The transmembrane domain and luminal C-terminal region independently support invariant chain trimerization and assembly with MHCII into nonamers

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Abstract

Background: Invariant chain (CD74, li) is a multifunctional protein expressed in antigen presenting cells. It assists the ER exit of various cargos and serves as a receptor for the macrophage migration inhibitory factor. The newly translated li chains trimerize, a structural feature that is not readily understood in the context of its MHCII chaperoning function. Two segments of li, the luminal C-terminal region (TRIM) and the transmembrane domain (TM), have been shown to participate in the trimerization process but their relative importance and impact on the assembly with MHCII molecules remains debated. Here, we addressed the requirement of these domains in the trimerization of human li as well as in the oligomerization with MHCII molecules. We used site-directed mutagenesis to generate series of li and DR mutants. These were transiently transfected in HEK293T cells to test their cell surface expression and analyse their interactions by co-immunoprecipitations.

Results: Our results showed that the TRIM domain is not essential for li trimerization nor for intracellular trafficking with MHCII molecules. We also gathered evidence that in the absence of TM, TRIM allows the formation of multi-sub-unit complexes with HLA-DR. Similarly, in the absence of TRIM, li can assemble into high-order structures with MHCII molecules.

Conclusions: Altogether, our data show that trimerization of li through either TM or TRIM sustains nonameric complex formation with MHCII molecules.

Keywords: Antigen presentation, MHCII, CD74, Nonamerization, Transmembrane domain, Trimerization domain, RXR
the human TM (aa 30–55) can trimerize in the spectroscopy and deletion studies have demonstrated the human TRIM to trimerize [36–38]. Second, infrared recombinant fragment have confirmed the capacity of magnetic resonance (NMR) spectroscopy studies on the MHCII/Ii complex will reach the endocytic path-

N-terminal 16 aa found in p35 and p43 encompass a cytoplasmic di-arginine (RxR) ER retention motif and a PKC-phosphorylatable serine [12–15]. In its native state, this serine is part of a sequence recognized by β-COP, a component of COPI vesicles which mediate retrograde transport of cargo proteins from the cis-Golgi to the ER [16]. However, phosphorylation of the serine triggers the association of 14-3-3β, which is part of a family of ubiquituous proteins that regulate various biological activities. It has been postulated that, binding of 14-3-3β to lip35 prevents recognition by β-COP and allows forward transport past the cis-Golgi [16, 17]. From the trans-Golgi, the MHCII/Ii complex will reach the endocytic pathway, either directly or after a short transit at the plasma membrane [18–22]. Once in endosomes, li is sequentially degraded, leaving CLIP into the groove of MHCII. This complex is recognized by the non-classical HLA-DM, which catalyzes the exchange of CLIP for a high-affinity peptide [23, 24].

Best characterized as a MHCII chaperone, recent studies have revealed that li is also engaged in a number of other immune functions [25–27]. For example, li regulates the trafficking of additional proteins, such as CD70, CD1 and MHCI [28, 29]. Interestingly, li has important biological properties that appear to be independent of its chaperoning activities. Indeed, a pool of li is displayed at the plasma membrane (thereby its CD74 designation) and serves as the receptor for MIF, a function hijacked by Helicobacter pylori [30, 31]. In light of its multifunctional nature, structural analyses are ongoing and key functional domains of li have been exposed. However, its crystal structure has yet to be determined, the major hurdle probably residing in the flexible nature of the membrane-proximal region [32].

