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ARTICLES

Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain

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Dendritic cells (DCs) can present extracellularly derived antigens in the context of major histocompatibility complex (MHC) class I molecules, a process called cross-presentation. Although recognized to be important for priming of T cell responses to many viral, bacterial and tumor antigens, the mechanistic details of this alternative antigen-presentation pathway are poorly understood. We demonstrate here the existence of an endolysosomal compartment in DCs where exogenously derived peptides can be acquired for presentation to T cells, and show that the MHC class I cytoplasmic domain contains a tyrosine-based targeting signal required for routing MHC class I molecules through these compartments. We also report that transgenic mice expressing H-2K^b with a tyrosine mutation mount inferior H-2K^b-restricted cytotoxic T lymphocyte responses against two immunodominant viral epitopes, providing evidence of a crucial function for cross-priming in antiviral immunity.

Cell-mediated immunity against viral pathogens is dependent on specific priming of naive T lymphocytes by 'professional' antigen-presenting cells. DCs are the most potent antigen-presenting cells known, mainly because of their high surface expression of MHC class I and class II molecules and costimulatory molecules, and their ability to efficiently process a wide variety of potentially antigenic proteins into peptides for presentation to both CD4$^+$ and CD8$^+$ T cells[1]. Unlike most other cell types, DCs are able to present exogenously derived peptides in the context of MHC class I molecules[2,3]. Over the years, such cross-presentation has increasingly become recognized to be necessary, if not essential, for a wide variety of CD8$^+$ T cell–mediated immune responses, including those to viruses, bacteria, allografts, tumor and self antigens[4–8]. Despite this, the mechanistic details underlying this alternative antigen-presentation pathway remain mostly undefined.

Evidence has supported two principal, nonmutually exclusive, models to explain how MHC class I molecules in DCs acquire exogenously derived peptide antigens. The first model, whose mechanism is dependent on the transporter associated with antigen processing (TAP), proposes that internalized antigens are normally routed from endolysosomal compartments directly into the cytosol, after which they follow the conventional pathway of proteosome digestion and TAP transport of peptides into the lumen of the endoplasmic reticulum[9–11]. Peptide binding to nascent MHC class I heavy chain and β_2-microglobulin then facilitates conformational changes that allow for secretory transport of trimeric MHC class I complexes out of the endoplasmic reticulum, through the Golgi apparatus and to the cell surface. The second model (TAP-independent) proposes that MHC class I molecules encounter and bind exogenously derived peptides in post-Golgi or endolysosomal compartments, much as do MHC class II molecules, before being transported to the cell surface for presentation to specific CD8$^+$ T cells[12–14]. Because the cytoplasmic domains of the invariant chain, MHC class II and CD1 molecules all contain endosomal trafficking motifs that direct their trafficking through endolysosomal compartments[15,16] and ultimately their acquisition of exogenous antigens[17,18], we sought to determine whether the MHC class I cytoplasmic tail had a similar function in antigen presentation.

Encoded by three separate exons (6, 7 and 8), the amino acid sequences of MHC class I cytoplasmic domains of evolutionarily distant species show a notable pattern of conservation (**Fig. 1a**) of one tyrosine residue (encoded by exon 6) and of two serine residues (encoded by exon 7), one of which is a known site of phosphorylation[19]. Splicing of exon 7 occurs naturally in some cell types[20], resulting in MHC class I molecules that cannot be internalized efficiently in lymphoblastoid cell lines[21].

Functional *in vitro* studies have shown the MHC class I cytoplasmic domain to be completely unnecessary for transport to the cell surface as well as for the ability of MHC class I molecules to acquire antigens through the conventional pathway and act as targets for allogeneic or specific cytotoxic T lymphocytes (CTLs)[22,23]. However, the sole report to our knowledge investigating the function of the cytoplasmic domain in the natural generation of MHC class I–restricted immune responses showed that transgenic mice expressing a glycosyl-phosphatidylinositol–anchored form of H-2D^b failed to mount an effective CTL response to an immunodominant H-2D^b-restricted influenza A virus epitope[24]. This result has indicated that the cytoplasmic and/or transmembrane MHC class I domains are much more important *in vivo*.

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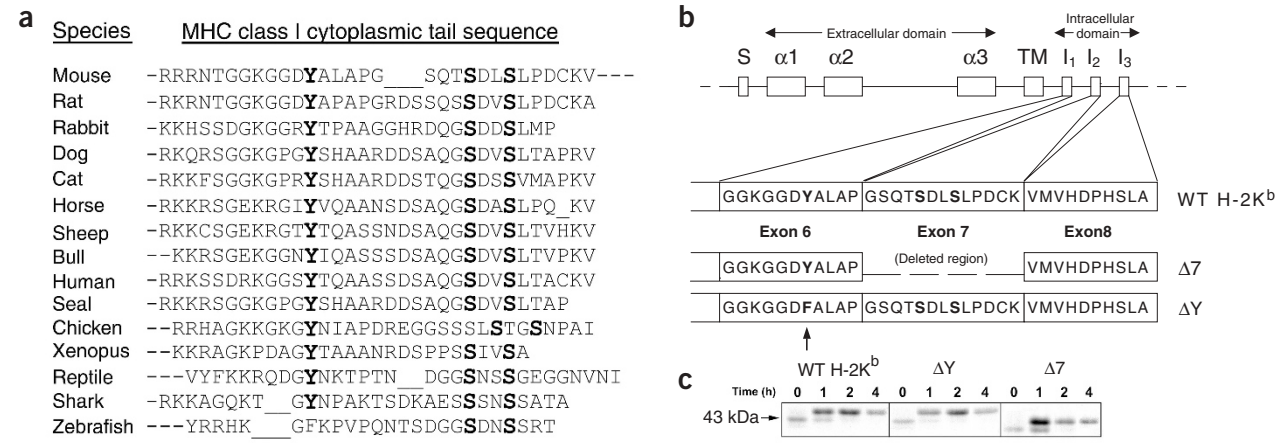

Figure 1 MHC class I cytoplasmic tail conservation and H-2K^b mutagenesis. (**a**) Aligned amino acid sequences of MHC class I cytoplasmic domains from 15 widely divergent vertebrate species. In bold, conserved tyrosine and serine residues. Species and MHC class I alleles (top to bottom): mouse (*Mus musculus*), H-2K^b; brown rat (*Rattus norvegicus*), RT1-AA; rabbit (*Oryctolagus cuniculus*), RLA 11/11; dog (*Canis familiaris*), DLA A9/A9; domestic cat (*Felis catus*), FLA-2; horse (*Equus caballus*), EX-MHC-B1; sheep (*Ovis aries*), SHP-MHCE; bull (*Bos Taurus*), BOLA BL3-6; human (*Homo sapiens*), HLA-A0101; harbour seal (*Phoca vitulina*), PLA-A1; chicken (*Gallus gallus*), B-FIV; African clawed frog (*Xenopus laevis*), Xen-MHC I; reptile (*Ameiva ameiva*), MHC I; leopard shark (*Triakis scyllium*), UAA; zebrafish (*Brachydanio rerio*), UBA-01. References for these amino acid sequences can be found in the **Supplementary Note** online. (**b**) Intron and exon structure of the gene encoding H-2K^b. The last three exons (6, 7 and 8) have been expanded to show the wild-type (WT) amino acid sequence of the cytoplasmic region of H-2K^b and the predicted amino acid sequences of the cytoplasmic H-2K^b mutants Δ7 and ΔY. S, signal sequence; TM, transmembrane domain. In bold, conserved tyrosine and serine residues. Upward arrow, single point mutation in ΔY (tyrosine to phenylalanine). (**c**) Pulse-chase experiment. Maturation rates of H-2K^b proteins are similar in splenic lymphocytes derived from transgenic mice expressing wild-type (WT) H-2K^b, ΔY or Δ7. H-2K^b proteins were immunoprecipitated with conformation-dependent antibody AF6-88.5 (at the times indicated above each lane) after pulse-labeling of cells with [^{35}S]cysteine and methionine. The Δ7 molecule, predicted to lack 13 amino acids, shows the expected increased mobility by SDS-PAGE compared with that of wild-type H-2K^b and ΔY molecules.

