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Important Role of Cathepsin S in Generating Peptides for TAP-Independent MHC Class I Crosspresentation In Vivo

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Summary

The immune system detects viral infections and mutations in parenchymal cells when antigens from these cells are crosspresented on MHC class I molecules of professional antigen-presenting cells (APC). Exogenous antigens are crosspresented through TAP-dependent (cytosolic) or poorly understood TAP-independent (vacuolar) pathways. The TAP-independent pathway is blocked by the cysteine protease inhibitor, leupeptin, but not by proteasome inhibitors, which is opposite to the effects of these agents on the TAP-dependent pathway. Dendritic cells lacking the cysteine protease cathepsin S lack the TAP-independent pathway. Mice whose APC lack cathepsin S have reduced crosspriming to particulate and cell-associated antigens, as well as to influenza virus. Cathepsin S-deficient phagosomes generate a class I-presented peptide poorly. In contrast, cathepsin S-sufficient phagosomes and recombinant cathepsin S produce the mature epitope. Therefore, cathepsin S plays a major role in generating presented peptides for the vacuolar pathway of crosspresentation, and this mechanism is active in vivo.

Introduction

To generate CTL responses in vivo, naive T cells must first be stimulated with antigenic peptides presented on MHC class I molecules of antigen-presenting cells (APC). If the APC is synthesizing antigens, e.g., from a viral infection, then it can directly process and present these antigens to T cells. However, to generate immune responses to antigens that are exclusively produced by nonprofessional APC, e.g., tumor, cellular, or tissue tropic viral proteins (Huang et al., 1994; Kurts et al., 1996; Li et al., 2001; Sigal et al., 1999), it is necessary for APC to acquire and present antigens from the extracellular environment, a process termed crosspresentation.

Macrophages and dendritic cells (DC) are the principal cells that crosspresent exogenous antigens in vitro and in vivo (den Haan et al., 2000; Heath and Carbone, 2001; Jung et al., 2002; Rock, 1996; Rock et al., 1990a; Shen et al., 1997; Watts, 1997). In this process, exogenous antigens are internalized into endocytic compartments (Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Pfeifer et al., 1993) and then can be presented on MHC class I molecules by at least two distinct pathways. One pathway involves the transfer of antigens from phagosomes into the cytosol (Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Rodriguez et al., 1999). The antigens are degraded by proteasomes into oligopeptides that are then transported by the transporter associated with antigen processing (TAP) into the ER (Kovacsovics-Bankowski and Rock, 1995) or back into the phagosome (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Antigenic peptides are then bound by MHC class I molecules and transported to the cell surface. Therefore, the phagosome-to-cytosol pathway is inhibited by proteasome inhibitors and by mutations that block TAP function.

The other crosspresentation pathway(s) is less well understood. Peptides are presumed to be generated within endocytic compartments, and, accordingly, this pathway is not affected by proteasome inhibitors (Song and Harding, 1996). However, the proteases that generate the MHC class I-presented peptides are unknown. Moreover, whether this "vacuolar" pathway is dependent on the TAP transporter is controversial (Chefalo et al., 2003). So far, this vacuolar pathway has been demonstrated clearly only in cell culture (Rock, 1996). TAP-independent crosspresentation has been observed in vivo (Norbury et al., 2001; Ruedl et al., 2002); however, whether it occurs by the vacuolar pathway is unknown.

Endocytic compartments contain many proteases (Chapman et al., 1997; Villadangos et al., 1999) that degrade internalized proteins and play key roles in class II antigen presentation. The cysteine proteases, cathepsin (Cat) B, L, and S, an asparagine-specific endopeptidase, and the aspartyl proteases, Cat D and E, may participate in the generation of MHC class II-presented peptides. However, in most cases the inhibition of any one protease does not block MHC class II presentation, presumably because lysosomal proteases are redundant (Nakagawa and Rudensky, 1999; Villadangos et al., 1999). In addition, Cat S in DC (Nakagawa et al., 1999; Shi et al., 1999), Cat L in thymic cortical epithelium (Nakagawa et al., 1998) and probably Cat F in macrophages (Shi et al., 2000) degrade the MHC class II-associated invariant chain (Ii), a critical step for its removal from MHC class II molecules. Whether these same endosomal proteases also play a role in the vacuolar crosspresentation pathway is unknown; however, they may be different because peptides presented on class I molecules are shorter and of a precise length, while those on class II are longer and of variable length.

Given the important role of crosspriming in generating CTL responses to virus, tumor, and transplants, it is important to understand how these pathways operate in vivo. Here we show that Cat S plays a key role in the TAP-independent crosspresentation pathway both in vitro and in vivo.

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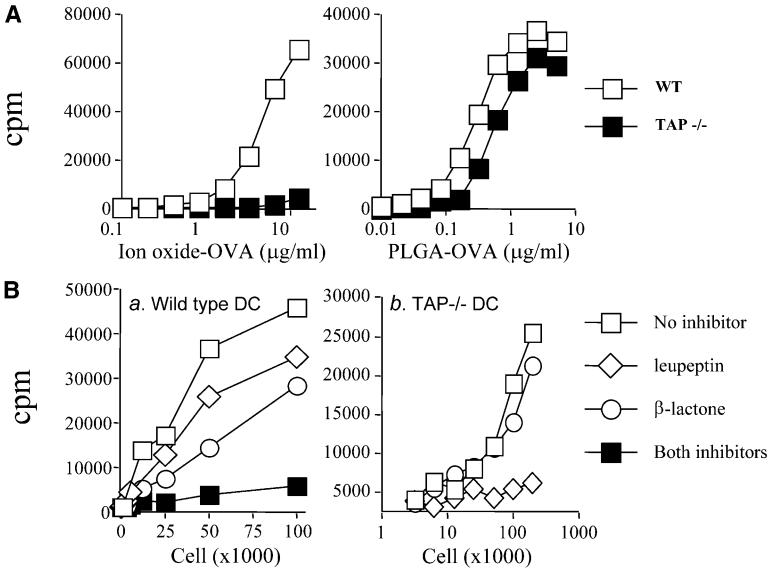

Figure 1. Particulate Antigens Can Be Cross-presented by a TAP-Dependent Mechanism and a TAP-Independent Pathway that Requires Cysteine Proteases

(A) TAP-dependent and -independent presentation of particulate antigens by professional APC. WT C57BL/6 and TAP$^{-/-}$ BM DC (5×10^4) were incubated with the indicated concentrations of iron oxide-OVA (left) or PLGA-OVA (right) and with RF33.70 T-T hybridoma (specific for K^b/SIINFEKL) (1×10^5) for 16 hr. The content of IL-2 in supernatants was measured using CTLL2 indicator cells.