Once translated and translocated into the ER, li rapidly trimerizes [33–35]. The structural basis for such self-association has been studied in mice and humans. Three regions of li have been shown to independently associate into trimers. First, a trimeric domain (TRIM) of 27 kDa (aa 118–192 of human p33/p41 encoded by exon 6) is located in the luminal region, just C-terminal of the CLIP region (Fig. 1a). Biochemical and nuclear magnetic resonance (NMR) spectroscopy studies on the recombinant fragment have confirmed the capacity of the human TRIM to trimerize [36–38]. Second, infrared spectroscopy and deletion studies have demonstrated that the human TM (aa 30–55) can trimerize in the absence of TRIM [39, 40]. Accordingly, using biophysical and computational methods, Dixon et al., demonstrated trimerization of the mouse TM in isolation [41]. Third, the group of Bakke has used NMR spectroscopy to demonstrate that a synthetic peptide, corresponding to the first N-terminal cytoplasmic 27 aa of hlip33, forms, in solution, an almost coplanar triple-stranded α-helical bundle in which two helices are parallel and one antiparallel [42].

While there is ample experimental and computational evidence that these different regions of li can trimerize, their relative importance remains debated. For one, the cytoplasmic region is not believed to play a role in self-association of full-length li because of its antiparallel nature. Rather, trimerization of this domain was proposed to facilitate sorting and promote endosomal retention as well as the generation of large endosomes [42, 43]. While the TM clearly self-associates, many groups have shown that it is not essential for trimerization to occur. Also, depending on the experimental system used, its deletion can slightly affect the association with HLA molecules [34, 37, 44, 45]. On the contrary, other experimental evidences point to the indispensable nature of TRIM for mouse or human li trimerization [34, 36, 44, 46]. Nevertheless, the TRIM-less mouse lip10 proteolytic product found in endosomes has been shown to remain trimeric [47]. Importantly, these p10/p12 poly-peptides of mice and humans are not only trimeric, they were also shown to remain associated with MHCII molecules as part of a nonameric structure [35, 47]. Thus, the relative roles of TM and TRIM in trimerization and the formation of high order structures with MHCIIls remain controversial. In humans, no study has yet concluded that TM is required, nor that TRIM is dispensable for li trimerization in the ER. While some data point to interactions between MHCII and both the TM and TRIM domains, their importance for the folding and nonamer formation remains to be fully characterized [37, 39].

Here, we have revisited these issues using a cellular system that allows assessing the capacity of li to trimerize and to associate into high-order structures with MHCIIls. Our data demonstrate that neither TM nor TRIM are essential for li trimerization. In addition, we show that any of these domains is sufficient to trigger the assembly into nonameric structures with MHCIIls, as long as the li moieties involved share the same domain. The importance of these trimerization regions for Ag presentation by MHCIIls and for li functions in general are discussed.

Results
Formation of li trimers in absence of the TRIM domain
Conflicting data exist in the literature regarding the importance of the TRIM motif of li in trimer formation as well as its relevance in the trafficking of MHCII-li complex [39, 41, 44, 46]. First, we asked if deletion of TRIM could affect the formation of li trimers in living cells. To address this question, we used a truncated version of p35 that preserves its glycosylation sites but lacks the three C-terminal α-helices forming the TRIM domain.
HEK293T cells were transiently transfected with either the wild-type (WT) p35, p35 lacking its endosomal sorting signals (p35LIML) or p35 lacking the TRIM motif and the sorting signals (p35LIMLTRIM). Mutation of the two leucine-based endosomal localization motifs favors the accumulation of Ii at the plasma membrane in the presence of MHCII molecules, thus providing a simple, indirect flow cytometry readout for ER egress [48–50]. It is important to stress that despite lacking strong sorting motifs, cell surface IiLIML and the IiLIML/MHCII complex are nevertheless internalized (Additional file 1: Figure 1) in endosomes, where Ii gets degraded [51, 52]. When associated with MHCIIIs, this passage of Ii into endosomes results in the formation of MHCII/CLIP complexes. In the absence of HLA-DM, these complexes are recycled to the plasma membrane and can be detected using a CLIP-specific mAb. For the flow cytometry detection of p35LIMLTRIM, which has lost both its luminal and cytoplasmic epitopes recognized by the BU45 mAb, a myc/His-tag (detection 9e10 mAb) was introduced at the C-terminal end (Fig. 1a).

