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N-Terminal Trimer Extension of Nominal CD8 T Cell Epitopes Is Sufficient to Promote Cross-Presentation to Cognate CD8 T Cells In Vivo

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Cross-priming is the process in which Ag-presenting dendritic cells (DCs) acquire, process, and present Ags scavenged from other cells, and use these cells to activate naive CD8 T cells. Cross-priming of cognate CD8 cells can result in either tolerance or immunity, depending upon the activation status of the Ag-presenting DC. Previous studies have shown that nominal peptide is inefficiently cross-presented and that proteins and large polypeptides that require proteasomal processing are the main source of naturally cross-presented Ags. In this study we show that N-terminal extension of nominal peptide by as few as three residues is sufficient to produce a substrate for TAP-dependent cross-presentation that is highly efficient in cross-priming murine CD8 T cells in vivo. On a molar basis, cross-priming with 3-mer-extended peptide is 20-fold more efficient than priming with intact protein. This method of peptide extension should prove of great value in facilitating in vivo studies of CD8 immunity and tolerance that rely on cross-presentation. The Journal of Immunology, 2007, 179: 8280–8286.

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3 Abbreviations used in this paper: DC, dendritic cell; HA, hemagglutinin; β2-M, β2-microglobulin; KO, knockout; ER, endoplasmic reticulum.

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KO and Táp-deficient mice (TAP−/−) were purchased from The Jackson Laboratory and maintained under sterile conditions at the Animal Breeding Facility at The Scripps Research Institute. Clone 4 TCR transgenic mice and OT-I TCR transgenic mice were housed under specific pathogen-free conditions. Experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Virus and infection**

Influenza virus A/PR/8/34 H1N1 (PR8) was grown in the allantoic cavity of 10- to 11-day-old hen’s eggs. Upon isolation, the allantoic fluid was titrated for HA activity using chicken RBC and stored at −70°C. Mice were infected i.p. with 500 HA units of PR8 virus.

**Peptides**

Peptides used in these studies included: the KdHA518–526 (IYSTVASSL) epitope and ILA-KdHA (ILAIYSTVASSL) hereafter referred to as ILA-KdHA, which extends the sequence of the nominal peptide by including three amino proximal residues that appear in the natural sequence of the protein. The A-KdHA, LA-KdHA, ALA-KdHA, AAA-KdHA, FLA-KdHA, and QILA-KdHA peptides extend the sequence of the nominal KdHA peptide by including the corresponding amino proximal residues. All peptides were synthesized at The Peptide Synthesis Core Facility at The Scripps Research Institute. Peptide purity was evaluated using reverse-phase HPLC analysis. All peptides were initially dissolved in DMSO at 10 mg/ml and stored at −70°C.

**Purification, CFSE labeling and adoptive transfer of TCR transgenic CD8 T cells**

Lymph nodes were harvested from TCR transgenic mice and CD8+ T cells were purified using MACS magnetic beads, according to the manufacturer’s instructions. Purity was always >90% as checked by flow cytometry. Purified CD8 T cells were labeled with the fluorescent dye CFSE. Briefly, cells were incubated with 10 μM CFSE at 37°C for 10 min, followed by two washes with ice-cold HBSS. A total of 3 × 10⁶ CFSE-labeled T cells in 0.2 ml of HBSS was injected i.v. into each recipient mouse.

**Loading of β2-M KO spleen cells with ILa-peptide**

ULA Ag-loaded spleen cells were prepared by osmotic shock using the method described by Steinman and coworkers (13). Briefly, 150 × 10⁶ splenocytes from β2-M KO mice were washed twice in RPMI 1640 and resuspended in 1 ml of hypertonic medium (0.5 M sucrose, 10% w/v polyethylene glycol 1000, and 10 mM HEPES in RPMI 1640 (pH 7.2)), containing the indicated concentration of corresponding peptides, for 10 min at 37°C. Then 13 ml of prewarmed hypotonic medium (40% H₂O, 60% RPMI 1640) was added, and the cells were incubated for an additional 2 min at 37°C. Immediately thereafter, the cells were pelleted by centrifugation and washed twice with ice-cold HBSS, and 30 × 10⁶ in 0.2 ml of ILa-loaded splenocytes was injected i.v. into each recipient mouse as a source of dying cells. In some experiments (where indicated), in addition to ILa-loaded splenocytes, mice were also i.p. injected with agonistic anti-CD40 Ab (FGK45, 150 μg) and polyinosinic-polycytidylic acid (200 μg; Sigma-Aldrich) at the same time.