(B) Effects of proteasome inhibitor and leupeptin on PLGA-OVA antigen presentation. (a) A combination of proteasome and cysteine protease inhibitors blocks exogenous antigen presentation on class I. WT BM DC were preincubated with no inhibitors, 100 μg/ml leupeptin, 2 μM β-lactone, or a combination of both inhibitors for 30 min. PLGA-OVA was then added at a concentration of 5 μg/ml for 5 hr in the continuous presence of inhibitors, and the cells were then fixed. They were then titrated and tested for their ability to stimulate RF33.70 hybridomas to produce IL-2. (b) Leupeptin inhibits exogenous antigen class I presentation by TAP-deficient DC. Similar to (a), except BM DC from TAP$^{-/-}$ mice were used instead of wild-type BM DC in the antigen presentation assay in the presence of 2 μM β-lactone or 100 μg/ml leupeptin. The results are expressed as the mean cpm [^{3}H]thymidine incorporation of cultures in duplicate, and variation between wells was <10%.

Results

TAP-Dependent and -Independent Crosspresentation

Why certain forms of antigens are crosspresented by the phagosome-to-cytosol versus vacuolar pathway is unknown. To study the vacuolar pathway, we searched for a form of ovalbumin that was presented in a TAP-independent manner. We empirically discovered that OVA incorporated into microspheres of poly-lactide poly-glycolide (PLGA), a biodegradable copolymer, was presented by TAP-deficient bone marrow (BM)-derived DC to an OVA-specific T cell hybridoma. The responses stimulated by the TAP-deficient DC were only slightly decreased compared to C57BL/6 wild-type DC (Figure 1A, right), which was in contrast to results with OVA iron oxide or latex beads where TAP function was absolutely required (Figure 1A, left) (Kovacsovics-Bankowski and Rock, 1995). These results indicate that PLGA-OVA can be presented by a TAP-independent pathway. When the concentration of antigen was more limiting and/or the incubation was shorter (e.g., APC fixed after 5 hr), the TAP-deficient APC still presented the antigen, but less well than wild-type cells (Figure 2B and data not shown). This suggested that PLGA-OVA might be presented through two distinct pathways, one TAP-dependent and one TAP-independent.

Two Crosspresentation Pathways Use Different Proteolytic Mechanisms

We used protease inhibitors to investigate the proteolytic processes involved in the TAP-dependent and TAP-independent crosspresentation pathways. In wild-type DC, a broadly active cysteine protease inhibitor, leupeptin, partially inhibited the crosspresentation of PLGA-OVA (Figure 1Ba). The concentration of leupeptin used was sufficient to inhibit cysteine proteases because it completely blocked the presentation of OVA on MHC class II (data not shown). These results indicated that cysteine proteases contribute to the presentation of exogenous PLGA-OVA, but leupeptin-insensitive proteases were also involved in this process. Since a portion of the response to PLGA-OVA was TAP dependent, we tested the effect of treating DC with the highly specific proteasome inhibitor, clasto-lactacystin β-lactone (referred to as β-lactone below). This agent also partially inhibited the presentation of PLGA-OVA (Figure 1Ba). The concentration of β-lactone used effective because it completely inhibited the generation of SIINFEKL-K^b complexes from OVA iron oxide beads and from vaccinia OVA recombinants; both of these sources of antigen are presented exclusively by the cytosolic pathway (Kovacsovics-Bankowski and Rock, 1995; Rock et al., 1994). In contrast, leupeptin had no effect on the presentation of these forms of antigen. Treatment with a combination of leupeptin and β-lactone almost completely inhibited the crosspresentation of PLGA-OVA (Figure 1Ba). Therefore, class I-presented peptides are generated from PLGA-OVA by two separate proteolytic pathways, one involving proteasomes and the other involving cysteine protease(s).

Proteasomes generate the peptides presented by the TAP-dependent pathway of crosspresentation (Kovacsovics-Bankowski and Rock, 1995). To test whether cysteine proteases generated the peptides presented by the TAP-independent pathway, we treated TAP-deficient DC with leupeptin. This agent almost completely abolished the TAP-independent presentation of PLGA-OVA (Figure 1Bb). In contrast, the proteasome inhibitor β-lactone had no effect (Figure 1Bb). Therefore, cysteine proteases are required for the TAP-independent pathway of presentation.

Cathepsin S Plays a Critical Role in Vacuolar Crosspresentation

Cysteine proteases are found in the cytosol (e.g., calpains) and in endocytic compartments (e.g., cathep-

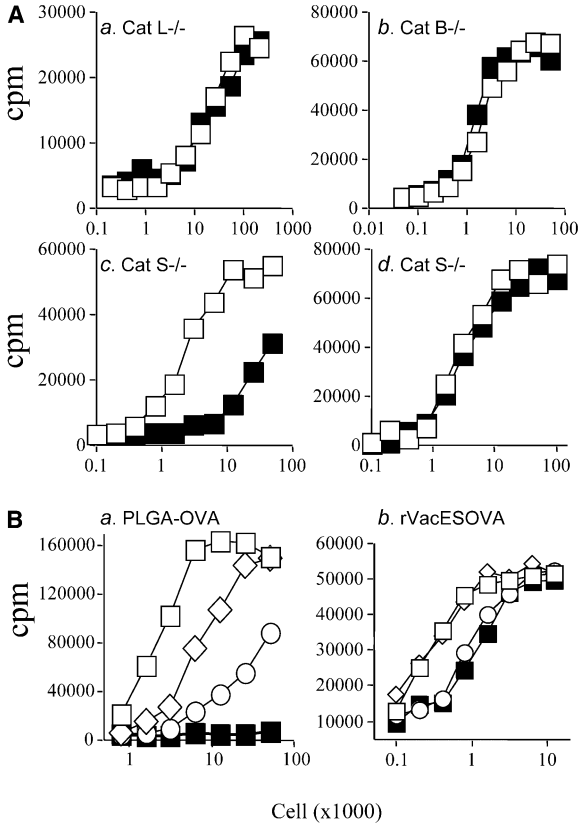

Figure 2. Cat S Is Required for the TAP-Independent Antigen Presentation on MHC Class I

(A) Cat S, but not Cat L and B, is involved in exogenous antigen presentation on MHC class I. BM DC from Cat $L^{-/-}$ (a), Cat $B^{-/-}$ (b), Cat $S^{-/-}$ (c) (solid squares) and C57BL/6 mice (open squares) were incubated with 5 μg/ml PLGA-OVA for 5 hr and then fixed. OVA SIINFEKL presentation on K^b was determined as described in Figure 1. (d) Similar to (c), except, instead of PLGA-OVA, 10 μg/ml of iron oxide bead OVA was used in antigen presentation assay.

(B) Cat S is required for TAP-independent antigen presentation on MHC class I. (a) BM DC from C57BL/6 (open squares), Cat $S^{-/-}$ (open diamonds), $TAP^{-/-}$ (open circles), and $TAP^{-/-}$Cat $S^{-/-}$ (solid squares) were compared for their ability to crosspresent PLGA-OVA antigen on class I K^b. The procedures were essentially identical to that described in (A). (b) Similar to (a), except the DC were infected with rVacESOVA instead of being incubated with PLGA-OVA.

sins). Since the presentation of peptides generated by the cysteine protease(s) is TAP independent, it was unlikely that they were produced in the cytosol. We therefore examined the role of various cathepsins in this pathway. Cat L-deficient (Figure 2Aa) and Cat B-deficient (Figure 2Ab) DC both crosspresented PLGA-OVA equivalently to their wild-type counterparts. Therefore, Cat B and L are dispensable for crosspresentation of PLGA-OVA.

