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MARCH9 mediated-ubiquitination regulates MHC I export from the TGN

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Running title: MARCH9 ubiquitination controls MHC I and CD1a transport.

ABSTRACT

Given the heterogeneous nature of antigens, MHC-I intracellular transport intersects with multiple degradation pathways for efficient peptide loading and presentation to cytotoxic T cells. MHC-I loading with peptides in the endoplasmic reticulum (ER) is a tightly regulated process, while post-ER intracellular transport is considered to occur by default, leading to peptide-bearing MHC-I delivery to the plasma membrane. We show here that MHC-I traffic is submitted to a previously uncharacterized sorting step at the trans Golgi network (TGN), dependent on the ubiquitination of its cytoplasmic tail lysine residues. MHC-I ubiquitination is mediated by the E3 ligase MARCH9 and allows MHC-I access to syntaxin 6 (Stx6)-positive endosomal compartments. We further show that MARCH9 can also target the human MHC I-like lipid antigen presentation molecule CD1a. MARCH9 expression is modulated by microbial patterns exposure in dendritic cells (DC), thus revealing the role of this ubiquitin E3 ligase in coordinating MHC-I access to endosomes and DC activation for efficient antigen cross-presentation.

Keywords: ubiquitin ligase, HLA, antigen presentation, dendritic cells.

INTRODUCTION

Immune surveillance by lymphocytes is a key mechanism for detecting and eliminating abnormal cells, particularly those infected by pathogens¹. CD8⁺ T cells scan the physiological state of cells by probing the repertoire of antigenic peptide presented by major histocompatibility complex class I (MHC I) on target cell surface². MHC I molecules bind a diverse array of peptides in the endoplasmic reticulum (ER)³, where multiple quality control steps allow the coordination of peptide loading and MHC I assembly, prior traffic through the secretory pathway and cell surface arrival^{4,5}. In addition to direct export of peptide-loaded MHC I to the plasma membrane, a regulated access to endosomes is important for the presentation of exogenous antigens to CD8⁺ T-cell. This process called “cross-presentation” is principally occurring in mouse CD8⁺/CD103⁺ and human BDCA3⁺ dendritic cells (DC)⁶.

Several E3 ubiquitin ligases of the membrane-associated RING-CH (MARCH) family have been attributed immune functions^{7, 8}. MARCH1 promotes MHC II and CD86 surface internalization in human monocyte-derived DCs (MoDCs), mouse DCs and B cells^{9,10}, and participate to tolerogenic activity of regulatory T cells (Treg) and Interleukin-10 (IL-10) exerted on professional APCs or monocytes^{11,12}. Ectopic over-expression of MARCH9 has also been shown to trigger the surface down-regulation of several immunology-relevant receptors, such as MHC class I, ICAM-1 and CD4 in transfected cell lines¹³, although the physiological relevance of these observations has not been further documented.

We confirm that MARCH9 over-expression prevents surface accumulation of HLA molecules, as well as the MHC I-like molecule CD1a, through the ubiquitination of lysine residues present in the cytoplasmic tails of these transmembrane proteins. We further show that endogenous MARCH9 functions at the Trans Golgi Network (TGN) level, where it facilitates neo-synthesized peptide-loaded MHC I complexes access to Syntaxin 6 (Stx6)- and secretory carrier membrane protein 3 (SCAMP3)-positive endosomal compartments. Thus, MARCH9 up-regulation in activated DCs contributes to the coordination of MHC I intracellular transport to endosomes, with the acquisition of specialized function, such as antigen cross-presentation in DCs.

RESULTS

MHC class I is naturally ubiquitinated in human cells.

To investigate whether MHC I can be naturally ubiquitinated, we immunoprecipitated peptide-loaded HLA-I molecules from different human cells using the conformation specific W6/32 monoclonal antibody (mAb) and revealed ubiquitin **by performing immunoblot** with the P4D1 mAb (Fig. 1A). A fraction of HLA-I molecules was found to be ubiquitinated in all cell types tested, and was comparatively enriched in monocyte-derived DCs (MoDC) extracts. HLA-I cytoplasmic tails contain 2 or 3 conserved lysine residues, which are known ubiquitination target sites for the Kaposi's sarcoma-associated herpesvirus K3 and K5 E3 ligases associated with immune evasion⁸. To confirm the relevance of these residues for endogenous ubiquitination, tagged HLA-I mutants lacking cytoplasmic tail lysine residues (K to R substitutions = Kless) were monitored for potential ubiquitination after ectopic expression in HeLa cells. After transfection, His-tagged HLA-A2 (His-A2) and HA-tagged HLA-B7 (HA-B7) molecules were correctly processed and transported to the HeLa cell surface **as observed by flow cytometry and immunoblot** (Fig. Sup 1). Ubiquitination was detected **by immunoblot using P4D1** both in His-A2 and HA-B7, but remained however undetectable in K-less mutants (Fig. 1B), confirming the endogenous ubiquitination of MHC I on these residues. We were however intrigued by the relative abundance of ubiquitinated-HLA in the MoDC extracts, and examined the variation of MHC I ubiquitination with respect to different MARCH E3 ligases expression in non-activated vs LPS-activated cells. HLA-I ubiquitination levels were moderately increased after 24h of LPS exposure (Fig. 1C). In parallel and solely among other E3 ligases tested⁹, MARCH9 **mRNA** expression **monitored by qPCR** was found to be up-regulated by 7 to 20 fold over 24h in response to LPS or poly I:C both in human MoDC and mouse GM-CSF BM-derived DC (Fig. 1D), while MARCH1 mRNA levels were down-regulated⁹. This result suggested that MARCH9, could regulate MHC I molecules transport in maturing DC through ubiquitination of their cytoplasmic tail. MARCH9 has been proposed to interfere with surface expression of both MHC I and ICAM-1 molecules when artificially over-expressed^{7,13}. MARCH9 up-regulation, associated with increased MHC I ubiquitination, would therefore be expected to impact

negatively on surface MHC I, by promoting their endocytosis. However biochemical examination of HLA-I indicated that both biosynthetic and surface levels were increased with activation (Fig. 1E), while endocytosis of peptide-loaded complexes, measured using [biotinylated W6/32 Fab uptake](#) and [flow cytometry](#), remained unchanged upon LPS exposure (Fig. 1E). Thus, in a physiological situation, endogenous MARCH9 does not act on surface MHC I endocytosis, and ubiquitination could therefore be required for a different step of MHC I intracellular transport.

