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A CD74-DEPENDENT MHC CLASS I ENDOLYSOSOMAL CROSS-PRESENTATION PATHWAY

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Abstract

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

Keywords

CD74; MHC class I; cross-presentation; cross-priming; dendritic cell

During primary immune responses, dendritic cells (DCs) are the principal antigen presenting cells (APCs) that initiate adaptive immune responses predominantly through cross-presentation and cross-priming of T cells. This involves extracellular antigen uptake, digestion of cell-associated antigenic fragments and presentation of proteolytic peptide products on both MHC class I and II molecules[1]. For MHC class I molecules, two main

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This work is dedicated to the memory of Dr. Ralph Steinman.

CONTRIBUTIONS

GB and KO designed, performed and analyzed experiments. ACS performed DC transfection experiments. ATR performed experiments and provided intellectual input. NL provided intellectual input. KBC performed experiments. WAJ conceptualized the research project, designed experiments, supervised the research and analyzed the data. GB, KO and WAJ wrote the manuscript.

pathways have been described that may explain how this process occurs: the cytosolic pathway[2–5] shown to function convincingly *in vitro*, and the vacuolar pathway, shown to play a major role *in vivo* for select antigens[6–8]. The phago-ER model of cross-presentation has been considered a dominant pathway of cross-presentation[9]. Subsequent data has disputed this conclusion[10]. One factor contributing to this controversy appears to be the over-interpretation of data that designate intracellular proteins as definitive markers of specific organelles that are often not exclusive but merely enriched during dynamic organelle biogenesis and partitioning. Furthermore, contrasting conclusions may have been inferred from studies using different forms of exogenous antigens and in studies using long-term DC cell lines versus those using 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[11]. Furthermore, membrane and cytosolic soluble NSF attachment proteins (SNAREs) that control donor and acceptor tethering and docking events during intracellular membrane fusion also appear to play a fundamental role in cross-presentation events [12]. However, the source of MHC class I in the cross-priming compartment, the mechanism of its transport and the site of peptide loading remain areas of active study[8,13].

Spontaneous internalization of recycling MHC class I into endosomes has been demonstrated[14,15]. Our previous results support a model in which MHC class I recycling from the plasma membrane to an endolysosomal loading compartment is facilitated through recognition of the tyrosine internalization signal found in the MHC class I cytoplasmic tail[8,13]. Therefore, recycling MHC class I molecules from the plasma membrane is one source of MHC class I for loading with exogenous antigens destined for participation in cross-presentation[8,13]. 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 phagosome and ER fusion[9]. An alternative and potentially complementary hypothesis is that the CD74 (invariant chain) molecule known to associate with MHC class II in the ER thereby preventing premature binding of peptides and mediating trafficking to the endocytic pathway through sorting signals present in the CD74 cytoplasmic tail[1,16], could bind MHC class I and deliver a fraction of the MHC class I to the vacuolar-endocytic compartment to function in cross-presentation [17,18]. This mechanism would coincidently place peptide-receptive MHC class I in the same or similar compartment with exogenous antigen and MHC class II molecules[19], the MIIC compartment, facilitating antigenic peptide loading and binding to MHC class I molecules. This pathway would link MHC class I transport to the vacuolar pathway, as it is unlikely that CD74 would be involved in the cytosolic route of MHC class I exogenous presentation[20,21].

The MHC class I interaction with CD74 and their coincident localization in the same compartment was previously demonstrated in human cell lines[17–19]. Although it was concluded on the basis of older paradigms, that a MHC class I-CD74 interaction was unlikely to control the fate of MHC class I transport to endosomes under physiological conditions[22], other contrasting studies demonstrated that CD74-transfected cells exhibited a substantial increase in surface expression of diverse MHC class I alleles suggesting that MHC class I-CD74 interaction might have functional significance[23]. Here, we have

investigated the immunological relevance of MHC class I interaction with CD74 *in vivo* and describe a clear and critical role for CD74 in cross-presentation of exogenous antigen and subsequent cross-priming by DCs.

RESULTS

CD74 is required for primary anti-viral responses

DCs can be directly infected and could therefore utilize classical MHC class I presentation to activate naïve $CD8^+$ T cells. However, during infection with a low viral titer, direct infection of DCs is less likely and DC cross-presentation is the dominant pathway responsible for generation of $CD8^+$ T cell responses[8,24]. In order to address the role of CD74 in cross-presentation to generate primary anti-viral immune responses, a low dose of Vesicular Stomatitis Virus (VSV) was used to infect wild type ($Cd74^{+/+}$) and CD74-deficient ($Cd74^{-/-}$) mice. $Tap1^{-/-}$ mice which are impaired in MHC I assembly and intracellular transport so lack $CD8^+$ T cells due to improper thymic selection, were similarly infected as a negative control (Fig. 1, Supplementary Fig. 1a)[25]. In this infection, anti-VSV primary and memory $CD8^+$ immune responses can be generated in the absence of $CD4^+$ T cells[26,27]. In this way, the role of CD74 in cross-presentation can be tested regardless of the role it plays in $CD4^+$ T cell responses. The percentage of $CD8^+$ T cells generated against the $VSVNP_{52\text{-}59}$ immunodominant epitope on MHC class I ($H\text{-}2K^b$) was detected following the VSV infection. $Cd74^{-/-}$ mice had a significantly reduced capacity (5.0% vs. 19.0%; $p<0.05$) to generate antigen specific $CD8^+$ T cells compared to $Cd74^{+/+}$ mice (Fig. 1a,b). This resulted in an immune response with reduced CTL killing capacity (Fig. 1c).

Bone marrow chimeras were constructed to further exclude a role for T cell help on cross-priming in the VSV infection model class[26,27]. Additionally, the chimeras would confirm whether the deficiency in generating immune responses is dependent on the hematopoietic-derived DCs ability to cross-present antigen and prime T cells. Normal levels of $CD8^+$ and $CD4^+$ cells were found in the periphery of $Cd74^{+/+} \rightarrow Cd74^{+/+}$ and $Cd74^{-/-} \rightarrow Cd74^{+/+}$ mice. However, reduced $CD4^+$ and somewhat increased $CD8^+$ cell numbers were seen in the $Cd74^{-/-} \rightarrow Cd74^{-/-}$ and $Cd74^{+/+} \rightarrow Cd74^{-/-}$ mice (Supplementary Fig. 1b,c). This indicated that positive selection in recipient $Cd74^{-/-}$ mice was impaired due to reduced levels of MHC class II in the $Cd74^{-/-}$ thymic epithelium.

To examine antiviral responses, chimeric mice were infected with a low titer of VSV and assessed for $VSVNP_{52\text{-}59}$ -specific $CD8^+$ T cell generation using tetramer analysis and a CTL killing assay (Fig. 2). $Cd74^{+/+} \rightarrow Cd74^{-/-}$ mice, with low $CD4^+$ T cell numbers, were able to produce $VSVNP_{52\text{-}59}$-specific $CD8^+$ T cells similar to wild-type $Cd74^{+/+} \rightarrow Cd74^{+/+}$ chimeras (1.1% vs. 1.2%, Fig. 2a) resulting in immune responses with comparable killing capacity (16.8% vs. 1.9%; $p<0.05$, Fig. 2b). However, the $Cd74^{-/-} \rightarrow Cd74^{+/+}$ mice were grossly impaired in the generation of $VSVNP_{52\text{-}59}$ specific $CD8^+$ cells (0.2%; $p<0.05$, Fig. 2a) despite having normal $CD4^+$ T cells resulting in diminished CTL killing responses (18.0% vs 4.5%; $p<0.05$, Fig. 2b). This suggests that the generation of VSV specific CTL responses is independent of $CD4^+$ T cell numbers. Importantly, bone marrow-derived APCs expressing CD74 were required and allowed $Cd74^{-/-}$ mice to produce a robust antiviral immune response comparable with that of $Cd74^{+/+}$ mice.