Cat S is a cysteine protease that is preferentially expressed in APC including DC, macrophages, and B cells. Interestingly, the crosspresentation of PLGA-OVA was reduced in Cat S-deficient BM DC (Figure 2Ac). In contrast, the presentation of OVA bound to iron oxide beads (BioMag OVA), which does not require cysteine proteases, was not affected by Cat S deficiency (Figure 2Ad). We also observed that antigen presentation by Cat $S^{-/-}$ DC was even more markedly reduced at low antigen doses (data not shown). Therefore, Cat S plays a role in crosspresentation, especially when antigen is limiting.

PLGA-OVA was still presented by Cat S-deficient DC, albeit at a reduced level (Figure 2Ac). To determine whether this Cat S-independent presentation was due to other cysteine proteases or presentation by the proteasome-TAP-dependent pathway, we analyzed WT, $TAP^{-/-}$, Cat $S^{-/-}$, and $TAP^{-/-}$Cat $S^{-/-}$ DC. As in the previous experiments, Cat S- and TAP-deficient DC showed reduced crosspresentation of PLGA-OVA as compared to the wild-type APC (Figure 2Ba). However, the presentation by TAP and Cat S double-deficient DC was almost completely abolished (Figure 2Ba). This result indicates that the residual presentation in Cat S-deficient DC was due to the coexisting TAP-dependent cytosolic pathway and not due to the participation of other endosomal proteases. More importantly, it demonstrates Cat S is a critical enzyme in TAP-independent presentation on MHC class I and that the presented peptides are indeed generated in endosomal compartments.

To investigate whether the loss of Cat S led to any unexpected impairment of the class I pathway, we analyzed the presentation of a SIINFEKL minigene targeted into the ER by a signal sequence; this construct is presented efficiently by $TAP^{-/-}$ cells. $TAP^{-/-}$Cat $S^{-/-}$ DC infected with a vaccinia recombinant expressing the minigene presented SIINFEKL as efficiently as did TAP-deficient DC (Figure 2Bb), even under limiting conditions. These results indicate that the loss of Cat S did not impair synthesis, assembly, or transport of MHC class I molecules.

Taken together, the experiments with inhibitors and Cat S ± TAP-deficient cells complement one another and indicate that there are two distinct and independent crosspresentation pathways, cytosolic and vacuolar. For the vacuolar pathway, the cysteine protease Cat S is an important protease, at least for the crosspresentation of PLGA-OVA.

Cat S Is Not Affecting Crosspresentation through Hydrolysis of Invariant Chain

In most cells, newly synthesized MHC class I molecules are not transported into endosomes. However, in APC the Ii chain binds to some class I molecules and directs their transport into endocytic compartments (Sugita and Brenner, 1995; Vigna et al., 1996). Therefore, it is possible that similar to the MHC class II antigen presentation pathway, the Ii chain is needed to deliver class I molecules into the vacuolar pathway and that Cat S is then required to remove the Ii chain. However, Ii-deficient DC crosspresented PLGA-OVA similarly to wild-type DC (Figure 3). Therefore the Ii chain is not required for crosspresentation by the vacuolar pathway. Moreover, Ii and Cat S double-deficient DC also had lower class I antigen presentation than that of Ii (single)-deficient DC or wild-type DC (Figure 3). This indicated that Cat S was still required for crosspresentation in Ii-deficient APC. This latter result also ruled out the possibility that the reduction in antigen presentation in Cat S-deficient cells was due to an accumulation of Ii chain in endosomes. Therefore, Cat S must be needed in the vacuolar pathway for something other than hydrolyzing the Ii chain.

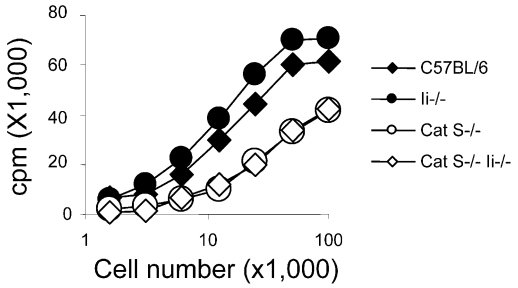

Figure 3. Cat S Is Not Affecting Crosspresentation through Hydrolysis of Invariant Chain

BM DC from wild-type C57BL/6, Cat $S^{-/-}$, $Ii^{-/-}$, or Cat $S^{-/-}Ii^{-/-}$ were incubated with 5 μg/ml PLGA-OVA for 5 hr, fixed, and then titrated at the indicated cell numbers in 96-well plates to which RF33.70 hybridoma cells were added (1×10^5/well). After 16 hr, supernatants were harvested and IL-2 production was determined using CTLL2 cells. The results are expressed as mean cpm of triplicate wells. The SD is less than 10%.

Cat S Generates the Correct Class I-Presented Peptide

It has been presumed in the vacuolar pathway that the class I-presented peptides were generated in phagosomes, although this had not been formally shown. Therefore, we tested whether isolated phagosomes could produce SIINFEKL when incubated with OVA. When peptides were generated from wild-type phagosomes and pulsed onto fixed APC, they stimulated SIINFEKL-specific OT-I T cells (Figure 4A). We next examined whether Cat S plays any role in this process. There was a marked reduction in the production of the OVA-presented peptide by phagosomes lacking Cat S (Figure 4A). Therefore, Cat S is necessary for the generation of this class I-presented peptide in phagosomes.

It was possible that in addition to Cat S, other phagosomal proteases were needed to generate SIINFEKL. Therefore, we next investigated whether purified recombinant Cat S by itself could generate SIINFEKL from OVA. An OVA peptide that could stimulate SIINFEKL-specific T cells was detected and shown to be SIINFEKL by HPLC (Figure 4B). Cat S produced SIINFEKL over a wide range in pH including near neutral conditions (Figure 4C). Therefore, Cat S is both necessary and sufficient for generating this class I-presented peptide.

