A CD74-dependent MHC class I endolysosomal cross-presentation pathway

Genc Basha1–3, Kyla Omilusik1,3, Ana Chavez-Steenbock1, Anna T Reinicke1,2, Nathan Lack1, Kyung Bok Choi1 & Wilfred A Jefferies1

Immune responses are initiated and primed by dendritic cells (DCs) that cross-present exogenous antigen. The chaperone CD74 (invariant chain) is thought to promote DC priming exclusively in the context of major histocompatibility complex (MHC) class II. However, we demonstrate here a CD74-dependent MHC class I cross-presentation pathway in DCs that had a major role in the generation of MHC class I–restricted, cytolytic T lymphocyte (CTL) responses to viral protein– and cell-associated antigens. CD74 associated with MHC class I in the endoplasmic reticulum of DCs and mediated the trafficking of MHC class I to endolysosomal compartments for loading with exogenous peptides. We conclude that CD74 has a previously undiscovered physiological function in endolysosomal DC cross-presentation for priming MHC class I–mediated CTL responses.

During primary immune responses, dendritic cells (DCs) are the principal antigen-presenting cells that initiate adaptive immune responses predominantly through cross-presentation and the cross-priming of T cells. This involves extracellular antigen uptake, digestion of cell-associated antigenic fragments and presentation of proteolytic peptide products on both major histocompatibility complex (MHC) class I and MHC class II molecules. For MHC class I, two main pathways have been described that may explain how this process occurs: the cytosolic pathway, shown convincingly to function in vitro; and the vacuolar pathway, shown to have a major role in vivo for certain antigens. The ‘phago-ER’ (endoplasmic reticulum–mediated phagocytosis) model of cross-presentation has been considered a dominant pathway of cross-presentation. Subsequent data have disputed that conclusion.

However, one factor that has contributed to this controversy seems to be the over-interpretation of data that designate intracellular proteins as definitive markers of specific organelles that are often not exclusive but merely undergo enrichment during dynamic organelle biogenesis and partitioning. Furthermore, contrasting conclusions may have been inferred from studies of different forms of exogenous antigens and in studies of long-term DC cell lines versus those of freshly isolate DCs.

In the vacuolar pathway, cathepsin S has been identified as a protease that generates antigenic peptides that are loaded onto peptide-receptive MHC class I molecules. Furthermore, membrane and cytosolic SNARE proteins, which control tethering and docking events for donors and acceptors during intracellular membrane fusion, also seem to have a fundamental role in cross-presentation events. However, the source of MHC class I in the cross-presentation compartment, the mechanism of its transport and the site of peptide loading remain areas of active study.

Spontaneous internalization of recycling MHC class I into endosomes has been demonstrated. Published results support a model in which the recycling of MHC class I from the plasma membrane to an endolysosomal compartment is facilitated by recognition of the tyrosine internalization signal found in the MHC class I cytoplasmic tail. Therefore, MHC class I molecules recycling from the plasma membrane are one source of MHC class I for loading with exogenous antigens destined for participation in cross-presentation. Likewise, transport of MHC class I from the endoplasmic reticulum (ER) to the endocytic compartment has also been proposed. This could occur by a mechanism involving fusion of the phagosome and ER. An alternative and potentially complementary hypothesis is that the chaperone CD74 (invariant chain), known to associate with MHC class II in the ER, thereby preventing premature binding of peptides and mediating trafficking to the endocytic pathway by sorting signals present in the CD74 cytoplasmic tail, could bind MHC class I and deliver a fraction of the MHC class I to the endocytic compartment to function in cross-presentation. This mechanism would coincidently place peptide-receptive MHC class I in the same compartment with exogenous antigen and MHC class II molecules (or a similar compartment), the MIIC compartment, facilitating antigenic peptide loading and binding to MHC class I molecules. Furthermore, this pathway would link MHC class I transport to the endocytic pathway, as it is unlikely that CD74 would be involved in the cytosolic route of exogenous presentation by MHC class I.

The interaction of MHC class I with CD74 and their coincident localization in the same compartment has been demonstrated in...
human cell lines. Although it was concluded on the basis of older paradigms that the MHC class I–CD74 interaction probably does not control the fate of the transport of MHC class I to endosomes under physiological conditions, other contrasting studies have demonstrated that cells transfected to express CD74 have much higher surface expression of MHC class I encoded by diverse alleles, which suggests that the MHC class I–CD74 interaction might have functional importance. Here we investigate the immunological relevance of MHC class I interaction with CD74 in vivo and describe a clear and critical role for CD74 in the cross-presentation of exogenous antigen and subsequent cross-priming by DCs.

RESULTS

Primary antiviral responses require CD74

DCs can be directly infected and could therefore use classical presentation by MHC class I to activate naive CD8+ T cells. However, during infection with virus at a low titer, direct infection of DCs is less likely and DC cross-presentation is the dominant pathway responsible for the generation of CD8+ T cell responses. To address the role of CD74 in cross-presentation to generate primary antiviral immune responses, we infected wild-type (Cd74+/+) and Cd74-deficient (Cd74−/−) mice with a low dose of vesicular stomatitis virus (VSV). We similarly infected mice deficient in the transporter TAP (Tap1−/−), which have impaired assembly and intracellular transport of MHC class I and thus lack CD8+ T cells due to improper thymic selection, as a negative control (Fig. 1a and Supplementary Fig. 1a). In this infection, the responses of primary and memory CD8+ T cells to VSV can be generated in the absence of CD4+ T cells. In this way, the role of CD74 in cross-presentation can be assessed regardless of its role in CD4+ T cell responses. We assessed the frequency of CD8+ T cells generated in response to the immunodominant epitope VSV nucleoprotein amino acids 52–59 (VSVNP52–59) presented on MHC class I (H-2Kb) after VSV infection. Cd74−/− mice had a significantly lower capacity to generate antigen specific CD8+ T cells than did Cd74+/+ mice (5.0% versus 19.0%; Fig. 1ab). This resulted in an immune response with a lower killing capacity of cytotoxic T lymphocytes (CTLs; Fig. 1c).