**Intracellular cytokine staining**

To assess the ex vivo production of IFN-γ in response to peptide Ag, single cell suspensions of purified lymphocytes from mouse spleen were prepared, and cells were incubated in complete RPMI 1640 medium with 1 μg/ml KdHA peptide and 1 μl/ml GolgiPlug solution (BD Pharmingen) for 6 h at 37°C. Cells were then incubated in 100 μl of 2.4 G2 hybridoma supernatant (American Type Culture Collection) for 10 min at 4°C to block Fc receptors. PE-conjugated anti-Thy1.1 and PerCP-conjugated anti-CD8 Ab were then added, and cells were incubated for an additional 30 min at 4°C. After two washes, intracellular IFN-γ staining was performed according to the manufacturer’s instructions using the Cytofix/Cytoperm Plus kit (BD Pharmingen) and allophycocyanin-conjugated rat anti-mouse IFN-γ. Cells were analyzed on a BD Biosciences FACSsort or LSRII flow cytometer, and data were analyzed using CellQuest or FlowJo software. All Abs for flow cytometry were purchased from BD Pharmingen.

**Results**

N-terminal modification of KdHA epitope significantly enhances its cross-presentation to clone 4 T cells in vivo

Our goal was to develop a method for in vivo induction of cross-presentation of the Kd-restricted HA peptide IYSTVASSL, which was based on Ag loading of spleen cells by osmotic shock (13). Upon injection in vivo, the apoptotic cells are cleared by cross-presenting DCs. The advantage of this method is that the concentration of Ag loading can be varied at will. In agreement with reports from several labs that have shown that nominal peptide or peptide-MHC complexes are not a source of Ag for cross-presentation (18, 19), we found this method was inefficient at inducing cross-presentation of nominal Ag (Fig. 1, left panels). Very high concentrations of the nominal peptide (500 μg/ml) were required to activate all of the naïve clone 4 cells.

Previously work of Reits et al. (15) showed that N-terminal but not C-terminal extension of peptides can enhance their stability and half-life within the cytosol. In the study, it was found that extension by 3 aa was sufficient to achieve optimal stability. Therefore we next attempted to enhance the ability of the peptide to be...
cross-presented to cognate clone 4 CD8 cells in vivo by extending the nominal 9-mer by adding three residues that are amino-proximal in the natural HA sequence, ILA (Fig. 1). By comparing the amount of nominal K\textsuperscript{d}HA peptide and the amount of ILA-K\textsuperscript{d}HA peptide required to achieve a comparable amount of division of clone 4 cells in vivo, it was determined that the efficiency of cross-presentation was increased by >10-fold by the addition of ILA-K\textsuperscript{d}HA to the peptide (Fig. 2B). The amount of proliferation achieved when lymphocytes were loaded with as little as 0.5 μg/ml ILA-K\textsuperscript{d}HA peptide could only be achieved with 10 μg/ml nominal K\textsuperscript{d}HA peptide used to load lymphocytes. In agreement with the early work by Carbone and Bevan (20), we observed a 10-fold decrease in the efficiency of T cells activation when isotonic, rather than hypotonic, conditions were used during the loading of peptide onto spleen cells (data not shown).

N-terminal extension by at least three residues is required to enhance cross-presentation of the K\textsuperscript{d}HA epitope to clone 4 CD8 T cells

To learn more about the types of N-terminal extensions that can successfully promote cross-priming, we synthesized K\textsuperscript{d}HA peptides extended at the N-terminal by different lengths. As shown in Fig. 2A, neither A-K\textsuperscript{d}HA nor LA-K\textsuperscript{d}HA enhanced cross-presentation, as the levels of clone 4 division induced by spleen cells loaded with these two peptides were comparable with that of the nominal 9-mer K\textsuperscript{d}HA peptide. However, when a third residue was added (ALA-K\textsuperscript{d}HA, FLA-K\textsuperscript{d}HA, or ILA-K\textsuperscript{d}HA) the efficiency of cross-presentation was greatly enhanced. This suggests that a minimum of three residues is required for efficient cross-priming. However, the presence of L at P2 was important, as extension with AAA did not enhance cross-priming. When four residues from the sequence of natural HA protein were used (QILA-K\textsuperscript{d}HA), this extended 13-mer also enhanced cross-presentation significantly. The results of a dose titration comparing each extension are provided in Fig. 2B.

N-terminal extension with ILA promotes TAP-dependent cross-presentation of the SIINFEKL epitope to OT-I CD8 T cells

To further test the generalization of the ILA-extension method, and to also determine whether the extended peptides required the presence of the TAP for cross-presentation by the APC, we next tested the K\textsuperscript{\alpha}-restricted 8-mer SIINFEKL epitope derived from OVA.
protein that is recognized by CD8 cells from OT-I mice. β2M KO spleen cells were loaded in vitro with ILA-SIINFEKL peptide or nominal peptide. As reported previously, cross-presentation of the nominal peptide by this method was inefficient, as assessed by proliferation of CFSE-labeled SIINFEKL specific OT-I cells (Fig. 3A). This is consistent with the observations of Norbury et al. (18) as well as Shen and Rock (19) that the OVA nominal peptide is inefficient for cross-presentation. The ILA-extended form of epitope was cross-presented 20-fold more efficiently as based on the percentage of proliferating cells. Moreover, cross-presentation of ILA-SIINFEKL is TAP-dependent, as OT-I cells were not activated by ILA-SIINFEKL loaded spleen cells in a TAP-deficient host (Fig. 3B).

