The p35 human invariant chain in transgenic mice restores mature B cells in the absence of endogenous CD74

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Abstract

The invariant chain (Ii; CD74) has pleiotropic functions and Ii-deficient mice show defects in MHC class II (MHC II) transport and B cell maturation. In humans, but not in mice, a minor Iip35 isoform of unknown function includes an endoplasmic reticulum-retention motif that is masked upon binding of MHC II molecules. To gain further insight into the roles of Ii in B cell homeostasis, we generated Iip35 transgenic mice (Tgp35) and bred these with mice deficient for Ii (Tgp35/miKO). Iip35 was shown to compete with mIi for the binding to I-Ab. In addition, classical endosomal degradation products (p20/p10) and the class II-associated invariant chain peptide (CLIP) fragment were detected. Moreover, Iip35 favored the formation of compact peptide–MHC II complexes in the Tgp35/miKO mice. I-Ab levels were restored at the plasma membrane of mature B cells but Iip35 affected the fine conformation of MHC II molecules as judged by the increased reactivity of the AF6-120.1 antibody in permeabilized cells. However, the human Iip35 cannot fully replace the endogenous Ii. Indeed, most immature B cells in the bone marrow and spleen of transgenic mice had reduced surface expression of MHC II molecules, demonstrating a dominant-negative effect of Iip35 in Tgp35 mice. Interestingly, while maturation to follicular B cells was normal, Iip35 expression appeared to reduce the proportions of marginal zone B cells. These results emphasize the importance of Ii in B cell homeostasis and suggest that Iip35 could have regulatory functions.

Keywords: invariant chain, Iip35, MHC, antigen presentation, transgenic mouse

Introduction

MHC class II (MHC II) molecules are responsible for exogenous antigen presentation to CD4⁺ T cells. The invariant chain (Ii or CD74) plays a key role in this process. During biosynthesis in the endoplasmic reticulum (ER) of antigen-presenting cells (APCs), three MHC II αβ heterodimers combine with a preformed Ii trimer, forming a nonamer complex (1). Acting as a chaperone, Ii assists in the folding of MHC II and prevents ER peptides from gaining access to the groove (2, 3). After ER egress, the di-leucine cytoplasmic motifs of Ii target the non amergic complex to the endocytic pathway (4–6). In more acidic compartments, Ii is degraded sequentially to Iip22 and Iip10, both of which contain a short sequence [class II-associated invariant chain peptide (CLIP)] that blocks the peptide-binding groove (7, 8). Upon the degradation of the p10 fragment by cysteine proteases, CLIP-MHC II complexes are generated (9). While some of these complexes may escape to the plasma membrane, most of the CLIP is released by the non-classical MHC II molecule HLA–DM (H2-DM in mice), which catalyzes the binding of high-affinity peptides found in endosomes (10–12). The stable peptide–MHC II complex (pMHC II) is then free to move to the plasma membrane to present nominal antigens to CD4⁺ T cells (13).

Early studies performed on Ii-null mice have highlighted the role of the chaperone in MHC II folding, trafficking and antigenic presentation. These mice showed aggregation and aberrant transport of MHC II molecules, which resulted in decreased cell surface expression (14–16). Additionally, instead of taking on the compact conformation typically seen in stable αβ heterodimers associated with antigenic peptides, most MHC II molecules in the Ii-null mice were in a...
molecules remained in a floppy state (17, 18). These maturation perturbations had profound consequences on thyenic selection. Indeed, the CD4+ compartment in Ii-deficient mice was found to be affected in terms of both absolute T-cell numbers and repertoire diversity (19, 20). Interestingly, the critical role of li in MHC II folding was shown to be allele-specific, I-Aβ being greatly affected in li-null mice (14, 21).

In addition to its impact on T-cell development, li expression has been shown to affect B lymphocyte homeostasis. Indeed, except for NOD mice, li-deficiency in all tested mice backgrounds resulted in reduced numbers of mature follicular (FO) B cells in secondary lymphoid organs (22, 23). Although this phenotype was attributed to a defect in B cell maturation, it was recently suggested that FO cells have a shorter life span in the absence of li (24). The molecular mechanism underlying this li effect remains controversial. While some have suggested that li is required to transduce signals and activate nuclear factor (NF)-κB (25), others have reported that it plays a role in preventing the toxic accumulation of free β chains or weakly associated MHC II heterodimers in the ER of B cells (24, 26, 27).

Several new functions have recently been attributed to li, some of which are not directly related to peptide loading. For instance, li’s folding and/or trafficking properties do not appear to be exclusive to classical MHC II molecules and would also benefit MHC I, the neonatal Fc receptor, CD1, and CD70 (28–31). As mentioned above, li has the ability to transduce signals following cleavage of its transmembrane domain and release of its cytoplasmic tail (25). In addition, at the cell surface, li serves as the receptor for both the macrophage migration inhibitory factor (MIF) and Helicobacter pylori (32, 33). More recently, li has been shown to interact with myosin to negatively regulate dendritic cells (DCs) motility in vivo (34). The most recent studies have been performed in mice and the results suggest that li strongly impacts the control of immune responses.