MARCH9 mediates MHC I and CD1a ubiquitination.

Over-expression of membrane associated E3 ligases over time, could affect their intracellular localization or unbalance enzyme/targets ratios, and might be misleading to define their specificity or precise site of activity. Thus, the discordant results obtained in transfected HeLa cells⁷ and in primary DCs could be the results of MARCH9 over-expression. We re-evaluated the specificity of human MARCH9 to target surface MHC I in comparison to other human MHC I like-molecules using different methods of ectopic expression. We first investigated the impact of MARCH9 and control E3 ligases on HLA I and on the lipid antigen presentation molecule CD1a. CD1a is the only member of the CD1 molecules family to lack a tyrosine residue in its cytoplasmic tail that serves as endocytic sorting signal¹⁴. CD1a appeared therefore to be an interesting target to evaluate MARCH9 specificity and its potential impact MHC I-like molecules transport. HeLa cells, stably expressing CD1a, were therefore transfected with plasmids coding for MARCH2 and MARCH9 fused with GFP, as well as the K3 KSHV ligase prior analysis by flow cytometry and microscopy. MARCH9-GFP expression was found to efficiently down-regulate both surface CD1a and HLA molecules, while MARCH2, used here as a negative control, had no effect, and, as anticipated, K3 was found to affect mostly HLA molecules and more moderately CD1a (Fig. 2A).

Ubiquitination of MHC I and CD1a was considerably increased upon MARCH9 and viral E3 ligases expression (Fig. 2B and 2C). MARCH9-dependent MHC I ubiquitination pattern was however distinguishable from those resulting from K3 and K5 over-expression, both in terms of number of conjugated ubiquitin moieties and

relative abundance (Fig. 2B). His-tagged mutants of HLA-A2, bearing lysine replacement in the cytoplasmic tail (Fig. 2B), were tested in parallel for their sensitivity to MARCH9 expression and internalization from the plasma membrane. Surface His-A2 WT molecules were affected both by MARCH9 and K3, while K-less mutants were not. Individual mutations in lysine residues did not significantly interfere with MARCH9- and K3-dependent surface clearance of the His-A2 mutants, at the exception of His-A2-K340R, which remained unaffected by K3 expression, confirming the identity of K340 in HLA-A2 as target residue this viral ligase¹⁵. MARCH9 specificity for given lysine residues appeared, however, to be relatively loose, which could be again a consequence of its ectopic overexpression¹⁵, since this E3 ligase endogenous expression is normally extremely low and finely regulated.

Upon [immunoblot](#) detection, CD1a ubiquitination was found to be induced by MARCH9 in a well defined molecular pattern, which was likely constrain by the shortness of CD1a cytoplasmic tail (-RKRCFC) (Fig. 2C), bearing only 3 residues being potentially post-translationally modified. Two cysteines (C325 and C327), that can be S-acetylated, with no reported impact on CD1 transport¹⁴, and a unique lysine (K323), which represents a potential ubiquitination site. CD1a mutants bearing distinct individual lysine-to-alanine and cysteine-to-alanine replacements were generated [by site directed mutagenesis](#) (Fig. 2C) and stably transfected HeLa cell lines were selected for equivalent WT CD1a or mutant expression (Fig. 2C). Upon MARCH9-GFP expression, surface expression of mutants CD1a lacking lysine 323 (No-K and No K/C) [was determined by flow cytometry and shown to remain insensitive](#) to MARCH9 activity, while WT and No-C constructs were efficiently cleared from the plasma membrane. At the sub-cellular level, No-K mutants were shown [by confocal microscopy](#) to be incapable of reaching intracellular compartments upon MARCH9 expression, while WT and No-C mutants co-localized with MHC I in EEA1 positive early endosome (Fig. 3), demonstrating that high levels of MARCH9 expression promotes surface CD1a and HLA-1 ubiquitination and triggers their accumulation in a subset of early endosomes.

Upon MoDC activation with LPS, endogenous CD1a, which normally transit through recycling endosomes¹⁶, accumulated massively at the cell surface (Fig. 3D).

However, in DCs activated for 1 or 2 hours, CD1a mostly co-localized with the mannose-6P-receptor and not with the early endosome marker EEA1, suggesting that a significant fraction of CD1a molecules traffic between the Golgi apparatus and the endosomes upon DC activation. However again and similarly to HLA-1, increased CD1a surface internalization and endosomal distribution in activated DCs was not correlated with MARCH9 expression levels. Although, MARCH9 is likely to act specifically on MHC I and MHC I-like molecules⁹, such as CD1a and mouse Mult1¹⁷, its uncontrolled ectopic expression results in a loss of subcellular distribution specificity, which impacts MHC like-molecules in a non-physiological manner and is likely to promote abnormal internalization from the surface.

We next evaluated how a more controlled expression of MARCH9 could act on MHC I transport. We used short-term (4-6h) **electroporation** of eGFP-tagged MARCH9 mRNA compared to traditional plasmid lipofection (Fig. 4A). **Electroporated** MARCH9-eGFP mRNA expression failed to alter HLA-I surface levels **measured by flow cytometry**, but instead, **as observed by confocal microscopy**, promoted HLA-I accumulation in **GFP-positive** vesicular structures, containing the ligase itself (Fig. 4B). Thus, as anticipated, controlled expression of MARCH9 does not promote massive surface HLA-I internalization, but instead seems to regulate a specific intracellular membrane trafficking step.