We constructed bone marrow chimeras to further exclude the possibility of a role in cell in cross-priming in the VSV infection mode. Additionally, we used the chimeras to confirm whether the deficiency in generating immune responses was dependent on the ability of the hematopoietic cell-derived DCs to cross-present antigen and prime T cells. For this we reconstituted Cd74−/− mice with Cd74+/+ bone marrow (Cd74+/+→Cd74+/+) or Cd74−/− bone marrow (Cd74−/−→Cd74+/+) and reconstituted Cd74−/− mice with Cd74+/+ bone marrow (Cd74+/+→Cd74−/−) or Cd74−/− bone marrow (Cd74−/−→Cd74−/−). We found normal amounts of CD8+ T cells and CD4+ T cells in the periphery of Cd74−/−→Cd74+/+ and Cd74−/−→Cd74−/− mice. However, we found fewer CD8+ T cells and somewhat more CD8+ T cells in Cd74+/+→Cd74−/− and Cd74+/+→Cd74−/− mice (Supplementary Fig. 1b,c). This indicated that positive selection in recipient Cd74−/− mice was impaired because of a lower abundance of MHC class II in the Cd74−/− thymic epithelium.

To examine antiviral responses, we infected chimeric mice with a low titer of VSV and assessed the generation of VSVNP52–59-specific CD8+ T cells by tetramer analysis and a CTL killing assay (Fig. 2). Cd74+/+→Cd74−/− mice, with low CD4+ T cell numbers, were able to produce VSVNP52–59-specific CD8+ T cells similar to wild-type Cd74+/+→Cd74+/+ chimeras (1.1% versus 1.2%; Fig. 2a), which resulted in immune responses with similar killing capacity (16.8% versus 1.9%; Fig. 2b). However, Cd74+/+→Cd74−/− mice were grossly impaired in the generation of VSVNP52–59-specific CD8+ T cells (0.2%; Fig. 2a) despite having normal CD4+ T cells, which resulted in lower CTL killing responses (18.0% versus 4.5%; Fig. 2b). This suggested that the generation of VSV-specific CTL responses was independent of CD4+ T cell numbers. Notably, bone marrow–derived antigen-presenting cells expressing CD74 were required and allowed Cd74−/− mice to produce a robust antiviral immune response similar to that of Cd74+/+ mice.

Depletion of CD4+ cells has no effect on anti-VSV responses

Next we eliminated the possibility that residual CD4+ T cells in the Cd74+/+→Cd74−/− chimeras that resulted from dysfunctional positive selection in Cd74−/− mice contributed to the efficiency of their antiviral immune responses. During the course of the infection, we depleted Cd74+/+→Cd74−/− chimeras of the CD4+ T cells by injecting them with the GK1.5 antibody to CD4 (anti-CD4). Although CD4+ T cells were almost completely undetectable relative to background, Cd74+/+→Cd74−/− chimeras depleted of CD4+ T cells generated significantly more CD8+ T cells specific for VSVNP52–59 than did Cd74−/−→Cd74+/+ chimeras (13.5% versus 4.1%; Fig. 2c), which resulted in an immune response with more lytic activity (14.0% versus 4.9%; Fig. 2d). Together these data confirmed that Cd74+/+→Cd74−/− chimeras mounted stronger anti-VSV responses than did Cd74+/+→Cd74−/− chimeras. This was independent of CD4+ T cells but was instead due to the reconstitution of Cd74−/− mice with wild-type DCs that were fully able to prime antiviral CD8+ T cell responses.

Cross-priming of cell-associated antigen is Cd74 dependent

To investigate the role of CD74 in the primary immune response to cell-associated antigen, we used lethally irradiated DCs pulsed with ovalbumin (OVA) or DCs with MHC class I mismatched to that of the host pulsed with OVA as a source of cell-associated antigen to activate CTLs in Cd74−/− mice, Cd74+/+ mice depleted of CD4+ cells, and Cd74−/− mice, as well as in reconstituted mouse chimeras. Mice with a wild-type immune system, challenged with cell-associated OVA, were able to induce proliferation of CD8+ T cells derived from mice with transgenic expression of the OT-I ovalbumin-specific TCR.

Figure 1 Cd74−/− mice generate weak antiviral primary immune responses. (A) Generation of VSVNP52–59-specific (H-2Kb–VSVNP) CD8+ T cells in spleens of Cd74+/+, Cd74−/− and Tap1−/− mice isolated 6 d after infection with a low titer of VSV (2 x 103 of a dose that infects 50% of a tissue culture cell monolayer or mouse), then stimulated for 5 d with VSVNP52–59). Numbers in quadrants indicate percent cells in each throughout. (B) Frequency of H-2Kb–VSVNP52–59–specific CD8+ T cells among cells obtained as in (n = 3 mice per genotype). (C) Standard 51Cr-release assays of CTLs generated after VSV infection and in vitro boosting as in (n = 3 mice per genotype). *P < 0.05 (Student's t-test). Data are representative of at least three separate experiments (mean ± s.d.).
(Supplementary Fig. 2) and to activate endogenous CTLs that were efficient at killing target cells pulsed with OVA amino acids 257–264 (OVA(257–264) (data not shown). However, with the same challenge of cell-associated OVA, mice with a hematopoietic system deficient in CD74 were much less able to stimulate the proliferation of OT-I CD8+ T cells and generated fewer endogenous CTLs that contributed to a lower killing ability (Supplementary Fig. 2 and data not shown).

**CD74-dependent cross-priming is independent of CD4+ T cells**

To focus specifically on DC cross-priming defects and eliminate the requirement for help from CD4+ T cells, we incubated Cd74−/− and Cd74+/+ DCs with OVA protein or OVA(257–264) peptide and injected those cells along with purified OT-I CD8+ T cells labeled with the cytosolic dye CFSE into T-cell–deficient recombination-activating gene 1–deficient (Rag1−/−) mice on a BALB/c background. We then assessed the ability of the DCs to cross-prime the OT-I T cells (Fig. 3a). Cd74−/− DCs induced much less proliferation of OT-I T cells than did Cd74+/+ DCs when incubated with OVA protein (18% versus 48%; Fig. 3b). However, when we pulsed the DCs with OVA(257–264) peptide, as a positive control for direct presentation, Cd74−/− DCs were as competent as Cd74+/+ DCs in activating the OT-I CD8+ T cells (59.5% versus 60.0%; Fig. 3b).

To address the possible confounding role of CD74 in the motility and homing of DCs28 from the site of injection to the spleen, we assessed the localization of CFSE-labeled DCs after intravenous injection (Fig. 3b and Supplementary Fig. 3). Cd74+/+ and Cd74−/− DCs injected intravenously into Rag1−/− mice localized equivalently to the spleen. Therefore, the diminished ability of Cd74−/− DCs to induce T cell proliferation was not due to differences in DC migration but was due to less ability to process and present antigen. We concluded that CD74 has a critical role in cross-presentation of cell-associated antigen by MHC class I and in the priming of CD8+ T cells in vivo and this is unrelated to CD4+ T cell help or CD4+-mediated motility of DCs.

