Université de Montréal

Structure-fonction de MARCH1, une E3 ubiquitine ligase régulant la présentation antigénique par le CMH II

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Thèse présentée à la Faculté de médecine
en vue de l'obtention du grade de Ph. D
en Microbiologie et Immunologie

Mai 2012

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse est intitulée:

Structure-fonction de MARCH1, une E3 ubiquitine ligase régulant la présentation antigénique par le CMH II

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a été évaluée par un jury composé des personnes suivantes :

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Résumé

Les molécules classiques du CMH de classe II sont responsables de la présentation de peptides exogènes par les cellules présentatrices d'antigène aux lymphocytes T CD4+. Cette présentation antigénique est essentielle à l'établissement d'une réponse immunitaire adaptative. Cependant, la reconnaissance d'auto-antigènes ainsi que l'élimination des cellules du Soi sont des problèmes à l'origine de nombreuses maladies auto-immunes. Notamment, le diabète et la sclérose en plaque. D'éventuels traitements de ces maladies pourraient impliquer la manipulation de la présentation antigénique chez les cellules dont la reconnaissance et l'élimination engendrent ces maladies. Il est donc primordial d'approfondir nos connaissances en ce qui concerne les mécanismes de régulation de la présentation antigénique.

La présentation antigénique est régulée tant au niveau transcriptionnel que post-traductionnel. Au niveau post-traductionnel, diverses cytokines affectent le processus. Parmi celles-ci, l'IL-10, une cytokine anti-inflammatoire, cause une rétention intracellulaire des molécules du CMH II. Son mécanisme d'action consiste en l'ubiquitination de la queue cytoplasmique de la chaîne bêta des molécules de CMH II. Cette modification protéique est effectuée par MARCH1, une E3 ubiquitine ligase dont l'expression est restreinte aux organes lymphoïdes secondaires.

Jusqu'à tout récemment, il y avait très peu de connaissance concernant la structure et les cibles de MARCH1. Considérant son impact majeur sur la présentation antigénique, nous nous sommes intéressé à la structure-fonction de cette molécule afin de mieux caractériser sa régulation ainsi que les diverses conditions nécessaires à son fonctionnement.

Dans un premier article, nous avons étudié la régulation de l'expression de MARCH1 au niveau protéique. Nos résultats ont révélé l'autorégulation de la molécule par formation de dimères et son autoubiquitination. Nous avons également démontré l'importance des domaines transmembranaires de MARCH1 dans la formation de dimères et l'interaction avec le CMH II.

Dans un second article, nous avons investigué l'importance de la localisation de MARCH1 pour sa fonction. Les résultats obtenus montrent la fonctionnalité des motifs de localisation de la portion C-terminale de MARCH1 ainsi que la présence d'autres éléments de localisation dans la portion N-terminale de la protéine. Les nombreux mutants utilisés pour ce projet nous ont permis d'identifier un motif ''VQNC'', situé dans la portion cytoplasmique C-terminale de MARCH1, dont la valine est requise au fonctionnement optimal de la molécule. En effet, la mutation de la valine engendre une diminution de la fonction de la molécule et des expériences de BRET ont démontré une modification de l'orientation spatiale des queues cytoplasmiques. De plus, une recherche d'homologie de séquence a révélé la présence de ce même motif dans d'autres ubiquitines ligases, dont Parkin. Parkin est fortement exprimée dans le cerveau et agirait, entre autre, sur la dégradation des agrégats protéiques. La dysfonction de Parkin cause l'accumulation de ces agrégats, nommés corps de Lewy, qui entraînent des déficiences au niveau du fonctionnement neural observé chez les patients atteints de la maladie de Parkinson. La valine comprise dans le motif ''VQNC'' a d'ailleurs été identifiée comme étant mutée au sein d'une famille où cette maladie est génétiquement transmise. Nous croyons que l'importance de ce motif ne se restreint pas à MARCH1, mais serait généralisée à d'autres E3 ligases.

Ce projet de recherche a permis de caractériser des mécanismes de régulation de MARCH1 ainsi que de découvrir divers éléments structuraux requis à sa fonction. Nos travaux ont permis de mieux comprendre les mécanismes de contrôle de la présentation antigénique par les molécules de CMH II.

Mots-clés : MARCH1, ubiquitination, autorégulation, dimérisation, localisation

Abstract

Classical MHC class II molecules are responsible for the presentation of exogenous peptides to CD4+ T cells, which is essential for the establishment of the adaptive immune response. However, problems with recognition of auto-antigens and the subsequent cell elimination are at the root of numerous autoimmune diseases. Manipulation of the antigen presentation pathway in order to eliminate cells that present self-antigens could serve as potential treatments of many autoimmune disorders. It is therefore essential to deepen our knowledge regarding the mechanisms regulating antigen presentation.

Antigen presentation is regulated both transcriptionally and post-translationally. Whereas many cytokines affect the latter, IL-10, an anti-inflammatory cytokine, causes the intracellular retention of MHC II molecules. This phenotype is the result of the ubiquitination of MHC II -chain cytoplasmic tail by MARCH1.

MARCH1 is an E3 ubiquitin ligase expressed in secondary lymphoid organs. Until recently, little was known about the structure-function and the targets of MARCH1. Considering its major impact on antigen presentation, we were interested to study this E3 ligase in order to reveal how it is regulated and what are the required conditions for its function.