The capacity of these individual molecules to homotrimerize was tested in transfected cells treated with the crosslinking agent DSP. After cell lysis, proteins were analyzed by WB using a polyclonal rabbit Ab recognizing the CLIP core sequence common to all constructions.
p35 and p35\textsubscript{LIML} were detected at various molecular weights, corresponding to monomers but mostly dimers and trimers (Fig. 1b, c). Interestingly, while p35\textsubscript{LIMLTRIM} also formed dimers and trimers, we noted that a substantial amount of monomers remained in these conditions. A densitometrical analysis of three independent experiments suggests that in the absence of TRIM, the formation of trimers is less efficient. While the proportions of dimers, which were shown to be disulfide-linked [53], appear to be independent of TRIM (Fig. 1b), the possibility remains that trimers forming in the absence of TRIM dissociate more easily upon cell lysis than WT li. This is in line with a previous report from Dixon et al., who observed different ratios of monomers to trimers for the li transmembrane domain depending on the detergent used for lysis [41]. Since the TM trimerization occurs within the membrane, disturbing its integrity affects the likelihood of observing high proportion of trimers. Interestingly, Dixon et al., did not see any dimers for the li transmembrane alone. To confirm that p35\textsubscript{LIMLTRIM} can form trimeric complexes in the absence of crosslinking reagent, we tested by co-IP its ability to associate with WT li. HEK293T cells were transiently transfected with either p35, p35\textsubscript{LIMLTRIM} or both p35 and p35\textsubscript{LIMLTRIM}. Cells were lysed and the TRIM mutant was immunoprecipitated using the 9e10 mAb against the myc tag (Fig. 1d). This mAb did not bring down p35 unless the p35\textsubscript{LIMLTRIM} molecule was co-expressed, in line with the above-described results of crosslinking experiments showing dimers and trimers of p35\textsubscript{LIMLTRIM} (Fig. 1b). Altogether, our data suggest that the TM is sufficient to allow the trimerization of li.

**li's TRIM motif is not necessary for binding to MHCII molecules and to egress the ER**

We next asked whether deletion of TRIM could prevent the interaction of li with MHCII. As p35 does not exit the ER on its own, we tested the capacity of DR to assist surface expression of p35 and p35\textsubscript{LIMLTRIM} As controls, we used li mutants devoid of their cytoplasmic tail (Δ20) and TRIM domain (Δ20\textsubscript{TRIM}) (Fig. 2a). These constructs were separately transiently expressed in HEK293T cells alone (Fig. 2b) or with DR (Fig. 2c). Cells were stained for the presence of li at the plasma membrane (surface) using BU45 (Fig. 2b, c, left panels) or 9e10 (Fig. 2b, c, right panels) mAbs. A fraction of the cells was permeabilized (total) before staining to ascertain expression of the li protein in conditions where surface expression was negative. The results clearly show that in the absence of DR, only the Δ20 constructs were gaining access to the plasma membrane. The li proteins that include a RxR motif are prevented from ER egress. However, DR rescued expression at the cell surface of all p35-based proteins, independent of the presence of TRIM. These results demonstrate that the TRIM domain is not required for li to associate with MHCII molecules.

Next, we ascertained that the li-MHCII interaction was genuine in the absence of TRIM and that the complex could interact with DM. While li can bind different regions of MHCII molecules [35, 37, 54, 55], the groove of DR is a major binding site that accommodates the CLIP\textsubscript{99-101} region, just like any other nominal Ag [56]. Indeed, cell surface staining with the CerCLIP.1 mAb revealed the presence of CLIP at the cell surface (Fig. 2d). Interestingly, upon co-transfection of the cell surface, DR was efficiently removed. These results show that in the absence of TRIM, both truncated p33 and p35 can still form trimers. When loaded with MHCII, they egress the ER and serve as substrates for lysosomal degradative enzymes that generate CLIP.

