated for 20 h, both the OT-I Bcl-2 and OT-I Bcl-x_L CTLs proceeded through multiple rounds of division, although they seemed to lag slightly behind the wild-type OT-I CTLs when measured at 72 h. These data show that although sustained signaling favors cellular survival, the limited clonal expansion induced by a brief stimulation cannot be overcome by expression of the anti-apoptotic factors Bcl-2 and Bcl-x_L. The observed difference in CTL accumulation, therefore, may not be solely due to protection from apoptosis.

Figure 2. Abortive expansion *in vivo* is not overcome by overexpression of Bcl-2 or Bcl-x_L.

(a) Naive OT-I cells were stimulated for 4–20 h with engineered fibroblast APCs and then transferred to empty wells. After 3 d *in vitro*, cells were harvested and the frequency of apoptotic cells of the total culture was determined by staining with allophycocyanin-conjugated annexin V (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol. Similar results were obtained in three separate experiments. **(b,c)** CFSE-labeled OT-I cells isolated from OT-I, OT-I Bcl-2 or OT-I Bcl-x_L mice were stimulated *in vitro* with MEC.B7.SigOVA cells. After a 4- or 20-h coculture, stimulated OT-I cells were harvested and transferred to empty wells or injected i.v. (3×10^6) into naive B6.PL mice (CD90.1⁺). As a control, unstimulated OT-I cells were cultured or transferred *in vivo* (unfilled histograms). Three days after initiation of the experiment, *in vitro* cultures **(b)** and splenocytes of recipient mice **(c)** were harvested and CFSE dilution of OT-I cells (filled histograms) was analyzed by FACS. Histograms reflect gating on CD8⁺CD90.2⁺ cells and are representative of three mice per group in experiments repeated three times with similar results.



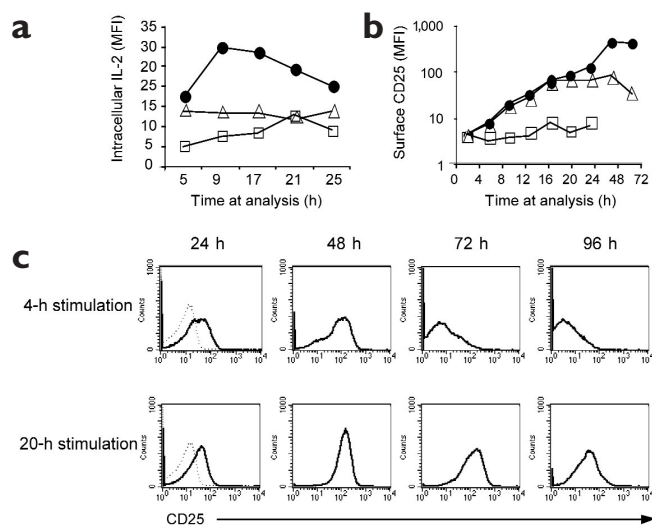


Figure 3. Sustained antigenic stimulation leads to increased IL-2 production and IL-2 receptor- α expression. Naive OT-I cells were stimulated with MEC.B7.SigOVA cells for 0, 4 or 20 h and then transferred to wells lacking APCs. (a–c) After various periods of culture, (a) IL-2 production and (b,c) IL-2 receptor- α (CD25) expression were determined by FACS analysis using phycoerythrin-conjugated antibodies to IL-2 (clone JES6-5H4) and CD25 (clone 3C7). The graph in (a) shows the geometric mean fluorescence (MFI) of IL-2 detected in OT-I cells stimulated for 4 h (Δ) or 20 h (\bullet) or left unstimulated (\square). The same symbols are used in b. In c, the dotted line in the $t = 24$ h panels represents CD25 expression on naive OT-I cells. Similar results were obtained in seven separate experiments.