In a first report, we have investigated the regulation of MARCH1's protein expression. Our results revealed its autoregulation via dimer formation and autoubiquitination. In addition, we have demonstrated the involvement of MARCH1's transmembrane domains for dimerization and MHC II interaction.

In a second article, we highlighted the importance of MARCH1 localization for its function. Our results indicated that localization motifs in the C-terminal portion of MARCH1 were functional and revealed the presence of some sorting elements in the N-terminal portion of the molecule. A panel of mutant were used and allowed us to identify a ''VQNC'' motif, located in the C-terminal cytoplasmic portion of MARCH1, in which the valine is central for the molecule's function. Indeed, point-mutation of the valine led to a

decrease in MARCH1 ability to relocate MHC II whereas BRET experiments revealed a modification in the spatial organization of the cytoplasmic tails. Moreover, a blast of sequence homology showed the presence or that same motif in others ubiquitine ligases, one of which is Parkin. Parkin is highly expressed in the brain and seems to be implicated in protein aggregates' degradation. It was reported that malfunction of Parkin leads to the accumulation of aggregates, called Lewy bodies, responsible for the neural functions deficiencies observed in patients with Parkinson disease. Interestingly, a family for which the sickness was genetically transmitted has a mutated valine in the VQNC motif. We believe that the importance of this motif is not restricted to MARCH1 and could be generalized to others E3 ubiquitin ligases.

This project enabled us to characterize the regulation mechanisms of MARCH1. In addition, we discovered various structural elements required for its function. Altogether, our data allows for a better understanding of the mechanisms controlling MHC II molecules antigen presentation.

Keywords: MARCH1, ubiquitination, autoregulation, dimerization, localization

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Abréviations

AP: *Adaptator protein*, protéine adaptatrice

CD: Cellule dendritique

CIITA: *Class II transactivator*, transactivateur de classe II

CLIP: *Class II associated invariant chain peptide*, peptide de la chaîne invariante associé au classe II

CMH: Complexe majeur d'histocompatibilité

CMH I: CMH de classe I

CMH II: CMH de classe II

CPA: Cellule présentatrice d'antigènes

c-MIR: *Cellular-MIR*, MIR-cellulaire

DIRT: *Domain in-between the RING and the first TM*, domaine entre le RING et le premier TM

EAE: Encéphalomyélite allergique expérimentale

ERAD: *ER associated degradation*, dégradation associée au RE

GAS: *Gamma IFN activated sequence*, séquence activée par l'IFN gamma

HECT: *Homologous au E6-AP carboxyl terminus, homologue du C-terminal de E6-AP*

IFN: Interféron

kK3: K3 de KSHV

kK5: K5 de KSHV

IL-10: Interleukine-10

LAMP-1: *Lysosome associated membrane protein-1*, protéine membranaire associée aux lysosomes-1

LB: Lymphocyte B

LPS: Lipopolysaccharide

LT: Lymphocyte T

LTc: LT cytotoxique

MARCH: *Membrane-associated RING-CH*, RING-CH associé à la membrane

MIIC: *MHC II compartment*, compartiment de CMH II

MLB: *Multilamellar bodie*, corps multilamellaire

MVB: *Multivesicular bodie*, corps multivésiculaires

mK3: K3 murin

PAMP: *Pathogen associated molecular pattern*, motif moléculaire associé aux pathogènes

RCT: Récepteur de LT

RE: Réticulum endoplasmique

RING: *Really interesting new gene*, nouveau gène très intéressant

Tfr: *Transferrin receptor*, récepteur de la transferrine

TGF: *Tumor growth factor,* facteur de croissance des tumeurs

Th: *T helper cell*, LT auxiliaire

TLR: *Toll like receptor*, récepteur de type toll

TM: Domaine transmembranaire

UBA: *Ubiquitin associated domain*, domaine associé à l'ubiquitine

UBC: *Ubiquitin conjugating domain*, domaine de conjugaison de l'ubiquitine

UBL: *Ubiquitin like domain*, domaine ressemblant à l'ubiquitine

UBP: *Ubiquitin binding protein*, protéine liant l'ubiquitine

UIM: *Ubiquitin interacting moti*f, motif interagissant avec l'ubiquitine

VIH: Virus d'immunodéficience humaine

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« Y'en aura pas de facile » Guy Daigneault

Remerciements

Je tiens d'abord à remercier mon directeur de recherche, le Dr. Jacques Thibodeau. Jacques, merci de m'avoir accueilli dans ton laboratoire alors que je connaissais très peu le domaine scientifique, d'avoir cru en moi et de m'avoir permis de devenir ce que je suis aujourd'hui. Sans ton aide constante, ta présence et nos innombrables et interminable échanges par courriels, mon expérience d'étudiante graduée n'aurait pas été aussi enrichissante. Tu es bien plus qu'un directeur de recherche pour moi.

Merci à tous mes collègues pour leur aide tout au long de cette aventure, particulièrement à Maryse Cloutier, Mammad Pezeshki, Annie Ladouceur, Nicola Raby et Maxime Raymond. Je tiens à remercier spécialement Jean-Simon Fortin qui fut non seulement mon mentor en matière de labo, mais aussi pour m'avoir montré à dessiner ainsi que pour son aide fort appréciée lorsqu'il vient le temps de commenter mes travaux. Merci aussi à Marie-Hélène Côté pour m'avoir tant appris et supportée dans les périodes difficiles.