Our study here extends these findings with the demonstration that the highly conserved MHC class I cytoplasmic tyrosine residue forms part of an intracellular targeting motif that is responsible for MHC class I endolysosomal trafficking in DCs leading to the acquisition and subsequent cross-presentation of exogenously derived peptide antigens. Furthermore, we show that transgenic mice expressing H-2K^b molecules with a tyrosine point mutation generate inferior CTL responses against two separate H-2K^b-restricted immunodominant viral epitopes, providing evidence that cross-presentation forms a crucial component of antiviral immunity *in vivo*.

RESULTS

H-2K^b mutagenesis and generation of transgenic mice

To explore whether the MHC class I cytoplasmic tail has a function in antigen presentation, we first used site-directed mutagenesis to introduce specific mutations into the mouse H-2K^b genetic sequence that encodes the cytoplasmic domain. Two mutants were generated (**Fig. 1b**): H-2K^b mutant Δ7 contains a complete deletion of exon 7 (encoding 13 amino acids of the tail, including the serine phosphorylation site); and mutant ΔY contains a single point mutation substituting a phenylalanine residue for the conserved tyrosine residue of exon 6. We sequenced these mutated gene constructs and the gene encoding wild-type H-2K^b and microinjected them separately into fertilized mouse oocytes to generate transgenic mouse lines expressing each of the H-2K^b transgenes. We bred transgenic mice onto an H-2^k background by backcrossing transgenic founders with C3H/He mice for a minimum of six generations. We also transfected mouse L cell fibroblasts (H-2^k) with the same H-2K^b DNA constructs.

Protein analysis showed that the Δ7 and ΔY mutant H-2K^b molecules had the predicted molecular weight based on the mutations made (**Fig. 1c**). Pulse-chase experiments of spleen-derived lymphocytes showed that Δ7 and ΔY molecules matured at rates similar to those of wild-type H-2K^b molecules in both wild-type H-2K^b transgenic and C57BL/6 control mice, as determined by their acquisition of glycosylation groups and resistance to endo-N-acetylglucoseaminidase H over time (**Fig. 1c** and data not shown). These data indicated that in lymphocytes, transport of nascent MHC class I complexes from the endoplasmic reticulum through the Golgi and to the cell surface was not affected by the cytoplasmic mutations. We obtained similar results with transfected fibroblasts (data not shown).

CTL responses *in vivo* require cytoplasmic tyrosine

To determine the function of the MHC class I cytoplasmic domain *in vitro* and *in vivo*, we evaluated viral antigen presentation both in cultured fibroblasts and in transgenic mice. As predicted, L cell fibroblast transfectants expressing similar amounts of wild-type, ΔY or Δ7 H-2K^b (**Fig. 2a**) showed similar abilities to act as targets for CTLs specific for a single viral peptide consisting of amino acids 52–59 of the vesicular stomatitis virus (VSV) nucleocapsid protein (called VSV N(52–59) here), either when pulsed with VSV N(52–59) or when infected with VSV for 6, 12 or 18 h (**Fig. 2b,c** and data not shown). This result confirms previous studies[22,23] and demonstrates that classical endogenous antigen processing and presentation in cultured fibroblasts is not affected by mutations to conserved regions of the MHC class I cytoplasmic tail.

The functional consequences of the H-2K^b cytoplasmic tail mutations were apparent only when we examined H-2K^b-restricted antiviral responses *in vivo*. Transgenic mice expressing similar amounts of wild-type H-2K^b, Δ7 and ΔY on peripheral blood leukocytes (**Fig. 2d**) generated notably dissimilar immune responses to viral infections. Whereas spleens from transgenic mice expressing wild-type H-2K^b or Δ7 and C57BL/6 control mice were visibly enlarged 5–6 d after