The availability of OT-I cells further allowed us to compare the efficiency of cross-presentation of the intact OVA protein with the ILA-extended peptide (Fig. 3A). When spleen cells were incubated with comparable molar concentrations of either OVA protein or ILA-SIINFEKL, and the cells were delivered to mice that received CFSE-labeled OT-I cells, it was observed that the ILA-extended epitope induced a similar percentage of OT-I proliferation at a concentration around 30-fold less than that of the intact OVA protein.

Cross-presentation of ILA-KKHA leads to activation of clone 4 T cells, and can be used to induce tolerance or immunity

It has been shown that depending on the maturation status of the Ag-presenting DCs, T cell activation may lead to either tolerance.

**FIGURE 3.** N-terminal extension with ILA significantly enhances cross-presentation of the SIINFEKL epitope to OT-I CD8 T cells by a TAP-dependent pathway. On day 0, 3 x 10⁶ CFSE labeled CD8 Thy1.1⁻ OT-I cells (specific for Kb-SIINFEKL epitope derived from OVA) were transferred (i.v.) into B6 mice (A) or B6 and B6 TAP⁻/⁻ mice (as indicated in B). The next day, splenocytes from β2M KO B6 mice were loaded with the indicated concentration of the OVA protein, SIINFEKL or ILA-SIINFEKL peptides (A) or 0.005 mg/ml ILA-SIINFEKL peptide (B) through osmotic shock, and 30 x 10⁶ peptide-loaded β2M KO cells were injected to each recipient mice. On day 4, mice were sacrificed and the CFSE dilution profile was analyzed by FACS to evaluate the division of CD8⁻ Thy1.1⁻ OT-I T cells.
or immunity. Cross-presentation of HA by quiescent DCs to clone 4 CD8 T cells in vivo leads to abortive activation in which CD8 T cells are activated but do not gain effector function (10). Therefore, we assessed effector function in clone 4 T cells following cross-presentation of ILA-KdHA to clone 4 T cells. As shown in Fig. 4A, in contrast to viral infection that induced robust cell division and IFN-γ production, cross-presented ILA-KdHA leads to only partial activation as characterized by cell division but without IFN-γ production. This phenotype is typical of clone 4 T cells undergoing tolerance after encounter with cross-presented Ags on resting DCs in the absence of inflammation. In contrast, cross-presented ILA-KdHA was able to induce effector function if the host also received inflammatory reagents in the form of anti-CD40 and polynosinic-polycytidylic acid (Fig. 4A).

We next evaluated the ability of the ILA-KdHA to delete and tolerate Kd-restricted T cells in vivo. Splenocytes were loaded in vitro with the ILA-KdHA peptide by osmotic shock and injected into recipient mice that had previously received Thy1.1+ clone 4 T cells. Four weeks later, mice were infected with influenza to promote expansion of residual clone 4 CD8 cells. With one dose of ILA-KdHA, almost all transferred clone 4 T cells were deleted and tolerated (Fig. 4B). These results indicate that cross-presentation of ILA-KdHA leads to deletion and tolerance of naive cognate CD8 T cells in mice.

Discussion

In this study, we demonstrate that N-terminal extension of nominal peptide by as few as three residues significantly increases the efficiency of cross-presentation of class I-restricted epitopes. This method works for the Kd-restricted HA epitope, the Kb-restricted SIINFEKL epitope and also for the Kd IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein) epitope (residues 206–214 from the endogenously expressed islet Ag IGRP restricted by Kd) recognized by the 8.3 TCR transgenic line (21, 22) (data not shown).