MARCH9 controls MHC class I export to Stx6⁺-endosomal compartments.

MARCH9-eGFP was detected **by microscopy** in at least two distinct sub-cellular localizations, in pericentriolar membrane compartments and in punctate vesicular structures distributed more peripherally (Fig. 4B). Pericentriolar MARCH9 co-localized with TGN46 and SCAMP3¹⁸, but not with AP1, nor with ERGIC53 (Fig. 4B). An overlapping distribution of the ligase with the Golgi matrix protein GM130 and p230, a golgin known to regulate TGN-to-early endosomes transport¹⁹, confirmed that MARCH9 mostly accumulates at the TGN level (Fig. 4B). Peripheral MARCH9, however, was extensively co-distributed with HLA-I and CI-M6P-R, in vesicles also strongly positive for Stx6, and lesser for SCAMP3 (Fig. 4B), but clearly negative for other known TGN markers. Extensive HLA-I distribution in this compartment was only observed upon MARCH9 mRNA expression and based on these observations, we propose that MARCH9 functions at the interface between

TGN and post-Golgi compartments, where a portion of the neo-synthesized peptide-loaded MHC I pool could be ubiquitinated to favor their delivery into Stx6-positive endosomes.

MARCH9 silencing delays cell surface arrival of peptide-MHC I complexes.

HeLa cell lines stably expressing control (HeLa-con) and specific shRNA to silence MARCH9 expression (Hela-MARCH9-KD) were generated. RNA silencing reduced MARCH9 mRNA levels by 60 to 90 % in Hela-MARCH9-KD (Fig. 5A). Endogenous MARCH9 protein levels are however too low to be detected with available methods and reagents²⁰ and could not be established in KD cells. We could however show, using [radioactive](#) ³⁵S-methionine pulse and chase labeling followed by immunoprecipitation, that tagged-MARCH9 has a 3h half-life in transfected cells (Fig. 5A). An ectopically expressed tagged version of this ligase was also efficiently down-regulated in Hela-MARCH9-KD cells, further suggesting that silencing of endogenous MARCH9 mRNA, efficiently impacts the E3 ligase protein levels. Surface HLA molecules were moderately reduced in MARCH9-silenced cells, while total expression levels and synthesis rate remained identical (Fig. 5B). The weak surface HLA-I internalization, monitored by [biotinylated W6/32 Fab uptake and flow cytometry](#), remained also unchanged in the MARCH9-KD cells, indicating that variations in HLA-I surface levels could not be attributed to a higher endocytic rate (Fig. 5C). ³⁵S-methionine pulse-chase labeling followed by HLA-I immunoprecipitation and endoglycosidase (Endo)-H treatment confirmed that MARCH9 silencing had no major effect on MHC I neo-synthesis, since only a minor delay in the acquisition of Endo-H resistance by HLA molecules in MARCH9 KD cells was observed [in immunoblots](#) (Fig. 5D).

We next compared the surface recovery of MHC class I-peptide complexes after acid stripping of both control and MARCH9-silenced cells to evaluate the rate of HLA-I transport from the ER to the surface (Fig. 5E). To synchronize the flow of peptide/MHC I molecules exiting the ER, a 2h pre-treatment with brefeldin A (BFA) was performed prior surface monitoring with W6/32 or anti-HLA-A2 specific mAbs. Recovery rate of surface HLA-I molecules was reduced by 30 to 35% in MARCH9-KD cells, suggesting that MARCH9 depletion affects HLA transport prior arrival to

the cell surface, while MHC I internalization remained unaffected (Fig. 5C). When His-A2 WT and K-less molecules were expressed comparatively, they displayed identical transport rates, and this independently of MARCH9 (Fig. 5E), suggesting that MARCH9 depletion recapitulates the functional consequences of lysine replacement in the HLA-I cytoplasmic tail. Thus, MARCH9 controls a secretory step requiring ubiquitination of HLA and likely to occur at the TGN level as pointed-out by the sub-cellular distribution of the E3 ligase.

MARCH9 controls MHC class I transit through TGN compartments.

HLA-I localization in MARCH9-silenced cells was further determined by confocal microscopy (Fig. 6A), using different post-ER marker such as Stx6 and SCAMP3, which define the TGN/endosome interface²¹. In control cells, HLA-I was poorly co-localized with Stx6 or SCAMP3 positive organelles, and found mostly in the ER and at the plasma membrane. Upon MARCH9 silencing, now, HLA-I extensively accumulated in Stx6/TGN46/SCAMP3-containing pericentriolar compartments, confirming that MARCH9-dependent ubiquitination is likely to regulate HLA-I export from the TGN to peripheral endosomal compartments. Insufficient ubiquitination, resulting from MARCH9 silencing, could thus impair this sorting step and promote the retention of a fraction of HLA-A2 molecules in the TGN.

In order to evaluate the functional impact of MARCH9, we had to turn to the mouse system, which was found more suitable for antigen presentation studies. DC development can be faithfully recapitulated in GM-CSF and Flt3L-supplemented bone-marrow cultures, which give rise to all identified subsets of dendritic cells, including the CD8⁺ cross-presenting DCs²². We used the bone-marrow of MARCH9^{-/-} mice to generate DCs and test functionally MHC I distribution in CD8⁺DCs. Confocal microscopy analysis of magnetically sorted and pl:C activated CD8⁺DCs indicated that MARCH9 inactivation impacts MHC I molecules distribution in mouse DCs and, like in silenced Hela cells, promotes their accumulation in TGN/Stx6⁺ compartments (Fig. 6B).