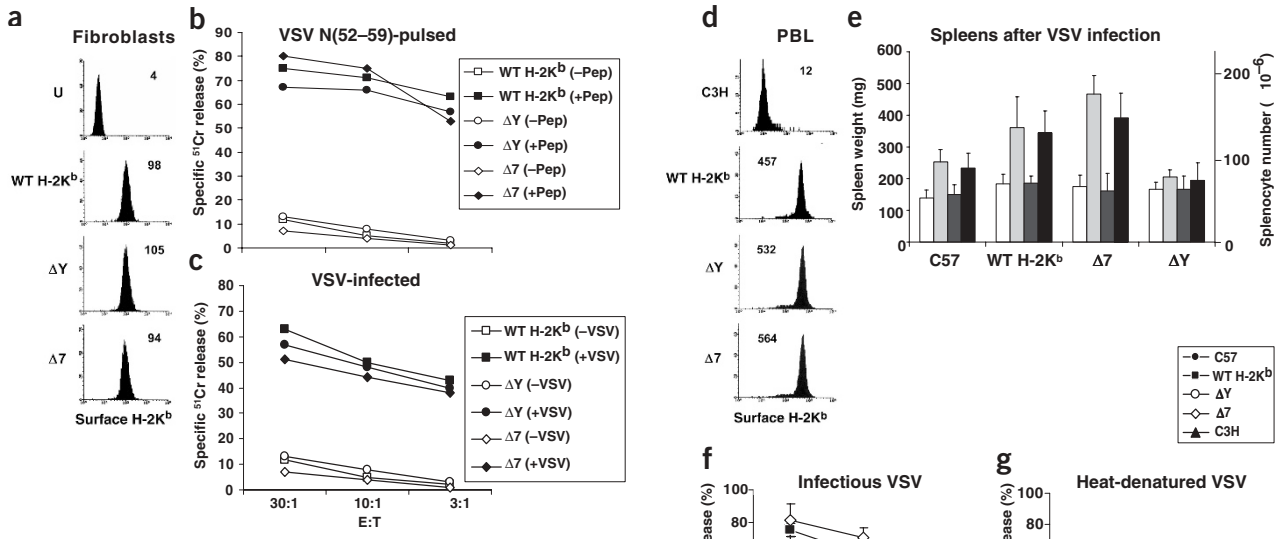

Figure 2 Viral antigen presentation in mouse fibroblasts and transgenic mice. (**a**) Surface expression of H-2K^b molecules in transfected L cell fibroblasts, as measured by flow cytometry. U, untransfected L cells. Numbers in histograms indicate mean fluorescence intensities. (**b**,**c**) Fibroblasts were either pulsed with VSV N(52–59) (1 M; + Pep; **b**) or infected with VSV (+VSV; **c**) for 18 h before being used as targets for VSV N(52–59)–specific CTLs in a standard ^{51}Cr-release assay. –Pep (**b**), unpulsed; –VSV (**c**), uninfected. (**d**) Surface expression of H-2K^b molecules in peripheral blood lymphocytes (PBLs) from transgenic mice expressing H-2K^b, as measured by flow cytometry. Numbers in histograms indicate mean fluorescence intensities. C3H, negative control mice. (**e**) Spleens from transgenic and control (C57) mice 6 d after intraperitoneal injection with VSV. Data represent spleen weights and splenocyte counts with (light gray and filled bars, respectively) or without (open and dark grey, respectively) VSV infection. (**f**–**i**) Antiviral CTL responses in transgenic mice expressing H-2K^b: At 6 d after intraperitoneal injection with infectious VSV (**f**), heat-inactivated VSV (**g**), infectious Sendai virus (**h**) or heat-inactivated Sendai virus (**i**), splenocytes were restimulated in culture for 5–6 d with 1 M of the appropriate H-2K^b-restricted peptide: VSV N(52–59) or Sendai NP(324–332). Specific CTL activity was measured with a standard cytotoxicity assay, with ^{51}Cr-labeled (H-2K^b-transfected L cell fibroblasts pulsed with the appropriate peptide as target cells. C3H, negative control mice; C57, positive control mice. Data represent ^{51}Cr release (mean and s.d.) of two mice per group, with all measurements obtained in triplicate. Data are representative of at least three separate experiments. E:T, effector/target ratio; WT, wild-type.

intraperitoneal injection of VSV, spleens from VSV-infected mice expressing ΔY had much lower spleen weights and splenocyte counts (**Fig. 2e**), indicative of a diminished response to VSV infection. Although C3H/He mice (H-2^k transgenic background) do not generate a detectable CTL response against VSV[25], H-2K^b-expressing mice normally generate a vigorous, immunodominant response against VSV N(52–59)[26]. Consistent with this, splenocytes from VSV-infected wild-type H-2K^b and Δ7 mice demonstrated strong VSV nucleocapsid peptide–specific lytic activity after 5 d of *in vitro* peptide stimulation. In contrast, CTLs derived from ΔY mice typically required 10- to 20-fold higher effector/target ratios to achieve comparable lysis of ^{51}Cr-labeled nucleocapsid peptide–pulsed target cells (**Fig. 2f**). We obtained similar results as early as 6 d after VSV infection, at which time anti-VSV CTL activity was detectable in splenocytes from both wild-type H-2K^b and Δ7 transgenic mice, but not in those of ΔY mice (data not shown).

To confirm this apparent deficiency in CTL priming with another viral system, we assessed CTL responses against Sendai virus in the H-2K^b-expressing transgenic mice. Sendai virus has the unique property of eliciting a H-2K^b-restricted CTL response when delivered in either an infectious or a heat-denatured form[27], a property not shared with VSV (**Fig. 2g**). As with VSV, ΔY-expressing mice infected with infectious Sendai virus mounted a much weaker CTL response against the immunodominant H-2K^b-restricted Sendai nucleoprotein epitope consisting of amino acids 324–332 (called NP(324–332) here) than did transgenic mice expressing wild-type H-2K^b or Δ7 and C57BL/6 control mice (**Fig. 2h**). This defect was greatest in ΔY mice immunized with heat-denatured Sendai virus (**Fig. 2i**). Because this last antigen must generate CTL solely through cross-presentation, this result indicated that the antigen-presenting cells of the ΔY strain had lost the capacity to present exogenous (nonreplicative) Sendai virus antigen in the context of H-2K^b molecules.

DC cross-presentation requires cytoplasmic tyrosine

We next evaluated whether inefficient cross-presentation by DCs may be responsible for the lack of H-2K^b-restricted antiviral CTL activity in ΔY transgenic mice. We examined bone marrow–derived and spleen-derived DCs from the different transgenic mouse strains for their ability to cross-present the well characterized H-2K^b-restricted ovalbumin epitope consisting of amino acids 257–264, OVA(257–264)[10]. We isolated immature bone marrow DCs and differentiated them in culture with granulocyte-monocyte