Merci à Serge Sénéchal dont l'appui est allé bien au-delà de son mandat. Tu es toujours prêt à aider dans tous les domaines. Tes conseils dans le domaine canin m'ont également été très utiles et j'ai apprécié travailler avec toi durant toutes ces années.

Merci également à mes nombreux collaborateurs pour ces projets variés auxquels j'ai eu la chance de participer: Dr. Steimle, Dr. Lamarre, Dr. Cohen et Dr. Mourad.

Comment ne pas remercier mes amis pour leur appui sans failles et leur compréhension. Marianne, Raphaël, Vanessa, Jessica, Francis, Mélanie et Diane, je vous promet que dorénavant, nous allons discuter de d'autres sujets que du labo Thibodeau et de toutes ces choses un peu indigestes auxquelles vous avez eu la gentillesse de vous intéresser.

Merci à mes parents qui n'ont jamais cessé de croire en moi. Je suis conscience et reconnaissante de tout le support que vous m'offrez et ce, de manière inconditionnelle. Merci à ma soeur qui est aussi ma meilleure amie. Toi, Simon et la toute nouvelle arrivée

Charlie êtes pour moi une source constante de réconfort. Votre présence dans ma vie a une valeur inestimable à mes yeux.

Merci à Jody pour ta simple présence. Ton désir de comprendre est l'une de tes plus grandes qualités, mais comme tu le dis si bien, il te manque la base pour mieux comprendre.

Merci à la science qui permet de transformer mes pires défauts, comme la curiosité maladive et l'acharnement, en qualités. Je remercie également EMBO que j'aime de tout mon coeur.

Finalement, je tiens à remercier les nombreux organismes subventionnaires : l'IRSC qui subventionne le projet, Rx et D, le COPSÉ, le Département de Microbiologie et Immunologie de l'Université de Montréal, la fondation Cole et la faculté des études supérieures pour l'attribution de bourses ainsi qu'Immunologie Montréal, la fondation Cole et la SCI pour les bourses de voyage. Je tiendrais également à remercier les membres du jury pour la lecture et l'évaluation de mon travail.

Mise en contexte: rôle de MARCH1 dans la réponse immunitaire

Le système immunitaire permet à l'organisme de se défendre contre les agents pathogènes. Il agit à la manière d'un système de contrôle de la qualité qui permet le maintient de l'homéostasie. L'immunité adaptative est une réaction spécifique à un antigène donné. Cette composante de l'immunologie permet d'élaborer une réponse immunitaire dirigée par exemple, contre un microorganisme en particulier et repose sur la présentation des antigènes à la surface cellulaire. Ceux-ci peuvent appartenir à l'organisme, ou encore à un agent pathogène lorsqu'il s'agit d'une infection. Les cellules infectées ou mutantes seront alors reconnues et éliminées par différents effecteurs des réponses immunitaires.

Un défaut de présentation antigénique ne permettra pas l'élimination des cellules infectées ce qui pourra alors avoir pour conséquence d'entraîner l'infection généralisée et éventuellement de causer la mort de l'organisme. D'un autre côté, une réponse immunitaire incontrôlée entraîne les cellules immunitaires à endommager les tissus de l'hôte. Il est donc primordial de réguler ce processus dans le but de protéger l'organisme contre les méfaits potentiels de son propre système de défense.

MARCH1 est un régulateur clé de la présentation antigénique. Il prévient l'expression de surface du CMH de classe II. Puisque la régulation des régulateurs est essentielle au contrôle de la présentation antigénique, nous nous sommes intéressé à la régulation de MARCH1. MARCH1 étant peu caractérisé au niveau de sa structure-fonction, nos travaux portent sur sa régulation et sur les divers éléments structuraux requis à son fonctionnement optimal.

Chapitre 1: Introduction

1.1. La Présentation antigénique

1.1.1 Concept général

Il existe deux grands types de réponses immunitaires; la réponse innée et celle adaptative [1,2]. Chacune comporte son rôle particulier, mais c'est la conjugaison des deux qui permet l'établissement d'un système de défense efficace contre les infections.

1.1.1.1. L'immunité innée

L'immunité innée se compose de mécanismes non spécifiques et constitue la première ligne de défense du système immunitaire. Elle survient dès les premières minutes suivant l'infection, permettant ainsi un contrôle rapide de celle-ci. Néanmoins, elle ne permet pas l'établissement d'une mémoire immunitaire, c'est-à-dire d'une réponse spécifique plus efficace dans l'éventualité d'une seconde infection par un même microorganisme pathogène [3]. Parmi les mécanismes de défense innée figurent les barrières physiques telles que la peau et le mucus qui limitent l'invasion de l'organisme par les agents pathogènes environnementaux [2].

L'immunité innée étant non spécifique, la reconnaissance des agents pathogènes se fait à l'aide de motifs moléculaires associés aux pathogènes (*pathogen associated molecular patterns*: PAMPS) qui sont reconnus par les récepteurs de type toll (*toll-like receptor*: TLR), de type RIG-I (*RIG-I-like receptor*), de type Nod (*Nod-like receptor*) ou encore ceux de type lectine (*lectin-like receptor*) [4]. Parmi ceux-ci figurent les lipopolysaccharides (LPS) bactériens et les ARN double-brins viraux [5]. Ainsi, en cas d'infection, les cellules du système immunitaire des tissus infectés reconnaîtront les PAMPs et enclencheront l'inflammation [5–7]. La sécrétion de cytokines pro-inflammatoires, telles que le TNFα et l'IL-1, permet le recrutement massif de cellules immunitaires au site d'infection [2,6,7].