In mice, alternative splicing of exon 6 in li mRNA leads to the translation of two isoforms of 31 kDa (lip31) and 41 kDa (lip41), respectively (35). The additional domain in lip41 serves as a chaperone for various cathepsins in the endocytic pathway (36). In humans, the use of two different start codons on each mRNA allows the production of a total of four isoforms termed lip33, lip35, lip41 and lip43, the last two comprising the cathepsin binding domain (37). The lip33 and lip35 isoforms are predominant, lip33 being about four times more abundant than lip35 (1, 38, 39). A type II protein, the lip35 isoform bears a 16-amino acid N-terminal cytoplasmic extension. This region contains an Arg-x-Arg (RxR) ER retention motif which is inactivated upon phosphorylation of two adjacent serines and association of MHC II molecules (39–44). Unlike lip33, lip35 is rapidly internalized, preventing any accumulation at the plasma membrane (45, 46). lip35/p43 isoforms are dominant and dictate the fate of mixed li trimers (38, 40).

While the physiological role of li’s RxR motif is unknown, some studies have determined that the deregulation of the lip33/lip35 ratio impacts peptide loading, ER exit rate and compartment localization. Newcomb and Cresswell (41) tracked proteolytic intermediates to show that lip33 and lip35 are differentially transported to endosomes and vary in their capacity to support peptide loading. In addition, Anderson and Roche (43) demonstrated that lip35 phosphorylation increases both its degradation and MHC II peptide loading, suggesting a possible post-translational regulation of antigen presentation.

li-null C57BL/6 mice present defects in I-Aβ folding and trafficking that lead to reduced antigen presentation. To further investigate the biology of li and its role in B cell homeostasis, we generated transgenic mice expressing exclusively the human lip35 isoform on both murine li-proficient and -deficient backgrounds.

Methods

Generation of Tg mice

The li cDNA encoding exclusively the lip35 isoform was generated by mutating the second methionine to an alanine (42). This cDNA was optimized at the Kozak sequence, cloned under the control of the murine li gene promoter in the pDOI6 vector, linearized and injected into FvB mouse embryos (47). Offspring were genotyped for the presence of lip35 by PCR using the following primers: 5′-GAAACTGACAGTCACCT-3′ and 5′-GAAATCTACAGCTCAAGG-3′. Three different founders (Tg35) were obtained and bred with C57BL/6 mice for 20 generations. To obtain the transgenic lip35 mice devoid of murine li (Tg35/mlKO), Tg35 mice were bred with C57BL/6-mliKO mice (Jackson Laboratories, Bar Harbor, ME, USA) for 20 generations. The mli gene was genotyped by PCR as described previously (27). All experiments were performed in accordance with the Canadian Council of Animal Care.

Antibodies and peptide

BU45 and Pin-1 mouse hybridomas produce IgG, mAbs that specifically recognize the C- and N-terminal parts of human li, respectively (38, 48). They were purified from cell culture supernatants and coupled to Alexa-647 (Invitrogen, San Diego, CA, USA). Likewise, In-1, a rat IgG2a mAb specific for the N-terminal part of murine li (BD-PharMingen, Mississauga, Canada), was coupled to Alexa-647. The D-6 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) recognizes the C-terminal part of human li. The M5/114 antibody which recognizes the I-Aβ chain was purified from cell culture supernatants, while the 30-2-FITC mAb specific for the CLIP/I-Aβ complex was purchased from Santa Cruz Biotechnology, Inc. CD11c-biotin, IgM-PercP, IgD-FITC and CD21-PE antibodies were purchased from BD-PharMingen and CD21-PE antibodies were purchased from BD-PharMingen. The α-IgM-PE, α-B220-FITC and α-I-Aβ (28-16-8S; mouse IgM) antibodies were purchased from InVitrogen. Antibodies against I-Aβ (AF6-120.1), IgD-PercP, CD19-PE and CD19-Alexa647 were purchased from Biolegend (San Diego, CA, USA), while the α-actin was obtained from Calbiochem (Mississauga, Canada). The rat anti-mouse H2-DM (2C3A) was a kind gift from Dr L. Karlsson. The mouse anti-I-Aβ KL295 hybridoma was purchased from ATCC and antibodies were purified from cell culture supernatant.

Secondary reagents, including streptavidine-PE, goat anti-mouse IgG peroxidase and goat anti-rat peroxidase, were purchased from Jackson ImmunoResearch, Inc. (West Chester, PA).
Grove, PA, USA), while the donkey anti-rat-APC and the anti-mouse Ig True blot were obtained from eBioscience (San Diego, CA, USA). The biotinylated CLIP peptide (81–105 segment of li) was purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA, USA).

Flow cytometry
Spleen and bone marrow (BM) were dissociated to obtain single cell suspensions. Red blood cells were lysed by incubating them for 5 min in 0.83% NH₄Cl. Cells (10⁶) were resuspended in PBS, FBS 3% and HEPES 10 mM and stained for 30 min on ice. For total staining, cells were permeabilized for 10 min in the same buffer containing 0.1% saponin. Cells were analyzed on a FACSCalibur® flow cytometer (Becton Dickinson, Mississauga, Canada).

Western blot analysis
Splenic DCs (BMDCs) were generated as described previously (49). Briefly, BM cells were cultivated for 7 days with GM-CSF 500 U mL⁻¹ (Invitrogen) and IL-4 (~250 U mL⁻¹), previously (49). Briefly, BM cells were cultivated for 7 days with GM-CSF 500 U mL⁻¹ (Invitrogen) and IL-4 (~250 U mL⁻¹), washed twice in complete media.

Isolated spleens were digested using 0.1% collagenase D and 20 μg mL⁻¹ DNAse for 45 min at 37°C. Cell suspensions were filtered on a 70-μm nylon filter (BD Falcon™) and analyzed on immunoblots.

BM-derived DCs
BM-derived DCs (BMDCs) were generated as described previously (49). Briefly, BM cells were cultivated for 7 days with GM-CSF 500 U mL⁻¹ (Invitrogen) and IL-4 (~250 U mL⁻¹), originating from P815.IL-4 hybridoma supernatant.