**No region other than TM or TRIM can support the formation of high-order complexes**

The above-described experiments demonstrate that the TM region of li can support the formation of trimers. Next, we confirmed the importance of TM using a different experimental system where the luminal β chain domains were covalently linked to the extracellular region of li, thereby eliminating transmembrane anchors (Fig. 3a). This linkage is possible because DRβ and li are type I and II proteins, respectively [57]. This single chain dimer (SCD) construct, when co-expressed with DRα, allows us to study the impact of different regions of a co-expressed li. Thus, we postulated that while p35 would retain this pseudo MHCII/li complex (DRα+βSCD), a TRIM-less p35 variant unable to associate with the li moiety of the SCD would have no impact on intracellular sorting.

First, we characterized the intracellular trafficking of βSCD. The covalent linkage of li and DRβ chain may prevent the problems encountered in a previous study where a TM-deleted form of li showed altered binding to MHCIIIs [34]. When co-expressed with DRα, WB analysis of cell lysates demonstrate that a fraction of the recombinant βSCD protein becomes EndoH resistant (Fig. 3b, open arrowhead). Interestingly, the anti-DRβ chain-specific mAb also detected a fully EndoH-resistant fragment (filled arrowhead) migrating slightly faster than the WT DRβ chain (arrow) (Fig. 3b). This fragment most likely represents the DRβ moiety of the SCD that remains following the degradation of li in endosomes. These observations suggest that the SCD is properly folded, exits the ER and crosses the Golgi en route to the endosomes where li is degraded. Indeed, Fig. 3c shows that this chimeric protein is well expressed at
the plasma membrane and ultimately generates CLIP/MHCII complexes (Fig. 3c, left panel), which serve as substrates for DM (Fig. 3c, right panel).

Then, the DRα + βSCD molecule was co-expressed with either p33 or p35. These WT li isoforms can form heterotrimers with the li moiety of the βSCD. Indeed, IP of the full-length li isoforms with the cytoplasmic tail-specific Pin.1 mAb showed the presence of both WT DRα and the recombinant βSCD, the latter being detected with the XD5 mAb directed at the β1 domain (Fig. 3d, e).

Interestingly, p35 prevents expression of DRα + βSCD at the plasma membrane, as shown by the absence of CLIP, MHCII and li on co-transfected cells (Fig. 3f–h, middle columns). This is due to the lack of a DRβ tail capable of masking the p35 ER retention motif [50, 58]. Importantly, a TRIM-less p35 could not prevent surface expression of DRα + βSCD, in line with the need for this domain in the interaction with the li moiety of βSCD (Fig. 3f–h, right columns). Finally, we repeated these experiments using WT p35 co-expressed with a βSCD devoid of its TRIM (Fig. 4a, b). Again, the lack of bidirectional TRIM-dominating interactions prevented the interaction between p35 and βSCD, as judged by the presence of the latter at the plasma membrane (Fig. 4c–e).
The TRIM domain of Ii is not required for the formation of nonameric complexes  

We next investigated whether TRIM is required to assemble multiple MHC class II molecules around a multimeric Ii scaffold. For this, we designed an MHCII trap consisting of a mutant MHCII molecule (DRKKAA) bearing a stringent KKAA cytoplasmic ER retention motif (which cannot be overcome in any ways) (Fig. 5a, left panel) [49]. We asked whether a TRIM-less Ii, once bound to DRKKAA, could catch and prevent ER egress of other co-expressed WT MHCIIIs, thereby confirming the formation of multimeric complexes comprising multiple Ii and MHCII molecules (Fig. 5a, right panel).