1.1.1.2. L'immunité adaptative

De son côté, l'immunité adaptative est spécifique à un antigène donné et dépend de la présentation antigénique. C'est l'immunité innée, de par l'inflammation, qui enclenche et localise l'immunité adaptative [3,5,8,9].

1.1.1.2.1. La présentation antigénique

Les cellules présentent continuellement à leur surface un inventaire des protéines qu'elles contiennent. Ces protéines sont dégradées en de courts peptides qui sont ensuite présentés à la membrane plasmique par les molécules du complexe majeur d'histocompatibilité (CMH) [10]. L'activation d'une cellule présentatrice d'antigènes (CPA) dépendra de la présence ou de l'absence d'un danger en fonction du peptide présenté ainsi que de la présence de signaux de dangers reçus dans le microenvironnement. Ceux-ci sont des composantes moléculaires relâchées par les cellules qui vivent un stress ou qui sont en processus de mort cellulaire. Ce signal de co-stimulation est essentiel à l'activation des CPA [11].

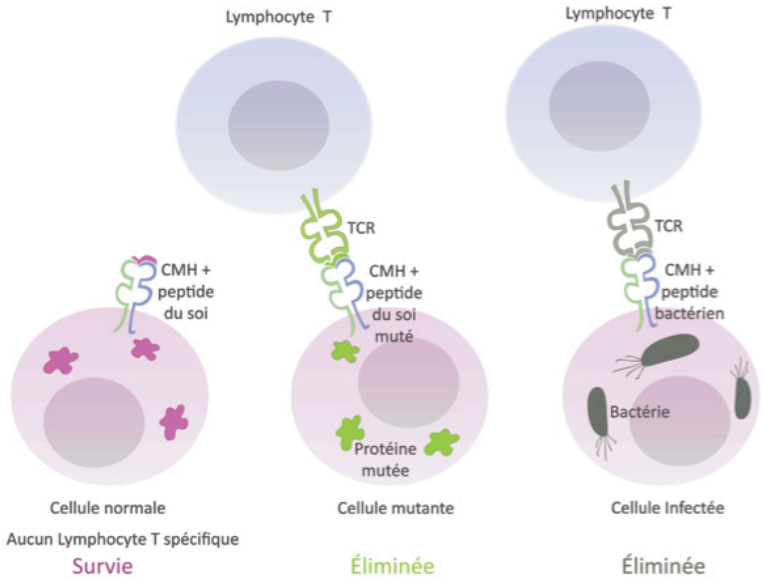

Figure 1.1. Identification des cellules mutantes et infectées à éliminer. Le schéma illustre la présentation antigénique de peptides dérivés de protéines normales, mutantes ou bactériennes. Les LTs reconnaissent les cellules à éliminer en fonction du peptide qui est présenté à la surface cellulaire.

1.1.1.2.2. Le Soi et le non-Soi

La présence d'un danger sera donc attribuable à la reconnaissance de peptides appartenant au non-Soi ainsi qu'aux signaux reçus du microenvironnement (voir Figure 1.1.) [2,12]. Le Soi étant l'ensemble des peptides habituellement exprimés par les cellules

alors que le non-Soi inclut les peptides exogènes ou mutés. Par exemple, des protéines endogènes mutantes, virales ou bactériennes appartiennent au non-Soi [13]. La présentation antigénique est cruciale pour l'établissement d'une réponse immunitaire et l'élimination des agents pathogènes, mais également pour la préservation de la tolérance au soi ainsi qu'à la prévention de réactions auto-immunes [13,14].

D'un point de vue évolutif, les premiers organismes ayant acquis la capacité à discerner le Soi par rapport au non-Soi étaient les vertébrés à mâchoire [1,5,8]. Ce sont donc les premiers à avoir développé une réponse immunitaire spécifique vis-à-vis un antigène reconnu. C'est l'immunité adaptative. Cette branche de l'immunité est également responsable du développement de la mémoire immunitaire qui permet une élimination plus rapide et efficace du microorganisme pathogène dans le cas d'infections subséquentes.

1.1.2. Les lymphocytes T

1.1.2.1. La stimulation

Les lymphocytes T (LT) expriment, à leur surface, un récepteur unique à chaque clone; le récepteur de cellule T (RCT) [15]. Le RCT reconnaît spécifiquement un peptide associé à une molécule du CMH. Cette reconnaissance se produit à la synapse immunologique.

La stimulation appropriée d'un LT nécessite trois signaux: l'interaction d'un RCT avec un complexe peptide-CMH qui lui est spécifique, la reconnaissance du CMH de classe I (CMH I) et II (CMH II) avec leurs co-récepteurs respectifs CD8 et CD4 et l'interaction des molécules de co-stimulation CD80 ou CD86 avec la molécule CD28 à la surface du LT [16]. Lorsque ces facteurs sont réunis, un signal d'activation est transmis via le multi-récepteur CD3 associé au RCT.

Les LTs réagissant contre des antigènes du Soi constituent un danger pour l'organisme. Au cours de leur maturation, ils feront face à deux types de sélection. Ils seront sélectionnés pour leur capacité à reconnaître un peptide dans le contexte du CMH et l'absence de reconnaissance du Soi.