Results

Generation of transgenic lip35 mice
The lip35 cDNA was obtained by mutating the second methionine-encoding triplet (42). This prevents internal translation initiation and production of the lip33 isoform. The cDNA was cloned under the control of the murine li gene promoter in the pDO16 vector and injected in mice embryos (47). We obtained three different founders which were bred with C57BL/6 to obtain Tgp35 mice. In addition, mice were bred with li-null C57BL/6 mice to generate animals that only express the human lip35 (Tgp35/nilKO). Most experiments were performed on the offspring of all founders and generated the same results. As expected, using the human li-specific Pin-1 mAb, western blotting revealed that lip35 was expressed in both Tgp35 and Tgp35/nilKO mice splenocytes (Supplementary Figure 1 is available at International Immunology Online).

Expression of lip35 in APCs
The pattern of lip35 protein expression in Tg mice was assessed by flow cytometry in different cell types. As seen in Fig. 1, lip35 was found in APCs, but its expression was heterogeneous. Moreover, as opposed to the endogenous mli, only a fraction of cells in any given APC population expressed lip35. Indeed, ~33% of splenic B cells, ~47% of splenic DCs and ~24% of BMDCs were positive for lip35 (Fig. 1A-D). Interestingly, the endogenous mli was more abundant in the Tgp35 B cells compared with wild-type (Fig. 1B, right panel) and this difference was significant (Fig. 1C, left panel). The analysis was also performed on cells double-stained for mli and lip35. The results confirmed the significant increase of mli in lip35⁻ mice versus lip35⁻ cells (Fig. 1C, right panel), suggesting that lip35 and mli compete for binding to MHC II molecules. While it has yet to be determined why the overall pattern of lip35 expression does not match that of the endogenous li, it remains that all three founders showed the same phenotype (data not shown).

Interaction of lip35 with murine li and MHC II molecules
The human li has previously been shown to bind murine MHC II molecules (50). Likewise, I-A⁻ co-immunoprecipitated lip35 from lysates of Tgp35 and Tgp35/nilKO splenocytes (Fig. 2A). Lip35 was also co-immunoprecipitated with the endogenous mli in Tgp35 cells (Fig. 2A). Accordingly, mli was co-immunoprecipitated with I-A⁻ and lip35 (hli) in Tgp35 cells (Fig. 2B). Ig light chains of antibodies used for immunoprecipitations were all recognized by the anti-rat secondary antibody used for blotting and are identified by a bracket.

We then asked if the presence of mli in Tgp35 mice could affect lip35 expression compared with Tgp35/nilKO mice. As seen in Fig. 2C, CD19⁺ splenocytes from both Tg mice expressed similar levels of lip35, based on the staining with BU45, a mAb which recognizes the C-terminal end of unprocessed hli. However, almost twice as many cells expressed lip35 in the Tgp35/nilKO mice compared with Tgp35 mice (51 versus 27%, respectively).

Next, we investigated whether lip35 could egress the ER and be processed to low-molecular weight intermediates. For these experiments, we used the highly sensitive D-6 mAb. Lip35 was immunoprecipitated from Tgp35/nilKO cells and analyzed on immunoblots (Fig. 2D). Two fragments of 22 kDa (p22) and 10 kDa (p10) were detected, a pattern consistent with the degradation of lip35 by pH-sensitive cysteine proteases in the endocytic pathway (2, 3). These results indicate that the initial steps in lip35 maturation are normal and that the RxR-retention motif can be masked by I-A⁻.
Fig. 1. Expression of lip35 in APCs. Cells of different origins were isolated, permeabilized with saponin and stained with either BU45 or In-1 and different lineage-specific mAbs. The percentage of cells expressing li is indicated. (A) The spleens were removed and digested with collagenase D and DNAse and then isolated on a 14% nycodenz gradient. Collected cells were stained with BU45 or In-1, anti-CD11c and I-Ab (28-16-8S). (B) Splenocytes were stained with BU45 or In-1, anti-CD19 and I-Ab (28-16-8S). (C) Statistical significance of the data obtained in panel B was determined using a Student's t-test. In the left panel, expression of mIi was measured on CD19+ cells. In the right panel, mIi expression was compared between CD19+ lip35− and CD19+ lip35+ spleen cells isolated from Tgp35 mice. Error bars indicate the standard deviation (SD). (D) BM was removed from tibia and femur and collected cells were cultured for 7 days in the presence of GM-CSF and IL-4. DCs were then isolated on a 14% nycodenz gradient and stained with BU45 or In-1 and anti-CD11b. Data are representative of at least five independent experiments.
allowing ER egress and trafficking to degradative endocytic compartments.

The pattern of lip35 degradation fragments suggests that p10 would eventually be cleaved by cathepsins in endosomes to generate CLIP. To test this, we used the 30-2 mAb raised against the human CLIP/I-A\(^{\alpha}\) complex (51). Although this mAb can cross-react with I-A\(^{\alpha}\) associated with the mouse CLIP on LPS-activated wild-type blasts (51), its reactivity is weak; no signal was detected in C57BL/6 mice under our experimental conditions (Fig. 2E). However, Tg cells accumulated intracellularly some 30-2 complexes, while stainings were negative at the plasma membrane (data not shown). The levels of CLIP/I-A complexes were monitored specifically in lip35\(^{+}\) cells stained for CD19, BU45 and 30-2 Abs (Fig. 2F). The fact that lip35\(^{+}\) spleen cells from the Tgp35/mIiKO display significantly more CLIP/MHC II complexes than the Tgp35 cells suggested again that the human and mouse Ii compete for binding to I-A\(^{\alpha}\).