CD4$^+$ cells depletion has no effect on anti-VSV responses

Next, to eliminate the possibility that residual CD4$^+$ cells in the *Cd74*$^{+/+}$→*Cd74*$^{-/-}$ chimeras that result from dysfunctional positive selection in *Cd74*$^{-/-}$ mice are contributing to the efficiency of anti-viral immune responses, during the course of the infection, the CD4$^+$ cells of *Cd74*$^{+/+}$→*Cd74*$^{-/-}$ chimeras were depleted with CD4 (GK1.5) antibodies. Although CD4$^+$ cells were virtually undetectable over background, CD4$^+$ cell depleted *Cd74*$^{+/+}$→*Cd74*$^{-/-}$ chimeras generated significantly more CD8$^+$ VSVNP$_{52\text{-}59}$ specific T cells than *Cd74*$^{-/-}$ →*Cd74*$^{+/+}$ chimeric mice (13.5% vs 4.1%; $p<0.05$; Fig. 2c) resulting in an immune response with increased lytic activity (14.0% vs. 4.9%; $p<0.05$; Fig. 2d). Taken together, these data confirm that *Cd74*$^{+/+}$→*Cd74*$^{-/-}$ chimeras mount stronger anti-VSV responses than *Cd74*$^{-/-}$ →*Cd74*$^{+/+}$. This is independent of CD4$^+$ cells but rather due to the reconstitution of *Cd74*$^{-/-}$ mice with wild-type DCs that were fully capable of priming anti-viral CD8$^+$ T cells responses.

Cross-priming of cell-associated antigen is CD74 dependent

In order to investigate the role of CD74 in primary immune response to cell-associated antigen, lethally irradiated ovalbumin (OVA)-pulsed DCs or mismatched MHC class I OVA-pulsed DCs were used as a source of cell-associated antigen to activate antigen-specific CTLs in *Cd74*$^{+/+}$, CD4$^+$-depleted *Cd74*$^{+/+}$ 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 OT-I-derived CD8$^+$ T cells (Supplementary Fig. 2) or activate endogenous H-2K^b-OVA$_{257\text{-}264}$ specific CTLs that were efficient at killing OVA$_{257\text{-}264}$-pulsed target cells (data not shown). However, with the same challenge of cell-associated OVA, mice with the hematopoietic system deficient for CD74 had a substantially reduced ability to stimulate proliferation of OT-I CD8$^+$ T cells and generated fewer endogenous CTLs contributing to a reduced killing ability (Supplementary Fig. 2 and data not shown).

CD74-dependent cross-priming is CD4 T cell independent

To focus specifically on DC cross-priming defects and eliminate extraneous factors including the requirement for CD4-help, *Cd74*$^{-/-}$ and *Cd74*$^{+/+}$ DCs incubated with OVA protein or OVA$_{257\text{-}264}$ peptide were injected with CFSE-labeled purified OT-I CD8$^+$ T cells into T cell deficient *Rag1*$^{-/-}$ mice on a BALB/c background. The ability of the DCs to cross-prime the OT-I T cells was assessed (Fig. 3a). *Cd74*$^{-/-}$ DCs incubated with OVA protein induced significantly less OT-I proliferation in comparison to the *Cd74*$^{+/+}$ DCs (18% vs. 48%; Fig. 3B). However, when *Cd74*$^{-/-}$ DCs were pulsed with OVA$_{257\text{-}264}$ peptide, as a positive control for direct presentation, *Cd74*$^{-/-}$ DCs were as competent as *Cd74*$^{+/+}$DCs to activate the CD8$^+$ OT-I T cells (59.5% vs 60.0%, Fig. 3B).

In order to address the possible confounding role for CD74 in DC motility and homing[28] from the site of injection to the spleen, we assessed the localization of CFSE-labeled DCs after injection intravenously[29] (Fig. 3b and Supplementary Fig. 3). *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs injected intravenously into *Rag1*$^{-/-}$ mice were found to localize equivalently to the spleen. Therefore, the reduced ability of *Cd74*$^{-/-}$ DCs to induce T cell proliferation is not due to differences in DC migration but due to reduced antigen processing and presentation

ability. We conclude that CD74 plays a critical role in MHC class I cross-presentation of cell-associated antigen and $CD8^+$ T cell priming *in vivo* and this is unrelated to $CD4^+$ T cell help or CD74-mediated DC motility and homing.

CD74-deficient DCs have impaired cross-priming ability

Spleen-derived DCs from different mouse strains were examined for their ability to cross-present the H-$2K^b$-restricted ovalbumin epitope $OVA_{257-264}$ *in vitro*. DCs were incubated with soluble OVA, with or without cytokines, and stained with an antibody specific for the H-$2K^b$-$OVA_{257-264}$ complex or co-cultured with B3Z, a T cell hybridoma that is activated following recognition of H-$2K^b$ in association with the $OVA_{257-264}$ peptide[30]. *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs had similar ability to internalize OVA and had comparable levels of total surface MHC class I (Fig. 4a,b). However, *Cd74*$^{-/-}$ DCs displayed substantially reduced levels of H-$2K^b$-$OVA_{257-264}$ complexes following OVA incubation compared to *Cd74*$^{+/+}$ DCs (Fig. 4b). It has been shown that cross-priming capacity of DCs is differentially regulated by inflammatory mediators that induce upregulation of costimulatory and MHC molecules, and reduce endocytosis[31,32]. This results in an increased capacity of T cell priming but lowers the ability of DCs to capture and present soluble antigens. To test T cell activation in a situation resembling *in vivo* conditions that involves co-stimulation, OVA-pulsed DCs were incubated with B3Z T cells with and without cytokines. In the presence of tumor necrosis factor (TNF) and interferon (IFN)-γ, *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs had an equal ability to upregulate costimulatory molecules CD80, CD86, and CD40 (Fig. 4c and data not shown) but *Cd74*$^{-/-}$ DCs had a much reduced capacity to activate B3Z T cells compared to *Cd74*$^{+/+}$ DCs (Fig. 4d). As expected, no T cell activation was detected following incubation with OVA-pulsed DCs derived from *Tap1*$^{-/-}$ in the presence of cytokines. These data support the conclusion that CD74 plays a role in T cell cross-priming and does not affect costimulatory molecule expression.

CD74 mediates endolysosomal MHC class I loading

To better understand the mechanism of the cross-presentation and priming deficiency at a molecular level, comparative immunofluorescent confocal microscopy (ICM) was used to determine the intracellular localization, trafficking and distribution of $OVA_{257-264}$ loaded MHC class I in *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs with and without TNF. Intracellular staining was performed with antibodies against H-$2K^b$-$OVA_{257-264}$ and the late endosome marker, LAMP1, following incubation with OVA protein. Colocalization with LAMP1 was detectable in a considerable number of the *Cd74*$^{+/+}$ splenic DCs staining positive for H-$2K^b$-$OVA_{257-264}$ complexes when no TNF was added to the culture (Fig. 4e,f). In the *Cd74*$^{-/-}$ and *Tap1*$^{-/-}$ DCs, some H-$2K^b$-$OVA_{257-264}$ complexes were identified; however, colocalization with late endosomes was minimal (Fig. 4e,f). The absence of loaded MHC I in the *Tap1*$^{-/-}$ DCs is consistent with TAP playing a role in cross-presentation, a mechanism that has been previously postulated[24,33]. Following treatment with TNF, *Cd74*$^{+/+}$ DCs demonstrated a significant increase in colocalization of H-$2K^b$-$OVA_{257-264}$ complexes with LAMP1 (Fig. 4e,f, $p<0.05$), but not with the ER marker GRP78, or Golgi marker Giantin (data not shown). In contrast, few H-$2K^b$-$OVA_{257-264}$ complexes were observed in late endosomal compartments in *Cd74*$^{-/-}$-derived DCs indicating that the H-$2K^b$-$OVA_{257-264}$ complex formation in late endosomes was reduced (Fig. 4f). Comparison of the ICM data