**Impaired cross-priming ability of CD74-deficient DCs**

We assessed the ability of spleen-derived DCs from various mouse strains to cross-present the H-2Kb-restricted ovalbumin epitope OVA(257–264) in vitro. We incubated DCs with soluble OVA, with or without cytokines, and either stained the cells with an antibody specific for the H-2Kb–OVA(257–264) complex or cultured the cells with B3Z, a T cell hybridoma that is activated after recognition of H-2Kb–OVA(257–264) complex. Cd74−/− DCs were as competent as Cd74+/+ DCs in activating the OT-I CD8+ T cells (59.5% versus 60.0%; Fig. 3b).

Figure 3 *Cd74−/− DCs are unable to cross-present cell-associated antigens in vivo to prime antigen-specific CD8+ T cells.* (a) Protocol: Cd74−/− or Cd74+/+ BMDCs pulsed with OVA protein or OVA(257–264) peptide are injected into Rag1−/− mice on a BALB/c background, along with purified CFSE-labeled OT-I CD8+ T cells (left), followed 3 d later by analysis of the proliferation of H-2Kb–OVA(257–264) complex or cultured the cells with B3Z, a T cell hybridoma that is activated after recognition of H-2Kb–OVA(257–264) complex. Numbers above bracketed lines indicate percent CFSE− (dividing) cells. Data are representative of two experiments.
in association with OVA(257–264)\(^3\), \(Cd74^{+/+}\) and \(Cd74^{-/-}\) DCs had similar ability to internalize OVA and had similar total surface expression of MHC class I (Fig. 4a,b). However, after incubation with OVA, \(Cd74^{-/-}\) DCs had a much lower abundance of H-2K\(^b\)-OVA(257–264) complexes than did \(Cd74^{+/+}\) DCs (Fig. 4b). It has been shown that the cross-priming ability of DCs is augmented by inflammatory mediators that induce the upregulation of costimulatory and MHC molecules and diminish endocytosis\(^3,13,32\). This results in a greater capacity for

Figure 4 Cross-presentation and cross-priming are defective in \(Cd74^{-/-}\) DCs. (a) Uptake of OVA–Alexa Fluor 488 by BMDCs incubated with OVA at 37 °C (dark gray filled curves) or 4 °C (light gray lines), assessed by flow cytometry. (b) Formation of H-2K\(^b\)-OVA(257–264) complexes on splenic DCs with (+OVA) or without (–OVA) incubation with soluble OVA (top), as well as total H-2K\(^b\) (green filled curves) above background (gray lines; bottom), measured by flow cytometry. (c) Expression of CD80 and CD40 on BMDCs incubated with medium alone (–OVA), OVA alone (+OVA) or OVA and interferon-γ (OVA + IFN-γ), assessed by flow cytometry. (d) Activation of B3Z T cells by spleen-derived DCs incubated with various concentrations of soluble OVA (horizontal axis) in the presence of the cell-signaling molecule GM-CSF alone (top) or GM-CSF plus TNF (middle) or interferon-γ (bottom), measured by chemiluminescence assay and presented as relative light units (RLU). (e) ICM of mature, spleen-derived DCs incubated with OVA or TNF (bottom) or without TNF (top), then stained with antibody to H-2K\(^b\)-OVA(257–264) (red) and anti-LAMP-1 (green), presented as optically merged images. Scale bar, 5 μm. (f) Quantitative analysis of the colocalization of H-2K\(^b\)-OVA(257–264) with LAMP-1+ late endosomes in the presence of TNF (top) and fluorescence of \(Cd74^{-/-}\), \(Cd74^{+/+}\) and Tap1\(^{-/-}\) DCs (>20 per strain) with and without treatment with TNF (bottom), presented as normalized individual pixels relative to total pixels. *P < 0.05 (Student’s t-test). Data are representative of two experiments (a–c,e,f; error bars (f), s.d.) or are from one experiment representative of three separate experiments with similar results (d; mean ± s.d. of triplicate samples).
T cell priming but diminished ability of DCs to capture and present soluble antigens. To assess T cell activation in a situation resembling in vivo conditions that involve costimulation, we incubated OVA-pulsed DCs with B3Z T cells with and without cytokines. In the presence of tumor necrosis factor (TNF) and interferon-γ, Cd74+/+ DCs and Cd74−/− DCs had an equal ability to upregulate the costimulatory molecules CD80, CD86, and CD40 (Fig. 4c and data not shown), but Cd74−/− DCs were much less able to activate B3Z T cells than were Cd74+/+ DCs (Fig. 4d). As expected, we detected no T cell activation after we incubated the cells with OVA-pulsed DCs derived from Tap1−/− mice in the presence of cytokines. These data supported the conclusion that Cd74 has a role in cross-presentation and does not affect the expression of costimulatory molecules.

**CD74 mediates endolysosomal MHC class I loading**

To better understand the mechanism of the cross-presentation and priming deficiency at a molecular level, we used comparative immunofluorescence confocal microscopy (ICM) to assess the intracellular localization, trafficking and distribution of OVA(257–264)-loaded MHC class I in Cd74+/+ and Cd74−/− DCs with and without TNF. We incubated cells with OVA protein and stained cells intracellularly with antibody to H-2Kb–OVA(257–264) and to the late-endosome marker LAMP1. Colocalization with LAMP1 was detectable in many of the Cd74+/+ splenic DCs that stained for H-2Kb–OVA (257–264) complexes when no TNF was added to the culture (Fig. 4e,f). We identified some H-2Kb–OVA(257–264) complexes in the Cd74−/− and Tap1−/− DCs; however, colocalization with late endosomes was minimal (Fig. 4e,f). The absence of loaded MHC class I in the Tap1−/− DCs was consistent with a role for TAP in cross-presentation, a mechanism that has been postulated before16,34. After treatment with TNF, Cd74−/− DCs had significantly more colocalization of H-2Kb–OVA(257–264) complexes with LAMP-1 (Fig. 4e,f) but not with the ER marker GRP78 or the Golgi marker giantin (data not shown). In contrast, we observed few H-2Kb–OVA(257–264) complexes in late endosomal compartments in Cd74−/− DCs (Fig. 4f), which indicated less formation of H-2Kb–OVA(257–264) complexes in late endosomes. Comparison of the ICM data indicated that in the presence of TNF, Cd74−/− DCs had significantly less OVA(257–264) loaded onto H-2Kb in the late endosomes than did Cd74+/+ DCs (62% versus 32%; Fig. 4f). These data suggested that in DCs, a Cd74-dependent MHC class I antigen-processing pathway exists that is required for the cross-presentation of exogenous antigens.

