Invariant chain with an AP3 interacting sorting signal is sorted to late endosomal compartments and may improve MHC class I loading and presentation.

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ABSTRACT

Invariant chain (li) is traditionally known as the dedicated MHCII chaperone. Recent reports have broadened our understanding about various tasks that li plays including its physiological role in MHCI cross-presentation. li bound MHCI via the MHCII scaffolding CLIP peptide may facilitate MHCI trafficking to the endosomal pathway. The sorting function of li depends on two leucine-based sorting signals present in the cytoplasmic tail that acts as binding sites for the adaptor proteins AP-1/AP-2. Here we increased the li cross-presentation potency by replacing these with an AP3 motif resulting an efficient transport of li from TGN to late endosomes. We also replaced the CLIP region of li with a therapeutically relevant peptide, MART-1. We found the li AP3mutant-MART1 construct was capable of loading MHCI and stimulate specific T-cell response more efficiently than the wild type counterpart. The results show that li with an AP3 binding sorting motif carrying peptide epitope(s) can

promote efficient antigen presentation to cytotoxic T cells (CTLs) independent of the ER located classical MHCI peptide loading machinery.

Introduction

Vaccination demands successful immune response that in turn depends on activation of both CD8⁺ and CD4⁺ T cells in the context of major histocompatibility complex I and II (MHCI and II) antigen presentation. The processing of the antigen and its subsequent presentation on MHCI or MHC II at the surface of antigen presenting cells (APCs) requires the coordinated action of different accessory molecules and chaperones. Many strategies have been described to obtain a robust immune response, such as the use of carrier proteins to improve peptide loading to MHC molecules, and targeting of antigens to “favorable” intracellular pathways where MHC reside ¹⁻⁷. MHCI and MHCII are not loaded by the same cellular machinery, and they are dependent on different trafficking signals. A successful MHC II antigen presentation largely depends on Ii and peptide loading in the endosomal pathway, while MHCI peptide loading is independent of Ii and occurs primarily in the endoplasmic reticulum ⁸.

The targeting to the endosomal pathway of MHCII relies on the two-leucine-based sorting signals Leu⁷/Ile⁸ and Met¹⁶/Leu¹⁷ of Ii ⁹⁻¹¹. These signals sequences are present in the cytoplasmic tail of Ii and bind the adaptor proteins AP-1 and AP-2 ^{12, 13}. These APs are generally found at the trans-Golgi network (TGN) (AP1) and plasma membrane (AP2) and act as coat proteins that bind the donor membrane in order to assemble a scaffold for vesicle budding ^{14,15}. The APs thus mediate sorting from the TGN to endosomes directly or via the cell surface. Upon entry into the endosomal pathway, Ii is sequentially degraded leaving the class II-associated Ii peptide (CLIP) bound to the MHCII groove. CLIP is subsequently exchanged for specific antigenic peptides in the later parts of the endosomal pathway prior to transport to the cell surface for presentation to CD4⁺ T cells. Several vaccination studies have

shown that replacement of the CLIP region with an antigenic peptide can lead to efficient MHCII loading and specific presentation to CD4⁺ T cells ^{1, 2, 16}.

The classical view is that MHCI encounters its (endogenous) antigenic peptides in the ER and this complex is transported to the PM and presents the antigen to the cytotoxic T cell ¹⁷. However, it was demonstrated a few years ago that MHCI like MHCII may be loaded in the endolysosomal pathway guided by li ¹⁸, but it was not shown where in the endosomal pathway this took place. An li-MHCI interaction was also demonstrated by van Luijn and coworkers who showed that CLIP efficiently binds to several MHCI molecules in leukemic cells ¹⁹. Furthermore, our recent study showed an li with CLIP substituted by a MHCI specific tumor antigen is efficient enough to load MHCI and activate T cell specific response in a proteasome/TAP/tapasin independent manner ²⁰. This strategy was found to be as efficient as exogenous loading of synthetic peptide *in vitro*, and thus identified a novel loading pathway for MHCI which may lead to novel vaccine strategies. In one of our earlier study, we have shown that it is possible to redirect a fusion protein with the li tail to late endosomes by introducing this AP-3 binding motif to the cytoplasmic tail of the li ²¹. Here we show that the introduction of AP-3 binding motif in li itself re-routed this molecule to proteolytic late endosomal compartments skipping the conventional trafficking route via the PM. As a consequence, this AP3 containing li protein had a dramatically shorter half-life than its wt counterpart. We have further investigated the potency of li-MHCI mediated antigen presentation with the li trafficking mutant designed to bind AP-3 ^{21, 22} where we replaced the CLIP region with the cancer relevant peptide MART-1 ^{20, 23, 24}. This mutant with CLIP/Mart-1 replacement was found to have an improved potency to activate CD8⁺ T-cells compared to the liwt and was able to increase the amount of peptide-MHCI on the plasma membrane (PM). Taken together, we find that an improved li-based antigen loading/presentation of this peptide may be achieved by routing the li and most likely also MHCI to a late stage of the endosomal pathway.