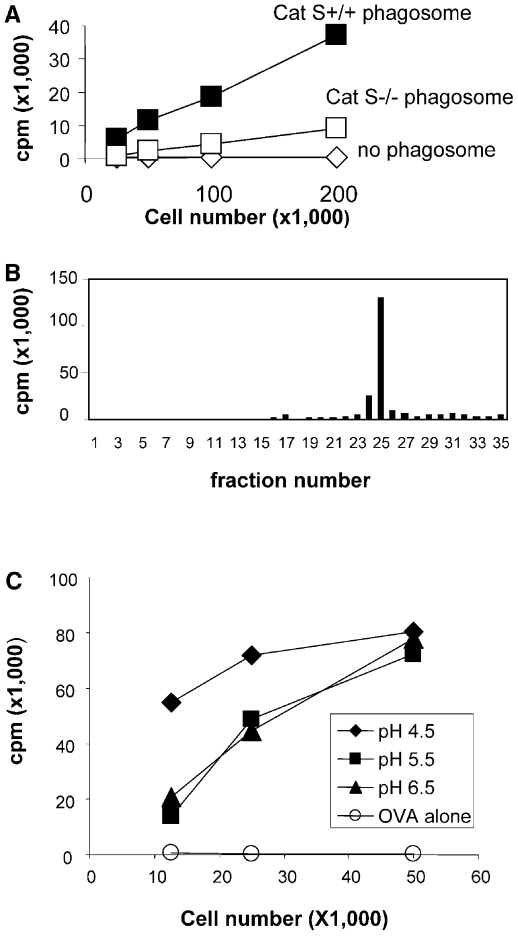

Figure 4. Cat S Can Generate the Correct Peptide for Presentation on MHC Class I

(A) Generation of an antigenic peptide by phagosomes requires Cat S. Peptides generated from OVA incubated with phagosomes from BM DC of C57BL/6 and Cat $S^{-/-}$ were pulsed onto DC2.4 cells and assayed for their ability to stimulate OT-1 T cells as described in the Experimental Procedures.

(B) Generation of SIINFEKL by Cat S. OVA was digested with recombinant Cat S, and the resulting peptides were separated by reverse phase HPLC. SIINFEKL was assayed as in (A). Under these conditions, SIINFEKL elutes in fraction 25.

(C) Cat S generates SIINFEKL over a wide range of pH. The peptides generated by Cat S at the indicated pH were pulsed onto fixed APC and assayed as in (A).

Role of Cat S in Antigen Presentation In Vivo

To address the contribution of the cytosolic and vacuolar pathways in generating CTL responses in vivo, C57BL/6, $TAP^{-/-}$, Cat $S^{-/-}$, or $TAP^{-/-}$Cat $S^{-/-}$ mice were injected with SIINFEKL+K^b-specific transgenic T cells (OT-I) labeled with the fluorescent dye CFSE. Since there could be a defect in CD4 T cell help in Cat S-deficient mice (Nakagawa et al., 1999; Shi et al., 1999), and this could potentially reduce the priming of CTL, CD4 T cells were depleted by injecting anti-CD4 (GK1.5) antibody in vivo. We verified by flow cytometry that $CD4^+$ T cells had been eliminated from the treated mice and had previously shown that OVA particles can prime $CD8^+$ T cells in the absence of $CD4^+$ T cells (Mintern et al., 2002; Rock and Clark, 1996). The recipients were then immunized with various doses of PLGA-OVA, and subsequently the proliferation of the OVA-specific T cells in lymphoid organs was analyzed by measuring their content of CFSE by flow cytometry.

Three days after injection, PLGA-OVA stimulated OT-I T cells to proliferate in the draining lymph nodes of wild-type mice in a dose-dependent manner (Figure 5 and data not shown). In $TAP^{-/-}$ or Cat $S^{-/-}$ mice, PLGA-OVA also stimulated OT-I T cells to proliferate but to a lesser degree than in wild-type controls (Figure 5A). There was also a marked reduction of OT-I T cells that had undergone proliferation in the spleen of Cat S-deficient mice at a later time point, i.e., day 5 (data not shown). Fourfold higher amounts of PLGA OVA stimulated similar amounts of proliferation in wild-type, $TAP^{-/-}$, and Cat $S^{-/-}$ mice at day 5 (Figure 5B). These results suggest

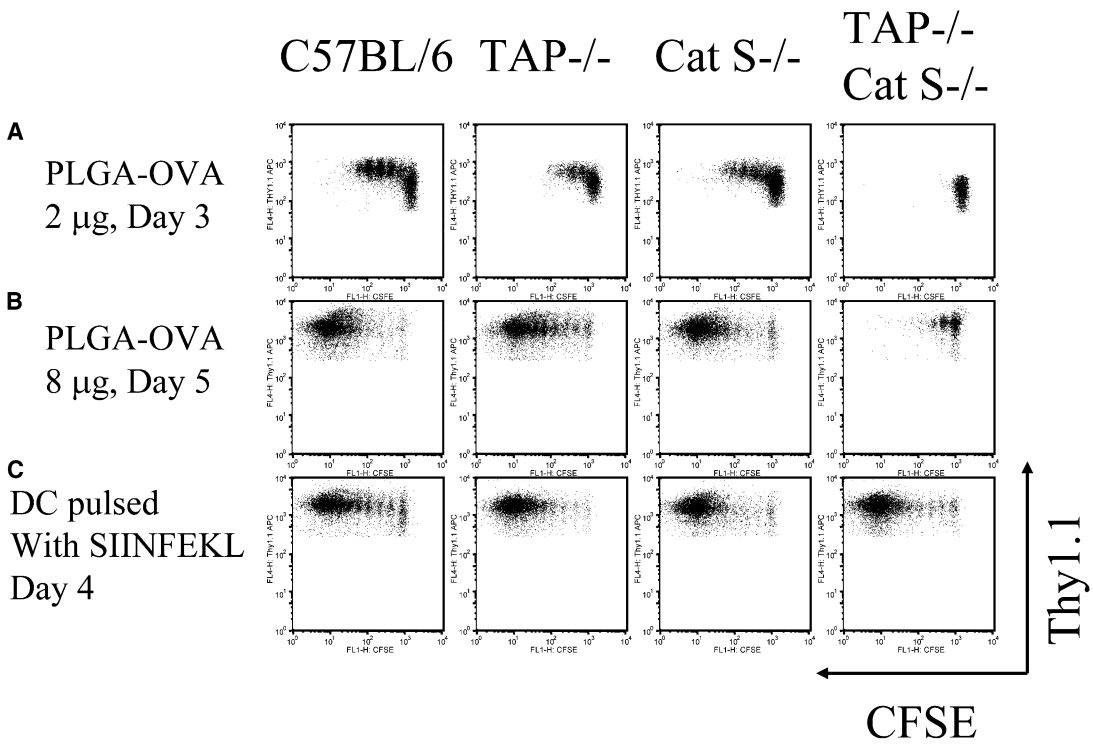

Figure 5. Proliferation of Adoptively Transferred OT-I T Cells upon PLGA-OVA Stimulation

CFSE-labeled OT-I T cells (4×10^6) were injected i.v. into the indicated mice. One day after transfer, the indicated amounts of PLGA-OVA (A and B) were injected s.c. on the left flank. In another experiment, C57BL/6 BM DC were pulsed with 10 μg/ml OVA SIINFEKL peptide and washed, and 1×10^6 cells were injected s.c. in the left flank (C). At the indicated time points, draining inguinal LN were harvested, and LN cells were stained with PerCP-CD8 and APC-Thy1.1, and analyzed by flow cytometry. CD8 and Thy1.1 double-positive T cells were analyzed for proliferation (CFSE dilution) using FlowJo software.

that in vivo, TAP-dependent and Cat S-dependent mechanisms contribute to T cells responses, which is similar to what was observed in vitro (Figures 1 and 2).