Cross-presentation is affected by MARCH9 inactivation in mouse DCs

Given the impact of MARCH9 deletion on MHC I intracellular distribution in mouse DCs, MHC I surface expression was monitored in different DC subsets and found to be equivalent in WT and MARCH9 $-/-$ cells (Fig. 7A). MHC I-restricted presentation was tested functionally in absence of the ligase by monitoring SL8 (SIINFEKL) peptide presentation after transfection of bm-DCs with mRNA coding for the model antigens eGFP-SL8 and eEGFP-OTII-SL8²³. Antigen expression and presentation was quantified by flow cytometry using direct eGFP fluorescence and surface staining with the 25D1.16 antibody specific for H-2K^b/SL8²⁴. As expected from the equivalent levels of surface MHC I found in WT and MARCH9 $-/-$ DCs (Fig. 7A), no difference in the ability of processing and presenting SL8 was detected in absence of MARCH9 (Fig. 7B).

Given its proposed role for MHC I targeting to Stx6 endosomes, we next evaluated if MARCH9-dependent sorting could facilitate cross-presentation (CP) in DCs. Ovalbumin coated-latex beads together with IL-2 release by “SIINFEKL” peptide-specific T cells (B3Z hybridoma)²⁵ were used to monitor CP by spleen DCs after incubation with various amount of antigen. Although, temperature-dependent phagocytic uptake of beads and direct presentation of antigenic SL8 peptide remained unaffected by MARCH9 inactivation (Fig. 7C), cross-presentation was reduced in MARCH9 $-/-$ cells (Fig. 7E). Interestingly, NOX2/gp91phox $-/-$ DCs, that have a deficit in phagosome acidification, display strongly reduced CP of phagocytosed antigens. Phagosomal pH was therefore measured in MARCH9 $-/-$ and wt bmDCs using the “PhagoFACS” technique²⁶ and was found equivalent in both cell types (Fig. 7D), indicating that MARCH9 deletion does not impact phagosome acidification. Hypothetically, the reduced CP of phagocytosed ovalbumin in MARCH9 $-/-$ cells, could be explained by a reduction of MHC I transport to endosomal compartments in absence of functional MARCH9. Supporting this concept, the impact of MARCH9 deletion was even more acute, when pI:C-dependent increase of soluble ovalbumin CP to primary OT1 cells, and normally mediated by CD8+ DCs, was found to be reduced after CFSE dilution monitoring of actively dividing T cells by flow cytometry (Fig. 7F).

DISCUSSION

Extensive work on viral ligases has demonstrated that ubiquitination induces MHC I degradation at the ER level or internalization from the surface of infected cells^{27, 8}. Ectopically-expressed MARCH9 has been shown to reduce surface MHC I by promoting endosomal targeting and degradation^{7,13}. We confirmed these observations and further demonstrated that CD1a is also a target of MARCH9, suggesting that this ligase could be specialized in controlling MHC I-like molecules traffic according to their expression patterns.

We demonstrated that MARCH9 localizes in the TGN, where it controls partially HLA-I export from this compartment. HLA-I sorting from the TGN appears to be the earliest transport step within the secretory pathway requiring ubiquitination for its regulation. Interestingly, upon vaccinia infection, mouse Kb class I molecules can segregate into preformed clusters that favor T cell activation, and which are first detected in the Golgi complex²⁸. Clustering has been shown to be compromised by Kb cytoplasmic tail removal, raising the possibility that ubiquitination could also control this phenomenon. As for CD1a, its cytoplasmic tail is extremely short (6 residues) and has generated considerable interrogations on the mechanisms allowing sorting to and recycling from endosomes¹⁴, ubiquitination of lysine 323, might thus contribute to the specialized sorting of this receptor.

Controlled MARCH9 expression promotes HLA-I accumulation in Stx6-positive endosomes. Conversely, MARCH9 silencing induces HLA-I retention in the TGN and delays its cell surface arrival. These data suggest that MARCH9 controls a previously uncharacterized TGN-to-endosome transport step, by which ubiquitinated HLA-I could access Stx6- and Scamp3- positive compartments. HLA-I intracellular accumulation suggests that unbalanced ubiquitination of HLA-I, impairs sorting to the cell surface or to an intermediate acceptor compartment. HLA-I haplotypes display polymorphism in the number and position of ubiquitin target lysine residues contained in their cytoplasmic tail (Fig. 2B). Thus, multiple ubiquitin ligases and their resulting ubiquitination patterns might represent a potent mechanism to sort differentially HLA-I in response to specific environmental cues. MARCH9 depletion, however, does not seem to overly affect the organization of

Stx6⁺ compartments, suggesting that its effect might be restricted to a limited number of cargo molecules, and its potency likely depends on the specific target/enzyme expression ratio observed in each individual cell type. Several mechanisms have been proposed to explain the molecular aspects of cross-presentation. So far, MHC I molecules have been described to enter the endocytic pathway exclusively after internalization from the plasma membrane, through direct fusion with the ER^{29,30,31} or via clathrin-independent mechanisms^{32,4}, some of which are regulated by members of the MARCH family³³. Early endosomal compartments containing both Rab14 and Stx6 play an important role in cross-presentation of both soluble and particulate ovalbumin by DCs³⁴. MARCH9-dependent MHC-I export through Stx6-positive compartments could therefore be part of the transport pathway used by neo-synthesized MHC I molecules to access ERC stores and cross-presentation-competent organelles. Microbial regulation of MARCH9 expression, argues for an adaptation of MHC I-like molecules sorting to the nature of the microbes detected and the cell subsets involved. MARCH9 regulation by different environmental cues might thus participate to the host protection against specific pathogens, and potentially prevent destruction of abnormal self-tissue.

METHODS

Monocyte-derived DCs.

MoDCs were generated from CD14⁺ peripheral blood monocytes as before described⁹. In brief, purified CD14⁺ cells were cultured for 6 days in RPMI supplemented with 10% FCS, nonessential amino acids, penicillin/streptomycin, recombinant human GM-CSF, and IL-4 (both from PeproTech). The cells were activated by LPS for indicated times.