RESULTS

Biochemical characterization of liR4RP₆/L₁₇A

The liwt sorting signal (Q₄RD₆)L₇I, found to bind the adaptors AP-1 and AP-2¹³, was replaced as shown in Figure 1A by an (R₄RP₆)L₇I motif, which is a strong AP-3 binder²² and found to mediate direct TGN to endosomal sorting²¹. We first tested whether the invariant chain with the AP3 motif, liR4RP₆/L₁₇A was a trimer. In addition to li wild type we included the double leucine mutant liL₇A/L₁₇A known to accumulate at the cell surface due to inactive sorting signals²⁵. As shown, all constructs were able to form trimers suggesting that the cytosolic tail mutations did not affect the trimer assembly (Fig. 1B). The abundance of mutant li trimers even in the reduced sample shows the efficiency of the mutant li in making stable trimers that did not compromise on its structural stability (Fig. 1B). However, the protein amount of the AP3 mutant was significantly less than the two others.

Arginine motifs may mediate ER retention and the li mutant (MDDRRPL₇I) could therefore affect li release from the ER thus reducing the total protein level of li^{26,27}. To test for this, we performed an Endoglycosidase H (Endo H) treatment, where passage through the Golgi apparatus prior to endosomal sorting is monitored by acquisition of Endo H resistance²⁸. Three li fractions were detected for the wild type and all the mutants of li (Fig. 1C). Thus, all constructs gained Endo H resistance indicating that despite the presence of the RRP amino acid sequence, the li mutant can egress the ER. Further to investigate why the level of liR4RP₆/L₁₇A is differed from liwt, we performed a pulse-chase experiment to monitor the half-life ($t_{1/2}$) of this protein. Transfected cells were pulsed with ³⁵Met/³⁵Cys containing media, and chased for various time points, followed by an immunoprecipitation of total li. As shown in Figure 1D, the $t_{1/2}$ of liwt was approximately three hours, whereas liR4RP₆/L₁₇A had a half-life closer to one hour, which suggests a faster kinetic to late endosomal compartments.

liL₇A/L₁₇A shown to accumulate at the plasma membrane¹¹ was not degraded after four hours and served as a control. As shown earlier, a TFR fusion protein with the liR4RP₆/L₁₇A cytosolic tail can be targeted directly to the late endosomes/lysosomes²¹. The short half-life

indicates that li with the liR4RP₆/L₁₇A tail also followed this pathway. To test for such a late endosomal proteolytic localization of liR4RP₆/L₁₇A, we added either the Cathepsin S inhibitor or the broad protease inhibitor Leupeptin, both taken up by endocytosis to the transfected cells. A combination of both cathepsin S and leupeptin were able to protect liR4RP₆/L₁₇A from degradation while the li L₇A/L₁₇A remained protected with either of the protease inhibitors (Fig. 1E). As expected, the li wild type, trafficking to the endosomal pathway via the PM^{29,30} was also protected by both Leupeptin and Cathepsin S. Interestingly, the liR4RP₆/L₁₇A mutant needed both the leupeptin and the cathepsin A inhibitor for maximal inhibition, most likely as this construct is targeted to late endosomes which are more difficult to reach by the endocytosed inhibitors than the wild type li which traffics via the PM.