We next tested whether there was a further reduction in the priming of OT-I T cells in TAP$^{-/-}$Cat S$^{-/-}$ recipient mice. PLGA-OVA stimulated little to no OT-I T cell proliferation at day 3 (Figure 5A), even at high doses and later time points (Figure 5B). In multiple experiments, only 7.2 ± 10% OT-I T cells proliferated in Cat S and TAP-deficient mice versus 91 ± 8% in wild-type mice, a reduction of >92%. These results mirror the in vitro studies (Figure 2Ba) and indicate that OVA is being presented by both TAP- and Cat S-dependent pathways in vivo. The small response to high doses of antigen in the TAP and Cat S double-deficient mice may indicate that some other protease(s) can generate SIINFEKL, but clearly this is a minor pathway.

To exclude the possibility that the loss of OT-I proliferation in TAP$^{-/-}$Cat S$^{-/-}$ mice was due to some defect other than crosspresentation (e.g., in T cell homing, survival, or other steps), we immunized these mice with wild-type DC pulsed with SIINFEKL peptide. Equivalent OT-I responses were observed in TAP$^{-/-}$Cat S$^{-/-}$, wild-type, and single knockout mice on days 3–5 (Figure 5C and data not shown). Therefore, the lack of response to PLGA-OVA in TAP$^{-/-}$Cat S$^{-/-}$ mice is due to a failure of antigen presentation and not due to other defects in the host environment.

Cat S Contributes to Crosspriming to Cell-Associated Antigen

Tumor and other cell-associated antigens are cross-presented by professional APC in vivo at least in part by the TAP-dependent pathway (den Haan et al., 2000; Huang et al., 1996; Li et al., 2001; Liu et al., 2002). To address the potential involvement of the vacuolar pathway in this process, we adoptively transferred OT-I cells into WT, Cat S$^{-/-}$, TAP$^{-/-}$, or Cat S$^{-/-}$TAP$^{-/-}$ mice (H-2^b) and then immunized these animals with an allogeneic (H-2^K) cell line (DAP) stably transfected with OVA (Shen and Rock, 2004). In this system the OT-I T cells can only be stimulated when the OVA made by the H-2^k cell line is crosspresented on host H-2^b APC. OT-I proliferation was clearly detectable in TAP$^{-/-}$ recipients, although at lower levels than that of B6 WT (Figure 6). The T cells were also stimulated to proliferate in Cat S$^{-/-}$ mice. However, they failed to proliferate in TAP$^{-/-}$Cat S$^{-/-}$ mice. Therefore, the Cat S-dependent vacuolar pathway is also involved in crosspresentation of cell-associated antigen in vivo.

Role of Cat S in Crosspriming to Viral Antigens

We next investigated the role of Cat S in TAP-independent viral antigen presentation in vivo. Wild-type mice were irradiated and reconstituted their hematopoietic systems with bone marrow from either WT, TAP$^{-/-}$, Cat S$^{-/-}$, or TAP$^{-/-}$Cat S$^{-/-}$ deficient mice. Four months

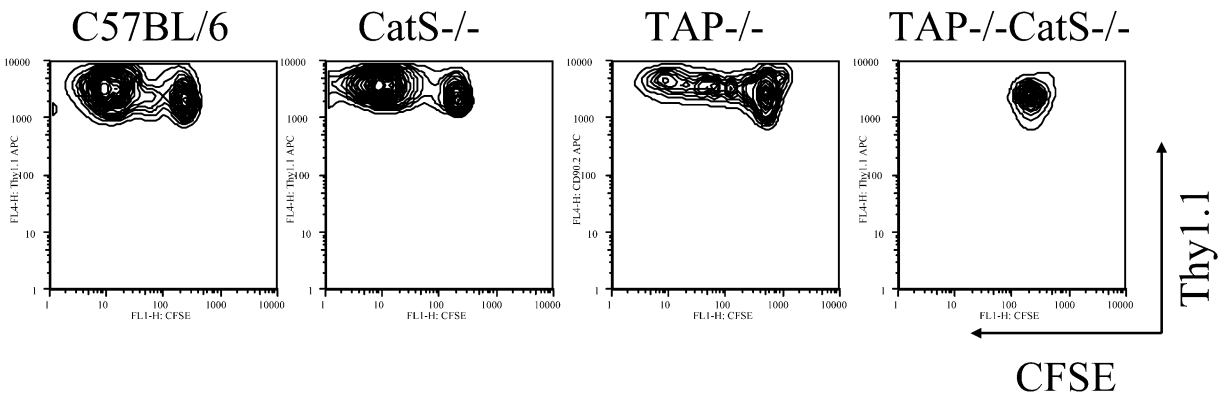

Figure 6. Cell-Associated OVA Is Crosspresented Using Both the Cytosolic and Vacuolar Pathways

CFSE-labeled OT-I T cells (4×10^6) were i.v. injected into the indicated mice. One day after transfer, 5×10^6 OVA-transfected allogeneic (H-2^k) DAP cells were injected s.c. on the left flank. On day 5, draining inguinal LN were harvested, and LN cells were stained with PerCP-CD8 and APC-Thy1.1 and analyzed by flow cytometry. CD8 and Thy1.1 double-positive T cells were analyzed for proliferation (FL-1) using FlowJo software.

after reconstitution, the chimeras were used for experiments. Since CD4 help is not required for CTL responses to influenza (Belz et al., 2002), CD4 T cells were eliminated from the chimeric mice with anti-CD4 antibody; therefore, any impairment of CTL responses in these treated mice would not be due to an effect of Cat S on CD4 T cell help.

We studied the generation of CTL to the influenza D^b-restricted $NP_{366-374}$ (ASNENMETM) epitope. When B6→B6 chimeras were infected with a low dose (25 HAU) of influenza virus and their spleens stimulated with ASNENMETM, CTL were readily detected in a ^{51}Cr release assay (Figure 7A). Consistent with our previous report (Sigal and Rock, 2000), there was no detectable CTL response in $TAP^{-/-}$→ B6 or $TAP^{-/-}$Cat $S^{-/-}$→B6 chimeras (Figure 7A). In contrast, CTL were primed in Cat $S^{-/-}$→B6 chimeras (Figure 7A), although the magnitude of the response was reduced compared to B6→B6 chimeras. Therefore, the response to this dose of influenza is largely TAP dependent, although there may be a contribution from Cat S dependent mechanisms.

As previously described (Sigal and Rock, 2000), higher doses (100 HAU) of influenza elicited CTL responses in B6→B6 chimeras (mean lytic unit [LU] = 911) and reduced but detectable ones in $TAP^{-/-}$→B6 chimeras (mean LU = 37) (Figure 7B). The CTL response in Cat $S^{-/-}$→B6 chimeras was also lower than that of B6→B6 chimeras (mean LU = 280) (Figure 7B). Most important, in $TAP^{-/-}$Cat $S^{-/-}$→B6 chimeras, CTL responses were markedly reduced to or slightly above background levels (mean LU < 1) (Figure 7B). Therefore, Cat S is also playing a role in the generation of CTL response to influenza virus in vivo.