Immunocytochemistry. HeLa cells were grown and transfected on coverslips in 24 wells-plates, while pDCs were incubated on 1% Alcyan blue-coated coverslips for 15 min. In both cases, cells were fixed in 3% PFA for 15 min at RT, permeabilized with 0,1% saponin in PBS/ 5%FCS/ 100mM Glycine for 10 min at RT and stained 1h with indicated primary antibody. All Alexa[®] secondary antibodies were from Molecular Probes, Inc (Eugene, OR). Immunofluorescence and confocal microscopy (using microscope model LSM 510; Carl Zeiss MicroImaging, Inc.)⁹. For images quantifications, pictures were treated using ImageJ and co-localization between markers was measured using Pearson coefficient calculated with the Coloc2 plugin.

Flow cytometry. Cells were harvested in PBS-5mM EDTA, washed in cold PBS with 1% FCS and stained using appropriate fluorochrome conjugated antibodies mixed in PBS with 1% FCS and 10% mouse serum for 30min at 4°C. After washes, cells were fixed in PBS with 2% PFA. Samples were read on FACS CANTO II (BD Bioscience) and analysed using FlowJo. Data were acquired onto BD LSR2 Fortessa on standardized analysis matrix using BD FACS DIVA 6.1.3 software.

Antibodies and immunodetection:

For Immunoprecipitation, anti-conformed HLA-I molecules monoclonal antibodies (w6/32) was used for all HLA-I IP performed in this study. M9-myc IP was done using rabbit polyclonal anti-MARCH9 antibody generated using specific M9 peptide as immunogen (sequence available upon request). Immunoblot: mouse anti-ubiquitin (clone P4D1, Covance); rat anti-HLA-I heavy chain (clone 3B10.7); Mouse anti-6His tag (AbD Serotec); mouse anti-HA tag (clone 12C5, AbCam); mouse anti-actin (Sigma). Immunofluorescence confocal microscopy: IgG1 mouse anti-6His tag (AbD Serotec); Rabbit anti-Syntaxin 6 (Proteintech Group Inc.); IgG2b mouse anti-

AP1 (Sigma); IgG1 mouse anti-ERGIC-53 (kind gift by S. Meresse, CIML, Marseille, France); rabbit anti-TGN46 (Serotec); IgG1 mouse anti-CI-M6P-R (clone 2G11, Calbiochem), rabbit anti-SCAMP3 (a kind gift of David Castle, University of Virginia, Charlottesville, Virginia); IgG2a mouse anti-conformed HLA-I (clone w6/32; home made from hybridoma); Rabbit anti-EEA1 (GeneTex); IgG1 mouse anti-GM130 (BD Bioscience); IgG1 mouse anti-p230 (BD Transduction Laboratories). FACS: Pacific-Blue conjugated mouse anti-conformed HLA-I (clone w6/32; Biolegend); Alexa-647 conjugated mouse anti-HLA-A2 (clone BB7.2; AbD Serotec); PE conjugated mouse anti-HLA-B7 (clone BB7.1, Millipore); PE conjugated mouse anti-CD71 (clone CY1G4, Biolegend). Anti-CD1a (clone B17 home made from hybridoma) was used for immunoprecipitation and confocal, BL6 clone (Beckman Coulter) and NA1/34 clone (Serotec) for cytometer and goat- (C19, Santa Cruz) and rabbit anti-CD1a (Abcam) were used for immunoblots.

Mice and dendritic cells.

Male C57BL/6, MARCH9 ^{-/-} C57BL/6 mice and OT-II mice were bred at the CIML animal facilities. Experiments were conducted in accordance with institutional guidelines for animal care and use. Protocols have been approved by the French Provence ethical committee (number 04/2005). MARCH9 ^{-/-} mice were the kind gift of Prof. Paul Lehner (University of Cambridge) generated by Genoway through targeted deletion of the first 5 exons of the MARCH9 gene on chromosome 10. BM-derived DCs from these mice, were differentiated *in vitro* from the bone marrow of 6-8 weeks old male mice, using either FLT3 ligand or GM-CSF. Flt3L-DCs were used for experiments between days 6 and 7. Flt3L was produced using B16-Flt3L hybridoma cells. Bone marrow progenitors were plated at 1.5×10^6 cells/ml, 4ml/well in 6 well plate, and cultivated with RPMI (GIBCO), 10% FCS (Sigma Aldrich), 100 units/ml penicillin and 100 μ g/ml streptomycin (GIBCO), 50 μ M β -mercaptoethanol (VWR) and Flt3L. For sorted Flt3L-DCs, cells were gently harvested at day 7 with cold PBS 2% FCS, centrifuged 1200 rpm 5 min at 4°C, counted and stained with for 30mins at 4°C. CD8⁺ dendritic cells were purified using a negative selection kit from Miltenyi. For splenic DCs, splenocytes were dissociated injecting Liberase TL, followed by 25 min incubation at 37 °C. Cells were washed using MACS buffer (PBS 1X + 1% FBS + 2mM EDTA) and passed in a cell strainer 70 μ m, then centrifuged 5 min at 450 RCF, 4°C. Red blood cells were lysed using the

commercial buffer from eBioscience. Dendritic cells were purified using CD11c⁺ positive selection kit from Miltenyi. Splenic DCs were then resuspended in the same medium as for Flt3L-DCs at 1. 10⁶cells/ml and plated in a 12 wells plate.