indicated that in the presence of TNF, *Cd74*$^{-/-}$-derived DCs had significantly less $OVA_{257-264}$ loaded onto H-2K^b (red) in the late endosomes (green) than in *Cd74*$^{+/+}$ DCs (62% vs 32%; $p<0.05$; Fig. 4f). These data suggest 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 fact that CD74-deficiency results in fewer H-2K^b-$OVA_{257-264}$ complexes in late endosomal compartments suggests that CD74 targets MHC class I from the ER into the endolysosomal pathway. Here, CD74 is presumably degraded and MHC class I is loaded with exogenous antigenic peptides. To examine this in more detail, the acidification of endosomes was blocked with chloroquine (CQ) and the CD74-mediated MHC class I cross-presentation pathway was assessed. Following CQ treatment, bone marrow-derived DC (BMDCs) had equivalent MHC I surface expression as untreated controls and displayed H-2K^b-$OVA_{257-264}$ when pulsed with $OVA_{257-264}$ peptide; however, when incubated with soluble OVA, CQ-treated DCs had significantly reduced amounts of surface H-2K^b-$OVA_{257-264}$ (Fig. 5 a–c). ICM analysis of BMDCs following CQ treatment (Fig. 5d,e) showed increased colocalization of H-2K^b (red) and CD74 (green). This indicates that CQ treatment increases the amount of endolysosomal MHC class I molecules presumably by blocking the dissociation of MHC class I and CD74 in the endolysosomes in a similar manner that was reported for the MHC class II pathway [34] and by inhibiting the degradation of recycling MHC class I. The end result is reduced loading of MHC class I with exogenous antigen and subsequently lower surface levels of H-2K^b/$OVA_{257-264}$. To confirm the finding that CD74 directs MHC class I to an endolysosomal compartment and unequivocally demonstrate that CD74 mediates MHC class I trafficking, CD74-deficient BMDCs were transfected with full length CD74 (FL) or CD74 lacking the cytosolic trafficking domain (Δ2–17) and the ability to present OVA protein or OVA peptide, a positive control that would bypass the need for processing, was assessed. As previously demonstrated, *Cd74*$^{-/-}$ DCs had impaired cross-priming ability inducing much less OT-I T cell proliferation compared to *Cd74*$^{+/+}$ DCs (Fig. 5f). As expected, when *Cd74*$^{-/-}$ DCs were reconstituted with full length CD74 cross-priming ability was restored and DCs could induce OT-I T cell proliferation with similar ability as wild-type DCs. However, when Δ2–17 CD74 lacking the endosomal trafficking motif was reintroduced into *Cd74*$^{-/-}$ DCs, cross-priming ability continued to be impaired (Fig. 5f) demonstrating that in the absence of CD74, MHC class I trafficking to endolysosome and cross-priming of OT-I T cells is reduced. Taken together, the data show that CD74 clearly influences MHC class I trafficking to the cross-priming compartment where efficient presentation of exogenous antigen takes place.

CD74 and MHC class I molecules form a complex in DCs

The interaction of CD74 with MHC class I in DCs as a prerequisite of targeting MHC class I to the cross-priming compartment was investigated at the molecular level. Spleen-derived DCs were isolated from *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ mice for analysis by ICM. DCs were stained with antibodies against H-2K^b (green) and CD74 (red). H-2K^b molecules were distributed at the cell surface and in the cytoplasm where they localized mainly to vesicular-like compartments. CD74 molecules colocalized markedly with these intracellular compartments

(Fig. 6a, top left). However, in *Tap1*$^{-/-}$ DCs, a reduced colocalization of H-2K^b with CD74 was observed, presumably due to the restricted overall availability of H-2K^b pool and their ability to traffic to the endolysosomes (Fig. 6a, top right).

To identify the compartment where these molecules colocalize, spleen DCs were co-stained with antibodies recognizing H-2K^b and LAMP1 that detects late endosomes. A considerable proportion of late endosomes contained H-2K^b in *Cd74*$^{+/+}$ DCs, confirming that a substantial amount of MHC class I molecules reside in the endocytic compartment [8,21]. In contrast, only a small fraction H-2K^b colocalized with late endosomes in *Cd74*$^{-/-}$ DCs (Fig. 6a, mid panel). This was confirmed by quantification of ICM images and suggests that significantly fewer MHC class I molecules were targeted to the endolysosomal compartment in *Cd74*$^{-/-}$ vs *Cd74*$^{+/+}$ DCs (73% vs 47%; Fig. 6b). Colocalization was even less evident in the *Tap1*$^{-/-}$ DCs possibly due to the impaired targeting of H-2K^b molecules to endolysosomes in the absence of TAP1. These data suggest that a substantial fraction of MHC class I molecules interact with CD74 facilitating their transport to the endolysosomal compartment of DCs likely from the ER. Demonstration of a direct molecular interaction between MHC class I and CD74 in DCs would further strengthen the argument that this is a yet undescribed pathway of antigen presentation in DCs. To this end, BMDCs from various knock-out and wild-type mice were ^{35}S-labelled, and MHC class I (H-2K^b), MHC class II (I-A^b) or CD74 bound complexes were co-immunoprecipitated and proteins in these complexes were identified based on apparent molecular weight. MHC class II co-immunoprecipitated the abundant 41 and 31 kDa isoforms of CD74 (Fig. 6c left, lane 1). The H-2K^b antibody also co-precipitated these same proteins corresponding to the CD74 isoforms (Fig. 6c left, lane 3) suggesting that at any one time, CD74 is bound to a fraction of the total pool of MHC class I molecules in DC. The two prominent proteins detected between 41 and 31 kDa may be components of a MHC I loading or transporting complex. Their sizes are consistent with the H-2DM or H-2DO but their identities have not yet been conclusively determined. The 41 and 31 kDa forms of CD74 were not present in the *Cd74*$^{-/-}$ DCs (Fig. 6c middle) demonstrating that they are indeed the previously reported isoforms of CD74 that have been shown to co-immunoprecipitate with MHC class I and MHC class II molecules. In addition, the 41 and 31-kDa CD74 isoforms were co-immunoprecipitated with H-2K^b in *Tap1*$^{-/-}$ DCs, suggesting that CD74 binding to MHC class I is not dependent on the peptide transporter function of the TAP molecule. Finally, the CD74 isoforms co-precipitated with MHC class I from *B2m*$^{-/-}$-derived DCs suggesting that CD74 can bind the folded β_2m-associated MHC class I complex (Fig. 6c right) and the β_2m-free MHC class I complex.

Immunoblotting was then performed to confirm the identity of the CD74 isoforms bound to MHC class I molecules. Immunoprecipitation with antibodies against I-A^b, H-2K^b and the exon-VIII region of the MHC class I molecule as well as an irrelevant antibody against transferrin receptor (TFR) was followed by blotting with a CD74 antibody (Fig. 6d). As expected, CD74 was found to associate with MHC class II (lane 1) but not with the irrelevant protein, transferrin receptor (lane 4). CD74 was definitively identified to be associated with MHC class I (lane 2,3) confirming that this interaction is clearly detectable and stable under the conditions used in this immunoprecipitation procedure.

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

Next, in order to unequivocally demonstrate the kinetics and origin of the MHC class I-CD74 interaction, we used biochemical means to further deduce the intracellular compartment where the CD74 and MHC class I interaction takes place. Proteins within the secretory pathway acquire Endo H resistance as they traffic from the ER through the Golgi compartment and there, undergo cleavage by mannosidase II [35]. It is well accepted that Endo H sensitivity acts as an indication that proteins are localized to the ER or in "transitional elements" between the ER and cis-Golgi. CD74-bound MHC class I was immunoprecipitated from *Cd74*$^{+/+}$ BMDCs with a CD74 or MHC class I antibody and treated with Endo H. Immunoblotting was performed with an MHC class I or CD74 antibody to visualize the Endo H sensitivity of the CD74-MHC class I complex. We clearly identified that the MHC class I associated with CD74 is Endo H sensitive (Fig. 6e,f). Furthermore, when treated with CQ, the amount of EndoH resistant CD74 associating with MHC class I was slightly increased as demonstrated by greater protein intensities (Fig. 6f). Overall, these data suggest that the CD74 interaction with MHC class I originates in the ER where the CD74 binds an 'immature' fraction of the MHC class I molecules and from here initiates trafficking to an endolysosomal compartment to mediate cross-presentation, T cell priming and primary immune responses[8,13].

CD74 does not affect MHC class I internalization

Lastly, to examine the source of MHC class I that binds CD74, the role of CD74-mediated MHC class I trafficking from the plasma membrane was examined. To determine if CD74 functions in surface receptor recycling, we followed the internalization of MHC class I in *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs. BMDCs were stained with an H-2K^{b} antibody and flow cytometric analysis was used to follow internalization over time. *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ DCs have very similar dynamics of MHC class I internalization (Fig. 6g). This indicates that CD74 is not interacting with MHC class I at the cell surface to cause internalization into an intracellular compartment for cross-presentation. This compliments our other studies that demonstrate a tyrosine-based motif in the cytoplasmic domain of MHC class I molecules is crucial for internalizing recycling MHC class I molecules into the endolysosomal cross-priming compartment from the plasma membrane[8,13] and thus reveals a unique and distinct pathway of CD74-dependent MHC class I trafficking.