As a control, we immunized the chimeric animals with a vaccinia virus containing a minigene encoding ASNENMETM with a signal sequence. The peptide expressed from this construct is cotranslationally transported into the ER and does not require TAP or vacuolar proteases for presentation. Similar CTL responses (LU ranging from 30 to 51) were generated in chimeric mice regardless of whether they had Cat $S^{-/-}$, $TAP^{-/-}$, and Cat $S^{-/-}TAP^{-/-}$ bone marrow (Figure 7C). Therefore, with the caveat that the minigene construct is a strong immunogen, these findings suggest that the ASNENMETM $CD8^+$ T cells in these animals were fully competent to respond to this peptide, and the APC, although defective in their ability to generate presented peptides by the cytosolic and vacuolar pathways, were otherwise fully functional.

To exclude the possibility that the differences we observed between groups were skewed by the in vitro stimulation of T cells, we assayed the CTL activity in influenza-infected chimeric mice during the primary immune responses in vivo. One week after infection, ASNENMETM-pulsed or unpulsed CFSE-labeled target cells were injected into mice, and the survival of the labeled cells was evaluated by flow cytometry (Barber et al., 2003). The killing of ASNENMETM-pulsed cells was reduced in both Cat S- and TAP-deficient BM chimeras and was almost completely abolished in TAP and Cat S double-deficient chimeras (Figure 7D). Therefore, these results confirm that Cat S is playing a role in the generation of the primary CTL response to influenza virus in vivo.

To further determine the generality of our findings, we investigated whether Cat S was involved in the generation of CTL responses to a third unrelated epitope, SSLENFRAYV, a D^b-restricted influenza peptide from the acid polymerase protein (PA). The killing of SSLENFRAYV-pulsed target cells in vivo was reduced in Cat S- and TAP-deficient BM chimeras and further reduced in Cat S and TAP double-deficient animals (Figure 7E).

On the basis of these data, we conclude that Cat S plays a major role in TAP-independent CTL response to influenza viral antigens in vivo. Moreover, since Cat S plays a critical role in the vacuolar but not the endogenous pathway of presentation, the Cat S-dependent component of the CTL response to influenza virus must be due to crosspresentation.

Discussion

The best-characterized mechanism of crosspresentation is the phagosome-to-cytosol pathway. In this pathway antigens internalized by macrophages and DC are transferred from endocytic compartments into the cyto-

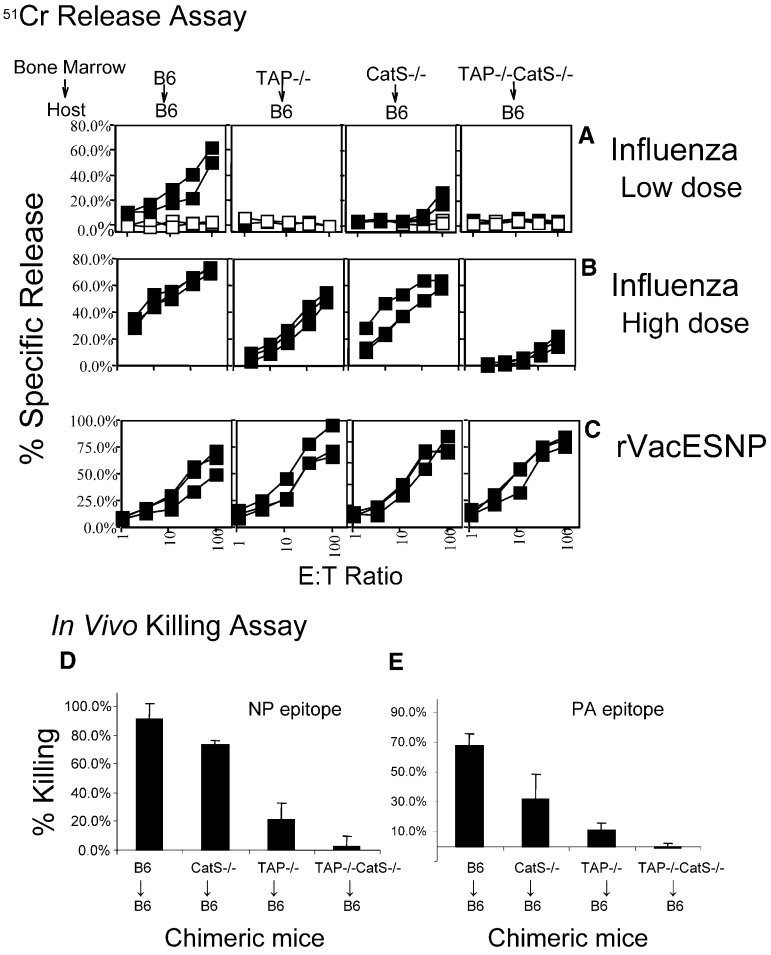

Figure 7. Effects of TAP and Cat S on Generation of Primary CTL Responses against Influenza Virus

(A–C) Detection of CTL cytotoxicity by ^{51}Cr release assay. The indicated BM chimeras were depleted of $CD4^+$ T cells using GK1.5 ascites and then injected i.p. with 25 HAU (A) or 100 HAU (B) influenza virus, or 20×10^6 pfu rVacESNP (C). Spleen cells were harvested and restimulated in vitro with 10^{-7} M influenza NP peptide, and then ^{51}Cr release assays were performed. EL4 cells pulsed with (solid) or without (open) NP peptide were used as targets. In (B) and (C), the killing of control EL4 targets (no peptide pulsing) was less than 10% and is not included in the figure for clarity. Results are expressed as % specific release. Each line is an individual mouse. Two or three mice were used in each experimental group.

(D and E) In vivo killing assay. The indicated BM chimeras (three to five mice in each group) were depleted of $CD4^+$ T and injected i.p. with 200 HAU influenza virus. Seven days after infection, B6.SJL ($Ly5.1^+$) spleen cells (target cells) were pulsed with NP or PA peptide or left unpulsed, and each target was then labeled with a different concentration of CFSE. The three target cells were mixed and i.v. injected into infected or uninfected mice. Ten hours later, blood was taken, and cells were stained with APC-Ly5.1 antibody and analyzed with flow cytometry. The percent of in vivo killing is expressed as mean % killing ± SD.

sol (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1995). The antigens are then degraded by proteasomes into oligopeptides that are transported by TAP into the ER (Rock and Goldberg, 1999) or back into phagosomes (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003) where they bind to MHC class I molecules. Other crosspresentation pathways have also been described that do not require proteasomes or newly synthesized class I molecules (Pfeifer et al., 1993; Song and Harding, 1996), but their underlying mechanisms were poorly understood. Often, these alternate pathways do not require the TAP; however, in some systems presentation was substantially lower without TAP (Song and Harding, 1996; Wick and Pfeifer, 1996). It had been uncertain whether this partial TAP dependence was because TAP participates indirectly (e.g., to allow export of class I molecules out of the ER to a crosspresenting compartment), because these antigens were being presented simultaneously by both TAP-dependent and -independent pathways, or because the two pathways were not truly distinct. Here we show that the two pathways are indeed distinct from one another and use fundamentally different mechanisms that can be distinguished by, among other things, TAP dependence.