Flow cytometry. Cells were harvested in PBS-5mM EDTA, washed in cold PBS with 1% FCS and stained using appropriate fluorochrome conjugated antibodies mixed in PBS with 1% FCS and 10% mouse serum for 30min at 4°C. After washes, cells were fixed in PBS with 2% PFA. Samples were read on FACS CANTO II (BD Bioscience) and analysed using FlowJo. For Spleen cells analysis, a 13 antibodies/14 colors/16 parameters panel including CD5, CD11b, CD11c, CD19, CD64, CD117, CD161, CD192, Ly6C, Ly6G, F4/80, MHCII, SigF was used to characterize the spleen cells of MARCH9^{-/-} and control mouse. 1x10⁶ cells were blocked with anti-CD16/CD32 antibody and stained with a 14 colors/16 parameters antibody panel including CD5, CD11b, CD11c, CD19, CD64, CD117, CD161, CD192, Ly6C, Ly6G, F4/80, MHCII, SigF was used to characterize the spleen cells of MARCH9^{-/-} and control mouse. Data were acquired onto BD LSR2 Fortessa on standardized analysis matrix using BD FACS DIVA 6.1.3 software.

Molecular biology and cell lines.

Constructs coding KSHV ubiquitin ligases K3-Flag and K5-HA were a gift from Laurent Coscoy (Berkeley, CA, USA). MARCH-9 cDNA was amplified by PCR from a HeLa cDNA library and cloned in frame with eGFP into the pEGFP-N1 vector (Clontech, Mountain View, CA). cDNAs encoding 4 mutants of human CD1a were generated by PCR using human CD1a in pcDNA3.1-zeo as a template DNA. CD1a was mutated in the cytoplasmic tail, deleting 5 of the 6 amino acids (CD1a noTail), substituting the lysine with arginine (CD1a noK), two cysteines with glycine (CD1a noC), or both lysine and cysteines (CD1a noK/C). The same sense primer 5'-AATGATATCGACCAATTTTTCTGAGAGAAGG-3' was used in the 4 PCR, while different antisense primers were used depending on the request mutation. In particular 5'-AATCTCGAGTTACCTGAACCAAAGCG-3' antisense primer was used for CD1a noTail, 5'-GGTGTGTCTTAACAGAAACAGCGTCTCCTGAACC -3' for CD1a noK, 5'-CATGGTGTGTCTTAACCGAAACCGCGTTTC- for CD1a noC and 5'-CATGGTGTGTCTTA ACCGAAACCGCGTCTC- for CD1a noK/C. The PCR products were inserted in pcDNA3.1Topo vector (Invitrogen) and following digestion

by XbaI they were cloned into CD1a-pcDNA3.1-zeo plasmid. The constructs were tested by PCR and sequenced. CD1a mutants were cloned in frame with eGFP into the pEGFP-N1 vector (Clontech, Mountain View, CA). CD1a-eGFP fusions were excised by restriction enzyme digestion and cloned into the pGEM4Z-A64 vector (kindly provided by E. Gilboa, University of Miami, Miami, Florida, USA) containing a synthetic poly-A tail. CD1a-eGFP mRNAs were generated by *in vitro* transcription (IVT) using the mMESSEAGEmMACHINE T7 kit (Ambion, Austin, TX) after SpeI vector linearization and according to the protocol provided by the manufacturer. Purification of IVT products were performed with RNeasy Mini kit (Qiagen). Quantity and purity of generated IVT products were determined by ultraviolet (UV) spectrophotometry and by formaldehyde/agarose gel electrophoresis. RNA was stored at -80°C in small aliquots. We generated stably transfected CD1a-HeLa cell lines, using Lipofectamine 2000 and selection in DMEM medium supplemented with 10% FBS and 500 $\mu\text{g}/\text{ml}$ Zeocine. In order to verify CD1a mutant expressed in each cell line, cDNA obtained from each CD1a cell line was tested by PCR, to amplify the cytoplasmic tail of CD1a, by appropriate primers and the PCR products were sequenced.

Measure of HLA-I surface endocytosis and recovery by acid stripping assay.

For recovery measurement, 2h brefeldin A (BFA)-treated cells were washed in PBS and resuspended in 1 ml of 0.2 M citric acid/0.2 M Na_2HPO_4 buffer (pH 3.0) and incubated on ice for 2 min. Cell suspensions was equilibrated by adding excess of ice-cold DMEM and washed two times. Then, cells were stained for flow analysis or resuspended in complete medium with or without drugs for indicated times. Cell surface HLA-I molecules were measured by FACS after staining with anti-HLA-I (w6/32) or anti-HLA-A2 (BB7.2) specific antibodies. For HLA-I surface endocytosis, cells were harvested, washed once in cold PBS and stained with biotinylated anti-HLA-I Fab (w6/32) for 30min at 4°C in PBS 1% FCS. After extensive washes, cells were incubated at 37°C in complete medium for indicated times, harvested, washed in ice-cold PBS, surface stained with conjugated anti-biotin fluorescent secondary antibodies for 15min at 4°C . Cells were fixed in 2% PFA prior Flow cytometry analysis.

Antigen presentation experiments.

Cross-presentation was assayed as described in³¹. Endotoxin free Ovalbumin (EndoFit™ Ovalbumin, Invivogen, Toulouse, France) was attached to 3mm latex beads by passive adsorption in PBS at 4°C, and extensive washes in PBS. Spleen DCs were allowed to phagocytose OVA beads for the indicated times. Alternately, DCs were loaded with soluble ovalbumin or specific OVA peptide as a control. Cells were fixed with 1% paraformaldehyde in 96 flat-well plates, washed, and cultured for 18 hr with the B3Z, CD8+ T cell hybridoma cells. Primary OT1 T cells were isolated from spleen and lymph nodes by magnetic depletion (Dyna, Invitrogen), labeled with 2 μ M CFSE where indicated, and co-cultured with 5000 DC at a 4:1 (T:DC) ratio. Phagocytosis and FACS pH measurement were performed as described²⁶, et al. For T cell hybridoma activation by endogenous antigen presentation, transfected bmDC were fixed mildly (0,25 % PFA, 2 min, RT), and cultured at 2:1 (T:DC) ratio B3Z cells for 24h. IL-2 production was determined by ELISA (eBiosciences).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software. we performed Student's t-test or Welch t-test (according the validity of homoscedasticity hypothesis).