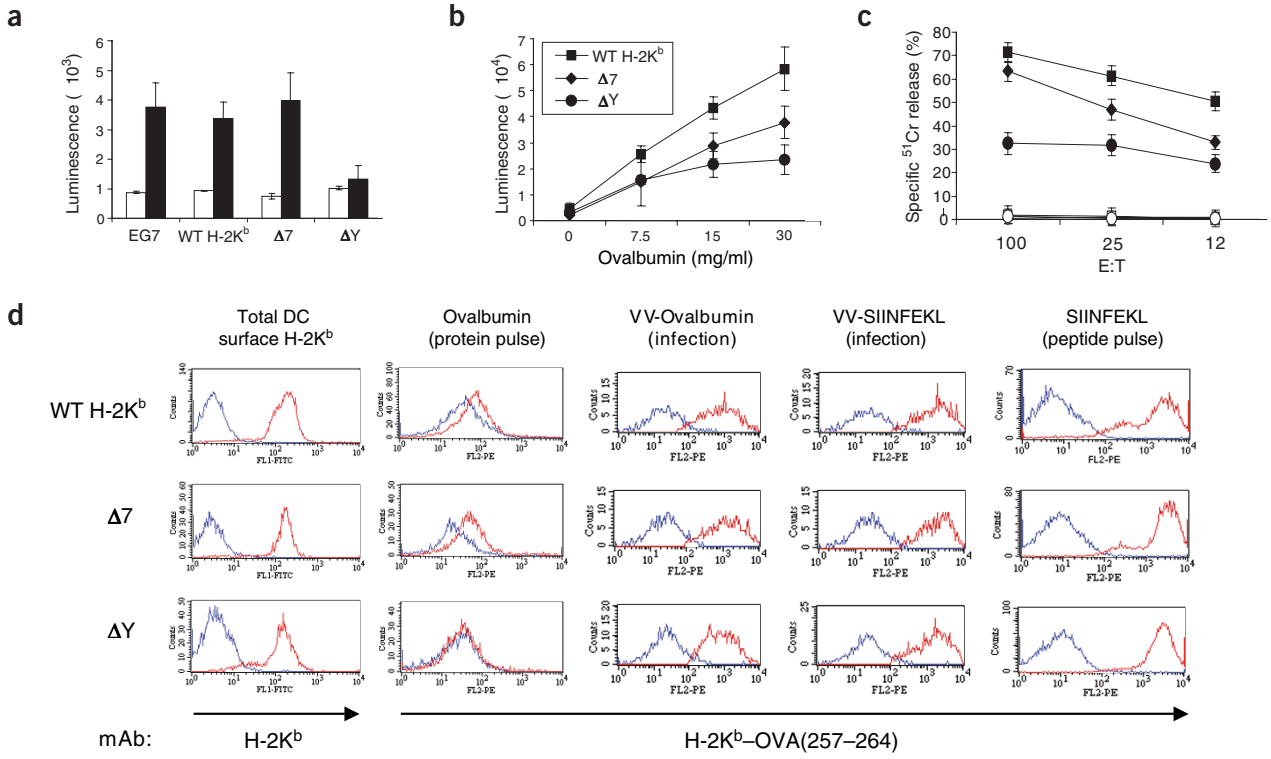

Figure 3 Cross-presentation of ovalbumin is defective in DCs derived from ΔY transgenic mice. (**a**) Activation of B3Z T cell hybridoma by immature bone marrow–derived DCs after 16 h incubation with (filled bars) or without (open bars) soluble ovalbumin (5 mg/ml). Activation of B3Z was measured with a chemiluminescence assay system to detect interleukin 2 promotor–driven β-galactosidase (lacZ) production. EG7, control cells (EL4 cells transfected with ovalbumin cDNA). Data represent means ± s.d. of results obtained from triplicate wells. Similar results were obtained in three separate experiments. (**b**) Presentation of exogenous ovalbumin by spleen-derived DCs, as measured by B3Z activation. Data represent means ± s.d. of results obtained from triplicate wells. Similar results were obtained in three different experiments. (**c**) Presentation of exogenous ovalbumin by splenic DCs, as measured by specific CTL lysis. $CD11c^+$ cells purified from spleens of $H\text{-}2K^b$ transgenic mice were incubated with ovalbumin, labeled with ^{51}Cr and exposed to activated OT-1 ($H\text{-}2K^b$-OVA-specific) CTLs at various effector/target ratios (E:T; horizontal axis). Data represent the means ± s.d. of specific ^{51}Cr release as measured in triplicate wells. Similar results were obtained in two separate experiments. (**d**) Presentation of $H\text{-}2K^b$–OVA(257–264) complexes by splenic DCs expressing similar amounts of surface $H\text{-}2K^b$ after overnight incubation with soluble ovalbumin (10 mg/ml), infection with vaccinia virus encoding ovalbumin (MOI 5) or vaccinia virus encoding OVA(257–264) (MOI 5), or incubation with synthetic OVA(257–264) (20 g/ml). DCs were stained with mAb 25.D1.16, specific for $H\text{-}2K^b$–OVA(257–264) complexes. DCs pulsed with an irrelevant protein (BSA) or peptide, or infected with an empty vaccinia virus, were used as negative controls (blue). Data are representative of three separate experiments. $P < 0.05$, all histogram peak shifts except for ovalbumin protein–pulsed ΔY-derived DCs (comparison of mean fluorescence intensities over the three experiments; *Z*-test, two-sided). WT, wild-type.

colony-stimulating factor (GM-CSF) as described[28]. After 16 h of incubation with soluble whole ovalbumin at a concentration of 5 mg/ml, we exposed DCs to B3Z, a T cell hybridoma that is activated by the recognition of $H\text{-}2K^b$ in association with OVA(257–264)[29]. DCs derived from transgenic mice expressing wild-type $H\text{-}2K^b$ or Δ7 efficiently activated B3Z cells after incubation with soluble ovalbumin, as did EG7 control cells (EL4 cells transfected with ovalbumin cDNA). However, we found no activation of B3Z cells after incubating them with ovalbumin-loaded DCs derived from transgenic mice expressing ΔY (**Fig. 3a**).

We also isolated DCs from the spleens of all transgenic mice with $CD11c^+$ magnetic beads before incubating the cells overnight with soluble ovalbumin at various concentrations and exposing them to B3Z cells. Again, DCs derived from transgenic mice expressing ΔY were considerably less efficient at activating B3Z cells than were those from transgenic mice expressing wild-type $H\text{-}2K^b$ (**Fig. 3b**). DCs derived from Δ7 mice elicited intermediate B3Z reactivity in these experimental conditions. We obtained similar results with a chromium-release assay to measure cytolysis of ovalbumin-pulsed DCs by activated CTL derived from OT-I mice that express a transgenic TCR specific for $H\text{-}2K^b$–OVA(257–264) complexes[30] (**Fig. 3c**).

To quantify cross-presentation more directly, we assessed DCs with a monoclonal antibody (mAb 25.D1.16) that specifically recognizes $H\text{-}2K^b$–OVA(257–264) complexes[31]. Expression of these complexes on the surfaces of DCs derived from both wild-type $H\text{-}2K^b$ and Δ7 mice was measurable with 25.D1.16 after overnight exposure to soluble ovalbumin (**Fig. 3d**). No $H\text{-}2K^b$–OVA(257–264) complexes were detectable, however, in DCs derived from transgenic mice expressing ΔY. Infection of these same DCs with a vaccinia virus expressing either whole ovalbumin protein or a 'minigene' encoding OVA(257–264)[32] showed that endogenous antigen presentation was intact and nearly equivalent in DCs from all three mouse strains (**Fig. 3d**). These experiments clearly demonstrated that the cytoplasmic tyrosine point mutation had abrogated the ability of $H\text{-}2K^b$ molecules specifically to cross-present the ovalbumin epitope OVA(257–264) in both bone marrow- and spleen-derived DCs.

Cytoplasmic tyrosine controls endolysosomal trafficking

To better understand this deficiency in cross-presentation, we used comparative immunofluorescence confocal microscopy to determine the intracellular localization of wild-type H-2K^b, Δ7 and ΔY molecules in bone marrow- and spleen-derived DCs. We generated transgenic bone marrow DCs as described[28] and collected them on day 9 to obtain a population of intermediate stage DCs that was >80% pure (**Fig. 4a**). We stained cells with conformation-dependent mAbs specific for H-2K^b or H-2K^k in addition to a mAb specific for the early endosome marker EEA-1. Immunofluorescence confocal microscopy showed a morphology typical of intermediate-stage DCs, with most cells having a rounded shape lacking cytoplasmic processes. H-2K^k molecules, endogenously expressed by all our transgenic mouse strains, localized at or near the cell surface in most cells examined (**Fig. 4b–d**). Similarly, most wild-type H-2K^b molecules were localized at the surface or inside vesicles near the cell surface (**Fig. 4e**). Both Δ7 and ΔY molecules, however, showed altered intracellular distributions compared with those of wild-type MHC class I molecules. The Δ7 molecules showed some surface distribution, but a substantial portion of the molecules localized to a single intracellular compartment (**Fig. 4f**). The ΔY molecules had an even greater redistribution to a single intracellular compartment in most cells examined, with a distinct down-regulation of surface staining (**Fig. 4g**). We found little or no colocalization of MHC class I molecules with EEA-1, providing further evidence that for intermediate stage DCs, MHC class I molecules reside in a compartment that is distinct from recycling early endosomes[33].