The mechanism by which presented peptides were generated in the TAP-independent crosspresentation pathways was unknown. We show that inhibitors of endosomal proteases block TAP-independent presentation in intact cells. Moreover, isolated phagosomes contain proteases that can generate a class I-presented peptide. Furthermore, this TAP-independent presentation is inhibited in cells genetically lacking the endosomal protease, Cat S. Similarly, isolated phagosomes lacking Cat S generate a class I-presented peptide very poorly. Together, these data demonstrate that class I-presented peptides are generated in phagosomes and that Cat S plays a critical role in this process.

These data indicate that, although the antigen initially enters endocytic vesicles in both crosspresentation pathways, its subsequent fate is very different. In one case it is hydrolyzed in the cytosol, while in the other it is cleaved in endosomal vesicles. Although the pathways are different, both are active in cloned DC and macrophages (data not shown) as well as populations of these primary APC, and therefore both pathways can operate simultaneously in the same cell. Having two distinct pathways involved in generating peptides, potentially in different amounts and including new sequences, could serve to increase overall the total number and diversity of peptides that are effectively presented and thereby broaden immune responses.

It is interesting that the loss of other cathepsins, including B, L, and D (data not shown) had no effect on crosspresentation. It is possible that other proteases

will be required for the presentation of other antigens (Fonteneau et al., 2003). However, since Cat S was required for the presentation of three unrelated antigens, OVA and influenza NP and PA, it may play an important role generally in this pathway. It is not clear why Cat S, but not other more abundant proteases, plays such a critical role in vacuolar crosspresentation; however, there are several possibilities that are not mutually exclusive.

Cat S is distributed throughout the entire endocytic compartments and might be the predominant protease in the specific vesicles that generate the presented peptides. Moreover, Cat S is different from other cathepsins because its activity is relatively pH insensitive (Shi et al., 1992). We show that it generates SIINFEKL from OVA over a wide range of pH. This property may be particularly important because class I molecules are unstable and bind peptides inefficiently in acidic conditions. Thus, it is possible that the vacuolar pathway operates in vesicles with more neutral pH, such as early endosomes (Ackerman et al., 2003), where Cat S will be the principal protease that is active. Interestingly, it was reported (Lennon-Dumenil et al., 2002) that DC and macrophages phagosomes preferentially fuse with vesicles enriched in Cat S, and perhaps antigen processing occurs in these vacuoles before they acidify. It is also possible that Cat S may be the only endosomal protease that can make the proper peptides for MHC class I presentation. We show that Cat S makes the proper cleavages in OVA to produce the epitope SIINFEKL in its mature form.

It has been unclear why certain antigens are presented exclusively by the cytosolic crosspresentation pathway while others use the vacuolar pathway. This may be because not all epitopes can be generated by endosomal proteases or are destroyed. While it is likely that this is a limiting factor in some situations, our data indicate that this cannot be the sole reason for this phenomenon. Thus, in a situation where both pathways could generate the exact same peptide (SIINFEKL) from the same protein (OVA), the factor that influenced which pathways were operative was whether the antigen was associated with PLGA or iron oxide (or latex) particles. Therefore, the physical form of the antigen must influence its accessibility to the two pathways. It has previously been shown that the chemical composition of particles strongly influences where they localize in endocytic compartments (Oh and Swanson, 1996). Similarly, different pathogens have been shown to localize into different, nonoverlapping endocytic compartments in DC (Cervi et al., 2004). Thus, we speculate that some particles, such as PLGA, localize in compartments where the vacuolar pathway is present (e.g., containing Cat S and higher pH) while others access different compartments where transfer to the cytosol occurs and Cat S activity is lower.

The TAP-independent pathway of crosspresentation has been primarily demonstrated in cells in vitro (Pfeifer et al., 1993; Reimann and Schirmbeck, 1999; Song and Harding, 1996; Wick and Pfeifer, 1996). In addition, there are also a few reports that this pathway is operative in vivo (Norbury et al., 2001; Ruedl et al., 2002; Sigal and Rock, 2000); however, the mechanisms by which this occurred were unknown. Our data show that the Cat S-dependent vacuolar pathway is not only active in vitro, but also in vivo. Moreover, we demonstrate that it contributes to the generation of immune responses under physiological conditions. However, its contribution to the overall immune response to these antigens appears to be less than the phagosome-to-cytosol pathway, at least with the antigens we have examined. Nevertheless, the vacuolar pathway may play a more significant role when APC are infected with viruses whose immune evasion genes block the cytosolic pathway, e.g., by inhibiting the TAP transporter. Moreover, during viral infections, antiviral cytokines, including IFNγ, are produced. IFNγ can further induce Cat S expression (Chapman et al., 1997) which in turn may augment vacuolar antigen presentation. Hence, the TAP-independent, Cat S-dependent pathway may play an even more important role in the generation of immunity to certain viral infections.

Our data gives further insights into the generation of immune responses to viral infections. Viral antigen presentation on MHC class I can occur when APC are infected (direct priming) and/or when they acquire exogenous antigens (crosspriming). In situations where a virus only infects parenchymal cells (Sigal et al., 1999) or blocks the function of infected DC (Shen et al., 2002), then crosspriming plays a critical role in the generation of immunity. However, in situations where the virus can infect both parenchymal cells and DC, the contribution to immunity of crosspriming versus direct priming is unknown. Influenza is a virus that can infect BM-derived APC including DC. Our finding that the generation of CD8 T cell responses to influenza viral infection is reduced in Cat S-deficient mice almost certainly indicates that crosspriming contributes to these responses. Since a portion of the response is also likely to be generated by the cytosolic crosspriming pathway, which is still operative in these mice, a substantial component of the immune response may be stimulated by crosspriming. We speculate that this is often likely to be the case with cytopathic viruses because they may kill or inhibit the function of infected APC in ways that interfere with their ability to traffic to lymphoid organs and/or stimulate T cells. In any case, these data provide further evidence that crosspriming is an important mechanism for the induction of viral immunity in vivo.

The physical form of antigen from cells that is crosspresented in vivo has not been known. It is possible that it is cellular protein (Serna et al., 2003; Shen and Rock, 2004), free peptide (Kleindienst and Brocker, 2003), peptide bound to heat shock proteins (HSP) (Srivastava, 2002), or even RNA (Freigang et al., 2003). Our finding that Cat S is required for vacuolar crosspresentation in vivo indicates that the form of antigen that is crosspresented is one that requires proteolysis. This implies that under physiological conditions the crosspresented antigen is acquired by APC as a protein or long peptide. These findings lend independent support to other emerging data that argue that the relevant form of antigen that is crosspresented in vitro (Serna et al., 2003) and in vivo (Shen and Rock, 2004) is polypeptide in nature and not a HSP-peptide complex.