First, we tested the control li Δ20TRIM variant, which is devoid of RxR and di-leucine cytoplasmic motifs. Transfected HEK cells were analysed for the expression of DR and Ii at the plasma membrane by flow cytometry (Fig. 5b–e). A fraction of the cells was also permeabilized and analysed for the expression of DR and Ii at the plasma membrane by flow cytometry (Fig. 5b–e).
to calculate the surface over total mean fluorescence intensity (MFI) ratio. This allows us to evaluate and to compare indirectly the efficiency of ER egress (Fig. 5b, d). When control DR was expressed as the sole source of MHCIIs, DR and IiΔ20 TRIM were both detected at the plasma membrane (Fig. 5b–d). Also, a substantial amount of DR/CLIP complexes were detected at the cell surface, confirming genuine association between MHCIIs and Ii (Fig. 5e, f). In contrast, when co-transfected with DRKKAA, very little Δ20 TRIM was able to make it to the plasma membrane (Fig. 5c, d) and, as those molecules that escaped retention by DRKKAA (Fig. 5b) trafficked on their own, no CLIP/DR complexes could be detected (Fig. 5e, f). Interestingly, when the two DR were co-expressed with Δ20 TRIM, we found some II and MHCIIs molecules at the plasma membrane (Fig. 5b–d). Also, the presence of CLIP demonstrates that DR/Δ20 TRIM complexes gained access to the endocytic pathway and thus were free of DRKKAA (Fig. 5e, f) These findings suggest that in the presence of Δ20 TRIM, WT DR most likely assemble independently from DRKKAA and can egress as pentamers (α1β1Δ20 TRIM3), or even trimers (α1β1Δ20 TRIM1). Thus, the use of control IIΔ20 TRIM could not inform on the capacity of IIΔ20 TRIM to assemble different MHCIIs into the same complex.

We then tested the impact of lip35LIMLTRIM in cells expressing DR. Our results show that DR was found at the plasma membrane together with II and CLIP (Fig. 5b–f). Again, this does formally demonstrate the formation of II trimers or trafficking of the complex in the form of a nonamer. As expected, DRKKAA could not rescue the ER egress of lip35LIMLTRIM as both molecules have retention motifs. Accordingly, no CLIP was present at the cell surface (Fig. 5e, f). Interestingly, when p35 LIMLTRIM was expressed with both DR and DRKKAA, class II, II and CLIP were not found at the cell surface (Fig. 5c–f). This is in stark contrast with the results obtained above using IIΔ20 TRIM. This result is in line with a model where lip35 LIMLTRIM does trimerize in the ER and stochastically associates with DR and DRKKAA. As it is likely that each and every p35 LIMLTRIM homotrimer recruited at least one DRKKAA molecule, this prevented surface expression of all MHCIIs species. Altogether, these results confirm that TRIM is not required for II and MHCIIs molecules to associate into multimeric structures.

The TRIM supports the scaffolding of nonamers in the absence of II’s TM
Experiments using truncated soluble molecules have demonstrated the rapid trimerization of II and the subsequent formation of complexes of variable stoichiometry with MHCIIs [45]. We have addressed in transfected cells the impact of deleting II’s N-terminal region, including TM, on the assembly with MHCIIs. To ascertain the efficient binding of the CLIP region into the peptide-binding groove of DR, II was covalently linked to the extracellular portion of DRβ (αSCD), as previously described (Fig. 6a) [50]. When expressed on its own in HEK293T cells, the αSCD remains EndoH sensitive and is most likely trapped in the ER (Fig. 6b). As expected, when co-transfected with the membrane-anchored DRβ, the αSCD is strongly expressed and a large proportion becomes EndoH-resistant (arrowheads, Fig. 6b). Also, an EndoH-resistant degradation product was detected (arrow, Fig. 6b), in line with the ER/Golgi egress of the αSCD/β complex and the eventual endosomal processing of the II moiety. Accordingly, CLIP, II and DR were all detected at the plasma membrane by flow cytometry (Fig. 6c–e, left
columns). However, when the αSCD was co-transfected with DRβKKAA chain instead of DRβ, the complex was not found at the cell surface (Fig. 6c–e). Interestingly, when αSCD, DRβ KKAA and WT DRβ were all co-expressed, there was no CLIP, Ii or DR at the plasma membrane (Fig. 6c–e, right columns). The data are compatible with a model where the αSCD first trimerizes [59] and the stochastic incorporation of the available DRβ chains will result in the ER retention of most nonamer-like complexes by DRβ KKAA. We conclude that the TM domain of Ii is not a prerequisite for the assembly of multimeric structures comprising multiple MHCII molecules. These data are in agreement with those of Cresswell and collaborators showing that the proteinase K digestion of Ii in MHCII/Ii complexes generates a C-terminal K3 fragment, which includes TRIM and by itself can retain the complex in its nonameric conformation [35].