**CD74 directs MHC class I from the ER to the endolysosomes**

The finding that Cd74 deficiency resulted in fewer H-2Kb–OVA(257–264) complexes in late endosomal compartments suggested that Cd74 targets MHC class I from the ER to the endolysosomal pathway. There, Cd74 is presumably degraded and MHC class I is loaded with exogenous antigenic peptides. To examine this in more detail, we blocked the acidification of endosomes through the use of chloroquine and assessed the Cd74-mediated MHC class I cross-presentation pathway. We found that bone marrow–derived DC (BMDCs) treated with chloroquine had surface expression of MHC class I equivalent to that of untreated control cells and displayed H-2Kb–OVA(257–264) when pulsed with OVA (257–264); however, after incubation with soluble OVA, chloroquine-treated DCs had much less surface H-2Kb–OVA(257–264) than untreated DCs had (Fig. 5a–c). ICM analysis showed that BMDCs had more colocalization of H-2Kb and Cd74 in the endolysosomes in a manner similar to that reported for the MHC class II pathway16 and by inhibiting the degradation of recycling MHC class I. The end result was less loading of MHC class I with exogenous antigen and subsequently less H-2Kb–OVA(257–264) on the cell surface. To confirm the finding that Cd74 directed MHC

---

**Figure 5** Inhibition of Cd74-mediated trafficking of MHC class I in DCs by treatment with chloroquine. (a) Formation of H-2Kb–OVA(257–264) complexes on BMDCs left untreated (−CQ) or treated with chloroquine (+CQ) and incubated with medium alone (−OVA) or with soluble OVA (+OVA; top) or OVA peptide (bottom), measured by flow cytometry. (b) Total H-2Kb (green) on BMDCs left untreated or treated with chloroquine; blue, background. (c) Surface H-2Kb–OVA(257–264) complexes on BMDCs treated as in a, presented as normalized mean fluorescence intensity (MFI) relative to that of untreated BMDCs, set as 100%. (d) ICM of mature BMDCs left untreated or treated with chloroquine, then costained with anti-H-2Kb (red) and anti-Cd74 (green), presented as optically merged images. Scale bar, 5 μm. (e) Quantification of the colocalization of H-2Kb with Cd74 in d, presented as normalized pixels relative to total pixels. (f) Proliferation of CFSE-labeled OT-I cells induced by Cd74−/− BMDCs reconstituted with full-length CD74 (+ FL) or truncated CD74 lacking the endolysosomal trafficking motif (+ Δ2-17) and incubated with soluble OVA protein or by Cd74+/+ BMDCs incubated with OVA(257–264) peptide (far right). Numbers in outlined areas indicate percent proliferating OT-I cells (CD8+CFSE−) relative to that induced by Cd74+/+ BMDCs (far left), set as 100%. Data are representative of two experiments (error bars, s.d.).
class I to an endolysosomal compartment and to unequivocally demonstrate that CD74 mediated the trafficking of MHC class I, we transfected CD74-deficient BMDCs with expression vectors for full-length CD74 or CD74 lacking the cytosolic trafficking domain and assessed their ability to present OVA protein or OVA(257–264) peptide, a positive control that would bypass the need for processing. CD74+/− DCs had impaired cross-priming ability and induced much less proliferation of OT-I T cells than did CD74+/+ DCs (Fig. 5f). As expected, cross-priming ability was restored in CD74+/+ DCs reconstituted with full-length CD74 and DCs were able to induce the proliferation of OT-I T cells with an ability similar to that of wild-type DCs. However, when we reintroduced CD74 lacking the endosomal trafficking motif into CD74−/− DCs, cross-priming ability continued to be impaired (Fig. 5f), which demonstrated that in the absence of CD74, there was less MHC class I directed to endolysosome and less cross-priming of OT-I T cells. Together these data showed that CD74 influenced the trafficking of MHC class I to the cross-priming compartment where efficient presentation of exogenous antigen takes place.

**Figure 6** CD74 controls the ER-to-endolysosome trafficking of MHC class I in DCs. (a) ICM of mature splenic DCs stained with anti-H-2Kβ (green) plus anti-CD74 (red; top) or anti-LAMP-1 (red; bottom). Scale bar, 5 μm. (b) Quantification of MHC class I in LAMP-1− compartments (50 DCs per mouse strain), presented as individual pixels/total pixels. (c) Immunoprecipitation (IP) of proteins from [35S]methionine-labeled Cd74+/+, Cd74−/−, Tap1−/− and β2-microglobulin-deficient (B2m−−) BMDCs with antibody to I-A−I-E (I-Aβ; left lane), anti-CD74 (middle lane) or anti-H-2Kβ (right lane). Arrows indicate 41-kDa (top) and 31-kDa (bottom) CD74 bands. (d) Immunoprecipitation of proteins from lysates of Cd74+/+ DCs with anti-I-Aβ, anti-H-2Kβ (conformationally dependent), antibody to the H-2Kβ cytoplasmic domain (e-VIII; conformationally independent) or antibody to the transferrin receptor (TFR), followed by immunoblot analysis with anti-CD74. Far right (WCL), immunoblot analysis of whole-cell lysates (control). (e) Immunoprecipitation of proteins from lysates of DCs with anti-CD74, followed by no digestion (−) or digestion with Endo H (+) and immunoblot analysis with anti–MHC class I. (f) Immunoprecipitation, with anti–MHC class I, of proteins from lysates of cells left untreated or treated with chloroquine or Endo H, followed by immunoblot analysis with anti-CD74. Numbers below the lanes indicate band intensity relative to that of cells not treated with CQ. (g) Internalization of MHC class I in DCs labeled with anti-H-2Kβ, evaluated over time by flow cytometry and presented as mean fluorescence intensity of DCs incubated at 37 °C relative to that of control DCs incubated at 4 °C. *P<0.05 (Student’s t-test). Data are representative of two experiments (a,b,e,f; mean ± s.d.), five experiments (c) or three experiments (d,e).

**CD74 and MHC class I molecules form a complex in DCs**

We investigated at the molecular level the interaction of CD74 with MHC class I in DCs as a prerequisite for the targeting of MHC class I to the cross-priming compartment. We isolated DCs derived from Cd74+/+ and Cd74−/− mouse spleens for analysis by ICM. We stained DCs with anti-H-2Kβ and anti-CD74 and found that H-2Kβ molecules were distributed at the cell surface and in the cytoplasm, where they localized mainly to vesicular-like compartments. CD74 molecules colocalized considerably with these intracellular compartments in Cd74+/+ DCs (Fig. 6a). However, we observed less colocalization of H-2Kβ with CD74 in Tap1−/− DCs, presumably due to the restricted overall availability of H-2Kβ (Fig. 6a).