We next examined spleen-derived DCs by immunofluorescence confocal microscopy to determine whether localization of Δ7 and ΔY molecules had been similarly altered and to identify the intracellular compartments where the molecules were localized at later stages of maturation. We isolated splenic DCs and cultured them for 48 h in the presence of GM-CSF and tumor necrosis factor-α to obtain a homogeneous population of mature DCs (**Fig. 5a**). Nonadherent cells were effectively enriched for DEC-205$^+$ cells, the DC subset shown to be the most efficient at cross-presentation[34,35]. We examined the three main known compartments of MHC class I trafficking (endoplasmic reticulum, Golgi apparatus and endolysosomes) for colocalization with MHC class I molecules. Cell morphologies of DCs were typical of the late stage of maturation, with most cells demonstrating dendritic processes in all populations examined. We noted little colocalization of MHC class I molecules with the endoplasmic reticulum marker GRP78 for any of the cells examined (data not shown), indicating that transport from endoplasmic reticulum to Golgi in DCs is independent of the MHC class I cytoplasmic tail, as has been described for other cell types[22]. Double-

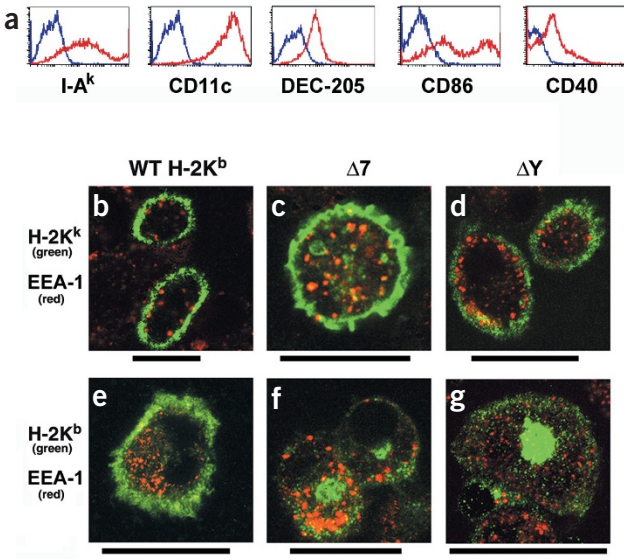

Figure 4 Aberrant intracellular trafficking of tyrosine-mutated H-2K^b molecules in bone marrow–derived transgenic mouse DCs. Intermediate-phenotype bone marrow DCs were collected on day 9 and cultured overnight on coated coverslips before being washed, fixed and permeabilized. (**a**) Histograms show intermediate DC phenotype. Red, specific staining for DC surface molecules; blue, isotype control mAb staining. (**b**–**g**) Cells were stained with an H-2K^k-specific mAb (**b**–**d**; green) or an H-2K^b-specific mAb (**e**–**g**; green), in addition to a mAb specific for EEA-1, a marker of early endosomes (red). These optically merged images are representative of most cells examined by immunofluorescent confocal microscopy. Scale bars represent 10 m.

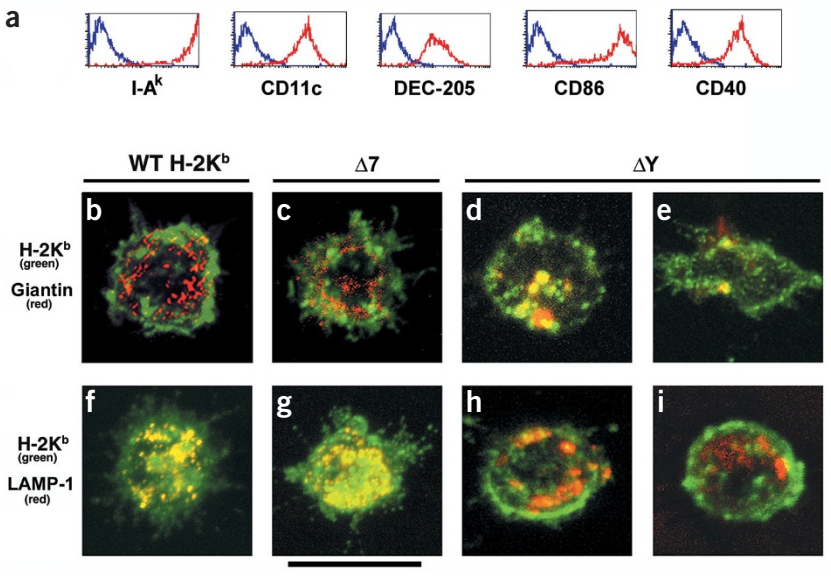

Figure 5 Cytoplasmic H-2K^b molecules with a tyrosine mutation do not traffic through endolysosomal compartments of spleen-derived DCs. Mature phenotype splenic DCs were purified from transgenic mouse spleens with CD11c magnetic beads, cultured, plated on coverslips, fixed and permeabilized. (**a**) Histograms show intermediate DC phenotype. Red, specific staining for DC surface molecules; blue, isotype control mAb staining. (**b**–**i**) Cells were stained with an H-2K^b-specific mAb (green) and either a mAb specific for the *cis*-Golgi marker Giantin (**b**–**e**; red) or a mAb specific for the endolysosomal marker Lamp1 (**f**–**i**; red). These optically merged images are representative of most cells examined by immunofluorescent confocal microscopy. Yellow, colocalization of green and red. Scale bar represents 10 m.

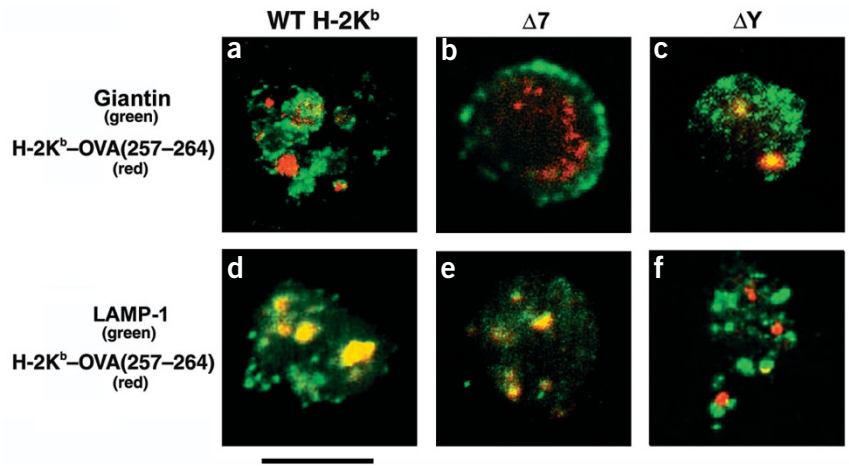

Figure 6 H-2K^b–OVA(257–264) peptide complexes are found in endolysosomal compartments of DCs after incubation with ovalbumin protein. After overnight incubation with ovalbumin (10 mg/ml), mature spleen-derived DCs were stained with a mAb specific for H-2K^b–OVA(257–264) complexes (red) and counterstained with either a mAb to the *cis*-Golgi marker Giantin (**a**–**c**; green) or a mAb specific for the endolysosomal marker Lamp1 (**d**–**f**; green). These optically merged images are representative of most of the cells examined by immunofluorescence confocal microscopy. Yellow, colocalization of green and red. Scale bar represents 10 m.

labeling for H-2K^b and the Golgi marker Giantin, however, showed that although there was no notable colocalization for wild-type H-2K^b and Δ7 molecules (**Fig. 5b,c**), ΔY molecules colocalized extensively with Giantin in approximately 70% of DCs examined (**Fig. 5d,e**).