Les LTs matures seront activés et entreront en processus d'expansion seulement si ils reconnaissent un antigène présent dans les organes lymphoïdes secondaires [17]. Ces antigènes, acquis dans les tissus périphériques, seront liés aux molécules de CMH à la surface des CPAs [17].

Ainsi débute la réponse immunitaire adaptative. Elle est nécessaire à l'élimination de l'antigène de manière ciblée ainsi qu'à la production d'anticorps et l'établissement de la mémoire immunitaire [18]. Les deux principaux types de LTs sont les cytotoxiques et les auxiliaires.

1.1.2.2 Les lymphocytes T cytotoxiques

Les LT cytotoxiques (LTc) sont responsables des réponses immunitaires à médiation cellulaire [19]. Ils présentent à leur surface le co-récepteur CD8 et interagissent spécifiquement avec les molécules du CMH I [10].

Les LTcs peuvent tuer les cellules portants le complexe CMH I-peptide spécifique à leur RCT. Cette mort cellulaire induite est médiée par la relâche de perforines, qui créent des pores, et de granzymes, qui y pénètrent. Les LTcs peuvent également induire la mort des cellules reconnues par la stimulation de Fas [19]. Pour médier leurs effets cytotoxiques, les LTcs nécessitent une pré-activation par un LT auxiliaire [20].

1.1.2.3 Les lymphocytes T auxiliaires

De leur côté, les LT auxiliaires (*T helper:* Th) sont caractérisés par la présence du co-récepteur CD4 [20]. Suite à la reconnaissance du complexe peptide-CMH qui lui est spécifique, le Th CD4+ se différenciera en l'un des nombreux sous-types de Ths dont les principaux sont Th1, Th2, Th17 et Tfh [21]. Le sous-type choisi dépendra majoritairement des signaux de co-stimulation reçus, ainsi que des cytokines présentes dans le micro-environnement [21]. Chaque sous-type accomplira différentes fonctions grâce, entre autre, à la sécrétion de nombreuses cytokines. Elles pourront alors recruter et activer les lymphocytes B (LB), les cellules dendritiques (CD), les macrophages, les LTcs, les mastocytes, les neutrophiles, les eosinophiles ainsi que les basophiles [21].

Les Th17s ont un rôle important à jouer dans la protection contre les infections opportunistes, mais sont souvent associés à des maladies auto-immunes causées par de l'inflammation excessive. Les Tfhs interagissent de manière prolongée ou transmettent un fort signal au BCR. Elles sécrètent peu de cytokines, mais ont la capacité unique de s'établir dans les follicules Bs. Ainsi, ce sont d'excellents stimulateurs de la production d'anticorps [22]. Les Th1s sont essentiels à l'élimination des agents pathogènes intracellulaire, mais sont également responsables de l'activation des LTcs, des macrophages et des cellules NKs [20]. De leur côté, les Th2s sont plutôt affectés à l'activation des LBs, ce qui engendrera la différenciation de ces derniers en plasmocytes, de même que leur expansion clonale et donc, la production massive d'anticorps spécifiques à l'antigène du non-Soi reconnu [20].

1.2. Le Complexe Majeur d'Histocompatibilité

1.2.1. Organisation génique

Chez l'humain, les gènes du CMH sont regroupés sur le chromosome 6 et se nomment antigène de leucocyte humain (*human leukocyte antigen*: HLA) [23]. Les molécules HLA-A, -B et C font partie du CMH I alors que HLA-DR, -DP et -DQ constituent le CMH II [14].

L'un des grands dogmes de l'immunologie est la dichotomie des antigènes présentés par les molécules du CMH I et II. Nous discuterons dans les sections suivantes de la théorie simpliste voulant que le CMH I présente les peptides dérivés d'antigènes endogènes alors que le CMH II présenterait plutôt ceux dérivés d'antigènes exogènes. Néanmoins, il est important de mentionner le phénomène de présentation croisée. Par exemple, la translocation des protéines du cytoplasme au réticulum endoplasmique (RE) par le transporteur TAP permettra la présentation d'antigènes du non-Soi par les molécules du CMH I [24]. À l'inverse, grâce à l'autophagie, des peptides du Soi pourront être présentés par les molécules du CMH II [25].

1.2.2. Le CMH de classe I

Le CMH I est exprimé par toutes les cellules nucléées du corps humain. La présentation antigénique par les molécules du CMH I vise essentiellement à éliminer les cellules cancéreuses ainsi que les cellules infectées [19]. Le CMH I est reconnu par les RCTs des LTcs [19].

1.2.2.1. La présentation antigénique par le CMH I

Les cellules cancéreuses possèdent les caractéristiques suivantes: prolifération illimitée, insensibilité aux signaux d'arrêt de croissance et d'entrée en apoptose, dérégulation du cycle cellulaire et instabilité génétique [26]. Ces caractéristiques sont le résultat de mutations ayant permis la transformation de la cellule. Certaines mutations transforment les molécules du Soi qui seront désormais reconnues comme étant du Soi dégénéré [27]. La présentation de ces peptides par les molécules du CMH I permettra l'élimination de la cellule mutante par les LTcs [19].