An important player in the release of CLIP from MHC II molecules is H2-DM. It has been suggested that mIi serves as a chaperone for H2-DM since its expression is reduced in Ii-deficient animals (23, 52). We assessed the levels of H2-DM in our Tg mice (Supplementary Figure 2 is available at International Immunology Online). As expected, splenocytes from Ii-null mice accumulated less H2-DM than wild-type mice. Interestingly, Tgp35/mIiKO cells did not up-regulate H2-DM, while levels in Tgp35 and wild-type mice were comparable. These results suggest that the accumulation of CLIP may be due to the reduced interaction between H2-DM and human Ii.

**lip35 generates compact MHC II molecules**

Previous reports have shown that most I-A\(^{\alpha}\)\(\beta\) chains in Ii-null mice remain in an immature state and migrate faster on SDS-PAGE (14–16). These immature products correspond to non-terminally glycosylated chains that have not gained access to the golgi and are sensitive to endoglycosidase H (14 and data not shown). Given that lip35 interacts with I-A\(^{\alpha}\) in the presence or absence of murine Ii, we tested its impact on the fine conformation of MHC II molecules. Splenocytes were lysed and analyzed on immunoblots under denaturing conditions. As for the mIiKO cells, the major \(\beta\) chain pool in the Tgp35/mIiKO cells was constituted of the faster migrating immature (labeled \(\beta\)'') species (Fig. 3A). Interestingly, in Tgp35 cells, expression of lip35 caused the accumulation of some immature \(\beta\)' chains despite the presence of the endogenous mIi.

Next, we addressed the effect of lip35 on the generation of compact I-A\(^{\alpha}\)–peptide complexes. Splenocytes were lysed and analyzed on immunoblots under non-reducing and non-boiled conditions using the conformation-dependent M5/114 mAb, which recognizes primarily I-A\(^{\alpha}\) \(\alpha\beta\) heterodimers. Several studies have shown that in the absence of Ii, especially in B6 mice, MHC II molecules can adopt a ‘floppy’ conformation, reflecting either an empty groove or loosely associated peptides (21, 53, 54). In addition, most I-A\(^{\alpha}\) molecules are misfolded and do not traffic properly to the cell surface, which in turn hinders antigenic presentation (14–16). As expected, in wild-type mice, all peptide/MHC II complexes were in a compact state (labeled ‘C’) of ~55 kDa under non-boiled conditions (NB) (Fig. 3B). These complexes readily dissociated upon boiling (B). The M5/114 mAb is primarily a conformer and usually does not detect the isolated 29 kDa \(\beta\) chain. As described previously, the few I-A\(^{\alpha}\) molecules that folded in Ii-deficient B6 mice adopt a floppy conformation (‘F’) and migrated slightly slower to ~65 kDa (14, 15). Moreover, a high-molecular weight band, corresponding to aggregated MHC II products that form in the ER in the absence of mIi (16), could be seen at the top of the gel (Fig. 3B, open arrow). To determine the impact of lip35 on the fine conformation of I-A\(^{\alpha}\), spleen cells were sorted with the D-6 mAb, which recognizes the C-terminus of Ii. Surprisingly, the non-boiled samples from Tgp35 cells also showed a high-molecular weight band (but no floppy forms), suggesting that I-A\(^{\alpha}\) molecules that fold in the absence of Ii or in the presence of lip35 can form aggregates (Fig. 3C). These most likely correspond to the \(\beta\)' chains observed under denaturing conditions (Fig. 3A). Interestingly, samples from the Tgp35/mIiKO showed principally the presence of compact forms. The band is weak, probably due to the low expression of lip35 in many cells (Fig. 2C). Still, there was no floppy heterodimers in these conditions. Upon boiling, the compact forms were lost. Although M5/114 is principally a conformer, it has been shown to detect reduced and denatured I-A\(^{\alpha}\)\(\beta\) chains on western blots (23). Interestingly, M5/114 somewhat recognized the isolated 29 kDa \(\beta\) chain in our non-reduced samples from the Tgp35 lysates, raising the possibility that a fraction of the molecules are improperly oxidized in the presence of lip35. Altogether, these results show that lip35 promotes the formation of compact complexes but generates also high-molecular weight aggregates.

**lip35 affects the loading of exogenous peptides**

While previous studies have shown that Ii-null cells display less MHC II molecules, their floppy or SDS-sensitive conformation allows for better binding of exogenous synthetic peptides (14–16). Given the lip35-mediated increase in compact MHC II I-A\(^{\alpha}\) heterodimers in Tgp35/mIiKO cells, we asked whether the loading of exogenous peptides could be affected. We used the synthetic human CLIP peptide, which forms a strong complex with I-A\(^{\alpha}\) (55, 56). Purified spleen cells were incubated with the biotinylated synthetic peptide before permeabilization and incubation with the lip35-specific mAb. Our results confirmed that Ii-deficient cells had strong peptide-binding abilities (Fig. 3D). In Tgp35/mIiKO cells, high lip35 expression strongly decreased the binding of the exogenous peptides. No effect of lip35 was observed in Tgp35 cells that already bind little exogenous peptides. Together, these results confirm the effect of lip35 on the fine conformation of I-A\(^{\alpha}\).