To identify the compartment in which these molecules colocalized, we also stained spleen DCs with anti-H-2Kβ and anti-LAMP-1 (to detect late endosomes). A considerable proportion of late endosomes contained H-2Kβ in Cd74+/+ DCs (Fig. 6a), which confirmed that a substantial amount of MHC class I molecules resides in the endocytic compartment. In contrast, only a small fraction of CD74−/− DCs reconstituted with full-length CD74 and DCs were able to induce the proliferation of OT-I T cells with an ability similar to that of wild-type DCs. However, when we reintroduced CD74 lacking the endosomal trafficking motif into Cd74−/− DCs, cross-priming ability continued to be impaired (Fig. 5f), which demonstrated that in the absence of CD74, there was less MHC class I directed to endolysosome and less cross-priming of OT-I T cells. Together these data showed that CD74 influenced the trafficking of MHC class I to the cross-priming compartment where efficient presentation of exogenous antigen takes place.

Figure 6. CD74 controls the ER-to-endolysosome trafficking of MHC class I in DCs. (a) ICM of mature splenic DCs stained with anti-H-2Kβ (green) plus anti-CD74 (red; top) or anti-LAMP-1 (red; bottom). Scale bar, 5 μm. (b) Quantification of MHC class I in LAMP-1− compartments (50 DCs per mouse strain), presented as individual pixels/total pixels. (c) Immunoprecipitation (IP) of proteins from [35S]methionine-labeled Cd74+/+, Cd74−/−, Tap1−/− and β2-microglobulin-deficient (B2m−−) BMDCs with antibody to I-A−I-E (I-Aβ; left lane), anti-CD74 (middle lane) or anti-H-2Kβ (right lane). Arrows indicate 41-kDa (top) and 31-kDa (bottom) CD74 bands. (d) Immunoprecipitation of proteins from lysates of Cd74+/+ DCs with anti-I-Aβ, anti-H-2Kβ (conformationally dependent), antibody to the H-2Kβ cytoplasmic domain (e-VIII; conformationally independent) or antibody to the transferrin receptor (TFR), followed by immunoblot analysis with anti-CD74. Far right (WCL), immunoblot analysis of whole-cell lysates (control). (e) Immunoprecipitation of proteins from lysates of DCs with anti-CD74, followed by no digestion (−) or digestion with Endo H (+) and immunoblot analysis with anti–MHC class I. (f) Immunoprecipitation, with anti–MHC class I, of proteins from lysates of cells left untreated or treated with chloroquine or Endo H, followed by immunoblot analysis with anti-CD74. Numbers below the lanes indicate band intensity relative to that of cells not treated with CQ. (g) Internalization of MHC class I in DCs labeled with anti-H-2Kβ, evaluated over time by flow cytometry and presented as mean fluorescence intensity of DCs incubated at 37 °C relative to that of control DCs incubated at 4 °C. *P<0.05 (Student’s t-test). Data are representative of two experiments (a,b,e,f; mean ± s.d.), five experiments (c) or three experiments (d,e).
molecules, which suggested that the binding of CD74 to MHC class I was not dependent on the peptide-transporter function of TAP. Finally, the CD74 isoforms precipitated together with MHC class I from β2-microglobulin-deficient DCs, which suggested that CD74 was able to bind the folded β2-microglobulin-associated MHC class I complex and the β2-microglobulin-free MHC class I complex.

We then used immunoblot analysis to confirm the identity of the CD74 isoforms bound to MHC class I molecules. We immunoprecipitated proteins with anti-I-Aβ, anti-H-2Kβ and antibody to the transferrin receptor (Fig. 6d). As expected, CD74 associated with MHC class II (I-Aβ) but not with the irrelevant protein transferrin receptor (Fig. 6d). We definitively identified CD74 as being associated with MHC class I (Fig. 6d), which confirmed that this interaction was detectable and stable under the conditions used in this immunoprecipitation procedure.

A MHC class I–CD74 complex forms in a pre-Golgi compartment

Next, to unequivocally demonstrate the kinetics and origin of the interaction between MHC class I and CD74, we used biochemical means to further deduce the intracellular compartment in which this interaction takes place. Proteins in the secretory pathway acquire resistance to endoglycosidase (Endo H) as they traffic from the ER through the Golgi compartment, and there they undergo cleavage by mannosidase II. It is well accepted that sensitivity to Endo H acts as an indication that proteins are localized to the ER or in ‘transitional elements’ between the ER and cis-Golgi. We immunoprecipitated CD74-bound MHC class I from Cd74+/+ BMDCs with anti-CD74 or anti–MHC class I and treated the immunoprecipitates with Endo H, followed by immunoblot analysis with anti–MHC class I or anti-CD74 to visualize the sensitivity of the MHC class I–CD74 complex to Endo H. We found that the MHC class I associated with CD74 was sensitive to Endo H (Fig. 6f). Furthermore, there was slightly more association of Endo H–resistant CD74 with MHC class I after treatment with chloroquine, as demonstrated by higher band intensities (Fig. 6f).

Overall, these data suggested that the interaction of CD74 with MHC class I originated in the ER, where CD74 bound an ‘immature’ fraction of the MHC class I molecules and from there initiated trafficking to an endolysosomal compartment to mediate cross-presentation, T cell priming and primary immune responses.

CD74 does not affect the internalization of MHC class I

Finally, to determine the source of the MHC class I that bound CD74, we investigated the role of CD74-mediated trafficking of MHC class I from the plasma membrane. To determine if CD74 functions in surface receptor recycling, we monitored the internalization of MHC class I in Cd74+/+ and Cd74−/− DCs. We stained BMDCs with anti-H-2Kβ and used flow cytometry to monitor internalization over time. Cd74+/+ and Cd74−/− DCs had very similar dynamics of MHC class I internalization (Fig. 6f). This indicated that CD74 was not interacting with MHC class I at the cell surface to cause internalization into an intracellular compartment for cross-presentation. This complemented published studies demonstrating that a tyrosine-based motif in the cytoplasmic domain of MHC class I molecules is crucial for the internalization of recycling MHC class I molecules into the endolysosomal cross-priming compartment from the plasma membrane and thus demonstrated a unique and distinct pathway of CD74-dependent MHC class I trafficking.