We found extensive colocalization of wild-type H-2K^b molecules with the transmembrane lysosomal protein Lamp1 in nearly all DCs examined (**Fig. 5f**), confirming the findings of others that MHC class I molecules normally reside in and traffic through endolysosomal compartments of DCs[33]. Similarly, Δ7 molecules demonstrated an intense overlap with Lamp1-positive compartments in nearly all DCs examined (**Fig. 5g**). In contrast, ΔY molecules showed only a modest degree of colocalization with Lamp1 in <25% of DCs, with the remainder demonstrating a complete lack of endolysosomal localization (**Fig. 5h,i**). Thus, the cytoplasmic tyrosine point mutation resulted in a considerable misrouting of MHC class I molecules, with only a fraction of molecules being targeted to endolysosomal compartments in most mature DCs. This misrouting did not alter surface expression of ΔY molecules appreciably, as H-2K^b expression in these DCs was similar to those of wild-type H-2K^b and Δ7 DCs, as determined by flow cytometry (**Fig. 3d**).

Exogenous peptide loading within DC endolysosomes

To identify the compartments in which H-2K^b–OVA(257–264) peptide complexes were localized within splenic DCs after overnight incubation with whole ovalbumin protein, we used intracellular staining with mAb 25.D1.16. We found no colocalization of H-2K^b–OVA(257–264) complexes with the endoplasmic reticulum marker GRP78 for any of the transgenic mouse–derived DCs (data not shown). Similarly, in DCs derived from wild-type H-2K^b and Δ7 mice, we found no colocalization of these complexes with the Golgi marker Giantin (**Fig. 6a,b**). However, DCs derived from ΔY mice demonstrated relatively sparse specific staining for H-2K^b–OVA(257–264), and the few complexes present were exclusively within Golgi compartments (**Fig. 6c**).

Of the DCs derived from wild-type H-2K^b and Δ7 spleens staining positive for H-2K^b–OVA(257–264) complexes, >80% demonstrated strong colocalization with Lamp1 (**Fig. 6d,e**). In contrast, we found no H-2K^b–OVA(257–264) complexes in Lamp1-positive compartments in DCs expressing ΔY molecules (**Fig. 6f**). These results indicate that endolysosomal (Lamp1-positive) compartments of DCs comprise the main sites for MHC class I loading of cross-presented ovalbumin peptide, and provide an explanation for the inability of H-2K^b molecules with mutated tyrosine to efficiently cross-present this epitope.

DISCUSSION

The results presented here provide evidence that the MHC class I cytoplasmic tail has a functional involvement in antigen presentation. Most previous studies designed to assess the function of the MHC class I cytoplasmic domain used *in vitro* systems and examined only the classical endogenous antigen-presentation pathway[22,23]. Our findings confirm those results and extend those studies with the use of a mouse model to evaluate the function of the MHC class I cytoplasmic domain *in vivo*.

The conserved MHC class I cytoplasmic tyrosine residue seemed to be pivotal for proper endolysosomal targeting in DCs and subsequent cross-presentation of exogenously derived antigens. Because DCs are unique in their ability to efficiently cross-present antigens to activate naive CD8$^+$ T cells, this indicates that the tyrosine-based endolysosomal targeting signal may be specific for DCs. Many other membrane proteins are dependent on cytoplasmic YXXØ (where Ø is a bulky, hydrophobic amino acid) tyrosine-based trafficking motifs for targeting to lysosomes[36,37]. Because the third amino acid position after the MHC class I cytoplasmic tyrosine residue (alanine) is also highly conserved, it is possible that the YXXA motif forms a trafficking signal analogous to those of other membrane proteins, although this remains to be demonstrated. CD1b and CD1d molecules provide relevant examples, as these nonclassical MHC class I molecules are dependent on a cytoplasmic tyrosine motif for their targeting to endolysosomal (Lamp1-positive) compartments, where they have been shown to acquire glycolipid antigens before mobilizing to the cell surface for presentation to natural killer T cells[16,17]. Adaptin proteins specifically recognize and bind to tyrosine motifs as the first step in the formation of coated transport vesicles[38]. DC-specific adaptin proteins may normally serve to link the MHC class I cytoplasmic tail to the endolysosomal vesicular transport pathway, and substitution of the single cytoplasmic tyrosine residue may be able to effectively abrogate this interaction[39].

Our data here support a model in DCs in which the cytoplasmic tyrosine motif normally targets a subset of MHC class I molecules to endolysosomal compartments, where they undergo peptide exchange and acquire exogenously derived antigens before mobilizing to the cell surface. The ultimate source of MHC class I molecules in these compartments remains to be determined, but recycling from the cell surface is a strong possibility, as both exon 7 and the cytoplasmic tyrosine residue regulate MHC class I endocytosis in lymphoblastoid cell lines[21,40]. Studies of a DC-like cell line have shown evidence of resident MHC class I molecules in the Golgi compartments of immature cells, but these have

been shown to mobilize directly to the cell surface after maturation[41]. The consistently high surface expression of ΔY molecules found in mature DCs indicates that this mobilization to the cell surface is generally intact. However, our finding of some ΔY molecules in the Golgi compartments of mature DCs indicates that a subset of MHC class I molecules may normally be sorted into endolysosome-bound vesicles directly from the Golgi. In the absence of a tyrosine motif, these molecules may be trapped in Golgi compartments *en route* to endolysosomes.

The small number of surface H-2K^b–OVA(257–264) complexes produced by cross-presentation compared with direct presentation probably reflects differences in the relative efficiencies of the two pathways and indicates that cross-presentation involves only a fraction of the total MHC class I pool present in DCs. Nonetheless, this modest amount of peptide presentation is immunologically relevant, as demonstrated by the considerable specific activation of T cells recognizing H-2K^b–OVA(257–264) complexes. The observation that H-2K^b–OVA(257–264) complexes were undetectable with mAb on the surface of DCs derived from ΔY mice, yet still activated T cells specific for H-2K^b–OVA(257–264) at a low level confirms that T cells are responsive to very small amounts of MHC class I–peptide complexes and that T cell recognition provides a much more sensitive, albeit less quantitative, assay for peptide presentation[31]. This acquisition of OVA(257–264) peptide by ΔY molecules may reflect endosome-to-cytosol transport of ovalbumin and subsequent loading of these MHC class I molecules in the endoplasmic reticulum through the classical endogenous antigen-presentation pathway. Alternatively, it may reflect a 'leakiness' of ΔY molecules to traffic through Lamp1-positive compartments. This latter possibility would not be unlikely, given the extremely conservative nature of the ΔY mutation (removal of a single oxygen atom) as well as the fact that tyrosine-based endosomal trafficking motifs do not seem to involve direct tyrosine phosphorylation[39].

The unexpected discovery that a point substitution to the cytoplasmic MHC class I tyrosine residue specifically abrogates cross-presentation in DCs and also impairs CD8$^+$ T cell priming in response to two separate viruses raises many immunological implications. Visualization of wild-type H-2K^b–OVA(257–264) complexes exclusively in Lamp1-positive compartments of DCs after incubation with ovalbumin protein provides evidence that these compartments comprise a chief site of exogenous antigen loading for MHC class I molecules. This evidence is strengthened by the fact that ΔY molecules, lacking the conserved cytoplasmic tyrosine residue and unable to traffic through these compartments, were substantially impaired in their ability to acquire and subsequently present the OVA(257–264) peptide at the DC surface.