DISCUSSION

The dichotomy of MHC class II molecules presenting exogenous peptides versus class I molecules displaying cytosolic peptides has been revised[6,8,36,37]. Not only does MHC class I cross-presentation demonstrate the blurring of this division, but it also shows that for specific cell types such as DCs this phenomenon plays a major role in generating primary immune responses *in vivo*[8]. In addition, the presentation of endogenously-derived peptides on MHC class II molecules demonstrates that MHC class I and II pathways possibly intersect and that they may share the same antigen-loading compartments[38]. Although CD74 is classically recognized as a major chaperone in MHC class II presentation, MHC class I and CD74 have also been shown to interact[17,18,39,40]. However, the physiological contribution of CD74 to MHC class I mediated immune responses *in vivo* has not been

investigated and the previous identification of CD74-MHC class I interaction was largely discounted as a biological curiosity. Here, we demonstrate that CD74 contributes significantly to MHC class I cross-presentation pathways in DCs. These studies demonstrate a major role for CD74-dependent cross-priming in the generation of responses against viral and cell-associated antigen.

To assess $CD4^+$ T cell independent CTL responses generated through DC cross-presentation, we used a low dose VSV infection model. Low viral doses mimic a physiological situation where the majority of DCs would presumably be spared from infection and other infected cells would act as antigenic peptide donors allowing the dissection of direct or endogenous presentation from cross-presentation. The observation that mice lacking CD74 are significantly impaired in their ability to generate MHC class I-restricted CTL responses, particularly against low viral doses where cross-priming is likely to dominate over direct priming by DCs, supports the conclusion that MHC class I cross-presentation is the primary mechanism by which antiviral $CD8^+$ T cell-mediated immunity is generated under physiological conditions *in vivo* [8,41]. We also confirmed the work of others and demonstrated that CTL responses against viruses such as VSV are CD4-independent[26,27] and thus independent of the function of MHC class II-CD74 complexes.

The generation of bone marrow chimeras made it possible to study the performance of myeloid $Cd74^{-/-}$ derived DCs on a different host background. These studies led to the conclusion that CD74's priming defect was of DC origin and indicated that the deficiency lies at the level of DC cross-presentation. Further, CD74-dependent cross-priming was revealed as an important MHC class I antigen presentation pathway as the absence of CD74 resulted in a greater than 50% decrease in the number of anti-VSV CTLs. In addition, the findings obtained by mouse chimeras support the observations that CD74-deficiency impairs the generation of primary immune responses against VSV, independent of reduced $CD4^+$ T cells [26,42]. This is in accordance with other recent data that demonstrate that in some cases, CD4 helper cells are required for secondary, but not primary CTL expansion[43]. Costimulation of the $CD8^+$ CTL by B7 molecules, along with TCR stimulation, can be sufficient to elicit $CD8^+$ CTL without T cell help[26]. Alternatively, it is entirely possible that two distinct lineages of $CD8^+$ CTLs precursors exist whereby the CD4 helper-independent population provides the predominant response to various viruses resulting in no loss of CTL function in the absence of $CD4^+$ T cells[42]. 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 thereby supporting a mechanism where MHC class I is transported from the ER to the endolysosome by CD74. Additionally, the deficient $CD8^+$ T cell activation by $Cd74^{-/-}$ DCs in $Rag1^{-/-}$ mice that completely lack $CD4^+$ T cells, unequivocally demonstrates that the defect in DC cross-priming function is due to the absence of CD74. In our studies, CD74 does not appear to play a role in DC homing and motility *in vivo* but does mediate a physiologically important CD74-dependent MHC-I dendritic cell cross-priming pathway.

Our studies also provide evidence of an association between MHC class I molecules and CD74 under physiological conditions in DCs. It also suggests that upon CD74 dissociation

in endolysosomes, the reassembly of MHC class I heavy chain with β_2m and antigenic peptides could then take place in the endolysosomal compartment[44]. In this context, we have directly demonstrated that the MHC class I-CD74 complex remains assembled in vesicular-like compartments identified as late endosomes. Furthermore, we have established that CD74 influences the presence of MHC class I in endolysosomes confirming previous observations that an MHC class I-CD74 interaction results in targeting of a subset of MHC class I molecules to the endolysosomal pathway[17].

In contrast to the cytoplasmic tail tyrosine mutants that affect MHC I recycling into the cross-priming compartment we previously described[8,13,45], it is unlikely that a stable interaction between CD74 and MHC class I molecules occurs at the plasma membrane as the absence of CD74 in DCs does not appear to influence MHC class I internalization. Our results support a model whereby both MHC class I recycling from the plasma membrane, directed by a tyrosine internalization signal in the cytoplasmic domain, and MHC class I trafficking from the ER through the binding of the CD74 chaperone contributes to the pool of peptide-receptive MHC class I in the endolysosomal pathway. Thus, in an analogous manner to 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 they transit to the cross-priming compartment. Indeed, two independent studies have shown that CD74 peptides, including a smaller peptide derived from the core CLIP peptide, the portion of CD74 bound in the MHC class II binding groove, can be eluted from MHC class I molecules[46,47]. Such peptides are therefore strong candidates for the MHC class I equivalents of CLIP (MRMATPLLM). This CLIP-derived (CLIPD) peptide may prevent premature peptide binding akin to MHC class II situation[46,48]. In this model, following CD74 digestion and removal, MHC class I could be loaded with high affinity cathepsin S-derived exogenous peptides[11] and progress to the cell surface where they could efficiently prime CD8 T cell precursors to become activated.

In summary, these and previous data[8,49] highlight the significance of the endolysosome as a principle compartment for cross-presentation in DCs and the present investigation formally establishes the structural and functional relevance of the CD74-MHC class I interaction on the intracellular routing of MHC class I molecules and cross-priming function of DCs. These observations define a new pathway for priming immune responses and therefore the complete elucidation of this process is of significant importance. These observations have considerable clinical significance and suggest that targeting vaccine candidates to the endolysosomes of DCs will enhance priming for both MHC class I and MHC class II antigens and thereby improve the immunogenicity and efficacy of vaccines.

METHODS AND MATERIALS

Mice

Cd74$^{+/+}$ (H-2K^b) mice were purchased from Charles River. *B2m*$^{-/-}$, *Tap1*$^{-/-}$, OT-1 T cell transgenic (H-2K^b), and *Rag1*$^{-/-}$(H-2K^d) mice were purchased from Jackson Laboratory. *Cd74*$^{-/-}$ (H-2K^b) mice were a gift from Diane Mathis (C.U. Strasbourg, France and The Harvard Stem Cell Institute, Boston, MA). For chimeric mice, donor bone marrow was depleted of mature T cells using a Thy1 antibody (Abcam) and injected (1×10^7) into

sublethaly irradiated (1200 rad) recipients. Peripheral T cells subsets were analyzed by flow cytometry following staining with CD8 and CD4 antibodies (Pharmingen). For CD4-depletion, prior to immunization and 48 hours prior to T cell assessment, mice were injected with 100 μg CD4 (GK1.5) antibody[50]. All studies followed guidelines set by the University of British Columbia's Animal Care Committee and the Canadian Council on Animal Care.

Viral infections

Vesicular Stomatitis Virus (VSV) was injected intraperitoneally at 1–2 × 10^5 $TCID_{50}$ (dose that infects 50% of a tissue culture cell monolayer). Six days post-infection, splenocytes were stained with a CD8 antibody (BD Pharmingen) and H-2K^b-$VSVNP_{52-59}$ or H-2K^b-$OVA_{257-264}$ iTAg Tetramer (immunomics-BeckmanCoulter) and analyzed using FACSCalibur (Becton Dickinson) and FlowJo software. Splenocytes were further cultured for 5 days with 1 μM $OVA_{257-264}$ (SIINFEKL) or $VSVNP_{52-59}$ (RGYVYQGL) peptide and tetramer staining was performed as above. Cytotoxicity assays were performed as described[8].

Uptake assay

Bone marrow DCs (BMDCs) were generated as described[8]. Cells were incubated with 30 μg/mL ovalbumin (OVA)-Alexa488 (Invitrogen) for 30 minutes on ice or at 37 °C. OVA uptake was analyzed by flow cytometry.