Discussion

Newly translated full-length Ii chains swiftly trimerize upon translocation into the ER [33, 36]. The need for such self-association is unclear. Data accumulated so far, including those presented here, lead to the conclusion that two distinct regions, highly conserved and encoded by separate exons, can mediate self-recognition of Ii. Besides its chaperone function, free Ii has been shown to accumulate at the plasma membrane, principally in APCs [60, 61]. At least three different functions of this pool of cell surface Ii/CD74 have been characterized. First, Ii serves as a receptor for MIF [30]. While both the ligand and receptor are trimeric, modeling studies point to a possible dodecameric structure where each Ii moiety binds a MIF trimer [62]. Future studies should address the need for these interactions in the generation of a signaling platform, which includes CD44, capable of activating MAPK and to trigger production of pro-inflammatory cytokines [63]. Crosslinking of CD74 also leads to the intramembrane cleavage and the release of the intracellular domain (ICD) [64, 65]. This short domain enters the nucleus and modulates the transcriptome of APCs [66]. While peptides corresponding to the cytoplasmic domain of Ii has been shown to trimerize [42], the structural basis underlying the nuclear activity of the ICD is unknown. In the context of full length Ii, the presence of three cytoplasmic tail was shown to be essential to endosomal targeting and for shaping endosomes morphology.
[43, 52]. As this activity of li is thought to be important for Ag presentation, it may explain in part the need for trimerization [67]. Thus, it is likely that a multi-functional li requires multiple trimerization domains, including an extracellular one (TRIM) to rigidify an otherwise unstructured membrane-proximal region and to create a MIF binding domain. While the exon 6b-encoded polypeptide is C-terminal to these trimerization sites, it does not appear to affect the overall stoichiometry [68]. However, the N-terminal extension of p35/p43 could modulate enlargement of endosomes or gene expression, two issues that will require further investigations. Also, the capacity of p35 to possibly interact specifically with a MIF binding domain. While the exon 6b-encoded polypeptide is C-terminal to these trimerization sites, it does not appear to affect the overall stoichiometry [68].

When considering the chaperone role of li, the need for trimerization in the context of MHCII transport is not readily apparent. Nonameric complexes (αβli)3 were first described in the early 1990s and are a direct consequence of li’s ability to form trimers [33]. However, in 2011, it has been proposed that due to structural constraints, li/ MHCII complexes can only exist as pentamers αβ(li)3 [69]. While our results confirmed that pentamers can to exit the ER, we have also clearly demonstrated that the ER retention motif of p35 promotes the formation of nonameric complexes [49, 50]. Indeed, there must be a direct interaction between p35 and the MHCII to inactivate the ER retention signal, thus forcing the addition of αβ heterodimers until all RxR motifs are matched [63, 70]. Mice don’t express p35 and we must envisage that an alternative regulatory checkpoint predominates. Early work by the group of Cresswell had shown that calnexin remains bound to the complex until the li trimer is fully saturated with MHCIIIs [71]. This mechanism may be more stringent in mice than in humans in preventing “premature” egress of pentamers and heptamers [72]. It is important to stress that in some experiments, we did not have direct or indirect evidence that li exited the ER as a trimer. For example, in Fig. 5, those complexes exiting to the plasma membrane could theoretically be formed over a dimer of liΔ20TRIM. Indeed, we have shown in Fig. 1b that such dimers of li (liΔ3) can be visualized on Western blots after crosslinking. Interestingly, these dimers have been described almost 40 years ago by Koch and Hammerling [53]. They were found to be disulfide-linked through the free intracytoplasmic cysteine residue near TM. Still, formation of these dimers or trimers is not mutually exclusive. Noteworthy, SCDs cannot form such dimers since they do not include the cytoplasmic cysteine of li.