Par ailleurs, lors d'une infection par un virus ou une bactérie, les protéines de ce dernier seront alors exprimées et soumises aux mêmes processus de contrôle de qualité que les protéines endogènes [28]. Ainsi, certains peptides viraux ou bactériens pourront alors interagir avec le CMH I pour ensuite être acheminés vers la surface cellulaire. Dans tous les cas, les complexes CMH I-peptide du non-Soi pourront alors être reconnus par les LTcs et la cellule infectée sera ensuite éliminée [19].

1.2.3. Le CMH de classe II

La présentation antigénique par le CMH II se fait par des cellules spécialisées nommées CPA professionnelles. La présentation antigénique par le CMH II aux LTs CD4+ est l'étape instigatrice de l'immunité adaptative. Sans elle, aucune réponse spécifique à un antigène donné ne pourrait se développer [10,29].

Il est maintenant reconnu qu'en plus de présenter les antigènes à la surface cellulaire, les molécules du CMH II servent également de récepteur à la transmission de

différents signaux [10,30,31]. La liaison des molécules du CMH II active une cascade ayant différents effets tels que la prolifération, la maturation et l'apoptose [31,32].

1.3. La présentation antigénique par le CMH II

1.3.1. Cellules présentatrices d'antigènes professionnelles

Les CPAs sont des cellules spécialisées dans l'internalisation, l'apprêtement et la présentation d'antigènes exogènes [33]. Les LBs, les macrophages et les CDs en sont les principales [18,23,34].

1.3.1.1. Les lymphocytes B

Les LBs expriment constitutivement de hauts niveaux du CMH II. Bien qu'elles internalisent également des antigènes via leurs récepteurs de type Fc et du complément, cette internalisation s'effectue principalement via leurs immunoglobulines de surface, ce qui limite leur capacité à présenter une vaste sélection d'antigènes [34,35].

1.3.1.2. Les macrophages

De leur côté, les macrophages ont une activité phagocytaire élevée et internalisent toutes les formes d'antigènes via les récepteurs: i) de types Fcγ, ii) de type polysaccharide et iii) du complément. De plus, ils expriment le CMH II, de même que les molécules co-stimulatoires, de manière constitutive. Néanmoins, cette expression est plus faible que chez les LBs et les CDs [34].

1.3.1.3. Les cellules dendritiques

La fonction première des CDs est la présentation antigénique. Elles ont une excellente capacité à endocyter du matériel exogène [17,34]. Au stade immature, elles expriment peu de CMH II à leur surface, mais la maturation induite par la reconnaissance de certains PAMPs cause la redistribution du CMH II à membrane plasmique, faisant ainsi des CDs matures d'excellentes CPAs [34,36,37].

1.3.2. Les molécules du CMH II

1.3.2.1. Les molécules classiques

Les molécules classiques du CMH II sont HLA-DR, -DP et –DQ. Elles sont responsables de la présentation de l'antigène à la surface cellulaire aux LTs CD4+. Les molécules classiques du CMH II sont très polymorphiques [23,29]. Ce sont des glycoprotéines hétérodimériques transmembranaires de type I formées par une chaîne α et une chaîne β [10,38].

La queue cytoplasmique de la chaîne β comporte un motif di-leucine qui est partiellement responsable de l'endocytose et du recyclage des molécules à la surface cellulaire [39].

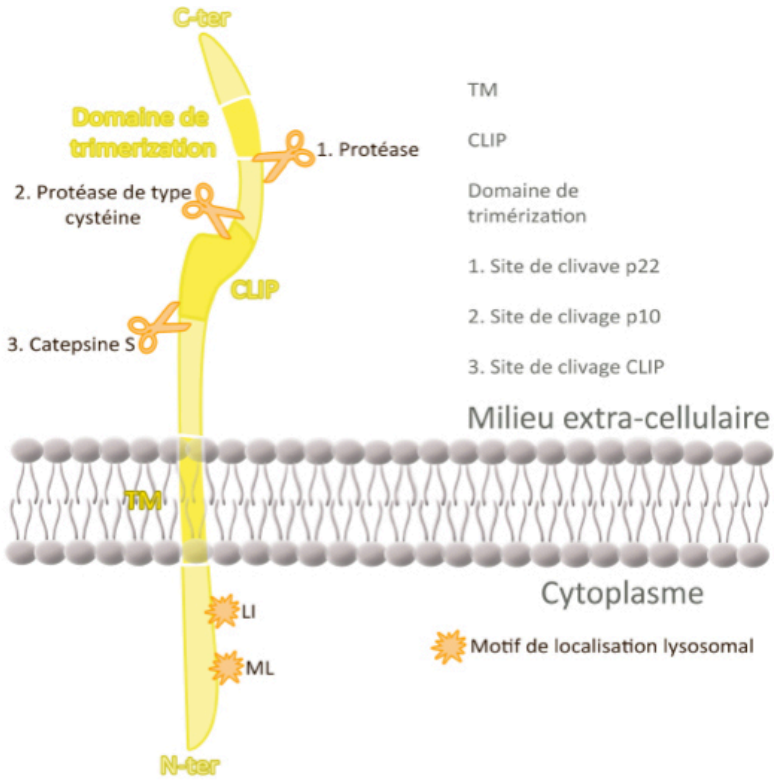

Figure 1.2. Représentation schématique de la chaîne invariante illustrant les différents domaines et motifs de celle-ci.