**lip35 increases MHC II surface expression**

In Tgp35/mIiKO cells, lip35 expression increased levels of MHC II compact forms, which were detected on immunoblots (Fig. 3C). Using flow cytometry, we next assessed the effect of lip35 on I-A\(^{\alpha}\) cell surface expression. As expected, I-A\(^{\alpha}\) expression was reduced at the plasma membrane of mIiKO
mice (MFV = 8.7) compared with wild-type mice (MFV = 26.2) (Fig. 4A and B). In C57BL/6 mice, Iip35 expression did not affect I-Ab levels. Interestingly, in Ii-null mice (Tgp35/mIiKO) spleen cells, Iip35 expression significantly increased surface expression of MHC II molecules (MFV = 15.1). Such an intermediate expression of MHC II molecules reflects, at least in part, the fact that only about one-third of CD19+ cells express Iip35 (Fig. 1B; data not shown).

We performed three-color flow cytometry to better evaluate the effect of Iip35 in B cells. Isolated spleen cells were stained for CD19 and MHC II molecules, permeabilized and stained for Iip35 using BU45. While MHC II levels were low at the surface of Ii-deficient B cells, the expression of Iip35 restored normal levels in a dose-dependent manner (Fig. 4C, right panel). While a defined population of cells (circled) expressing high levels of Iip35 and low levels of I-A^b was apparent, these results still confirm that Iip35 is functional in assisting the folding and trafficking of I-A^b in a fraction of mouse B cells.

The absence of mIi alters the conformation of I-A^b

Although we have shown that lip35 can restore I-A^b surface expression, the possibility remains that it also causes the intracellular accumulation of MHC II molecules. This was first tested by flow cytometry. Splenocytes were permeabilized and stained with the AF6-120.1 mAb specific for a conformational epitope on I-A^b (57). Surprisingly, we found significantly less MHC II molecules in wild-type mice (MFV = 17) than in their Ii-deficient counterparts (MFV = 41.2) (Fig. 5A and B). To our knowledge, such a dramatic phenotype has not been reported before on the H-2^k background (23). Other I-A-specific mAbs, including M5/114, originally used to demonstrate reduced MHC II molecules in Ii-deficient mice, were also tested (14, 15). While the results confirmed that I-Ab levels in Ii-null mice were reduced, both at the plasma membrane and in intracellular compartments (Fig. 5C–F), they emphasized the peculiar phenotype revealed by the AF6-120.1 mAb. Interestingly, the presence of Iip35 exacerbated this phenotype as the Tgp35/mIiKO cells (MFV = 95) are more reactive to this mAb than mIiKO (MFV = 41.2) (Fig. 5A and B). Importantly, the expression of Iip35 in mIi^+ cells (Tgp35) also increased the staining of AF6-120.1. To confirm that the increased reactivity of this mAb in transgenic mice was caused by lip35, mature IgD^high splenocytes were analyzed with AF6-120.1 before (surface) or after (total) permeabilization (Fig. 6). Compared with wild-type, splenocytes from...
Ii-deficient animals demonstrated little reactivity with AF6-120.1 at the cell surface (Fig. 6A). Most of the AF6-120.1 material was detected only after permeabilization (Fig. 6B). Even though Iip35 expression restored I-Ab surface levels in a dose-dependent manner in Tgp35/mIiKO cells, upon permeabilization, these cells showed impressive amounts of AF6-120.1-reactive β chains compared with wild-type or mIi-deficient cells. Iip35-expressing Tgp35 cells also showed a strong intracellular accumulation of MHC II products in the presence of mIi.

The increased AF6-120.1 reactivity could be due either to an accumulation of I-Aβ-related products, to a serological modification in the absence of mIi or to the cross-reactivity of the mAb against an unknown antigen uncovered in mIi-negative cells. lip35-expressing Tgp35 cells also showed a strong intracellular accumulation of MHC II products in the presence of mIi.

The increased AF6-120.1 reactivity could be due either to an accumulation of I-Aβ-related products, to a serological modification in the absence of mIi or to the cross-reactivity of the mAb against an unknown antigen uncovered in mIi-negative cells. To help distinguish between these possibilities, immunoprecipitations using AF6-120.1 were performed using spleen cells from wild-type, mIiKO and Tgp35/mIiKO mice, these last two exhibiting the strongest reactivity to this mAb. The samples were blotted with the KL295 mAb, which recognizes the denatured I-Aββ chain. Approximately, a 28-kDa band was detected in all cell lysates and was immunoprecipitated principally from the mIiKO and Tgp35/mIiKO cells, in line with the strong AF6-120.1 staining in these mice (Fig. 6C). No other band of lower molecular weight was observed, showing that the AF6-120.1-reactive material includes an intact I-Ab β chain. Of note, the total amount of β chain detected by KL295 in the different cell lysates is very similar, showing that the knock-out and transgenic mice do not accumulate more of I-Ab. Altogether, these results suggest that the conformational AF6-120.1 epitope on I-Ab β chain is restricted to a subset of molecules and that it can be down-modulated by mIi. On the other hand, the expression of lip35 appears to favor the conversion of I-Ab molecules to the AF6-120.1+ conformation.

lip35 is expressed in immature BM B cells

In mice, Ii was shown to have a strong impact on B cell homeostasis, as evidenced by the reduced survival of mature cells in the spleen of Ii-null animals (22). As Ii and MHC II do not appear to be required for B cell maturation (27), we
hypothesized that expression of lip35 in the BM progenitors would not affect B cell development. BM cells were isolated, permeabilized and stained for B220 and Ii. In Tgp35 mice, more cells (63%) expressed the lip35 transgene compared with endogenous mIi (44%) (Fig. 7A). This suggests that the pDOI-6 promoter which regulates lip35 may be turned on at an earlier stage of differentiation. Similar results were obtained in Tgp35/mIiKO mice (data not shown). Interestingly, as observed in splenocytes (Fig. 1B and C), the amount of endogenous mIi (MFV = 88 in the wild-type) increased in Tgp35 (MFV = 194), suggesting again that mIi is retained in the ER and not efficiently processed due to the presence of the competing lip35. It should be noted that a small population of B220+ Ii+ cells were negative for BU45 (Fig. 7B, right panel). These cells most likely express mIi, given that all I-A+ cells in the BM are mIi+ (data not shown).