Cross-presentation assays

BMDCs were generated as described[8] or splenic DCs were isolated using CD11c$^+$ magnetic beads (Miltenyi Biotech). DCs were incubated with OVA (Worthington) for 15 hours and where indicated 100 μM chloroquine. DCs were stained with Fc block (PharMingen) then H-2K^b, CD80, CD86, CD40 (PharMingen) or H-2K^b-$OVA_{257-264}$ antibodies (25.D1.16; a gift from John Yewdell, NIH, USA) and analyzed by flow cytometry. For cross-priming assays, DCs were incubated with OVA, 15 ng/mL GM-CSF (Sigma) and 10 ng/mL TNF-α or IFN-γ (R&D Systems). Activation of B3Z T cells (gift from Nilabh Shastri, Berkeley, USA) was assessed as described[8].

For *in vivo* studies, *Cd74*$^{+/+}$ and *Cd74*$^{-/-}$ BMDCs were incubated with 10 mg/ml OVA or $OVA_{257-264}$ peptide for 2 hours and injected (1 × 10^7 cells) intravenously into *Rag1*$^{-/-}$ BALB/c mice. After 24 hours, OT-I T cells were labeled with 2.5 μM CFSE (Molecular Probes) and injected intravenously (5×10^6 cells). Proliferation of OT-I T cells in the spleen was assessed 3 days later by CFSE dilution using flow cytometry. To confirm localization to spleen, CFSE-labeled DCs were injected intravenously into *Rag1*$^{-/-}$ BALB/c mice. After 2 hours, the presence of CFSE-positive cells in the spleen was assessed using flow cytometry.

Confocal microscopy

Spleen-derived DCs were isolated, fixed and permeabilized as described[8]. For analysis of cross-presentation, DCs were incubated with 5 mg/mL OVA for 10 hours with or without 10 ng/mL TNFα. As indicated, DCs were treated with 50 μM chloroquine for 72 hours prior to processing[34]. Cells were stained with antibodies against H-2K^b (BD Biosciences), CD74 (Fitzgerald), LAMP-1 (Santa Cruz Biotechnology) or H-2K^b-$OVA_{257-264}$. Rabbit anti-mouse

or goat Alexa-488 or 568, or goat anti-mouse Alexa-488 (Molecular Probes) were used as secondary antibodies. Images were acquired using a Nikon-C1, TE2000-U ICM and EZ-C1 software. Data were analyzed using ImageJ.1, Open*lab* and Adobe Photoshop. The relative fluorescence intensity of individual colors is expressed as percent of total fluorescence intensity.

Proliferation assay

C3H-derived BMDCs (H-2K^k) were incubated for 15 hours with 10 mg/ml OVA and injected (5×10^6 cells) intraperitoneally. OT-I T cells were labeled and intravenously injected as above. Proliferation of OT-I T cells was assessed by CFSE dilution 3 days later using flow cytometry.

Transfection

Immature BMDCs were transfected with full-length murine CD74 (p31 isoform; FL) or CD74 lacking amino acids 2–17 (Δ2–17) in the pBabe vector (a kind gift from Idit Shachar of the Weizmann Institute of Sciences, Israel) using the Amaxa Mouse Dendritic Cell Nucleofector Kit. One day post-electroporation, DCs were incubated with 20mg/ml OVA or 1 μM $OVA_{257\text{-}264}$ peptide for 8 hours then with OT-I CFSE labeled CD8$^+$ T cells for 3 days. CFSE dilution was assessed by flow cytometry.

Immunoprecipitation

BMDCs were starved in methionine and cysteine-free media for 1 hour, pulsed with 300 uCi/mL [^{35}S]methionine for 30 minutes then lysed in 0.5% Nonidet P-40 buffer (120 mM NaCl, 4 mM $MgCl_2$, 20mM Tris-HCl pH 7.6) containing protease inhibitor cocktail (Roche) and 40 μg/mL PMSF. Where indicated, DCs were incubated with 100μM chloroquine overnight before lysis. Cell lysate were precleared overnight with normal rabbit serum and Protein A-sepharose (Pharmacia). Immunoprecipitation was performed using the H-2K^b (AF6.88.5, BD Pharmingen) antibody recognizing fully-folded MHC class I, exon-VIII antibody (a kind gift of Professors David Williams and Brian Barber, University of Toronto, Canada) that recognizes all MHC class I, I-A/E antibody (M5/114.15.2, Becton Dickinson CA), CD74 antibody (ln-1, Fitzgerald) or transferrin receptor antibody (Invitrogen). Samples were analyzed on a 10–12% SDS polyacrylamide gel electrophoresis (PAGE). The gels were fixed, enhanced with Amplify (Amersham Biosciences), dried and exposed to Kodak XMR autoradiographic film. Alternatively, samples were transferred to a nitrocellulose membrane and immunoblotted with a CD74 or MHC class I antibody (KH95; SantaCruz Biotechnology). Endoglycosidase H_f digestions were performed per manufacturer's protocol (New England Biolabs). Whole cell lysate was blotted as a positive control. Donkey anti-mouse IgG (Li-Cor Biosciences) or goat anti-rat IgG (Invitrogen) were used as a secondary antibody. Blots were visualized using the Odyssey Infrared Imaging.

MHC class I internalization

BMDCs were stained with Fc block (BD PharMingen) then labeled with biotinylated H-2K^b (AF6-88.5) antibody for 30 minutes at 0°C. Samples were placed at 37°C or 0°C. At indicated times, DCs were fixed in 2% paraformaldehyde, labeled with streptavidin-PE then

examined by flow cytometry. Data were analyzed using FlowJo software to calculate the amount of internalized MHC class I.

Statistical analysis

Student's t-test was used to compare the difference between populations. The difference was considered statistically significant if $p < 0.05$ (two-tailed).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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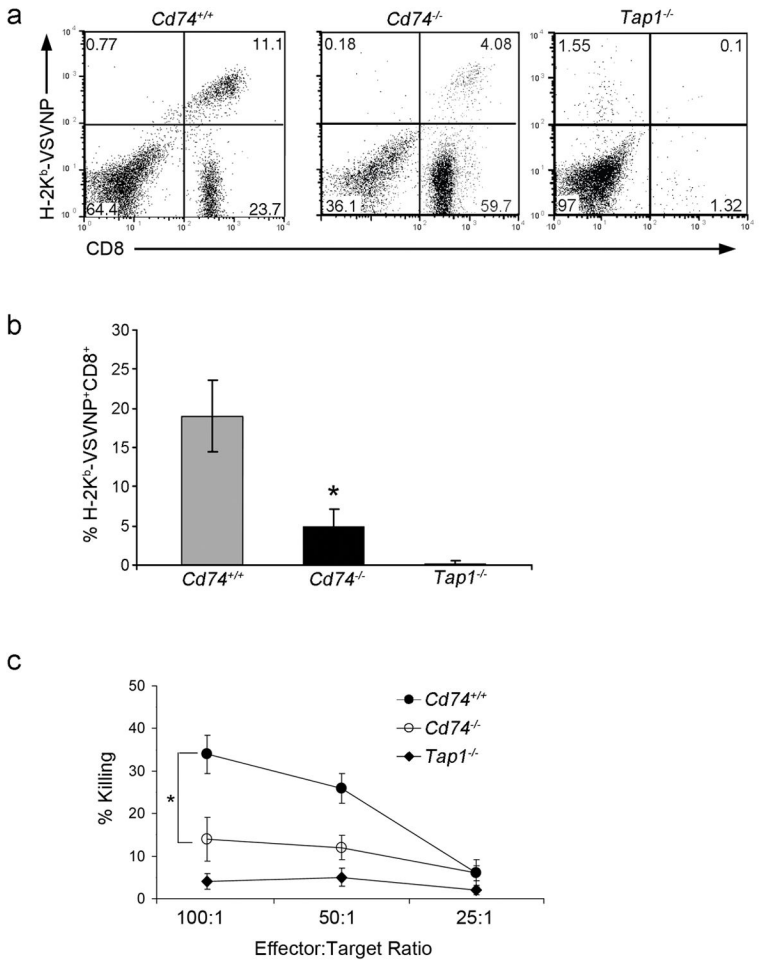

Figure 1. *Cd74*$^{-/-}$ mice generate weak antiviral primary immune responses

Cd74$^{+/+}$, *Cd74*$^{-/-}$ and *Tap1*$^{-/-}$ mice were infected with a low titer of VSV (2×10^5 $TCID_{50}$ or dose that infects 50% of a tissue culture cell monolayer/mouse). (a) Six days following viral infection, splenocytes were isolated and after a 5-day stimulation with $VSVNP_{52\text{-}59}$, the number of $VSVNP_{52\text{-}59}$-specific $CD8^+$ T cells generated was assessed. Percentages of $VSVNP_{52\text{-}59}$-specific $CD8^+$ T cells in representative mice are shown. (b) Mean percentages (± SD) of H-$2K^b$-$VSVNP_{52\text{-}59}$-specific $CD8^+$ T cells of three mice are shown. (c) Standard ^{51}Cr-release assays were performed using CTLs generated following VSV infection and *in vitro* boosting. Error bars represent SD. Data are representative of at least three separate experiments. * $p<0.05$.