AP-1 is located at the TGN and AP-2 is a plasma membrane adaptor¹⁴, AP-3 is involved in binding and sorting of proteins to late endosomes and detected both at the TGN and between early and late endosomes and is therefore believed to be involved both in sorting from TGN to late endosomes and endosomal maturation³¹. To further study the pathway of our AP3 binding construct, we performed RNAi depletion of AP-3 and investigated the effect on the protein level. As shown in Figure 1F, AP-3 depletion resulted in a dramatic accumulation of both liR4RP₆/L₁₇A and liwt was also protected from degradation, but less. Together with the protective effect of the protease inhibitors in the endosomal pathway, such a strong protective influence of AP-3 depletion on liR4RP₆/L₁₇A is in line with a hypothesis that liR4RP₆/L₁₇A is sorted directly from TGN to the late proteolytic pathway. The control liwt is less affected as it is sorted via the PM and only affected by the endosomal maturation inhibition caused by the AP3 inhibition.

Subcellular distribution of liR4RP₆/L₁₇A

The subcellular distribution and trafficking of liR4RP₆/L₁₇A was further investigated using live cell confocal imaging approach. Madine Derby Canine kidney (MDCK) cells were chosen for

their high tolerance for laser exposure. The cells were transfected with either liwt or liR4RP₆/L₁₇A N-terminally fused with red fluorescent protein mCherry, together with early and late endosomal markers, GFP-Rab5 or GFP-Rab7a respectively. To measure trafficking via the PM the cells were incubated with anti li-antibody, M-B741-Alexa647, one hour prior to imaging. It is furthermore known that liwt imposes enlargement of endosomes and causes a delay in endosomal maturation^{29, 32-34}. Due to this delay, at early time points the antibody reach primarily early endosomes, but gradually throughout the next 2-4 hours, the antibody was also seen in late endosomes^{20, 29}. We also observed enlarged endosomes in the cells transfected with liwt (Fig 2A and B) and colocalization with GFP-Rab5 and GFP-Rab7a, distributing almost equally (55-60%) within early and late compartments. In addition, more than 60% of liwt found to colocalize with M-B741-Alexa 647 (Fig. 2C), confirming trafficking via PM.

In contrast, mCherry-liR4RP₆/L₁₇A showed a 75% colocalization with the late endosomal marker GFP-Rab7a and less than 10% with GFP-Rab5 (Figure 2A and B). This cellular distribution indicated that the li mutant followed a direct sorting route to late endosomes circumventing the cell surface. In further support, we demonstrate that only 12% of mCherry-liRRP/L₁₇A colocalized with M-B741-Alexa 647 (Fig. 2C), which corroborates direct sorting to late endosomes, and is in line with our biochemical characterization of liR4RP₆/L₁₇A. Because of its rapid turnover (Fig. 1D), and the accumulation in late endosomal compartments, liR4RP₆/L₁₇A, did not delay endosomal maturation. The residual uptake of M-B741 was most likely due high expression level of the mutant li in some of the cells being missorted to the PM. We confirmed our observations with li transduced SupT1 cells. In this assay, the li membrane expression was monitored by staining cells without prior permeabilization and later analyzed by flow cytometry (Fig. 2D). This demonstrated that the li mutant did not appear on the cell surface. Finally, we used live confocal imaging and confirmed that the liR4RP₆/L₁₇A mutation did not affect the previously described MHCI-li association²⁰(Sup Fig. 1).

Soluble T cell receptors detect peptide loaded HLA-A2 from liR4RP₆/L₁₇A

Since the liR4RP₆/L₁₇A mutant is re-routed to a degradation trafficking pathway, we tested whether such a CLIP replaced li construct combined with this tail mutation could still load MHCI. To this end, we first detected that the peptide-MHC complex of cell expressing different constructs with a soluble T cell receptor (sTCR)³⁵ specific for MART1p. sTCRs have a low affinity for their target, however, we were able to detect li-MART1p, inserted in the CLIP region of liR4RP₆/L₁₇A (Fig. 2E). Although very low, this result suggests that the peptide was well loaded on the MHCI molecule. As a control, cells expressing HLA-A2 single-chain trimer (SCT) combined with MART-1 peptide (SCT-M1) were used and showed an expected saturating signal. Taken together, our data support an improved antigen loading ability of liR4RP₆/L₁₇A over the li wild type construct. In addition, the trafficking of li to the plasma membrane does not seem to be required to get an efficient loading.