Previous studies have presented two alternative views of the form of substrate that is required for cross-presentation. One view suggests that short peptides associated with cellular heat shock proteins are the predominant source of material that is available for cross-presentation by professional APCs (23). Our studies have not addressed the possibility that extended peptides may appear in the cytosol in association with heat shock proteins. Both the ILA-extended peptide and nominal HA peptide are in the size range known to bind to heat shock proteins (24). However, if such protection occurs, then it is unclear why the extended peptide is much more efficiently cross-presented than the nominal epitope. Another view suggests that intact proteins or soluble protein fragments that are larger than nominal peptide are the main form of Ag delivered by the donor cell for cross-presentation by the APC. The size of the KdHA peptide for 6 h at 37°C, followed by intracellular staining for IFN-γ. FACS analysis was then performed to evaluate both cell division and IFN-γ production by the CD8+ Thy1.1+ clone 4 T cells. Percentage shown in upper left quadrant denotes IFN-γ+ cells within divided clone 4 T cells. B. A total of 1 × 10^6 Thy1.1+ CD8+ clone 4 T cells were transferred into BALB/c mice. The next day, mice received either mock-loaded β2M KO cells or ILA-KdHA-loaded β2M KO cells. Four weeks later, both groups of mice (3 mice/group) were infected with 500 HA units of PR8 virus. One week later, both groups of mice were sacrificed, and the percentage of Thy1.1+ clone 4 T cells among CD8+ T cells in the spleen was determined in each group. Results shown were from one representative mouse in each group. C. The mean and SD of the absolute number of clone 4 T cells in each group of mice described in B were quantified.
fragments remains unresolved, and may include large post-proteasomal products; however, nominal peptide does not appear to have activity (17–19). None of these previous investigations have examined the minimum length of cellular peptide required to achieve efficient cross-presentation. In light of these past studies, it was surprising to find that by extending nominal CD8 epitopes at the N-terminal with as few as three residues, the efficiency of cross-presentation is increased 10- to 30-fold.

We find that cross-presentation of ILA-SIINFEKL is TAP-dependent. Currently, the detailed mechanisms involved in cross-presentation remain unresolved (2, 7, 25). At least two different pathways have been described and are distinguishable based on the role of TAP. In the TAP-dependent pathway, internalized protein is transferred from phagosome to the cytosol (26). Once in the cytosol, the exported Ag is degraded into oligopeptides by proteasomes and tripeptidyl peptidase II (27–29), and transported by TAP to MHC class I molecules in the endoplasmic reticulum (ER) or phagosomes (30). In the ER, peptides can be further trimmed by the ER amino peptidase ERAP1 (31). The fact that cross-presentation of ILA-extended SIINFEKL is dependent on TAP indicates that the extended peptide must go through the cytosol during a certain stage of the processing steps in the host DC, after which it is transported into the ER or phagosome for loading onto MHC class I molecules. There is also a TAP-independent pathway of cross-presentation that is insensitive to proteasome inhibitors (32). In this pathway, polypeptides are processed directly in the phagosome. It is unclear why the ILA-modified peptides do not take advantage of such a direct route.

There are at least two junctions at which ILA may enhance cross-presentation. Reits et al. (15) has shown that cytosolic amino acid and ILA-modified polypeptide were completely degraded in cytosolic extracts primarily by thimet oligopeptidase. The half-life of the peptide by 6- to 7-fold. The first three residues were particularly important in enhancing stability, and may explain why ILA but neither IL nor A was sufficient to enhance cross-priming. Beyond 15 aa, peptides become substrates for trimming by tripeptidyl peptidase II. Secondly, it has been shown that N-terminal extension of nominal peptide by even one or two residues can significantly increase the affinity of the peptide for TAP (33). Taken together, the N-terminal extended peptides may have both a longer half-life in the peptidase-rich cytosol and also enhanced affinity for the TAP. These two factors may contribute to the mechanism by which ILA may enhance the efficiency for cross-presentation.

We also took advantage of OT-I cells to compare the efficiency of cross-presentation of intact OVA protein with that of nominal peptide and ILA-extended peptide. On a molar basis, the intact protein was 3- to 10-fold more efficient than nominal peptide. This result is consistent with the results of Yewdell and colleagues (18) and Shen and Rock (19), both of which reported the inefficiency with which peptide is cross-presented. Comparison of the efficiency of cross-presentation of the ILA-extended peptide and the entire OVA protein indicated a 10- to 30-fold advantage to using the extended epitope. This may reflect the fact that protein processing can be inefficient. Although both ILA peptide and intact protein need to enter the cytosol to be transported by TAP into the ER, the protein, but presumably not ILA peptide, requires proteasomal degradation. This may result in the production of a precursor that is less efficiently transported to the ER than ILA peptide.

It is also of interest to note that there exists a preference of amino acid at position 2 from the N-terminal. For example, AAA that is less efficiently transported to the ER than ILA peptide. This may result in the production of a precursor. It is unclear why the ILA-modified peptides do not take advantage of such a direct route.

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are spared. Further studies are needed to characterize the peptide preference of substrates in more detail.

In summary, we have established a novel method that can efficiently target a known CD8 T cell nominal epitope to the cross-presentation pathway. Depending on the presence or absence of inflammatory signals, this method can be easily used for induction of immunity or tolerance.

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Disclosures

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References