The role of autocrine IL-2

The discrepancy between the extent of clonal expansion of CTLs *in vitro* as compared to *in vivo* is consistent with the action of a required autocrine growth factor whose impact on clonal expansion is regulated by the duration of stimulation. Such a factor could accumulate as a ‘parakine’ in culture supernatants and drive expansion of briefly stimulated OT-I CTLs *in vitro*, but could not do so as an ‘autokine’ *in vivo*, where clonal expansion of the transferred CTLs would be essentially autonomous. A longer stimulation, in contrast, would result in higher expression of this cytokine, its receptor or both and thereby enable autonomous clonal expansion of OT-I cells *in vitro* or *in vivo*. IL-2 is a logical candidate for such an autocrine growth factor, as it is produced and consumed by activated CD8⁺ T cells and can drive their proliferation²¹. To examine the influence of sustained signaling on IL-2 responses in OT-I CTLs, we measured intracellular IL-2 protein and surface IL-2 receptor expression at various times in populations that had been stimulated for either 4 or 20 h. OT-I CTLs stimulated for 20 h produced consistently more IL-2 for a longer time than OT-I cells that had been stimulated for 4 h (Fig. 3a). Expression of the high-affinity IL-2 receptor (CD25) initially increased progressively in both populations, but diverged starting shortly before cellular division began, approximately 24 h after initiation of priming (Fig. 3b). Surface CD25 expression continued to increase on the daughter cells generated from the original 20 h-stimulated OT-I CTLs, reaching amounts an order of magnitude higher than on the progeny of the 4 h-stimulated OT-I cells by 48–72 h after initiation of priming (Fig. 3b,c). Simultaneous measurement of the additional IL-2 receptor subunits CD122 and common γ -chain (γ_c) on the OT-I CTLs showed no difference in their surface expression patterns (data not shown). These results indicate that the capacities both to produce and to respond to IL-2 were increased in CTLs that had received sustained antigenic signaling, which may explain the discrepancy

between the capacity of briefly stimulated OT-I cells to proliferate extensively *in vitro*, versus abortively *in vivo*. Consistent with this, we found that providing exogenous IL-2 as a subcutaneous bolus *in vivo* drives 4 h-stimulated OT-I CTLs through many additional rounds of division (see Supplementary Fig. 1 online).

Expansion and migration of effector CTLs

The capacity to mediate destruction of target cells in the periphery is a key task of CTLs. We examined the influence of sustained signaling on the clonal expansion and migration of effector CTLs using two approaches. The first involves visualizing primary cytotoxicity by monitoring the specific eradication of an adoptively transferred target population of OVA(257–264)-pulsed splenocytes that have been differentially labeled with CFSE (CFSE^{hi}) so as to be distinguishable from a cotransferred reference population (CFSE^{lo}) pulsed with a control peptide²². We measured the frequency of each target cell population among spleen cells recovered 16 h after transfer to mice that had received naive, 4 h- or 20 h-stimulated OT-I CTLs (Fig. 4). Although both the 4 h- and 20 h-stimulated CTLs could specifically eradicate the OVA(257–264)-pulsed (CFSE^{hi}) target cell population, the 4 h-stimulated OT-I CTLs had a substantially reduced killing efficiency. This indicates that the functional capacity of the primary CTL effectors is compromised by the brief stimulation and subsequent abortive expansion.

In a second approach, we assessed the effector capacity of these CTLs using a transgenic mouse model of autoimmune diabetes. RIP-mOVA mice express a membrane-bound form of OVA in the pancreatic beta cells, where it can serve as a target antigen for CTL-mediated destruction of islets, leading to diabetes²³. Transfer of a limiting number of naive OT-I CTLs (10^6 cells) to RIP-mOVA mice led to a low incidence of diabetes (16%), which is mediated *via* cross-presentation of OVA by endogenous bone marrow-derived APCs, as previously reported²⁴ (Table 1). Whereas transfer of 10^6 , 4 h-stimulated, OT-I CTLs did not induce diabetes in RIP-mOVA mice, the same number of 20 h-stimulated OT-I CTLs produced diabetes in most RIP-mOVA recipients (92%). Histological examination of isolated pancreases showed peri-insular infiltrates of CD8⁺ CTLs in RIP-mOVA mice that had received naive or 4 h-stimulated OT-I CTLs, but substantial lymphocytic invasion and frank islet destruction were observed only in mice that received 20 h-stimulated OT-I CTLs (data not shown).

Briefly stimulated CTLs show decreased cytotoxicity *in vivo*.

OT-I cells were stimulated for 0, 4 or 20 h with MEC.B7.SigOVA cells and were later injected (1×10^6) i.v. into naive C57BL/6 mice. On the fifth day, CD90.1⁺ target cells were prepared as follows. Splenocytes from naive B6.PL mice were harvested and split into two populations. One part was labeled with 5 μ M CFSE (CFSE^{hi}), while the other part was labeled with 0.5 μ M CFSE (CFSE^{lo}). Next, the CFSE^{hi} cells were incubated for 90 min at 37 °C with OVA(257–264) (SIINFEKL) while the CFSE^{lo} cells were incubated with a control peptide (VNIRNCCYI). The target cells were washed five times, and the two populations were mixed at a 1:1 ratio and injected (5×10^6 cells each) i.v. into C57BL/6 recipient mice. The next day, splenocytes of recipient mice were isolated and *in vivo* killing of target cells was analyzed by FACS. Histograms represent gating on CD90.1⁺ target cells and are representative of eight to ten mice in four separate experiments. The percentage of specific target cells killed is shown in the upper right corner of each panel.