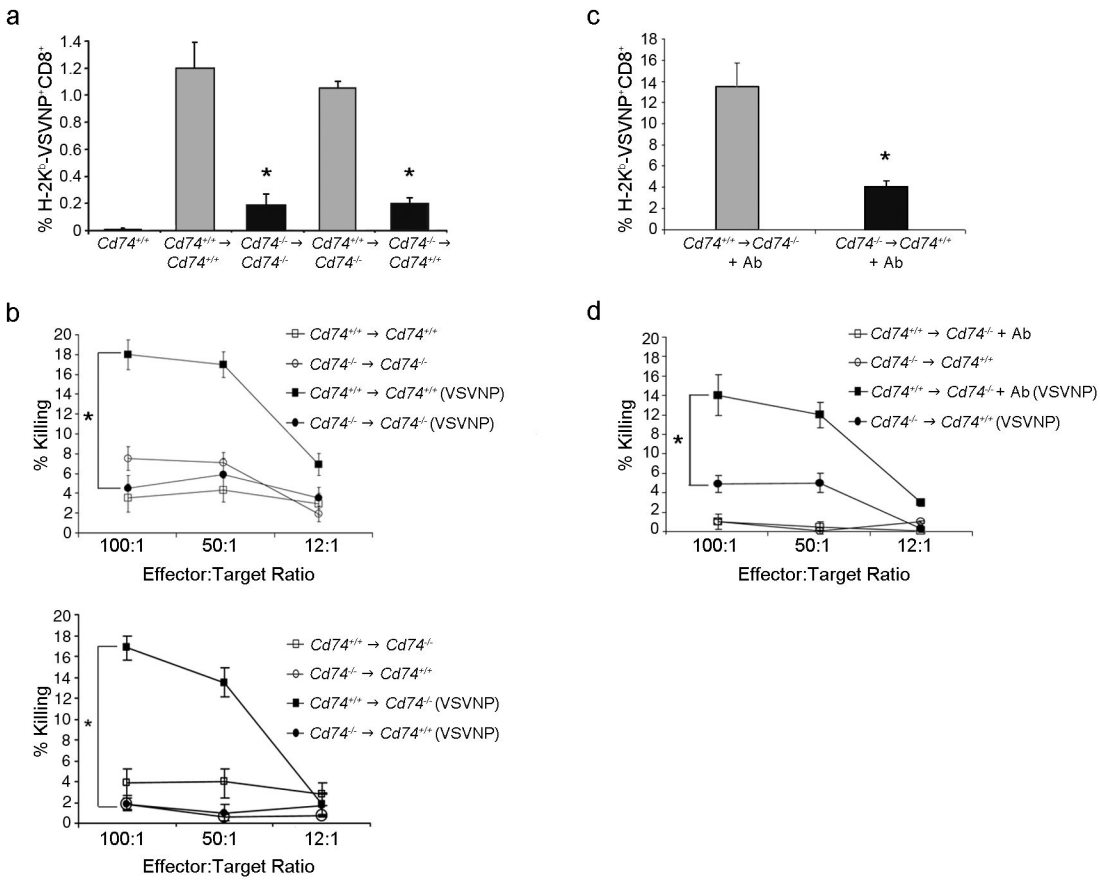

Figure 2. The deficiency of *Cd74*$^{-/-}$ mice to elicit primary immune responses resides in their APCs and is independent of CD4$^+$ T cells

(a) Chimeras were injected with 1 × 10^5 $TCID_{50}$ VSV and splenocytes were assessed for the generation of $VSVNP_{52\text{-}59}$ -specific CD8$^+$ cells. The mean percentage (± SD) of three mice assayed following *in vitro* boosting with VSV $NP_{52\text{-}59}$ peptide is shown. (b) Cytotoxicity assays were performed using CTLs generated following *in vitro* boosting. Data are representative of at least three separate experiments. (c) *Cd74*$^{+/+}$→*Cd74*$^{-/-}$ chimeras were depleted of CD4$^+$ cells by intravenous injection of a CD4 (GK1.5) antibody (+Ab) then assessed for immune function. Mice chimeras infected with VSV were evaluated for the generation of H-2K^b-$VSVNP_{52\text{-}59}$-specific CD8$^+$ T cells. The mean percentage (± SD) of tetramer$^+$CD8$^+$ cells in the spleen of three mice is shown. (d) The lytic activity of the immune response was also assessed. Error bars represent SD. * $p < 0.05$.

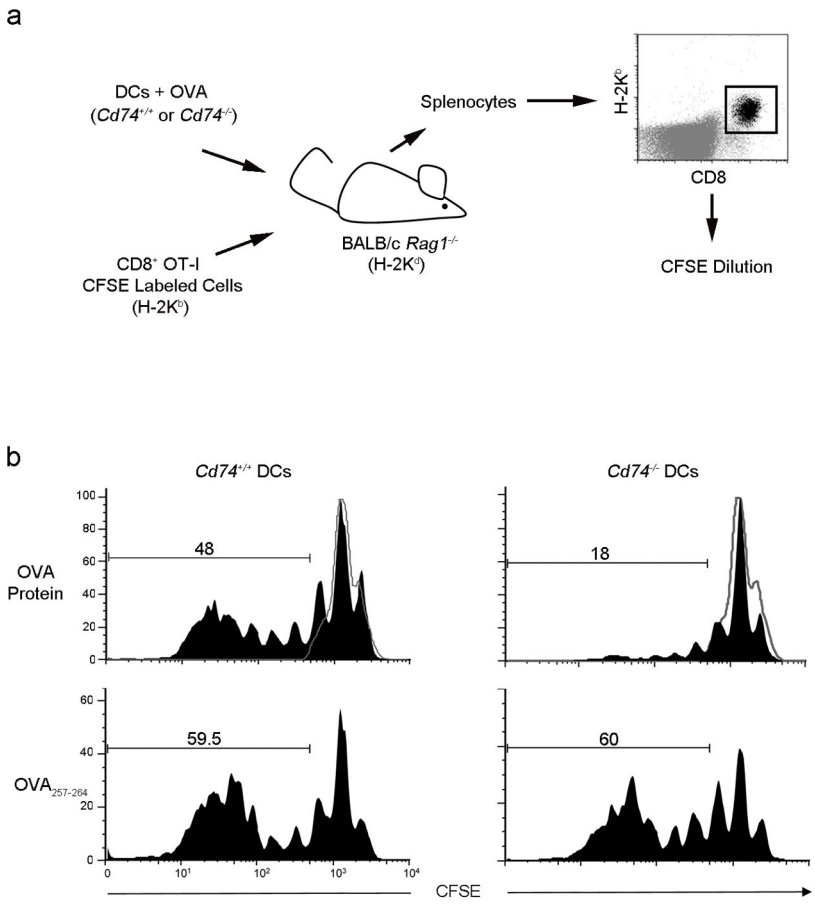

Figure 3. *Cd74*$^{-/-}$ DCs are unable to cross-present cell-associated antigens *in vivo* to prime antigen-specific CD8^{+} T cells

(a) OVA protein or $OVA_{257-264}$ pulsed *Cd74*$^{-/-}$ or *Cd74*$^{+/+}$ BMDCs were injected with purified CD8^{+} OT-I CFSE-labeled T cells into *Rag1*$^{-/-}$ mice on a BALB/c background. Three days later, H-2K^{b}CD8^{+} T cells were assessed for proliferation. (b) Black histograms represents proliferating OT-I derived T cells from the spleens of representative mice (n=3). Grey histograms represent unproliferating OT-I T cells.