Cells expressing li carrying tumor-associated epitopes efficiently load HLA-A2 and specifically activate CD8⁺ T cells

We have previously shown that li interacts with MHCI, and that the human MHCI allele, HLA-A*02:01 (HLA-A2) colocalized with li throughout the endosomal pathway. We have also demonstrated that CLIP-replaced li efficiently activated antigen specific CTLs when expressed in HLA-compatible APC²⁰. We therefore compared the ability of the liR4RP₆/L₁₇A mutant to load HLA-A2 peptides with the CLIP-replaced li construct (Fig. 3A). J76 cells stably expressing MART-1 specific TCR (DMF5)³⁵ were incubated with HLA-A2 positive cells expressing different li constructs. IL-2 secretion was used as a read-out for specific TCR stimulation; SCT-MART1 and SCT with an irrelevant peptide (SCT-irr) expressing cells were used as controls. When MART-1 peptide was loaded on HLA-A2 utilizing liR4RP₆/L₁₇A as carrier, the intensity of the stimulation was almost equal to the stimulation observed with SCT-M1, confirming an increase in peptide loading compared to liwt (Fig. 3B). In order to support these data, we performed a DC priming study using autologous donor cells. To this

end, we assessed the priming ability of DC transfected with li mutant (li17R4RP₆/L₁₇A MART-1) compared to liwt MART-1 or the MART-1 peptide. We found that the mutant li (li17R4RP₆/L₁₇A MART-1) was significantly more efficient and superior to peptide at priming primary CD8⁺ T cells, whereas liwtMART-1 seemed to be improved but did not reach significance (p=0,052, Fig. 3C), hence at this stage can be considered equal to peptide loading. In addition the li17R4RP₆/L₁₇A MART-1 was not significantly superior to liwtMART-1 (p=0.53, not shown). Taken together we can at this stage only conclude that the new construct is functional in DCs, but we might require to test more peptides before we can reach the same conclusions as in the Jurkat system (Fig. 3B). This is in agreement with our previous data where we show that liwt construct performed as efficiently as peptide loaded cells ²⁰. A possible explanation could be that a mutant li and wild type bind differently to MHCI. However, by co-immunoprecipitation experiments we found no difference in MHCI binding to the mutated li (li17R4RP₆) as compared to binding to wild type li (Sup Fig. 2). Together these data support the proposition that liR₄R₆P/L₁₇A improved the loading of peptide placed in CLIP region for MHCI mediated antigen presentation.

DISCUSSION

Previous studies have revealed that neither MHCI nor MHCII are AP3 dependent for trafficking and antigen presentation ³⁶. The kinetics of li transport and degradation are also unaffected in cells lacking AP-3 ³⁷. Through introduction of the AP-3, instead of the AP1/AP2 binding motif, we successfully re-routed the li towards late endosomal compartments. In addition to the re-routing of li, the insertion of AP3 binding motif increased the kinetics of trafficking of li to the late endosomal compartment. Overall, our effort of bringing mutations in the li created a strict and direct sorting pathway with improved MHCI peptide loading efficacy. Here, we characterized the sub-cellular distribution of liR₄R₆P/L₁₇A and compared with the liwt. Equal distribution of liwt to the early and late endosomes was observed

whereas liR4RP₆/L₁₇A was found to be mainly colocalizing with late endosomal markers. The direct sorting to endosomes also overcomes the property of delayed endosomal maturation substantiating a faster antigen processing.