We also measured the impact of lip35 on I-A\(^\delta\) cell surface expression. The I-A\(^\delta\) profile gated on B220+ Ii+ cells can be seen in Fig. 7C. While mIi+ cells expressed I-A\(^\delta\) in wild-type mice (MFV = 101), the presence of lip35 in Tgp35 cells caused a marked reduction in the amount of I-A\(^\delta\) displayed at the plasma membrane (MFV = 27). These results demonstrate that lip35 acts as dominant-negative and cannot replace the endogenous murine lip31.

**T1 cells hIi\(^{high}\) do not up-regulate MHC II levels**

Based on the aberrant phenotype of BM B cells observed in Tgp35 mice, we asked if lip35 could allow proper migration and maturation of B cells in secondary lymphoid organs. In wild-type and Tgp35 animals, ~95% of immature CD21\(^{low}\) IgD\(^{low}\) cells (T1) expressed the endogenous mIi (data not shown). We evaluated the expression of lip35 in T1 cells with four-color flow cytometry using mAbs specific for CD21, IgD, I-A\(^\delta\) and Ii (Fig. 8A). lip35 was expressed in 79.9% and 61.6% of T1 B cells in Tgp35/mIiKO and Tgp35 mice, respectively. As seen in the BM and total splenocytes, high levels of lip35 did not appear to entirely restore I-Ab expression at the plasma membrane. Thus, we analyzed the impact of lip35 on BU45\(^{neg}\), BU45\(^{int}\) and BU45\(^{high}\) T1 cells (Fig. 8B and C). In both Tg mice, the BU45\(^{high}\) cells expressed less surface I-A\(^\delta\), defining a sub-population of cells that most likely do not fully support ER egress of lip35. These BU45\(^{high}\) cells are reminiscent of the peculiar population seen in Fig. 4 (circled). Together, these results show that most T1 cells in the spleen express...
Fig. 5. The absence of mli alters the conformation of I-A\textsuperscript{b}. Splenocytes were stained for I-A\textsuperscript{b} either after (total; A, B, D and F) or before (surface; C and E) permeabilization with the following mAbs: AF6-120.1 (A and B), 28-16-8S (C and D) and M5/114 (E and F). Data are representative of at least three independent experiments. (B) Compilation of normalized AF6-120.1 total expression from four different experiments. One-way ANOVA test $P$-value is indicated by asterisks (*$P < 0.05$; **$P < 0.01$).
high levels of lip35, but do not up-regulate MHC II at the cell surface.

We then asked whether the proportions of T1 B cells were normal in Tg mice. Based on IgD and CD21 expression, the presence of lip35 did not affect the overall distribution of different B cell subsets, either on the wild-type or on the li-null backgrounds (data not shown). However, given the low percentages of lip35+ cells and the heterogeneous protein expression levels (Fig. 1B), we analyzed the data in the context of the level of BU45 staining. It has been reported that in the spleen, the percentage of T1 cells increases in the absence of li, most likely due to the reduction of the FO compartment (22, 23). In absolute numbers, all mice appeared to have similar T1 compartments (data not shown), and our data corroborate the increased percentage of T1 cells in mli-null mice (Fig. 9A). The proportion of T1 cells increased from 11.2% in wild-type cells to 23.8% in mliKO cells and this difference was statistically significant (Fig. 9D). However, BU45+ cells were significantly enriched in the T1 population of Tg animals and accounted for ~50% of CD19+hi(BU45) splenocytes (Fig. 9B and D). These proportions are much higher than

![Fig. 6. Mature B cells accumulate MHC II molecules with an AF6-120.1+ conformation. (A) Splenocytes were stained for IgD and I-A\(^{\alpha}\) (AF6-120.1) before permeabilization and stained for lip35 (BU45). (B) Cells were stained for IgD, permeabilized and stained for I-A\(^{\alpha}\) (AF6-120.1) and lip35 (BU45). Cells were gated on the IgD\(^{\text{high}}\) population. Data are representative of at least three independent experiments. (C) Splenocytes were lysed and I-A\(^{\alpha}\) was immunoprecipitated with AF6-120.1. Samples were boiled and analyzed on SDS–PAGE (12%) under reducing conditions using KL295.](https://academic.oup.com/intimm/article-abstract/24/10/645/686483)
those observed in mIiKO mice, suggesting that these T1 Tg cells do not mature properly.

In the hIi int cell population of Tgp35/mIiKO, the number of T1 cells was found to be similar to wild-type (Fig. 9C and E). Although T1 numbers in the Tgp35 (18.7%) were not as low as those in the wild-type (11.2%), they were clearly and statistically lower than those seen in mIiKO (23.8%). Altogether, these results suggest that Iip35 restores the maturation/survival of a high proportion of splenic B cells.