1.3.2.2. La chaîne invariante

La chaîne invariante est une glycoprotéine transmembranaire de type II [38,40]. Il existe quatre différentes isoformes issues de deux différents codons d'initiation et de leur épissage alternatif: p33, p35, p41 et p43 [41]. L'isoforme p33 est la plus courante, suivie de l'isoforme p35. Ce dernier possède un motif de rétention au RE de type

RXR et ne peut sortir du RE que lorsque ce signal est masqué par la queue cytoplasmique de la chaîne β du CMH II [42,43].

Peu après sa synthèse dans le RE, la région des acides aminés 87 à 107, le peptide de la chaîne invariante associé au CMH II (*class II associated invariant chain peptide*: CLIP), s'insère dans la niche peptidique du CMH II. Ceci prévient la liaison de peptides endogènes présents dans le RE [40,44,45]. La chaîne invariante possède une autre fonction: elle chaperonne les molécules de CMH II en assurant leur repliement adéquat ainsi que l'appariement approprié des chaînes α et β des différents isotypes [33,38,46].

Deux portions de la chaîne invariante assurent sa trimérisation: la région transmembranaire ainsi qu'un domaine de trimérisation situé dans la partie luminale de la molécule (voir Figure 1.2.) [23,40].

De plus, la région cytoplasmique possède un motif de type di-leucine qui permet de localiser la molécule dans les compartiments endosomaux (voir Figure 1.2.) [47]. C'est d'ailleurs la chaîne invariante qui dirige le CMH II dans ces compartiments [42].

1.3.2.3. Les molécules non classiques

Il existe deux molécules non classiques du CMH II: HLA-DM et -DO [14]. Ces molécules sont peu polymorphiques et ne possèdent pas la capacité de lier des peptides [48,49]. Comme les molécules classiques, ce sont des glycoprotéines transmembranaires hétérodimériques possédant une chaîne α et une chaîne β.

HLA-DM détient deux rôles: la relâche du CLIP ou d'autres peptides ainsi que la stabilisation de HLA-DR [50,51]. En effet, sa liaison avec le CMH II facilite la relâche des peptides de faible affinité et leur remplacement par d'autres ayant une plus haute affinité pour la niche [52]. Les molécules du CMH II classiques étant très instables lorsque dépourvues de peptide, cette association permet la stabilisation de la molécule et prévient son agrégation [52].

La queue cytoplasmique de la chaîne β de HLA-DM possède un motif de localisation lysosomale de type tyrosine [53–56]. Ce type de motif est reconnu par les protéines

adaptatrices (*adaptator protein*: AP) et permet la relocalisation endosomale de la molécule [57–59]. C'est grâce à ce motif que HLA-DM et HLA-DO sortent du RE [60]. En effet, sans l'aide de HLA-DM, HLA-DO reste captif dans le RE [49].

De son côté, HLA-DO possède également deux rôles: il stabilise HLA-DM et en régule négativement l'activité [50]. Lorsqu'il interagit avec HLA-DM, HLA-DO prévient l'action de ce dernier. Ainsi, l'échange peptidique est inhibé. Il en résulte une augmentation des complexes CMH II-CLIP au détriment des complexes CMH II-peptide [29].

La queue cytoplasmique de la chaîne β de HLA-DO encode un motif di-leucine. Des travaux réalisés par notre groupe de recherche ont démontré l'importance de ce motif pour la localisation du complexe HLA-DO/-DM [61].

L'expression de HLA-DO est restreinte aux LBs, à certaines CDs ainsi qu'aux cellules épithéliales thymiques [60].

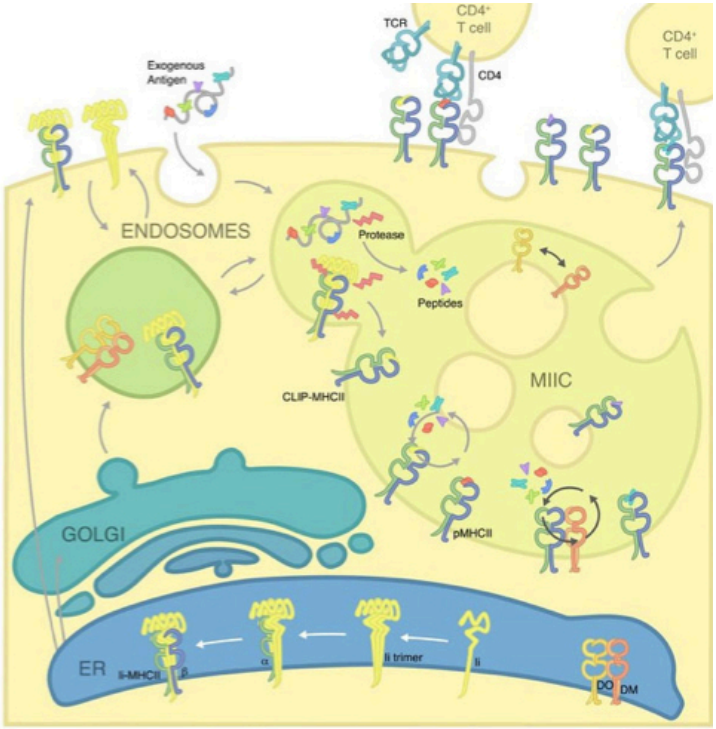

Figure 1.3. Représentation schématique du transport cellulaire des molécules de CMH II. Dessiné par Jean-Simon Fortin

1.3.3. Le transport des molécules du CMH II

Les molécules du CMH II sont synthétisées dans le RE, où trois chaînes α et β s'associent avec un trimère de chaîne invariante, formant ainsi un complexe nonamérique (voir Figure 1.3.) [33]. Il y a ensuite deux voies de sortie du RE. Le complexe peut passer par le réseau trans-golgien pour ensuite se retrouver dans les endosomes ou alors aller directement à la membrane cytoplasmique. Le transport via la voie exocytaire est le mécanisme par défaut

[42]. De la surface cellulaire, la protéine adaptatrice AP-2 reconnait (reconnaît) les deux motifs leucine de la chaîne invariante et redirige le complexe vers la voie endocytaire [62].