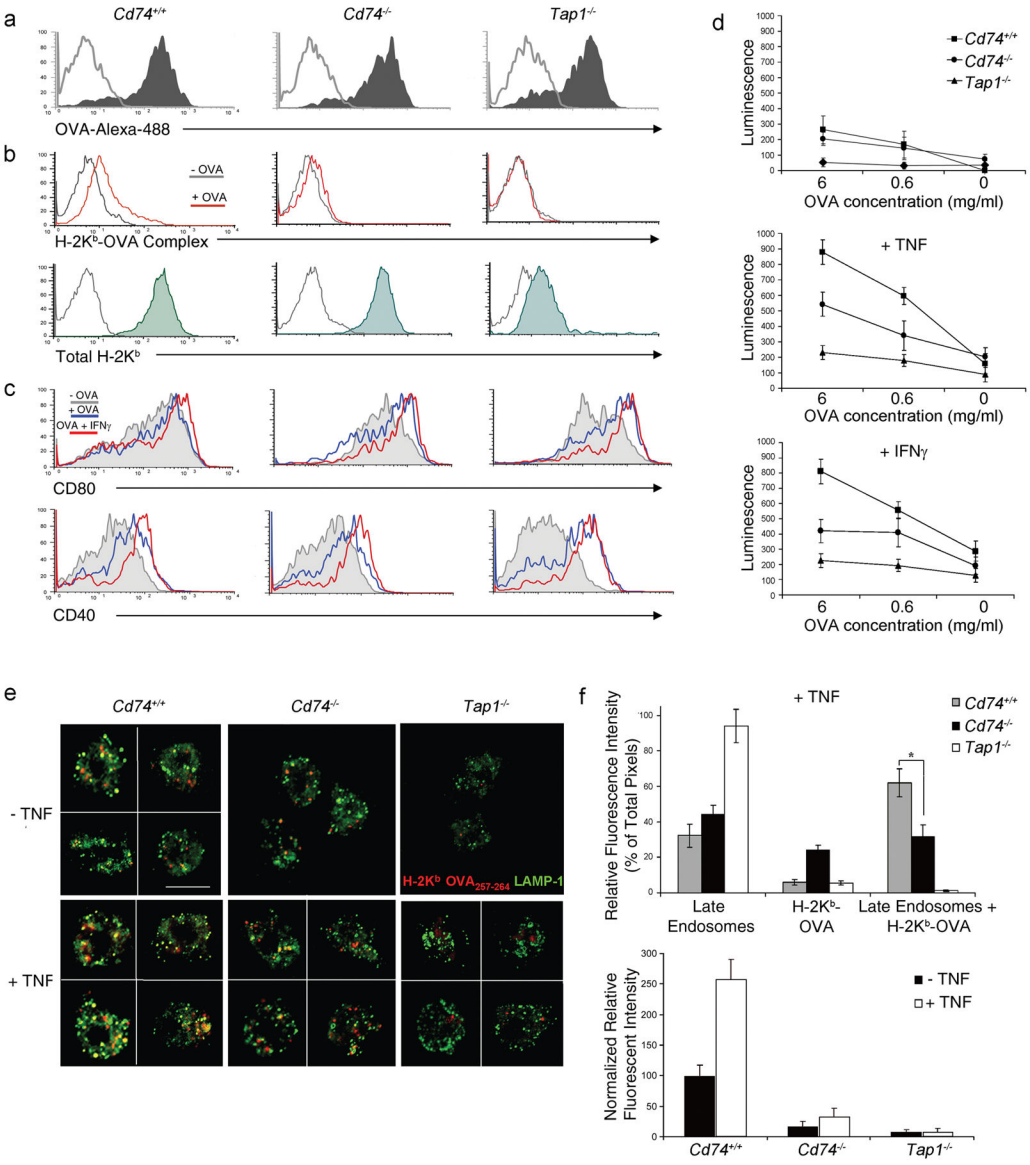

Figure 4. Cross-presentation and cross-priming is defective in *Cd74*$^{-/-}$-derived DCs

(a) BMDCs were incubated with OVA-Alexa-488 and uptake (shaded) was assessed over background (grey) by flow cytometry. (b) Formation of H-2K^b-OVA$_{257-264}$ complexes on splenic DCs with (red) or without (grey) incubation with soluble OVA as well as total H-2K^b (shaded) above background (grey) was measured by flow cytometry. (c) Costimulatory receptors CD80 and CD40 on BMDCs were assessed by flow cytometry following incubation with OVA (blue), OVA+IFNγ (red) or media alone (grey). (d) Spleen-derived DCs were incubated with soluble OVA as indicated, in the presence of GM-CSF or GM-CSF and TNF or IFN-γ. Activation of B3Z T cells was measured using a chemiluminescent assay. Data depict means (± SD) of triplicate samples for each OVA concentration. Similar results were observed in 3 separate experiments. (e) Mature spleen-derived DCs incubated with OVA were costained with H-2K^b-OVA$_{257-264}$ specific antibody (red) and LAMP-1 (green). The figure shows optically merged images representative of the majority of cells

examined by ICM. Scale bar, 5 μm. (f) Quantitative assessment was performed comparing H-2K^{b}-OVA$_{257-264}$ colocalization with LAMP-1^{+} late endosomes with or without TNF (top panel). Quantitative assessment comparing fluorescence between *Cd74*$^{+/+}$, *Cd74*$^{-/-}$ and *Tap1*$^{-/-}$ DCs with TNF treatment (bottom panel). For each analysis >20 DCs per mouse strain were examined. Graph depicts normalized individual color pixel percentages per total pixels ± SD. * $p < 0.05$.