The demonstration that ΔY molecules were able to efficiently acquire and present two different endogenously synthesized peptides (in VSV-infected fibroblasts and in DCs infected with vaccinia virus–ovalbumin), but were unable to effectively cross-present two exogenous antigens (DCs pulsed with whole ovalbumin and CTL priming *in vivo* with heat-inactivated Sendai virus) shows that only cross-presentation, and not direct presentation, was affected by the tyrosine point mutation. Furthermore, the observation that mice expressing ΔY molecules were substantially impaired in their ability to generate CTL responses against two infectious viruses generating well-characterized, H-2K^b-restricted immunodominant epitopes (VSV and Sendai virus) supports previous studies indicating that cross-presentation may be the main mechanism by which antiviral CD8$^+$ T cell-mediated immunity is generated naturally *in vivo*[4].

The source of antigenic peptides remains a central question in cross-presentation. Peptides capable of binding to MHC class I molecules may be generated directly within endolysosomal compartments by cathepsins and other proteolytic enzymes. Internalized ovalbumin, however, is transported efficiently into the cytosol of DCs through an endosome-to-cytosol route[10,42]. Further evidence of a cytosolic component to ovalbumin processing comes from studies showing that cross-presentation of OVA(257–264) is subject to proteosomal blockade[43,44]. Given those results, our data support a third, alternative model to explain cross-presentation, which incorporates the phenomenon of endoplasmic reticulum–mediated phagocytosis in macrophages[45,46]. This model proposes the existence of phagosomal compartments, generated through fusion of the endoplasmic reticulum with endolysosomal vesicles[47], which are positive for both Lamp1 and TAP. Similar fusion events of the endocytic pathway with the classical endogenous antigen-presentation machinery of DCs could explain many aspects of cross-presentation, including its dependence on proteosomes and TAP, as well as many of our results. This model does not, however, fully explain the apparent sequestration of exogenously acquired ovalbumin from endogenously synthesized ovalbumin demonstrated in our ΔY model. Although much remains to be determined regarding the nature of endolysosomal antigen processing, the identification of an MHC class I endolysosomal targeting motif provides a critical step in delineating the mechanisms behind cross-presentation.

The human immunodeficiency virus 1 protein Nef causes down-regulation of MHC class I molecules by sequestering them in Golgi compartments, a property that is directly dependent on the MHC class I cytoplasmic tyrosine residue[48]. Our results raise the possibility that Nef interferes with the same pathway that normally controls MHC class I endolysosomal targeting in DCs. Because DCs are a known reservoir of human immunodeficiency virus, infected DCs may be particularly susceptible to MHC class I misrouting by Nef[49], which *in vivo* could lead to a deficiency in DC cross-presentation of opportunistic pathogen-associated antigens.

These results provide a compelling justification for the high level of conservation shown by the MHC class I cytoplasmic tyrosine motif, an evolutionary event that itself may emphasize the importance of cross-presentation *in vivo*. Furthermore, the establishment of this model should now allow for direct assessment of the function of cross-priming in fundamental immunological events such as tolerance to self and tumors, and the generation of autoimmune responses.

METHODS

Mutagenesis of the gene encoding H-2K^b. Mutations were introduced into the mouse gene encoding H-2K^b with a PCR-based protocol[50]. The primers containing mismatches and deletions used for generating the mutant constructs were as follows. For the tyrosine-to-phenylalanine codon substitution in exon 6 (underlined) at position 321 (ΔY mutant), 5′-AAAAGGAGGGGACTTCGCTCTG GCTCCA-3′ was used, and for the complete deletion of exon 7 (residues 326–338; Δ7 mutant), 5′-TTTGTTTTGTTTTACC:TGACACTCTAGGGTCT-3′ was used (colon, location of deleted sequence). The 'bookend' primers[50] used for the mutation of exon 6 (Y→F) were 5′-TCTGAGTTTTCTCTCAGCCTCCTTTAG AGTGT-3′ and 5′-TAAAACAAAACAAATGTATATGCATATGTACAT-3′. The bookend primers used for the deletion of exon 7 were 5′-ACATTGGAGTGAAG TTGGAGATGATGGGA-3′ and 5′-AACATCAGACACTTGACTAAAGAGAA CT-3′. PCR reactions consisted of 30 cycles of denaturation at 95 °C for 30 s followed by annealing and extension at 60 °C for 60 s, with 0.25 M primers, 200 M dNTPs and Pfu polymerase (Stratagene) in the manufacturer's buffer. PCR fragments were digested with restriction enzymes and the cut fragments were gel-purified and ligated into the linearized pK^b vector. The validity of these constructions was confirmed by DNA sequencing.

Transgenic mice. The DNA constructs described above were purified on cesium chloride gradients, and the genes encoding wild-type and mutant H-2K^b were subsequently excised from their original cloning vector, pBR322, by an *Eco*RI

digestion followed by agarose gel purification. DNA was diluted to a concentration of 1 g/ml and was microinjected into fertilized mouse oocytes (derived from a C3H/He C57BL/6 mating), which were then transplanted into the uteri of pseudo-pregnant female mice. Transgenic offspring were originally selected by Southern blot analysis. These founder mice were tested for increased peripheral blood leukocyte H-2K^b expression by flow cytometry compared with that of H-2$^{b/k}$ control mice, indicative of transgene expression. Three founder mice from each group were chosen and subsequently bred to C3H/He mates for a minimum of six generations. All transgenic mouse studies were approved by the Committee on Animal Care at the University of British Columbia using the guidelines set out by the Canadian Council on Animal Care.

Peripheral blood leukocyte isolation and flow cytometry. Peripheral blood leukocytes were obtained from mice by a tail bleed. Blood (50–100 l) was initially mixed with 1 M heparin (75 l) and then PBS (825 l) before being separated on Ficoll gradients by centrifugation. AF6.88.5, 28.14.8S and 16.3.1N conformation-dependent mouse mAbs (of the IgG2a isotype) were specific for extracellular domains of H-2K^b, H-2D^b and H-2K^k, respectively. Cells (1 10^5 to 2 10^5) were stained with the appropriate MHC class I–specific primary antibody and then were incubated with goat antibody to mouse IgG2a (anti-IgG2a) conjugated to biotin (Southern Biotechnology) followed by streptavidin–fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories). Stained peripheral blood leukocytes were fixed, and leukocyte-gated cells were analyzed by flow cytometry (Becton Dickinson).

Immunoprecipitation. Spleen-derived lymphocytes were incubated for 20 min in methionine- and cysteine-free media, pulsed for 20 min with [^{35}S]methionine (300 uCi/ml), and chased in medium containing excess methionine (0.5 mM) and cysteine (0.5 mM). At various time points, lymphocytes (1 10^7) were collected, washed in cold PBS and lysed for 20 min at 4 °C in 1% Nonidet-P40 buffer (120 mM NaCl, 4 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.6) containing phenylmethyl sulfonyl fluoride (40 g/ml). After cell nuclei had been centrifuged, cell lysates were precipitated with trichloracetic acid and samples with equal amounts of incorporated radioactivity were immunoprecipitated with mAb AF6.88.5. Proteins were separated on a 10–15% gradient gel in reducing conditions by SDS-PAGE. The gel was then fixed, enhanced, dried and exposed to autoradiographic film.