In summary, we have shown that peptides presented by the TAP-independent crosspresentation pathway are generated by proteolysis in phagosomes. We have iden-

tified Cat S as a key protease in this pathway and show that it functions by generating the crosspresented peptide. We show that this pathway is truly separate from the TAP-dependent one and contributes to the presentation of cellular and viral antigens in vivo.

Experimental Procedures

Mice

C57BL/6, B6.129S2-*Abcb2*^{tm1Arp} (C57BL/6 TAP$^{-/-}$), and B6.129S6-*Ii*^{tm1Liz} (C57BL/6 Ii$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6. SJL (Ly5.1^{+}) mice were purchased from Taconic (Germantown, NY). Cat S (Shi et al., 1999), L (Nakagawa et al., 1998), and B (Deussing et al., 1998) deficient mice were provided by Dr. Hal Chapman (UCSF, San Francisco) and Dr. Hidde Ploegh (Harvard Medical School) and were backcrossed to C57BL/6 background. OT-I T cell transgenic mice were made available by Dr. William Heath at the Walter and Eliza Hall Institute and crossed with B6.PL-*Thy1*^{a}*/Cy* (Jackson Laboratory) to express the Thy1.1 marker. The preparation of BM chimeras was essentially as described (Sigal et al., 1999).

Cell Lines and Virus

RF33.70 (Rock et al., 1990b), DC2.4 (Shen et al., 1997), and DAP cells (L cells) and its OVA-stable transfectants (Shen and Rock, 2004) have been described. Recombinant vaccinia viruses, rVacESOVA and rVacESNP (encoding SIINFEKL and ASNENMETM with signal sequences, respectively), were originally provided by Dr. J. Yewdell (NIAID, Bethesda, MD). Influenza virus was A/PR/8/34 strain.

Antigen Microspheres

Amine-terminated iron BioMag beads (1 μm in diameter) were purchased from Polysciences, Inc. (Warrington, PA). Covalent conjugation of OVA to beads was performed according to the manufacturer's protocol. OVA incorporated into microspheres of biodegradable copolymer polylactide polyglycolide (PLGA-OVA) was made and provided by Corixa Cooperation (Seattle, WA).

Reagents

Chicken ovalbumin and leupeptin were purchased from Sigma (St. Louis, MO). Proteasome inhibitor, clasto-lactacystin β-lactone (Craiu et al., 1997), was kindly provided by Dr. J. Adams (ProScript, Cambridge, MA). Recombinant human Cat S was kindly provided by Dr. M. Brown (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). OVA SIINFEKL, influenza NP ASNENMETM, and PA SSLENFRAYV peptides were obtained commercially or from Tom Vedvick at Corixa and Dr. Ray Welsh at UMass Medical School.

Preparation of BM-Derived DC and Macrophages and Isolation of Phagosomes

BM cells were cultured with 10 ng/ml GM-CSF and 5 ng/ml IL-4 to generate DC. To generate BM macrophages, 10% DAP cell supernatant which contains M-CSF was added to cultures. Both DC and macrophages were used after 6 or 7 days of culture. The isolation of phagosomes was performed essentially as described (Desjardins et al., 1994).

Antigen Presentation Assays

Antigen presentation assays with T cell hybrids were performed essentially as described before (Shen et al., 1997). In addition to T cell hybridomas, antigen presentation assays were also performed using OT-I T cell. OVA digest (<10 K flowthrough) or HPLC peptide fractions were assayed by incubating them with fixed DC2.4 cells in serum-free media for 2 hr at 37°C. The cells were then washed and incubated with OT-1 T cells. The proliferation of OT-I cell was assayed by measuring the incorporation of [^{3}H]thymidine into DNA (cpm).

Digestion of OVA Using Recombinant Cat S and Isolated Phagosomes

OVA (500 μg) was incubated with cathepsin S (10 μg) or isolated phagosomes at 22°C in 50 mM sodium acetate, 2.5 mM EDTA, 2.5 mM TCEP (Tris [2-carboxyethyl] phosphine hydrochloride) (Pierce) (pH 4.5 to 6.5) at 22°C for 16 hr. The cleavage of OVA was confirmed by SDS-PAGE analysis of the reaction products (data not shown). The digested materials were separated by Centricon 10 concentrators. In some experiments, the flowthrough (<10 kDa) was further separated by reverse phase HPLC using a C18 column. The fractions were washed, dried, and resuspended in PBS and used to pulse fixed APC.

OT-I Adoptive Transfer and Proliferation Assay In Vivo

OT-I transgenic T cells were labeled with 1 μM CFSE (Molecular Probes) at 37°C for 30 min, and then 2.5–4 × 10^{6} labeled cells were transferred i.v. to mice. One day after the transfer of T cells, mice were immunized s.c. in the left flank with various dose of PLGA-OVA or DAP cells (H-2^{k}) stably transfected with OVA in 100 μl PBS. At the indicated time points, draining and nondraining lymph nodes and spleens were harvested. Cells were stained with PerCP-CD8 and biotin-Thy1.1 followed by APC- streptavidin (all from BD Pharmingen) and analyzed by flow cytometry. The CFSE fluorescence (FL1) in Thy1.1 and CD8 double-positive populations (transferred OT-I T cells) was analyzed with FlowJo software.

Influenza and Recombinant Vaccinia CTL Assays

Either a low dose (25 HAU) or high dose (100 HAU) of influenza virus, or 20 × 10^{6} pfu rVacESNP virus was injected i.p. into chimeric mice. CD4 cells were depleted in all mice by three daily i.p. injections of GK1.5 anti-CD4 ascites fluid. More than 95% of the CD4^{+} T cells were depleted as shown by staining and flow cytometry of spleen cells (data not shown). Fourteen days after influenza infection or seven days after rVacESNP infection, spleen cells were restimulated with 1 × 10^{-7} M of influenza NP (ASNENMETM) peptide. On day 5 or 6 of the restimulation, a ^{51}Cr release assay was carried out to determine the CTL cytotoxicity. EL4 cells were labeled and pulsed with NP peptide and used as targets. In all experiments, the spontaneous release is less than 10% of the total release. All experiments were repeated at least three times with similar results.

In Vivo Killing Assay

The in vivo killing assay was carried out as described (Barber et al., 2003). Seven days after infection with 200 HAU of influenza virus (i.p.), mice were i.v. injected with a mixture of three different target cells (7.5 × 10^{6} each). Target cells were B6.SJL strain (expressing Ly5.1 congenic marker) spleen cells pulsed with influenza NP or PA peptides or unpulsed, and then labeled with 1.0, 0.25, or 0.06 μM CFSE, respectively. Ten hours after the injection of target cells, blood was taken and RBC lysed and stained with APC-conjugated anti-Ly5.1 antibody. The elimination of Ly5.1 and CFSE-positive target cells was measured by flow cytometry and analyzed using FlowJo software. The percent killing was calculated as follows: 100 − {[(% peptide pulsed in infected/% unpulsed in infected)/(% peptide pulsed in uninfected/% unpulsed in uninfected)] × 100}.

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