DISCUSSION

The dichotomy of the presentation of exogenous peptides by MHC class II molecules versus the display of cytosolic peptides by MHC class I molecules has been revised. Cross-presentation by MHC class I not only demonstrates the blurring of this division but also shows that for specific cell types such as DCs, this phenomenon serves a major role in generating primary immune responses in vivo. In addition, the presentation of endogenously derived peptides on MHC class II molecules demonstrates that the MHC class I and class II pathways possibly intersect and that they may share the same antigen-loading compartments. Although CD74 is classically recognized as a major chaperone in presentation by MHC class II, CD74 and MHC class I have also been shown to interact. However, the physiological contribution of CD74 to MHC class I–mediated immune responses in vivo has not been investigated, and the identification of MHC class I–CD74 interaction has been largely discounted as a biological curiosity. Here we have demonstrated that CD74 contributed substantially to MHC class I cross-presentation pathways in DCs. Our studies have identified a major role for CD74-dependent cross-priming in the generation of responses to viral and cell-associated antigens.

To assess CD4+ T cell–independent CTL responses generated through DC cross-presentation, we used a model of infection with a low dose of VSV. Low viral doses mimic the physiological situation in which most DCs would presumably be spared from infection and other infected cells would act as antigenic peptide donors; this allows the delineation of direct or endogenous presentation versus cross-presentation. The observation that mice lacking CD74 were considerably impaired in their ability to generate MHC class I–restricted CTL responses, particularly to low viral doses at which cross-priming probably dominates over direct priming by DCs, supported the conclusion that cross-presentation by MHC class I is the main mechanism by which antiviral CD8+ T cell–mediated immunity is generated under physiological conditions in vivo. We also confirmed other published work and demonstrated that the responses of CTL to viruses such as VSV are independent of CD4+ T cells and thus independent of the function of MHC class II–CD74 complexes.

The generation of bone marrow chimeras made it possible to study the activity of CD74+/− myeloid cell–derived DCs on a wild-type host background. Those studies led to the conclusion that the priming defect of CD74 was of DC origin and indicated that the deficiency was at the level of DC cross-presentation. Furthermore, we identified CD74–dependent cross-priming as an important MHC class I antigen-presentation pathway, as the absence of CD74 resulted in more than 50% fewer anti-VSV CTLs. In addition, the findings obtained through the use of mouse chimeras supported the observation that CD74 deficiency impaired the generation of primary immune responses to VSV independently of the lower abundance of CD4+ T cells. This is in accordance with other published data demonstrating that in some cases, CD4+ T cells are required for secondary CTL population expansion but not for primary population expansion. Costimulation of CD8+ CTLs by B7 molecules, along with stimulation of the T cell antigen receptor, can be sufficient to elicit CD8+ CTLs without T cell help. Alternatively, it is entirely possible that two distinct lineages of CD8+ CTL precursors exist whereby the CD4+ T cell–independent population provides the predominant response to various viruses, which results in no loss of CTL function in the absence of CD4+ T cells.

We found that the expression of a form of CD74 lacking its endosomal targeting signal failed to complement DC cross-presentation and priming of T cells. However, reconstitution with a wild-type CD74 molecule containing a functional endosomal targeting signal
restored cross-priming, which supported the proposal of a mechanism whereby MHC class I was transported from the ER to the endolysosome by CD74. Additionally, the deficient activation of CD8+ T cells by CD74−/− DCs in Rag1−/− mice that completely lack CD4+ T cells unequivocally demonstrated that the defect in DC cross-priming function was due to the absence of CD74. In our studies, CD74 did not seem to have a role in DC homing and motility in vivo but did mediate a physiologically important pathway for the cross-priming of CD8+ T cells by DCs.

Our studies have provided evidence of an association between MHC class I molecules and CD74 in DCs under physiological conditions. They also suggested that after dissociation of the MHC class I–CD74 complex in endolysosomes, reassembly of the MHC class I heavy chain with β2-microglobulin and antigenic peptides could then take place in the endolysosomal compartment.45 In this context, we have directly demonstrated that the MHC class I–CD74 complex remained assembled in vesicular-like compartments identified as late endosomes. Furthermore, we have established that CD74 influenced the presence of MHC class I in endolysosomes, which confirmed published observations that an MHC class I–CD74 interaction results in the targeting of a subset of MHC class I molecules to the endolysosomal pathway17,18.

The tyrosine internalization signal in the MHC class I cytoplasmic tail that has been described before8,13,48 targets recycling MHC class I into the cross-priming compartment. In contrast to that mechanism, it is unlikely that a stable interaction between CD74 and MHC class I molecules occurs at the plasma membrane to direct the recycling of MHC class I, as the absence of CD74 in DCs did not seem to influence the internalization of MHC class I. Our results support a model whereby both the recycling of MHC class I from the plasma membrane, directed by a tyrosine internalization signal in the cytoplasmic domain, and the trafficking of MHC class I from the ER through binding to the CD74 chaperone contribute to the pool of peptide-receptive MHC class I in the endolysosomal pathway. Thus, in a manner analogous to that used by MHC class II molecules, the MHC class I–CD74 complex is formed in the ER and may be held in a conformation that masks peptide binding as it transits to the cross-priming compartment. Indeed, two independent studies have shown that CD74 peptides, including a smaller peptide derived from the core MHC class I–associated CD74 peptide CLIP (MRM TPALLM), which is the portion of CD74 bound in the MHC class II–binding groove, can be eluted from MHC class II molecules.47,48 Such peptides are therefore strong candidates for the MHC class I equivalents of CLIP. This CLIP-derived peptide may prevent premature peptide binding akin to MHC class II situation7,19. In this model, after digestion and removal of CD74, MHC class I could be loaded with high-affinity cathepsin S–derived exogenous peptides11 and progress to the cell surface, where they could efficiently prime CD8+ T cell precursors to become activated.

In summary, our results here and other published data6,50 emphasize the importance of the endolysosome as a principle compartment for cross-presentation in DCs, and our investigation here has formally established the structural and functional relevance of the MHC class I–CD74 interaction on the intracellular routing of MHC class I molecules and cross-priming function of DCs. Our observations have defined a previously unknown pathway for the priming of immune responses; future studies should completely elucidate this process. Our results are of considerable clinical relevance and suggest that targeting vaccine candidates to the endolysosomes of DCs would enhance priming for both MHC class I and MHC class II antigens and thereby improve the immunogenicity and efficacy of vaccines.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/

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

ACKNOWLEDGMENTS
We thank D. Mathis (Institut de Génétique et de Biologie Moléculaire et Cellulaire and The Harvard Stem Cell Institute) for CD74−/− (H-2Kb) mice; N. Shastri (University of California, Berkeley) for the B3Z T cell hybridoma; J. Yewdell (US National Institutes of Health) for antibody 25.D1.16; I. Shachar (Weizmann Institute of Sciences) for the CD74 constructs; and B. Barber and D. Williams (University of Toronto) for antisera directed toward the region of H-2Kb encoded by exon 8. Supported by the Canadian Institutes of Health Research (K.O.; and MOP-77631 and MOP-86739 to W.A.J.).