Recent studies have shown the role of li in trafficking of MHCI to the endosomal pathway and its implication in cross presentation^{18, 20}. The description of li as a vehicle to perform antigen presentation has brought this molecule to the doorstep of the clinic not only as a target for immunotherapy³⁸ but also as a vector for increasing immune reactions towards specific oncogenic antigens²⁰. Genetic exchange of the CLIP region with a peptide antigen substantially loads MHCI and presents the antigen on the cell surface. Our results showed that the antigenic peptide carried by liR4RP₆/L₁₇A can be detected by T cell carrying a specific TCR. Additionally, the antigen detection by the sTCR supports the specificity and the robustness of the liR4RP₆/L₁₇A mutant loading. Taken together, our data show that the modification in trafficking induced by the liR4RP₆/L₁₇A mutation can improve peptide loading and thus MHCI-peptide levels at the cell surface. In this study, liR4RP₆/L₁₇A carrying MART-1 antigen was shown to be equally efficient in activating specific TCR carrying cells in comparison to liwt. In addition, we found that liR4RP₆/L₁₇A mutant was also competent to load enough peptide onto DC in order to prime primary naive T cells. These studies confirm the capacity of MHCI loading in the endosomal pathway and establish that the loading may take place in the proteolytic later parts of this pathway. Additional studies will be necessary to determine if such and AP3 binding mutation will be advantageous *in vivo*, for instance to improve immunotherapy using modified li as an immunization vector.

METHODS

Recombinant cDNA constructs

cDNA encoding human lip33 wt⁹, was subcloned into the pcDNA3 expression vector at *KpnI*-*Bam*HI. Human lip33 mutants, li L₁₇A and li L₇A L₁₇A, in the PSV51L expression vector

have also been described ²⁵. *KpnI* and *BamHI* restriction sites were introduced up and downstream of the li sequences respectively by PCR and the following primers were used: li-*KpnI* forward 5' AGAGA GGGTACCGTCATGGATGACCAGCGCGAC 3'. li-*BamHI* reverse - 5' AGAGAGGGATCCTCACATGGGGACTGGGCCCAG 3'. The li mutants were thereafter subcloned into pcDNA3 at *KpnI*-*BamHI*, behind the T7-RNA polymerase promoter, and li L₁₇A was subsequently used as template for PCR quick change mutagenesis (all reagents used were included in the kit; QuickChange® Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA)) in order to generate the AP-3 binding motif RRP ²¹. Primers used: L17A RRP sense 5' CCGTCATGGATGACCGTCGTCCTTATCTCCAACAATG 3' and L17A RRP anti-sense 5' CATTGTTGGAGATAAGGGGACGACGGTCATCCATGACGG 3'. All primers were purchased by Eurofins MWG Operon (Ebersberg, Germany). mCherry-li was made by cloning liwt in frame to the C terminal end of mCherry without the stop codon in pcDNA3 (a kind gift from Terje Espevik, NTNU, Trondheim, Norway). mCherry- liR₄R₆P/L₁₇A was purchased from GenScript (Piscataway, NJ, USA). HLA-A2-GFP has been described and transfections were carried out as already described ²⁰. GFP-Rab5 and GFP-Rab7 were supplied by Cecilia Bucci ³⁹. All CLIP-antigenic peptide constructs (liMART1) were cloned by site direct mutagenesis of the liwt and liR₄R₆P/L₁₇A construct subcloned in pENTR vector (Invitrogen, Oslo, Norway). The mutagenesis to change the CLIP peptide (MRMATPLLM) into antigenic peptides (MART1: ELAGIGILTV) was performed as described ²⁰. After sequence verification, these constructs were recombined into a Gateway-converted pCI-pA102 ⁴⁰

Cell culture, transfections and RNA interference

HEK293 cells, human epithelial HeLa-Oslo and Madin Darby Canine Kidney (MDCK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Bio Witthaker, Walkersville, MD, USA). All media were supplemented with heat-inactivated 10% fetal calf serum (FCS, HyClone, Logan, UT, USA). J76 were a kind gift from Miriam Hemskerk, (Leiden University

Medical Center, Leiden, The Nederland) SupT1 from Martin Pule (UCL, London, Great Britain), both cell lines were grown in RPMI+10% fetal calf serum. All cells were grown in a 5% CO₂ incubator at 37°C. Transient transfections were performed with either lipofectamine 2000 reagent from Invitrogen (Hek293 cells, MDCK) or with FuGENE 6 (ProMega) (HeLa), both according to manufacturer's protocols. For siRNA interference (RNAi) we used the following oligonucleotides; the sense μ 3A, 5'-GGAGAACAGUUCUUGCGGC-3' and the antisense 5'-GCCGCAAGAACUGUUCUCC-3' oligos, for negative control a scrambled sequence was used, sense: 5' ACUUCGAGCGUGCAUGGCUTT 3' and antisense scrambled control 5' AGCCAUGCACGCUCGAAGUTT 3'. All of the oligos were from Eurofins MWG Operon (Ebersberg, Germany) and are previously described ^{36, 41}. Transfection of HeLa with siRNA was performed as previously described ⁴².