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FIGURE LEGENDS

Figure 1. HLA-I is ubiquitinated on cytoplasmic tail lysines and MARCH9 is up-regulated during DC activation. **A.** lysates from human B (Daudi and Raji), T (Jurkat), monocyte (U937), and HeLa cell lines as well as primary MoDC, were immunoprecipitated with the anti-HLA-I w6/32 mAb. Samples were analyzed by western blot using anti-ubiquitin (P4D1) anti HLA-I ab (3B10.7). Asterisks correspond to unspecific bands and arrowheads to ubiquitinated form of MHC I. **B.** HeLa cells were transfected with HLA-A2 (His-tagged, His-A2) or HLA-B7 (HA-tagged, HA-B7) molecules, WT or mutated on all lysines of the cytosolic domain (Kless). Lysates were IP with w6/32 and samples were submitted to SDS-PAGE and western blot with anti-ubiquitin, anti-HLA-I and anti-tag antibodies. **C.** LPS-activated MoDC lysates were IP with w6/32 and analyzed by SDS-PAGE and western blot for ubiquitination. **D.** qPCR for MARCH1 and MARCH9 mRNA expression in human MoDCs stimulated with LPS for the indicated time. qPCR for MARCH9 mRNA expression in mouse GM-CSF DCs exposed to LPS or pl:C for the indicated time (n=3 in duplicates). **E.** Analysis of HLA-I during MoDC activation with LPS. (left) FACS monitoring of surface peptide-loaded HLA-I levels, (center) measure of HLA-I neo-synthesis with ³⁵S (met) labeling followed by IP and (right) fluorescent-labeled W6/32 Fab uptake during time. Mean ± SEM of n=3 independent experiments.

Figure 2. MARCH9 promotes HLA-I and CD1a ubiquitination. **A.** HeLa cells were stably transfected with CD1a. After over-expression of KSHV ubiquitin ligases K3, K5 or different tagged versions of MARCH9 and MARCH2 (-eGFP or -Myc) HLA-1 and CD1a surface expression and ubiquitination was monitored. **A)** Flow cytometry staining demonstrates that MARCH9-eGFP over-expression decreases both HLA-1 and CD1a surface levels, one representative experiment of n=3, o, re **B.** (Left) overexpression of MARCH9 induces a different pattern of HLA-I ubiquitination, than K3 or K5 expression, while MARCH2 has no measurable activity on HLA-I. Amino acid sequences alignment of HLA-A2 and HLA-B7 cytosolic

domains. Lysines (K, red) were replaced by arginine residues (R) to create different K-mutants (e.g. K-less). Mutants were co-transfected with MARCH9-GFP and K3, prior surface levels monitoring with HLA-I antibodies. As expected K-less mutants remained insensitive to MARCH9 expression, while surface expression of the other mutants was decreased. **C.** (Left) Like HLA-I, CD1a is ubiquitinated upon over-expression of MARCH9 in stably expressing HeLa cells. Amino acid sequence alignment of the cytoplasmic tail of CD1a and mutants constructs. In mutants lacking the lysine (noK and noK/C), K was replaced by an arginine residue (bold R) and in constructs lacking cysteines (noC and noK/C), C was replaced by glycine (bold G). After transfection, individual clones were selected to express similar levels of CD1a, as demonstrated by western blot for CD1a and actin. MARCH9 expression induced a strong decrease in CD1a surface levels, except for mutants lacking the lysine residue (noK and noK/C). Mean \pm SEM of n=3 independent experiments. Student's t test. *p<0.05, **p<0.01.

Figure 3. Ectopic expression of MARCH9 influences CD1a distribution. A. HeLa cells were stably transfected with different CD1a constructs. Cells transiently transfected with MARCH9-GFP (green) were analyzed by confocal microscopy for CD1a or HLA-I intracellular distribution. MARCH-9 expression induces HLA-I and CD1a accumulation in intracellular organelles. **B.** MARCH9 induces CD1a (red) and HLA-1 (green) accumulation in early endosomal vesicles positive for EEA1 (blue). [Calculation with IMAGEJ of Pearson's coefficient values above R=0.5 indicate a significant co-localization among indicated staining.](#) **C.** CD1a-expressing HeLa cell lines (WT, noK or noC) were transiently transfected with MARCH9-GFP and analyzed by confocal microscopy. MARCH9 induced the intracellular accumulation of CD1a WT and noC (red), with a slight enrichment in the area of the Golgi and a strong co-localization with the ligase. Replacement of the lysine residue in the cytoplasmic tail of CD1a (noK, red), prevented its intracellular accumulation in response to MARCH9 expression (green). **D.** Endogenous CD1a distribution in immature MoDC (left) or 24h LPS-activated (center). LPS promotes the near to complete re-localization of CD1a at the plasma membrane. Upon short LPS activation (4H, right), CD1a (red) is mostly localized at the cell surface and intracellular vesicles positive for the M6P-R (green) and not EEA1 (blue). Scale Bars, 10 μ m.

Figure 4. MARCH9 accumulates in TGN-associated compartments and targets HLA-I to syntaxin 6 positive compartments. **A.** Comparison of HLA-I surface levels (Flow cytometry) upon plasmid cDNA-driven (24h, upper panel) or mRNA-driven (6-8h, lower panel) MARCH9-eGFP expression in HeLa cells. After mRNA transfection, HLA-I surface levels are not affected by the presence of MARCH9. **B.** Confocal microscopy analysis of MARCH9-eGFP mRNA transfected HeLa cells (enlarged areas are indicated by square). Pericentriolar MARCH9-eGFP co-localized mostly with TGN area positive for TGN46, p230, and SCAMP3, while peripheral vesicular containing MARCH9-eGFP and HLA-I molecules were strongly positive for Stx6 and CI-M6P-R. Scale bars, 10 μ M. Calculation with IMAGEJ of Pearson's coefficient values above R=0.5 indicate a significant co-localization among indicated staining in entire cells. However, "Peri" indicates that R was calculated for peripheral areas and "TGN" indicates the R for co-localization in the Golgi/TGN area.