Iip35 restores the mature B cell compartment

In the absence of li, mice display a perturbed mature B cell compartment. Despite normal precursor numbers in li-/- mice, studies have shown that the number of mature B cells in the spleen is greatly reduced (22–24). Our data demonstrated that up to the T1 stage, although MHC II levels were not fully restored, a large fraction of B cells continued to express Iip35. We next tested if the Iip35+ T1 cells could develop into FO cells. Analyses were performed on both Iip35int and Iip35high cells. Although the bulk of BU45high cells could be seen in the T1 subset, some were also found in other sub-populations. As seen in Fig. 9A, ~80% of wild-type mice cells had matured through the T2–FO stages. In contrast, this population was markedly reduced (47%) in li-null mice (Fig. 9A and D). The hIiint cells from Tg mice showed the same proportions of these mature cells as the mIiKO, suggesting that fewer cells expressing high levels of Iip35 acquire a mature phenotype. Alternatively, these cells may mature, but may not receive appropriate survival signals. Interestingly, as others have reported, we found that mIiKO mice have a more important marginal zone (MZ) compartment (24). However, although the Tg hIiint cells had a smaller percentage of mature FO cells, there was no concomitant increase in the MZ population (Fig. 9B and D). In fact, there were even less MZ cells in Tg mice compared with wild-type. These results demonstrate that MZ and FO B cell defects in mIiKO mice are independent and can be dissociated. Different mechanisms have recently been proposed to control these phenotypes (24).

When the proportions of mature B cells were analyzed in the context of hIiint cells, high proportions of FO cells were found in Tg mice (Fig. 9C and E). These B cells accounted for ~80% of CD19+liint splenocytes in Tg animals, a proportion similar to that observed in wild-type mice. A four-color flow cytometry analysis using CD19, IgD, IgM and hIi markers confirmed that Iip35 restores the compartment of mature FO B cells (Supplementary Figure 3 is available at International Immunology Online). However, the proportion of MZ cells was reduced in the context of Iip35 expression (Fig. 9C–E). In accordance with the slightly higher proportion of hIiint T1 cells in Tgp35 (Fig. 9C and E), our results suggest that cells expressing both mouse and human li chains mature less efficiently.

Fig. 7. Expression of Iip35 in immature BM B cells. (A) BM was removed from tibia and femur and collected cells were stained with anti-B220, permeabilized and stained with either BU45 (hIi, left panel) or In-1 (mIi, right panel). (B) Cells were stained for I-Aβ (28-16-8S) and B220 before permeabilization and staining with BU45. (C) Data were expressed as histograms to compare the expression of MHC II molecules in the B220+Ii+ populations. Data are representative of at least three independent experiments.
Discussion

The biology of Ii is complex. This chaperone appears to intervene in processes as diverse as antigen presentation, signal transduction and cell motility. In humans, the benefits conferred by the expression of Ii remain to be discovered. It is remarkable that such a short 16-amino acid extension at the N-terminal of Ii has the potential of adding multiple layers of complexity in the regulation of antigen presentation. Indeed, Ii is particular as it encompasses phosphorylation sites, an ER-retention motif and 14-3-3 binding sites. Moreover, Ii is dominant and dictates the intracellular trafficking behavior of Ii isoforms in mixed trimers. We have expressed this Ii isoform in mice in order to perturb the function of the endogenous mIi and get new insights into the function of these chaperones.

The Tg p35 mice were crossed onto the II-null background to obtain a Tg p35/mIIKO line with p35 representing the sole chaperone for I-Ab. Of note, Honey et al. (55) have previously reported that the human Ii was functional in mIi-proficient and -deficient mice. However, they used a genomic DNA fragment to generate Tg animals, which resulted in the predominant expression of Ii isoforms in mixed trimers. We have expressed this Ii isoform in mice in order to perturb the function of the endogenous mIi and get new insights into the function of these chaperones.

The Tg p35 mice were crossed onto the II-null background to obtain a Tg p35/mIIKO line with p35 representing the sole chaperone for I-A\(^\alpha\). Of note, Honey et al. (55) have previously reported that the human Ii was functional in mIi-proficient and -deficient mice. However, they used a genomic DNA fragment to generate Tg animals, which resulted in the predominant expression of Ii isoforms. Although not fully characterized, these mice did not show any aberrant phenotype, even when crossed on a mIi-null background. This suggests that the human Ii33 and the mouse Ii31 are interchangeable.

As one of the most important roles of Ii is the chaperoning of MHC II molecules, we first characterized biochemically the interaction of p35 with I-Ab. The fact that specific endosomal degradation products such as p10 were detected on immunoblots confirms that some of the p35 molecules are efficiently chaperoning I-A\(^\alpha\). CLIP/I-A\(^\alpha\) complexes were also detected in permeabilized cells but not at the plasma membrane, indicating that they must have encountered H2-DM along the endocytic pathway (Fig. 2).

Ii-deficient cells show a defect in the association of newly synthesized I-A\(^\alpha\) \(\alpha\) and \(\beta\) chains, which resulted in reduced M5/114 staining of permeabilized cells (14, 21, 23). The presence of p35 in Tg p35/mIIKO cells favored the association of \(\alpha\) and \(\beta\) chains based on intracellular M5/114 staining (data not shown) as well as the formation of compact forms of I-A\(^\alpha\) to the detriment of floppy heterodimers (Fig. 3C). Interestingly, the presence of p35 in Tg p35 cells expressing the endogenous mIi caused the accumulation of some immature I-A\(^\alpha\) molecules and the apparition of aggregates (Fig. 3A and C). Although this had no significant impact on the overall cell surface display of MHC II molecules, it is reminiscent of the situation in II-deficient mice and suggests that some I-A molecules are trapped by those p35 chains that do not egress the ER. In Tg p35 cells, as judged by the strong increase in AF6-120.1 mAb reactivity, there is clearly a large body of MHC II molecules that bind p35.
It is intriguing that lip35 exacerbates the phenotype of Ii-deficient cells regarding the reactivity of the AF6-120.1 mAb. Although Ii-deficiency on the I-A^k background caused the overt accumulation of MHC II, this is not the case for I-A^b. Indeed, western blot and flow cytometry experiments using conformation-independent antibodies showed no increase in
the levels of I-A<sup>β</sup> α and β chains in Ii-negative spleen cells (23) (Fig. 6C). Together, these results demonstrate that the effect of lip35 on the recognition of intracellular MHC II molecules by the AF6-120.1 mAb is conformational. Of note, the increase in I-A<sup>β</sup> expression at the surface of mature lip35<sup>+</sup> B cells in mIi-deficient animals (Fig. 4C) is not the mere reflection of the increased overall pool of AF6-120.1<sup>+</sup> molecules since it was also observed with the 28-16-8S and M5/114 mAbs (data not shown).