Antibodies and reagents

M-B741 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Labeling of antibody was with Alexa-647 performed according to manufacturer's protocol (Invitrogen/Molecular Probes, Carlsbad, CA, USA). Anti-actin was purchased from AbCam, (Cambridge, UK). The anti AP-3 antibody is affinity-purified rabbit antiserum directed at its μ subunit and was a kind gift from Professor Margaret S. Robinson (Cambridge, UK). The secondary antibodies: sheep anti-mouse- and sheep-anti rabbit-HRP were acquired from Invitrogen/Bio-Rad (Hercules, CA, USA). Anti-FLAGM2 monoclonal antibody was purchased at Sigma-Aldrich (Oslo, Norway). Soluble DMF5 TcR was prepared as described by Walseng *et al.* ³⁵.

Biochemical analyses

Metabolic labeling was performed using S₃₅-labeled Cysteine/Methionine (Perkin Elmer, Waltham, MA, USA). Cells were seeded to 60%-70% confluence in 6-well plates; washed three times in Cys/Met-free DMEM; incubated in Cys/Met-free DMEM for 45 min followed by

a 30 minutes pulse with Cys/Met-free DMEM supplemented with 50 μ Ci S₃₅. For the pulse chase assay, the cells were washed three times in DMEM containing 2mM L-glutamine, primocin, and 30% FCS and chased for indicated time periods. Immunoprecipitations were done at 4 °C over night with 1-2 μ g ml⁻¹ antibody in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tx100) supplemented with the protease inhibitor cocktail Protease Arrest (G-Biosciences, St. Louis, MO, USA). Antigen-antibody complexes were captured with Protein G-coupled Dynabeads (Invitrogen) and re-suspended in gel loading buffer containing 2% SDS, 125mM TrisHCL, 20% glycerol and 5% β -mercaptoethanol, or its non-reducing β -mercaptoethanol free counterpart. The samples were boiled for 5 min at 95° C , loaded onto 4-20% Tris-HEPES-SDS gels (Pierce, Rockford, IL, USA), and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Antibody incubation was done in 5% skim milk (BioRad, Hercules, CA, USA) at room temperature and immunoprecipitated protein was detected using Amersham® ECL Plus Western Blot Detection System (GE Healthcare, Buckinghamshire, UK). Radioactivity, however, was detected directly on ECL films (GE Healthcare, Buckinghamshire, UK). For the experiments including protease inhibitors, the same procedure was followed as for metabolic labeling. During the 30 min S₃₅-Cys/Met pulse, 20 nM Cathepsin S Inhibitor (Merck Chemicals Ltd., Nottingham, UK) and/or 100 μ M Leupeptin (SIGMA ALDRICH) were added. The procedure was then continued as described above. For Endo H digestion, the beads were resuspended in 0.1 M sodium phosphate buffer (pH5.5) containing protease inhibitor as described above. The samples were divided into two and incubated for 15 minutes at room temperature with, or without 0,5 mU of Endo H (SIGMA). After the Endo H treatment, the samples were boiled at 95°C, and loaded onto gels as described above.

Flow cytometry

Indicated samples were acquired using a BD LSR II flow cytometer and the data were analysed using FlowJo software (Treestar Inc., Tilburg, The Netherlands).