AUTHOR CONTRIBUTIONS
G.B. and K.O. designed, did and analyzed experiments; A.C.S. did DC-transfection experiments; A.T.R. did experiments and provided intellectual input; N.L. provided intellectual input and bioinformatics; K.B.C. did experiments; W.A.J. conceived of the research project, designed experiments, supervised the research and analyzed the data; and G.B., K.O. and W.A.J. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://www.nature.com/natureimmunology/ Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Guagliardi, L.E. et al. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. Nature 343, 133–139 (1990).
2. Kovacsics-Bankowski, M. & Rock, K.L. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science 267, 243–246 (1995).
3. Ackerman, A.L.; Kyrtisis, C., Tampe, R. & Cresswell, P. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc. Natl. Acad. Sci. USA 100, 12899–12904 (2003).
4. Guermonprez, P. et al. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425, 397–402 (2003).
5. Houde, M. et al. Phagosomes are competent organelles for antigen cross-presentation. Nature 425, 402–406 (2003).
6. Pfeffer, J.D. et al. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature 361, 359–362 (1993).
7. Song, R. & Harding, C.V. Roles of proteasomes, transporter for antigen presentation (TAP), and β2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. J. Immunol. 156, 4182–4190 (1996).
8. L. S. and G. G. et al. Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. Nat. Immunol. 4, 1065–1073 (2003).
9. Gagnon, E. et al. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110, 119–131 (2002).
10. Touret, N. et al. Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. Cell 123, 157–167 (2005).
11. Shen, L., Sigal, L.J., Boes, M. & Rock, K.L. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity 21, 155–165 (2004).
12. Cebran, I. et al. Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. Cell 147, 1355–1368 (2011).
13. Bashla, G. et al. MHC class I endosomal and lysosomal trafficking coincides with exogenous antigen loading in dendritic cells. PLoS ONE 3, e3247 (2008).
14. Chiu, I., Davis, D.M. & Strominger, J.L. Trafficking of spontaneously endocytosed MHC proteins. Proc. Natl. Acad. Sci. USA 96, 13944–13949 (1999).
15. Reid, P.A. & Watts, C. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. Nature 346, 655–657 (1990).
16. Bakke, O. & Dobberstein, B. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. Cell 63, 707–716 (1990).
17. Sugita, M. & Brenner, M.B. Association of the invariant chain with MHC class II: preference for HLA class I/β2-microglobulin heterodimers, specificity, and influence of the MHC peptide-binding groove. J. Immunol. 147, 707–716 (1991).
18. Vigna, J.L., Smith, K.D. & Lutz, C.T. Invariant chain association with MHC class I: a sorting signal for endosomal compartments. Cell 63, 707–716 (1990).
19. Kleijmeer, M.J. et al. Antigen loading of MHC class I molecules in the endocytic tract. Traffic 2, 124–137 (2001).
20. Zwickey, H.L. & Potter, T.A. Antigen secreted from noncylcotic Listeria monocytogenes is processed by the classical MHC class I processing pathway. J. Immunol. 162, 6341–6350 (1999).
21. MacAry, P.A. et al. Mobilization of MHC class I molecules from late endosomes to the cell surface following activation of CD34-derived human Langerhans cells. Proc. Natl. Acad. Sci. USA 98, 3982–3987 (2001).
22. Tourne, S. et al. Biosynthesis of major histocompatibility complex molecules and generation of T cells in II TAP1 double-mutant mice. Proc. Natl. Acad. Sci. USA 93, 1464–1469 (1996).
23. Reber, A.J., Turnquist, H.R., Thomas, H.J., Lutz, C.T. & Solheim, J.C. Expression of invariant chain can cause an allelic-dependent increase in the surface expression of MHC class I molecules. Immunogenetics 54, 74–81 (2002).
24. Vitalis, T. et al. Using the TAP component of the antigen-processing machinery as a molecular adjuvant. PLoS Pathog. 1, e36 (2005).
25. van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L. & Tonegawa, S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4+ T cells. Cell 71, 1205–1214 (1992).
26. McAdam, A.J., Farkash, E.A., Gewurz, B.E. & Sharpe, A.H. B7 costimulation is critical for antibody class switching and CD8+ cytotoxic T-lymphocyte generation in the host response to vesicular stomatitis virus. J. Virol. 74, 203–208 (2000).
27. Marzo, A.L. et al. Fully functional memory CD8 T cells in the absence of CD4 T cells. J. Immunol. 173, 969–975 (2004).
28. Faure-Andre, G. et al. Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. Science 322, 1705–1710 (2008).
29. Benvenuti, F. et al. Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. Science 305, 1150–1153 (2004).
30. Shastri, N. & Gonzalez, F. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. J. Immunol. 150, 2724–2736 (1993).
31. Sallusto, F., Cella, M., Danielli, C. & Lanzavecchia, A. Dendritic cells use macrophage mannose receptor and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. 182, 389–400 (1995).
32. Brossart, P. & Bevan, M.J. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. Blood 90, 1594–1599 (1997).
33. Merzougui, N., Kratzer, R., Saveanu, L. & van Endert, P. A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. EMBO Rep. 12, 1257–1264 (2011).
34. Loss, G.E. Jr. & Sant, A.J. Invariant chain retains MHC class II molecules in the endocytic pathway. J. Immunol. 150, 3187–3197 (1993).
35. Stockinger, B. et al. A role of Ia-associated invariant chains in antigen processing and presentation. Cell 56, 683–689 (1989).
36. Kornfeld, R. & Kornfeld, S. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54, 631–664 10.1146/annurev.bi.54.070185.003215 (1985).
37. Rock, K.L., Gamble, S. & Rothstein, L. Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science 249, 918–921 (1990).
38. van Lith, M., van Ham, M. & Neefjes, J. Stable expression of MHC class I heavy chain/HLA-DO complexes at the plasma membrane. Eur. J. Immunol. 33, 1145–1151 (2003).
39. Nuchtern, J.G., Biddison, W.E. & Klausner, R.D. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature 343, 74–76 10.1038/343074a0 (1990).
40. Cerundolo, V., Elliott, T., Elvin, J., Bastin, J. & Townsend, A. Association of the human invariant chain with H-2 Db class I molecules. Eur. J. Immunol. 22, 2243–2248 (1992).
41. Powis, S. J. CLIP-region mediated interaction of Invariant chain with MHC class I molecules. FEBS Lett 580, 3112–3116 (2006).
42. Sigal, L.J. & Rock, K.L. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. J. Exp. Med. 192, 1143–1150 (2000).
43. Butler, R.M., Holmes, K.L., Hugin, A., Frederickson, T.N. & Morse, H.C. III. Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. Nature 328, 77–79 10.1038/328077a0 (1987).
44. Jansen, E.M. et al. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature 421, 852–856 (2003).
45. Machold, R.P. & Ploegh, H.L. Intermediates in the assembly and degradation of class I major histocompatibility complex (MHC) molecules probed with free heavy chain-specific monoclonal antibodies. J. Exp. Med. 184, 2251–2259 (1996).
46. Reinicke, A.T., Omlusik, K.D., Bassa, G. & Jefferies, W.A. Dendritic cell cross-priming is essential for immune responses to Listeria monocytogenes. PLoS ONE 4, e7210 10.1371/journal.pone.0007210 (2009).
47. Luckey, C.J. et al. Differences in the expression of human class I MHC alleles and their associated peptides in the presence of proteasome inhibitors. J. Immunol. 167, 1212–1221 (2001).
48. Krüger, T. et al. Lessons to be learned from primary renal cell carcinomas: novel tumor antigens and HLA ligands for immunotherapy. Cancer Immunol. Immunother. 54, 826–836 (2005).
49. Busch, R., Cloutier, I., Sekaly, R.P. & Hammerling, G.J. Invariant chain protects in vivo human invariant chain with H-2 Kb class I molecules. Nature 318, 1143–1150 (2000).
50. Savina, A. et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126, 205–218 (2006).
ONLINE METHODS