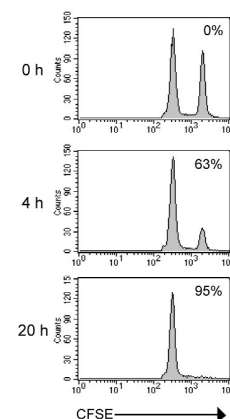


Table 1. Induction of diabetes by OT-I CTLs

Duration of antigenic stimulation <i>in vitro</i> ¹	Diabetes incidence ²
0 h	2/12
4 h	0/11
20 h	11/12

¹Naive OT-I cells were primed *in vitro* with engineered fibroblast APCs. After 4 or 20 h of stimulation, OT-I cells were harvested and 1×10^6 cells were injected i.v. into naive RIP-mOVA mice. ²Development of diabetes was monitored by daily blood glucose measurements. Mice with blood glucose values of more than 250 mg/dl on two consecutive days were considered diabetic.

Taken together, these results indicate that sustained antigenic stimulation is required to enable a limited number of CTLs to undergo sufficient expansion *in vivo* to mediate destruction of peripheral target cells.

Discussion

Our results show that the duration of antigenic stimulation during primary CTL activation can determine whether subsequent *in vivo* clonal expansion will be abortive or extensive. As the stimulus leading to either of these fates is delivered to the CTL before its first cell division, the observed differences in clonal expansion must be the result of a cell-intrinsic developmental program initiated during priming. This program is not invariant and can apparently be modified or 'tuned' by the duration of antigenic stimulation. These observations build on our previous studies of CTL programming, which showed that a brief stimulus was sufficient to commit a CTL to clonal expansion and functional differentiation *in vitro*¹¹. By monitoring proliferation and cytotoxicity *in vivo*, we now show that a longer stimulus is required to confer on CTLs the capacity for autonomous clonal expansion and optimal effector function. These data indicate that the program guiding CTL development is dynamic and instructional, involving the sequential acquisition of discrete functional capacities once specific thresholds of activation are reached.

The engineered fibroblast APC used in these studies represents only one set of antigenic conditions and is not meant to faithfully model *bona fide* APCs that express a wider variety of costimulatory and cytokine molecules. Nonetheless, our studies show that the sensory apparatus used by CTLs to receive inductive signals is able to integrate differences in their duration into programmed responses that are distinct in their outcomes yet begin with a commitment to proliferation. Depending on the duration of stimulation, however, cell-intrinsic factors can regulate the extent and magnitude of the subsequent clonal expansion. One of these seems to involve regulation of the α -chain of CD25 and thereby the capacity of CTLs to sustain IL-2-driven proliferation²⁵. Thus, our data indicate that programming involves a persistent change in macromolecular synthesis that survives the dilution effects of multiple rounds of cell division. It is notable that, even when CTLs receive a brief priming stimulus in our system, the cell cycle is not engaged until about 24 h after the initial contact with APCs, whereupon multiple rounds of division can occur at the rate of once every 5–6 h¹¹. The nature of the programming that takes place before the first cell cycle might relate to preparing the cell for this rapid cycle time, either by synthesizing required molecules in abundance or by ensuring their continued synthesis throughout the clonal expansion period. This might extend to the biosynthesis of complex organelles such as mitochondria and the endoplasmic reticulum whose absence would represent a significant block to further division of daughter cells. The identity of the molecules able to 'count' the size or duration of an antigenic stimulus, and of the signaling pathways

responsible for programming the subsequent response, remain key questions for future studies.

How might dynamic regulation influence CTL development under physiological conditions *in vivo*? By using signal duration as a variable, the immune system can select clones that display a greater degree of 'fitness' for full expansion and could delete the repertoire of 'unfit' clones, as has been suggested previously⁶. This would ensure that immune responses are focused on peptide-MHC complexes that are either more abundantly or more stably expressed on APCs, or on those for which higher-affinity T cell repertoires exist. The density of adhesion and costimulatory molecules on such APCs would also be expected to influence the duration of stimulation and thereby the regulation of clonal expansion. This could result in immune tolerance by actively purging the peripheral repertoire of clones that recognize unstable or lower-avidity peptide-MHC complexes as well as those presented by nonprofessional or immature APCs²⁶. In this context, we have shown that CTL priming leading to destruction of pancreatic islet cells and induction of diabetes requires sustained signaling of a limiting number of CTLs. It follows that naturally occurring autoimmunity mediated by CTLs would require similar priming conditions. Therefore, at least some forms of self-tolerance may persist through mechanisms to limit the duration of stimulation rather than its simple presence. Finally, our findings have relevance for vaccination strategies, as they predict that both antigen availability and the functional longevity of APCs will profoundly influence the extent and magnitude of CTL responses. A future challenge will be to identify the molecular and genetic components through which the CTL differentiation program can be modified and to determine the potential for their strategic manipulation.