Generation of virus-specific CTLs. VSV- or Sendai virus–specific CTLs were generated by *in vivo* priming followed by *in vitro* restimulation. Mice were infected by intraperitoneal injection of virus: VSV (1 10^7 '50% tissue culture–infectious dose', or $TCID_{50}$) per mouse (or 3 10^7 $TCID_{50}$ per mouse for heat-inactivated VSV); Sendai virus (1.5 10^7 '50% egg-infectious dose units', or EID_{50}) per mouse (or 5 10^7 EID_{50} per mouse for heat-inactivated Sendai). The $TCID_{50}$ and EID_{50} are the endpoint dilutions of the virus that infect 50% of inoculated tissue culture cells and embryonated chicken eggs, respectively. Heat inactivation of viruses was accomplished by incubation of virus preparations for 2 h at 65 °C before infection of mice. Heat inactivation was confirmed experimentally by infection of susceptible target cells. At 6 d after viral infection, mice were killed and spleens were collected. Splenocytes were cultured at a density of 2.5 10^6 cells/ml for 5 d in RPMI 1640 medium supplemented with 10% FCS (HiClone), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (50 M), L-glutamine (2 mM) and 1 M of the appropriate H-2K^b-restricted peptide: VSV N(52–59), RGYVYQGL, or Sendai NP(324–332), FAPGNYPAL.

Antiviral cytotoxicity assays. Cytotoxicity was assessed with a standard ^{51}Cr-release assay. Target cells (H-2K^b-transfected L cell fibroblasts) were incubated with 1 M peptide and labeled for 1.5 h with sodium chromate (100 Ci; Amersham), then washed and resuspended in RPMI 1640 complete medium. Effector cells were incubated for 4 h at 37 °C with target cells (1 10^4 cells per well in 96-well plates) at various effector/target ratios. Spontaneous ^{51}Cr release by labeled cells was measured in the absence of CTL, and maximum release was quantified by lysis of target cells in 2.5% Triton X-100 detergent. All experiments were done in triplicate, and specific ^{51}Cr release was calculated with the following equation: Percent specific ^{51}Cr release = [(experimental release – spontaneous release) / (maximum release – spontaneous release)] 100%. Spontaneous background ^{51}Cr release never exceeded 15% of maximum release.

DC culture. DCs were derived from mouse bone marrow precursors by culture *in vitro* with GM-CSF (20 ng/ml) as described[28]. Bone marrow DCs were collected on day 7 and day 9 to obtain immature and intermediate stage DC populations, respectively. Splenic DCs were isolated with CD11c magnetic beads and were either used immediately or cultured for 48 h in GM-CSF and tumor necrosis factor-α (10 ng/ml) to induce full maturation. Nonadherent splenic DCs were used, providing for the enrichment of DEC-205$^+$ DC subsets capable of efficient cross-presentation[34,35]. All DCs were analyzed by flow cytometry for cell surface expression of CD11c, DEC-205, CD40, CD86 and I-A^k to assess purity and stage of maturation.

Detection of cross-presentation of ovalbumin. B3Z cells express a TCR that specifically recognizes OVA(257–264) (SIINFEKL) in the context of H-2K^b in addition to carrying a β-galactosidase (lacZ) construct driven by nuclear factor of activated T cells elements from the interleukin 2 promoter[29]. An assay with Galacton-Star as a chemiluminescent substrate was used for the detection of lacZ activity in B3Z lysates. Bone marrow DCs (day 7, immature phenotype) were incubated for 16 h with whole ovalbumin (5 mg/ml) before being washed and incubated overnight with B3Z cells at a cell ratio of 1:1. DCs were also isolated from the spleens of transgenic mice expressing H-2K^b with CD11c$^+$ magnetic beads (Miltenyi Biotech), and were exposed to varying concentrations of ovalbumin for 16 h before being incubated with B3Z cells as described above. OT-I mice express a transgenic TCR that specifically recognizes H-2K^b in complex with OVA(257–264)[30]. For the generation of CTLs specific for H-2K^b–OVA, 1 10^7 EG7 cells (EL4 cells transfected with ovalbumin cDNA) were irradiated (20,000 rad) and cultured for 6 d in complete RPMI 1640 medium with 1 10^7 spleen cells from OT-I mice. Spleen-derived DCs were incubated for 16 h with ovalbumin (5 mg/ml) and labeled for 1.5 h with sodium chromate (^{51}Cr) in RPMI 1640 medium before being exposed to OT-I effector CTLs for 4 h at 37 °C. The percentage of specific lysis was calculated as described above. After overnight incubation with 5 mg/ml of soluble ovalbumin or control antigen BSA, Fc receptors of splenic DCs were blocked with 2.4G2 FcγIII/II blocker (PharMingen), then cells were stained with mAb 25.D1.16, specific for H-2K^b–OVA(257–264) complexes[31], or IgG1 isotype control antibody followed by phycoerythrin-conjugated rat anti-mouse IgG1. DCs were then washed and analyzed by flow cytometry.

Infection of DCs. Splenic DCs were isolated from all mouse strains as described above and infected at a multiplicity of infection (MOI) of 5 for 2 h with either empty recombinant vaccinia virus, recombinant vaccinia virus expressing full-length ovalbumin or recombinant vaccinia virus expressing a 'minigene' encoding OVA(257–264)[32]. After culture in complete RPMI medium for 16 h, Fc receptors were blocked in DCs and cells were stained with mAb 25.D1.16 or IgG1 isotype control antibody, followed by phycoerythrin-conjugated rat anti-mouse IgG1. DCs were then washed and analyzed by flow cytometry.

Confocal microscopy. Bone marrow– and spleen-derived DCs were isolated as described above. Bone marrow–derived DCs with an intermediate phenotype (day 9) were isolated and plated on polylysine-coated coverslips for 16 h. Next, cells were washed with PBS, fixed with paraformaldehyde (2%) and permeabilized with saponin (0.1%). DCs were double-stained with mAb AF6-88.5 (anti-H-2K^b) or mAb 36-7-5 (anti-H-2K^k; PharMingen) and a rabbit mAb specific for EEA-1, a marker of early endosomes. Spleen-derived CD11c$^+$ cells with a mature phenotype were plated overnight on coverslips, fixed and permeabilized as described above, and double-stained with mAb AF6-88.5 and Giantin (Calbiochem) or Lamp1 (Santa Cruz Biotechnology) rabbit mAbs. Secondary Alexa-488 green–conjugated goat anti-mouse and Alexa-568 red–conjugated goat anti-rabbit (Molecular Probes) were used as detection reagents. Alternatively, mature DCs were stained with conjugated mAb 25.D1.16 to detect H-2K^b–OVA(257–264) complexes after incubation with soluble ovalbumin (10 mg/ml) or control protein antigen BSA. Isotype control antibodies were used in all confocal microscopy experiments to confirm specificity of antibody stainings. Coverslips were examined by confocal microscopy, and *Z*-series' of multiple images were acquired from DCs representative of most cells in each culture. Figures are shown as optically merged 'green-plus-red' images.

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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