Mice. C57Bl/6 (H-2Kb) mice were from Jackson Laboratory. C3H/He mice (H-2Kb) were a gift from D. Mathis. For chimeric mice, donor bone marrow was depleted of mature T cells with anti-Thy-1 (MRC OX-7; Abcam) and injected (1 × 10^6 cells) into sublethally irradiated recipients (1,200 rads). Peripheral T cell subsets were analyzed by flow cytometry after being stained with anti-CD8 (53-6.7; BD Pharmingen) and anti-CD4 (GK1.5; BD Pharmingen). For depletion of CD4+ cells, before immunization and 48 h before T cell assessment, mice were injected with 100 µg anti-CD4 (GK1.5)51. All studies followed guidelines set by the University of British Columbia’s Animal Care Committee and the Canadian Council on Animal Care.

Viral infection. VSV was injected intraperitoneally (at 1 × 10^3 to 2 × 10^4 of a dose that infects 50% of a tissue culture cell monolayer). At 6 d after infection, splenocytes were stained with anti-CD8 (53-6.7) and H-2Kb–VSVNP(52–59) or H-2Kb–OVA(257–264) iTag tetramer (Immunoomics–Beckman Coulter) and analyzed with a FACSCalibur (Becton Dickinson) and FlowJo software. Splenocytes were further cultured for 5 d with 1 µM OVA(257–264) (SIINFEKL) or VSVNP(52–59) (RGYVYQGL), followed by tetramer staining as described above. Cytotoxicity assays were done as described8.

Uptake assay. BMDCs were generated as described5. Cells were incubated for 30 min at 4°C or at 37°C with OVA–Alexa Fluor 488 (30 µg/ml; Invitrogen). OVA uptake was analyzed by flow cytometry.

Cross-presentation assay. BMDCs were generated as described5 or splenic DCs were isolated with CD11c+ magnetic beads (Miltenyi Biotec). DCs were incubated for 15 h with OVA (Worthington) and, where indicated, with 100 µM chloroquine. DCs were then stained with Fc Block (PharMingen), then with anti–MHC class I (AF6.88.5; BD Pharmingen), antibody to sequence encoded by exon 8 that recognizes all MHC class I (from D. Williams and B. Barber), anti-MHC class I (KH95; Santa Cruz Biotechnology) or anti-LAMP-1 (N19; Santa Cruz Biotechnology). Samples were digested endoglycosidase Hf (P0703; New England Biolabs) according to the manufacturer’s protocol. Whole-cell lysates were analyzed by immunoblot as a positive control. Donkey antibody to mouse rat immunoglobulin G (A21096; Invitrogen) were used as secondary antibodies. Blots were visualized by Odyssey Infrared Imaging.

Immunoprecipitation. BMDCs were incubated for 1 h in methionine- and cysteine-free medium, then were pulsed with for 30 min with [35S]methionine (300 µCi/ml), then lysed in 0.5% (vol/vol) Nonidet P-40 in buffer (120 mM NaCl, 4 mM MgCl2 and 20 mM Tris-HCl, pH 7.6) containing a protease inhibitor ‘cocktail’ (Roche) and phenylmethyl sulfonyl fluoride (40 µg/ml). Where indicated, DCs were incubated with 100 µM chloroquine overnight before lysis. Cell lysates were preclarified by incubation overnight with normal rabbit serum and protein A–Sepharose (Pharmacia). Anti-H-2Kb recognizing fully folded MHC class I (AF6.88.5; BD Pharmingen), antibody to sequence encoded by exon 8 that recognizes all MHC class I (from D. Williams and B. Barber), anti-body to CD103 (3A11; BD Pharmingen), anti-CD4 (GK1.5; BD Pharmingen), anti–MHC class I (KH95; Santa Cruz Biotechnology) and anti–MHC class II (H-2Kb) were transferred to a nitrocellulose membrane and visualized by Odyssey Infrared Imaging.

MHC class I internalization. BMDCs were stained with Fc Block (BD Pharmingen), then were labeled for 30 min at 0°C with biotinylated anti–H-2Kb (AF6–88.5; BD Pharmingen). Samples were incubated at 37°C for 0°C. At the appropriate time, 5 × 10^6 cells. The proliferation of OT-I T cells was assessed 3 d later by flow cytometry. Data were analyzed with FlowJo software for quantification of internalized MHC class I.

Statistical analysis. Student’s t-test was used for comparison of the difference between populations. Differences were considered statistically significant when the P value was less than 0.05 (two-tailed).

51. Rashid, A., Auchincloss, H. Jr. & Sharon, J. Comparison of GK1.5 and chimeric rat/mouse GK1.5 anti-CD4 antibodies for prolongation of skin allograft survival and suppression of alloantibody production in mice. J. Immunol. 148, 1382–1388 (1992).