Beyond the debate regarding the stoichiometry of the complexes leaving the ER, the need for multimerization of MHCII in Ag presentation remains nebulous. At one extreme, li was even shown to be dispensable for MHCII assembly/trafficking in some cell lines and knockout mice [6, 73, 74]. This is certainly non-physiological as MHCIIIs and li are co-expressed and the latter is usually found in vast excess [75]. Few studies have addressed the importance of TRIM and TM in Ag presentation. Deletion experiments of either domain have produced variable results and stoichiometry of the resulting complexes has not always been thoroughly monitored. On one hand, Germain has shown that truncation of li after CLIP does not alter MHCII assembly, trafficking and peptide acquisition, suggesting that TRIM is not a prerequisite for Ag presentation [76]. However, this study did not address the trimeric nature of the truncated li. On the other hand, in mice, Koch and collaborators found that li oligomer formation through the C-terminal region is needed for HEL presentation [46].

No study has tackled the systematic comparison of Ag presentation efficiency using liαβ, liαβ or (liαβ)3 complexes. The difficulty resides in our capacity to generate structurally comparable complexes of defined stoichiometry. In our recent studies, we made use of the αCD and the results suggested that the TM of li is not required in living cells for the formation of li/MHCII complexes of variable stoichiometry. Here, we have confirmed these results and extended the conclusions to the TRIM of hi.
Fig. 6 (See legend on previous page.)
Also, we have shown that no region other than the TM or TRIM (or even MHCIIIs) promote Ii self-association. By using SCDs devoid of TRIM, we were able to compare the trafficking of Ii,αβ, with WT nonameric Ii,αβ, complexes. While we have not monitored Ag overlap extension for p35LIML, Δ20 and βSCD, giving TRIM domain (aa 128–216 in p33) were created by PCR.

P. Cresswell, Yale University).

rabbit anti-CLIP (CLIP region of Ii) (a kind gift from Dr (DM); 9e10 (myc tag) (Biolegend, San Diego, CA) and the tail of DRα) XD5 (DRβ); CerCLIP .1 (CLIP); MaP .DM1 (mic tail of hIi), L243 (HLA-DR); DA6.147 (cytoplasmic [58, 82]: BU45 (C-terminal region of hIi); Pin.1 (cytoplasmic 1, αβ)1 complex. While li chaperones DP4 to the endocytic pathway, more studies will be needed to determine if this peculiar stoichiometry intervenes in the association of these alleles with autoimmune diseases [78].

Conclusion
In conclusion, the purpose of the two distinct trimerisation domains of Ii in the chaperoning of MHCIIIs remains an open question. As mentioned above, it is possible that the luminal TRIM serves some MHCII-independent functions and that the structural features required for li to chaperone other cargos are dependent on TRIM. Future structure–function studies addressing the interaction of li with other molecules, such as CD70 and possibly CD1d, should shed light on this issue [79–81].

Methods
Plasmids and mutagenesis
pBud DR, pBud DM, pcDNA3.1 DRα, pBud αSCD, pcDNA3.1 DRβ, and pcDNA3.1 DRβKKAA, pcDNA3 li, pcDNA3 p33, pcDNA3 p33LIML, pcDNA3 p35, pcDNA3 p35LIML, and pBud Δ20 Ii have been described previously [48–50, 58, 82]. The β single-chain dimer (βSCD) linking the luminal domain of DRβ (aa 1–199) to li’s luminal region (aa 57–232) using a (Gly3(Ser)1(Gly)3 linker was created has described for αSCD [50]. Mutants lacking the TRIM domain (aa 128–216 in p33) were created by PCR overlap extension for p35LIML, Δ20 and βSCD, giving rise to the pBud p35LIMLTRIM, pBud Δ20TRIM and pBud βSCDTRIM respectively.