Figure 5. MARCH9 silencing delays cell surface arrival of HLA-I molecules. Lentiviral vector expressing shRNA targeting specifically MARCH9 were used to generate HeLa or HeLa stably expressing HLA-A2 (HeLa-A2) cell lines knock-down for MARCH9 (HeLa con and HeLa M9-KD). **A.** (top) M9 mRNA level was analyzed using RT-qPCR in HeLa con and HeLa M9-KD (n=3). (bottom) MARCH9-myc transfected HeLa cells were radiolabelled with [35S]methionine and [35S]cysteine for 15 min and chased for different times prior immunoprecipitation and SDS-PAGE separation. **B)** HLA-I and TfR surface levels monitored by flow cytometry in HeLa con and HeLa M9-KD (Thin lines=isotype control. Measure of HLA-1 synthesis after immunoprecipitation (IP) with w6/32 conjugated beads of [35S] labeled material from HeLa con and HeLa M9-KD. Neosynthesis rate of HLA-I molecules was not affected in M9-KD cell lines. Cell lysates of indicated cell lines were also western blotted for HLA-I level. Actin immunoblot is shown for equal loading control. **C)** Representative flow cytometry quantification of w6/32 antibody (Fab) uptake by M9-KD (dotted line) or control (filled line) HeLa or HeLa-A2 cell lines. Cells were stained with w6/32 Fab for 30 min at 4°C, warmed at 37°C for indicated times, and stained

with a secondary anti-mouse antibody at 4°C before FACS analysis. Kinetic of surface HLA-I internalization was strikingly similar in M9-KD and control cell lines. Results are representative of n=3 independent experiments and error bars indicate the SD of triplicate performed for the experiment shown. **D.** Control and M9-KD HeLa cell lines were radiolabelled with [35S]methionine and [35S]cysteine for 15 min and chased for different times. HLA-1 was immunoprecipitated using w6/32 conjugated beads and endoH treatment was performed. Samples were analyzed by SDS-PAGE separation and phosphorimager. HLA-I endoH sensitive form is shown by * and ** marks endoH resistant form. Results are representative of n=3 independent experiments. **E. (Left)** Control or M9-KD HeLa or HeLa-A2 cell lines were acid stripped. After washes, cells were cultured for different times with or without BFA and HLA-I surface recovery was monitored by FACS using w6/32 Ab or anti-HLA-A2 specific Ab for HeLa-A2 cell lines. Graph represented percentage of surface recovery normalized to HLA-I level before acid wash and HLA-I level just after acid wash was subtract. In both cell lines (HeLa and HeLa-A2) MARCH9 silencing affected HLA-I surface recovery. **(Right)** Indicated cells were transfected with His-A2-WT or Kless c-DNA. 16h after transfection cells were acid stripped and treated as above. HLA-A2-Kless recovery at cell surface was significantly reduced compared to WT molecule and reached the level of recovery obtained in HeLa-M9-KD cell line. Interestingly, lysine mutation has no impact on the His-HLA-A2 recovery in HeLa-M9-KD. Data are from one representative experiment out of n=3 performed. Error bars indicate the SD of triplicate.

Figure 6. MARCH9 silencing induces HLA-I accumulation in TGN-related intracellular compartments. A. Control and M9-KD HeLa cell lines were stained for HLA-I using W6/32 Ab (red) and intracellular compartment markers using specific Ab (green). Confocal microscopy analysis showed an increase of HLA-I intracellular accumulation in Stx6, SCAMP3 and TGN46 compartments in M9-KD compared to control cells. [Calculation with IMAGEJ of Pearson's coefficient values above R=0.5 indicate a significant co-localization among indicated staining. in the entire cells.](#) **B.** Confocal microscopy analysis of Syntaxin 6 (Stx6, green) and MHC I (Kb) (red) subcellular localization in WT and MARCH9 -/- Flt3L-derived DCs

stimulated with pl:C for 2h. Increased co-localization of Stx6 and MHC I molecules in the TGN (arrow heads) of MARCH9 ^{-/-} cells compared to WT (arrows) is indicated by a Pearson's coefficient of 0.62. Scale bars, 10 μ M.

Figure 7: Cross-presentation is inhibited in MARCH9-deficient cells.

A. MHC I surface detection by FACS in WT and MARCH9 ^{-/-} spleen CD11C⁺ DCs subsets, including B220⁺ plasmacytoid DCs. **B.** Monitoring of direct MHC I presentation after WT and MARCH9 ^{-/-} bmDC transfection of eGFP-SL8 fusion mRNAs and surface detection of SL8-K^b complexes using the 25D1.16 antibody. **C.** Ova-coated latex beads phagocytic uptake in WT and MARCH9 ^{-/-} spleen CD11c⁺ DCs measured by FACS at 4°C and 37°C after 1h. **D)** Kinetics of phagosomal pH after a 20 min phagocytosis pulse with pH-Sensitive (FITC) and insensitive (A647) fluorochrome-labelled beads (3 μ m) and different times of chase in DCs. Results are shown as a ratio of FITC/A647 MFI, which remains unaffected by MARCH9 deletion. **E)** *In vitro* cross-presentation of OVA coated beads (left panel) or control OVA peptide (SL8, right panel) by WT and MARCH9 ^{-/-} spleen CD11c⁺ DCs to B3Z SL8-specific T cells was monitored through IL-2 production. **F.** *In vitro* cross-presentation of soluble ovalbumin (left panel) and control OVA peptide (SL8, right panel) to primary OT1 cells by WT and MARCH9 ^{-/-} Flt3L-derived DCs, stimulated or not with poly(I:C). The results shown in B,D and E (mean \pm SEM) are representative of n=5 experiments or n=3 experiments for C and F.