Spinning disk – and confocal laser scanning microscopy

MDCK cells were grown to 70% confluence in 35 mm microwell dishes (MarTek, Ashland, MA, USA). The cells were then transfected with; GFP-Rab5, GFP-Rab7a, li constructs and HLA-A2-GFP/ β 2m. 1h prior to imaging, the cell medium was exchanged with complete DMEM without phenol red, and cells were incubated with M-B741-Alexa 647 to a final concentration of 1 μ g/mL. Live imaging was performed to eliminate fixation artifacts. Confocal images were acquired on an Olympus FluoView 1000 inverted microscope equipped with Plan/Apo 60/1.10 NA oil objective (Olympus, Hamburg, Germany). Constant temperature was set to 37 °C and CO₂ to 6% by an incubator enclosing the microscope stage. Fluorochromes were excited with 488nm, 543nm and 647nm lasers. All image acquisition was done by sequential line scanning to eliminate bleed-through. Live films were acquired using an Andor Revolution XD Spinning Disc microscope with PlanApo 60x1.42 NA oil immersion objective, as this microscope provides an ideal platform for high speed, high signal to noise imaging, with low bleach rate and low photo-toxicity. Three lasers were used; 488nm, 561nm, and 640 nm, and 4 frames per minute were acquired for the total of 25 min. Images was processed with ImageJ (NIH, USA) and Illustrator (Adobe systems Inc., San Jose, CA, USA).

***In vitro* generation of Dendritic cells for antigen presentation and T-cell priming assay**

Immature dendritic cells (DCs) were generated essentially as described in Subklewe, *et al.*⁴³ Briefly, monocytes obtained from leukapheresis product (REC Project no: 2013/624-15) were cultured for 2 days in CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with GM-CSF and Interleukin-4 (IL-4) in Ultra-low attachment cell culture flasks (Corning). The immature DCs were electroporated with either mRNA encoding for mutant li17R4RP₆/L₁₇A MART-1 or wild type li (liwt) carrying MART-1 peptide. Cytokines facilitating maturation were used (IL-1 β , IL-6, TNF- α , IFN- γ (all from PeproTech, Rocky Hill, NJ), prostaglandin E₂ (PGE₂), and TLR7/8 agonist R848 (MedChem Express, Sweden))⁴⁴ and

cultured for 24h. Mature DCs were used in T cell priming experiment. DCs electroporated with wild type li mRNA (no CLIP replacement) and DCs loaded with MART-1 peptide (10 µM) were included as negative and positive control, respectively, in the priming assay. Briefly, the distinct DC populations were cultured with autologous PBMCs at 1:10 DC:T cell ratio. On day 3, T cell cultures were supplemented with IL2 and IL7. On day 8, T cell cultures were re-stimulated with DCs and 10 days later T cells were stained with MART-1 dextramer (Immudex, Copenhagen, Denmark) to assess the presence of MART-1 antigen-specific T cells in the cultures.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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FIGURE LEGENDS

Figure 1: Biochemical characterization of liwt and liR₄RP₆/L₁₇A

(A) li constructs used in this study. The mutations done to li N-terminal cytoplasmic tail are indicated. CLIP: class II Invariant chain peptide; TM: transmembrane domain; TRI: trimerization domain; position 113 and 119: N-linked glycosylation positions. (B-E) For biochemical assays, HEK293 cells were transfected with li constructs as indicated. Twenty-four hours later, cleared cell lysates and immune complexes were collected, treated and loaded onto 4-20% SDS-PAGE gels and transferred to PVDF membranes for western blot (WB) analysis. All experiments were repeated three times. (B) Lysates were split into two, where half was treated with reducing sample buffer and boiled at 95 °C, while the other was subjected to non-reducing (without β-mercaptoethanol) sample buffer. Lysates were probed with anti-li (M-B741) and anti-actin antibodies. (C-E) Transfected cells were metabolically labeled with ³⁵S-Methionine and Cysteine for 30 minutes, washed three times in DMEM and lysed. Radioactivity was detected directly on ECL films. (C) Lysates were divided into two

where half was subjected to a 15 minutes Endo H treatment prior to immunoprecipitation (IP) with M-B741. Three li fractions were found for all constructs; the light /Endo H sensitive ER fraction, the premature Endo H resistant fraction with only one glycan and the fully mature double N-linked glycosylated mature fraction of li. **D)** Lysates were collected at time points 0, 0.5, 1 and 4 hours after 30 min of the radioactive pulse, and were immunoprecipitated with M-B741. The radioactivity was further quantified using the tool in ImageJ software, and the graph depicts the protein levels and half-life of the different li constructs, as indicated. The data shown in the graph are obtained from three independent experiments. **(E)** After the pulse, cells were incubated for 30 minutes in the presence of: none, one or the other, or both leupeptin and Cathepsin S inhibitor, 24h post-transfection. Lysates were probed with M-B741 and anti-actin. The protein levels were quantified using imageJ. **(F)** HeLa cells treated with either scrambled siRNA (control) or siRNA directed against AP-3, were transfected with li constructs as indicated. Lysates were run onto 10% SDS-PAGE gels and transferred to PVDF membranes, where they were probed with anti μ -AP-3, M-B741 and anti-actin antibodies and proteins were quantified using imageJ.