Abs, immunoprecipitation (IP) and Western blot (WB)
The following mouse mAbs were described previously [58, 82]: BU45 (C-terminal region of hIi); Pin.1 (cytoplasmic tail of hli), L243 (HLA-DR); DA6.147 (cytoplasmic tail of DRα) XD5 (DRβ); CerCLIP1 (CLIP); MaPDM1 (DM); 9e10 (myc tag) (Biolegend, San Diego, CA) and the rabbit anti-CLIP (CLIP region of Ii) (a kind gift from Dr P. Cresswell, Yale University).

Alexa Fluor 488- or 633-coupled goat anti-mouse secondary Abs (Invitrogen, Burlington, ON) were used for flow cytometry. For WB, Peroxidase-AffiniPure goat anti-mouse IgG (H + L) and Peroxidase-AffiniPure goat anti-mouse Fc specific (Jackson Immunoresearch, West Grove, PA) were used. For IPs, cells were lysed at 4 °C in 1% Triton-X100. Lysates were analyzed as controls and all samples were subjected to reducing SDS-PAGE. Proteins on immunoblots were detected by chemiluminescence (Roche Applied Science, Laval, Qué.). For crosslinking experiments, cells were lysed in 1% Triton and 400 μg/mL DSP (dithiobis (succinimidyl propionate)) (Sigma Aldrich, St-Louis, MO). For Endo H resistance assays, total lysates were treated with Endo H (New England Biolabs), according to the manufacturer’s recommendations. Proteins were analyzed in non-reducing conditions by SDS-PAGE.

Cell lines and flow cytometry
For transient expression, HEK293T cells were transfected using polyethylenimine (Polyscience, Warrington, PA) and stained after 48 h. To determine surface expression, live cells were stained on ice and analyzed by flow cytometry on a FACSCalibur or FACSScantiII. To determine total expression of MHCII and li, cells were fixed in 4% paraformaldehyde, permeabilized, and stained, as described previously [82]. Forward and side scatter gating strategy was used to gate on single cells.

Abbreviations
αSCD: Alpha single chain dimer; βCOP: Protein complex coatmer beta subunit; βSCD: Beta single chain dimer; DSP: Dithiobis (succinimidyl propionate); ER: Endoplasmic reticulum; HEK: Human embryonic kidney cells; HEL: Hen egg-white lysozyme Ag; hIi: Human Ii; ICD: Intracellular domain; Ii: Invariant chain; IP: Immunoprecipitation; MFI: Mean fluorescence intensity; MHCI: MHC class I; MHCII: MHC class II; MIF: Macrophage inhibiting factor; NMR: Nuclear magnetic resonance; PCR: Polymerase chain reaction; PKC: Protein kinase C; TM: Transmembrane domain; TRIM: Trimerization domain; WB: Western blot; WT: Wild type.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12865-021-00444-6.
Acknowledgements
We thank Dr. P. Cresswell for Abs and Dr H. Khalil for making the p35LIMLTRIM and Δ20TRIM constructs.

Authors’ contributions
MC planned experiments, conducted most experiments, analyzed data and wrote the manuscript. JSF generated molecular constructs used in this study. JT planned experiments, analyzed data and wrote the manuscript. All authors have read and approved the manuscript.

Funding
This research was funded by a Discovery grant from the National Science and Engineering Research Council of Canada (NSERC; Grant Number RGPIN-2020-07205) to JT. JT holds the Saputo Research Chair.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 25 August 2020   Accepted: 20 July 2021
Published online: 12 August 2021

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