Methods

Mice. CTLs were isolated from C57BL/6 RAG-1^{-/-} OT-I, OT-I Bcl-2⁺¹⁹ or OT-I Bcl-X_L⁺²⁰ mice. These mice were bred and housed under sterile conditions in the vivarium of the La Jolla Institute for Allergy and Immunology and were used at approximately 8–12 weeks of age. For adoptive transfer experiments, recipients were either sex-matched C57BL/6 or RIP-mOVA mice between 6–12 weeks of age. All mice were maintained under specific pathogen-free conditions in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

OT-I purification and CFSE labeling. OT-I cells were purified from spleens and lymph nodes of OT-I mice by antibody-directed complement lysis (two incubations of 30 min each at 37 °C) with antibodies to heat-stable antigen (J11d), class II MHC (M5/114 and CA-4.A12), macrophages (M1/70) and dendritic cells (33D1). They were then cross-linked with mouse anti-rat κ (MAR18.5). The resulting cells were >95% pure OT-I CTLs (CD8⁺, V α 2⁺, V β 5⁺) with a naive phenotype (CD25⁻, CD44⁻, CD62L⁺, CD69⁻). Purified OT-I cells were resuspended at a concentration of 10⁷ cells/ml in 0.1% BSA in PBS, and CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 5 μ M. After 10 min of incubation at 37 °C, labeling was quenched with ice-cold PBS. Cells were washed once before *in vitro* culture or transfer into recipient mice.

OT-I activation. The engineered fibroblast APC line MEC.B7.SigOVA was generated by transfecting the adherent C57BL/6 mouse embryo cell line MEC-1 with B7-1 and a minigene encoding the OVA(257–264) peptide, preceded in frame by the signal recognition particle-dependent leader sequence of the adenovirus E3/19K protein (Sig), essentially as described¹⁶. The SigOVA minigene thus directs efficient H-2K^b-restricted presentation of the OVA(257–264) peptide. Expression of B7-1 and K^b by the fibroblast APCs was confirmed by flow cytometry. The fibroblast APCs were seeded at 100,000 cell per well in 24-well plates and cultured overnight. The next day, wells were washed twice to remove nonadherent cells and cell debris. OT-I cells (0.5 \times 10⁶) were added to the monolayer of APCs and plates were centrifuged at 900g for 1 min. After various periods of coculture, the nonadherent OT-I cells were gently removed and transferred to empty wells or injected intravenously (i.v.) to recipient mice.

FACS analysis. Using standard protocols, cells were stained for FACS analysis in cold PBS containing 0.5% BSA and 0.05% sodium azide with fluorescein isothiocyanate-conjugated anti-V β 5.1, 5.2 TCR (MR9-4), phycoerythrin-conjugated anti-V α 2 TCR (B20.1) biotinylated anti-CD8 α (53.6.7) and anti-CD25 (3C7) (all from BD Pharmingen, San Diego, CA). Biotinylated antibodies were detected with streptavidin-conjugated allophycocyanin (Caltag, Burlingame, CA). For intracellular detection of IL-2, a commercially available kit

featuring phycoerythrin-conjugated antibodies to IL-2 (clone JES6-5H4) was used according to the manufacturer's protocol (BD PharMingen). To detect intracellular IL-2, cytokine secretion was inhibited by addition of GolgiPlug (BD PharMingen) 5 h before harvesting of the cells. Cells were analyzed on a FACScalibur cytometer (Becton Dickinson, San Jose, CA) using Cell Quest software. For *in vitro* experiments, 20,000–50,000 live events were collected, and for *in vivo* experiments, 400,000–800,000 live events were collected. CFSE fluorescence was detected with gating on CD8⁺ V α 2⁻ cells.

In vivo cytotoxicity assay. *In vivo* cytolytic activity was determined using B6 splenocyte target cells differentially labeled with the fluorescent dye CFSE²². The CFSE^{hi} labeled cells were used as targets and pulsed with OVA(257–264) (0.5 μ g/ml; 90 min at 37 °C, 5% CO₂), whereas the CFSE^{lo} labeled cells were pulsed with E1B(192–200) peptide (0.5 μ g/ml) to serve as the internal control. Peptide-pulsed target cells were extensively washed to remove free peptide and then coinjected *i.v.*, in a 1:1 ratio of CFSE^{lo} to CFSE^{hi} cells, into B6 mice that had received 1 million naive, 4 h- or 20 h-stimulated OT-I CTLs 5 d before. At 16 h after injection of target cells, spleens were removed and the ratio of CFSE^{lo}/CFSE^{hi} cells was determined by flow cytometry. The values in the upper right corner of each panel represents the percentage loss of OVA(257–264)-pulsed (CFSE^{hi}) target cells.

Note: Supplementary information is available on the Nature Immunology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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