The fact that lip35 expression reduces the loading of exogenously supplied peptides in the absence of mIi is in line with a conformational change of I-A<sup>β</sup>. It is puzzling that the cells which were clearly affected are those T1 immature cells that express the most lip35 but which do not strongly up-regulate surface I-A<sup>β</sup>. When compared with mIiKO cells that express similar amounts of surface I-A<sup>β</sup>, the proportion of MHC II molecules that make it to the plasma membrane in those T1 cells have clearly a different conformation. Still, the cells expressing intermediate levels of lip35 do not bind more exogenous peptides but since they express more I-A<sup>β</sup> at the surface, we conclude that the pool of MHC II molecules reaching the plasma membrane are compact and refractory to peptide exchange.

Our results demonstrate that lip35 can egress the ER to restore MHC II levels in some B cells. In this context, it was interesting to determine the impact of lip35 on B cell homeostasis. In the BM, more B220<sup>+</sup> cells express lip35 compared with the endogenous mIi (Fig. 7). These lip35<sup>+</sup> cells showed a marked reduction in expression of surface I-A<sup>β</sup>, even in Tgp35 cells in which mIi is also expressed. The reasons for this dominant-negative effect remain to be investigated, but a likely explanation is that lip35 is not phosphorylated in these cells, causing the ER retention of all associated molecules. Such a scenario would explain the increased expression of mIi as its ER retention would prevent degradation in endosomes.

Matza et al. (25) have clearly demonstrated that Ii-driven signaling is critical for the establishment of a normal mature B cell compartment in the spleen. Interestingly, NF-κB activation necessitates regulated intramembrane proteolysis in the endocytic pathway and the release of the N-terminal 42-amino acid Ii region. In support of this model, transgenic mice expressing only the short 1-82 N-terminal region of Ii had a normal B cell compartment (58). Thus, it appears that MIF binding is not a prerequisite for NF-κB activation by Ii. We do not know if lip35 does bind MIF, but we propose that it undergoes proteolysis in endosomes and initiates signaling events. However, many BM and T1 cells in Tg mice appear to express high levels of lip35 without a concomitant increase in I-A<sup>β</sup> surface expression. This indicates that lip35 is not processed in all cells and accumulates presumably in the ER where it cannot signal. It will be interesting to determine if we can modulate ER egress of lip35 in these T1 cells, allowing their maturation into FO cells. If this cell population represents a specific subset of T1 cells remains to be established.

Our transgenic mice will be useful in the future to refine the mechanisms involved in B cell maturation and survival. Also, we will deepen the characterization of the I-A<sup>β</sup> fine structure in lip35+ cells and the associated peptide repertoire in the context of thymic selection of CD4<sup>+</sup> T cells. The capacity of lip35 to chaperone other ligands such as CD1d will be investigated as well.

Upheaval of the MHC II pathway in mice by the introduction of hlip35 allowed us to shed new light on the biology of Ii. Some of the effects observed here cannot be formally ascribed to the peculiar structure of lip35. For example, the inability of lip35 to rescue H2-DM expression may be a characteristic of all human Ii isoforms and a thorough characterization of Tg mice expressing mainly lip35 will be needed to resolve such issues (55). On the other hand, the incapacity of lip35 to rescue I-A<sup>β</sup> surface expression in immature B cells is clearly due to specific structural characteristics of lip35. Indeed, mIi-deficient Tg mice expressing preferentially hlip35 did not show any defect in MHC II surface expression and the fine conformation of their I-A<sup>β</sup> molecules appeared normal based on their reduced capacity to bind synthetic peptides compared with mIi-deficient animals (55). Future studies should address the role of lip35 on the fine structure of MHC II molecules in defined B cell subsets and in other cell types as well. In addition, other MHC II alleles should be tested by crossing our mice on different H-2 backgrounds. For example, even in the presence of II, alleles such as I-A<sup>β</sup> appear to preferentially fold as SDS-unstable dimers rather than compact or floppy forms (21).

In conclusion, it is tempting to speculate that B cell homeostasis in humans could be regulated through the modulation of lip35 ER egress. Given the importance of PKCs in the activation of B cells, in autoimmunity and in the phosphorylation of lip35, a complex interplay of factors including Ii may operate in various B cell pathologies (39, 59, 60).

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

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**Abbreviations**

APC, antigen presenting cell; BM, bone marrow; DCs, dendritic cells; ER, endoplasmic reticulum; FO, follicular B cells; II, Invariant chain; IP, immunoprecipitations; MIF, mean fluorescence value; MIF, macrophage migration inhibitory factor; mIi, murine Ii; MZ, marginal zone B cells; T1, transitional 1 B cells; T2, transitional 2 B cells; pMHC II, peptide–MHC II complexes.
References