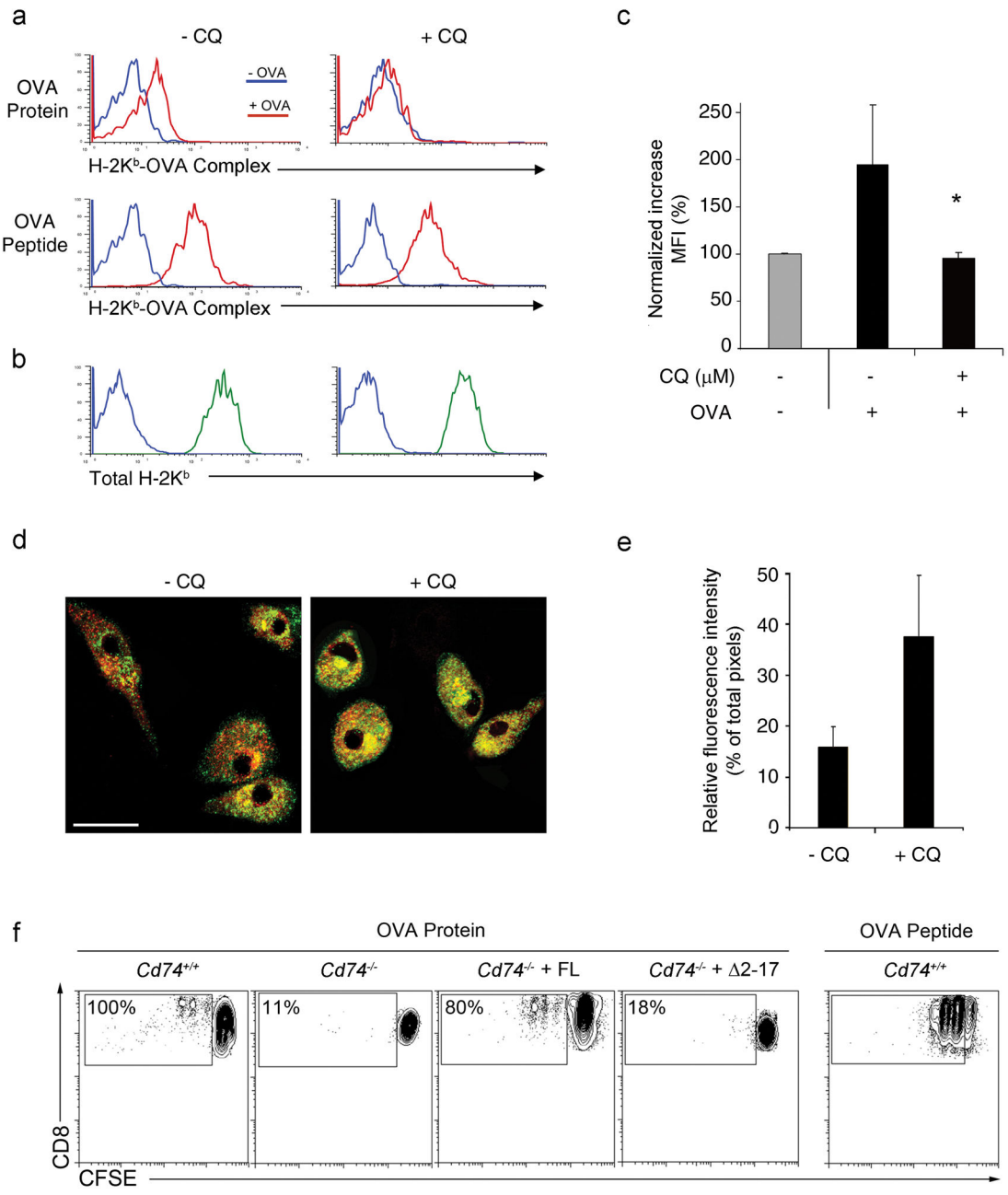

Figure 5. Inhibition of CD74-mediated MHC I trafficking in DCs by treatment with chloroquine

(a) BMDCs were treated with CQ and the formation of H-2K^b-OVA$_{257-264}$ complexes on DCs following incubation with soluble OVA (red; top panel) or OVA peptide (red; bottom panel) compared to media alone (blue) was measured by flow cytometry. (b) Total H-2K^b (green) above background (blue) was also assessed. (c) The fold increase in surface H-2K^b-OVA$_{257-264}$ complexes following incubation with soluble OVA was quantified. Graphs depict normalized increases in mean fluorescence intensity (MFI) ± SD. * $p < 0.05$. (d) Mature BMDCs treated with CQ were costained with H-2K^b (red) and CD74 (green) antibody. The figure shows optically merged images representative of the majority of cells

examined by ICM. Scale bar, 5 μm. (e) Quantitative assessment comparing H-2K^{b} colocalization with CD74. Graph depicts normalized individual color pixel percentages divided per total pixels ± SD. (f) *Cd74*$^{-/-}$ BMDCs reconstituted with full length (FL) CD74 or truncated (Δ2–17) CD74 lacking the endolysosomal trafficking motif were incubated with soluble OVA and assessed for the ability to induce proliferation in CFSE-labeled OT-I cells. Percentages represent the proportion of proliferating OT-I cells normalized to *Cd74*$^{+/+}$ controls.

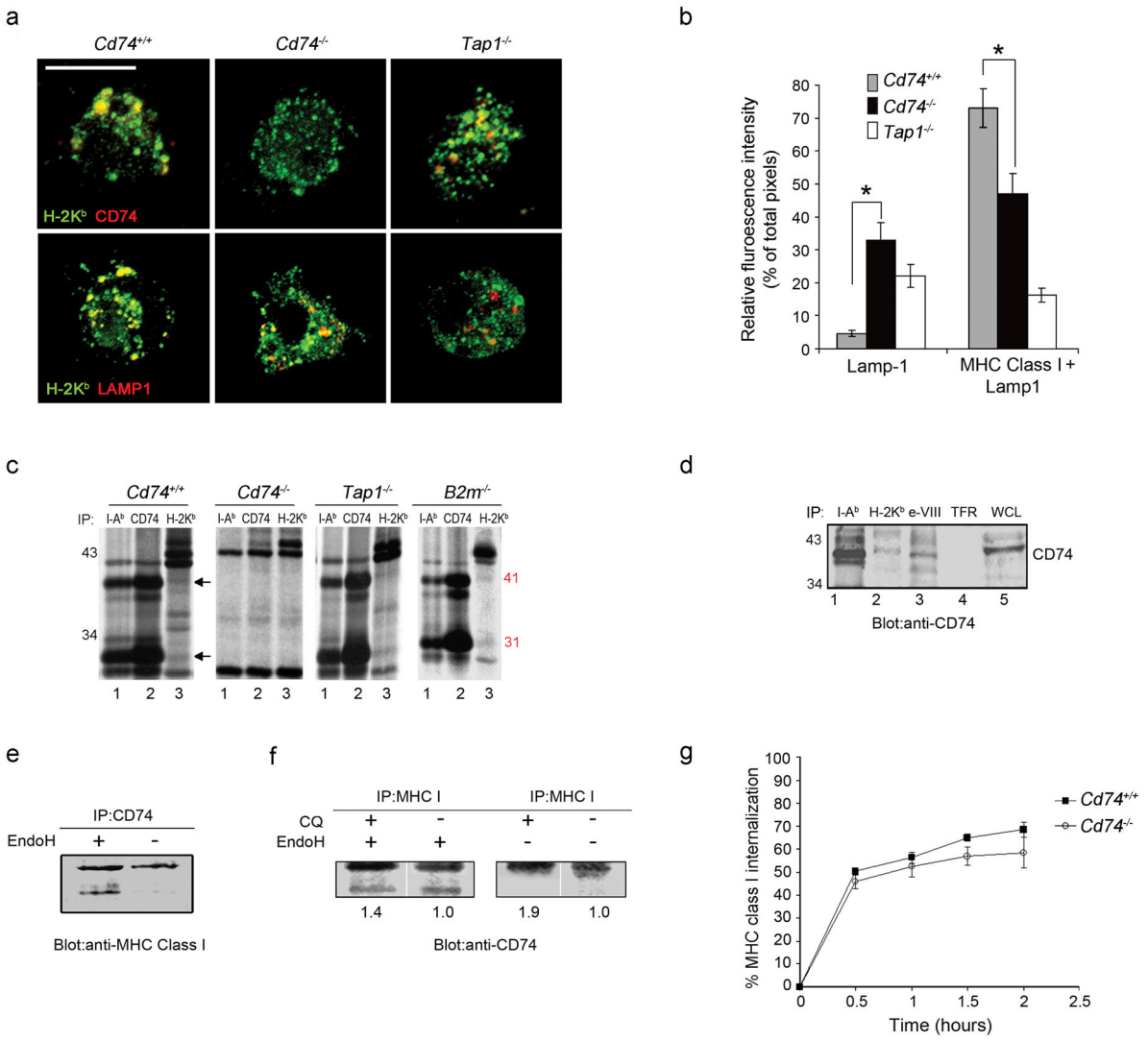

Figure 6. CD74 controls MHC class I ER-to-endolysosome trafficking in DCs

(a) Mature splenic DCs were stained with H-2K^b (green) and CD74 (red) or LAMP1 (red) antibodies. Representative images as examined by ICM are shown. Scale bar, 5 μm. (b) Quantitative assessment of MHC class I in LAMP$^+$ compartments was performed (50 DCs per mouse strain). Graphs depicts individual color pixel percentages per total pixels (mean ± SD) (c). Immunoprecipitation using H-2K^b, I-A/I-E and CD74 antibodies was performed on [^{35}S]methionine-labeled BMDC. The CD74 41 and 31 k-Da protein bands are indicated. (d) Immunoprecipitation with antibodies against I-A^b, H-2K^b (conformationally dependent), H-2K^b cytoplasmic domain (e-VIII; conformationally independent) or transferrin receptor (TFR) was performed with *Cd74$^{+/+}$* DC lysates. The identity of the co-immunoprecipitated proteins was confirmed by blotting with a CD74 antibody. Whole cell lysate (WCL) was blotted as a control. (e) DC cell lysates were immunoprecipitated with a CD74 antibody and digested with endoglycosidase H. Immunoblotting with a MHC class I antibody was used to assess the MHC class I fraction precipitated by CD74 antibody and to visualize the acquisition of EndoH resistance of the MHC class I subset interacting with CD74. (f) Cells

were treated with CQ to enhance CD74 and MHC I interaction and cell lysates were immunoprecipitated with a MHC class I antibody. Western blotting with a CD74 antibody was used to assess the CD74 protein fraction associating with MHC class I. (g) DCs labeled with a H-2K^b antibody were evaluated overtime for MHC class I internalization measured by flow cytometry as a reduction in mean fluorescence intensities over time. * $p < 0.05$.