Figure 2: Intracellular distribution of liwt and liR4RP₆/L₁₇A

(A) MDCK cells were transfected with mCherry-liwt or mCherry-liR4RP₆/L₁₇A in combination with GFP-Rab5 or **(B)** GFP-Rab7a as indicated. 24 h post-transfection, the cells were incubated with M-B741-Alexa 647 (anti li antibody) for 1h prior to the live cell imaging at 37°C using the Olympus FluoView 1000 inverted microscope equipped with Plan/Apo 60/1.10 NA oil objective. All samples were analyzed in media without phenol red. The images show representative cells and were processed using ImageJ. Three independent experiments were performed, in total 15 cells per condition. Scale bar 15 μ m. The graph shows the quantification of the co-localization of li in GFP-Rab5 positive vesicles and is shown as mean + SEM of five different cells and indicated *p*-value was determined by unpaired *t*-test *** *P* <

0.001. **(C)** The graph shows the quantification of M-B741-Alexa 647 uptake by the li constructs, as indicated, and is shown as mean + SEM of five different cells and indicated *p*-value was determined by unpaired *t*-test *** $P < 0.001$. This graph is representative of three independent experiments, where in total 15 cells were analyzed using ImageJ software. **(D)** SupT1 cells were transduced with liwt or liR4RP₆/L₁₇A constructs or left untreated (NT) and stained without permeabilization with anti-li antibodies. Presence of membrane li was detected by Flow cytometry. This staining is representative of two separate experiments. **(E)** SupT1 (HLA-A2+) cells were transduced with the indicated constructs. Around 10⁵ cells were incubated with FLAG-tagged sTcR (estimated at 10 ng/mL) for 30 minutes at RT. Anti-FLAG antibody was finally used to detect sTcR and the binding was analyzed by flow cytometry. This experiment was repeated once with similar result.

Figure 3: Antigen presentation with trafficking mutant increases MHCI-presentation and naive T-cell priming.

(A) li constructs used in this study. The mutations done to li N-terminal cytoplasmic tail are indicated. MART1p: MART1 peptide (ELAGIGILTV); CLIP: class II li peptide; TM: transmembrane domain; TRI: trimerization domain. **(B)** SupT1 cells expressing HLA-A2 in combination with the indicated li construct (MART1p=MART1 peptide, irr = irrelevant peptide) or a single chain trimer (SCT) construct with MART1p or an irrelevant peptide as controls. J76 cells expressing DMF5 (HLA-A2-MART1p specific TCR) were co-incubated for 20 hours with the presenting cells and IL-2 level in the supernatant was detected by ELISA. The values are given as ng mL⁻¹ of IL-2, and error bars are ±SD from two parallels, *p*-value was determined by unpaired *t*-test, this experiment was repeated once with similar results. **(C)** Dendritic cells (DCs) expressing HLA-A2 in combination with the indicated li constructs (mutant li17R4RP₆/L₁₇A carrying MART-1 peptide or wild type li, (liwt), carrying MART-1 peptide) were generated as wells as a wild type li construct with unmodified CLIP region (liwtCLIP) and DCs loaded with MART-1 peptide as controls. Autologous T cells were primed

with the presenting cells (DCs) and co-incubated for 8 days. On day 9, T cells were restimulated with DCs and the presence of MART-1 specific T cells was detected by MART-1 dextramer staining 19 days later. The values are given as frequency (%) of CD8+MART-1 dextramer+ T cells and error bars are standard deviations (n=5), *P*-value was determined by unpaired *t*-test with Welch's correction and analysis was performed by GraphPadPrism software (GraphPad Software, San Diego, CA, US).

Figure 1

A

B

C

D

E

F

Figure 2

D

E

Figure 3