De leur côté, HLA-DM et HLA-DO s'associent également dans le RE. Les complexe HLA-DO/-DM migrent ensuite via le système trans-golgien vers les endosomes où se retrouve également le complexe nonamérique [49]. La rencontre de HLA-DO, -DM et du CMH II dans les mêmes compartiments est essentielle à l'édition du répertoire peptidique par HLA-DM (voir Figure 1.3.).

1.3.4. Les compartiments de classe II

Dans les compartiments endosomaux, la fusion avec des vésicules acides ainsi que l'activation de pompes à protons V-ATPases contribue à leur acidification [63]. Cette acidification permet la maturation des endosomes en endosomes tardifs et en lysosomes.

Les molécules de CMH II s'accumulent dans des compartiments dits compartiments de classe II (*MHC II compartiments*: MIICs) [23,64]. Ces compartiments sont fortement apparentés aux lysosomes en termes de pH et de composantes. Ils sont d'ailleurs positifs pour les marqueurs LAMP-1 (*lysosome associated membrane protein-1*) et CD63 [65].

Il existe différentes morphologies de MIICs. On distingue principalement les corps multilamellaires (*multilamellar bodies*: MLB) et les corps multivésiculaires (*multivesicular bodies*: MVB) [66].

C'est dans les MIICs que le chargement peptidique aura lieu [67]. En effet, il y a une forte activité protéolytique, ce qui entraîne la dégradation séquentielle de la chaîne invariante par les cystéines et aspartiques protéases [68]. La cathepsine S, dont l'expression est majoritairement restreinte aux CPAs, avec l'aide d'autres protéases comme la cathepsine L, permet le clivage de la chaîne invariante en fragments de 22 kDa, puis de 10 kDa pour finalement ne laisser que la portion CLIP dans la niche peptidique [23,69].

1.3.5. Le chargement peptidique

Les MIICs contiennent les peptides qui seront chargés sur les molécules de CMH II. Ces peptides sont issus de la dégradation de molécules acquises par autophagie ou encore par phagocytose [70,71]. Les autophagosomes et les phagosomes fusionnent avec les MIICs, ce qui en permet la libération du contenu et sa dégradation subséquente.

Suite à la dégradation de la chaîne invariante, HLA-DM interagit avec le CMH II, favorisant ainsi le retrait du peptide [72]. De plus, tel que mentionné plus tôt, les molécules de CMH II vides sont très instable, alors HLA-DM chaperonne les molécules de CMH II jusqu'à ce qu'un peptide de haute affinité s'insère dans la niche peptidique [50]. La présence de HLA-DO prévient l'action de HLA-DM [73]. Néanmoins, l'activité de HLA-DO est dépendante du pH. Il sera actif à un pH de 5.5, mais inactif à un pH de 5.0 [74]. De cette manière, HLA-DM pourra être actif dans les MIICs.

Les molécules sont ensuite acheminées à la surface cellulaire [10]. De la surface, ces dernières sont recyclées, ce qui permet l'internalisation des molécules et le remplacement de leurs peptides [75,76]. Le recyclage des molécules de CMH II et le remplacement de leurs peptides permettent de présenter continuellement des peptides associés aux antigènes récemment incorporés.

1.4. La régulation de la présentation antigénique

La présentation antigénique étant essentielle à l'établissement d'une réponse immunitaire, il est primordial de réguler ce processus. De plus, une réponse exagérée et incontrôlée constitue un danger pouvant mener jusqu'à l'auto-immunité [13]. La présentation antigénique est donc régulée tant au niveau transcriptionnel que post-traductionnel.

1.4.1. La régulation transcriptionnelle

1.4.1.1. Le transactivateur de classe II

La régulation de l'expression génique du CMH II est effectuée par le transactivateur de classe II (*class II transactivator*: CIITA) [77]. Ce co-activateur transcriptionnel régule presque exclusivement les gènes associés à la présentation antigénique par le CMH I et II. CIITA est exprimé spécifiquement par les CPAs et les cellules épithéliales thymiques [78]. Il est également possible d'induire son expression chez les LTs activées et/ou suite à une stimulation à l'interféron-γ (IFNγ) [77]. De plus, son expression est régulée négativement suite à la différenciation des LBs en plasmocytes et la maturation des CDs [14].

Certains microorganismes pathogènes ont développé des mécanismes d'évasion du système immunitaire qui touchent directement CIITA. Par exemple, le virus d'immunodéficience humaine (VIH), *Toxoplasma* et *Mycobacterium* inhibent l'expression de CIITA et des gènes du CMH [77]. Ceci constitue une stratégie d'évasion du système immunitaire.

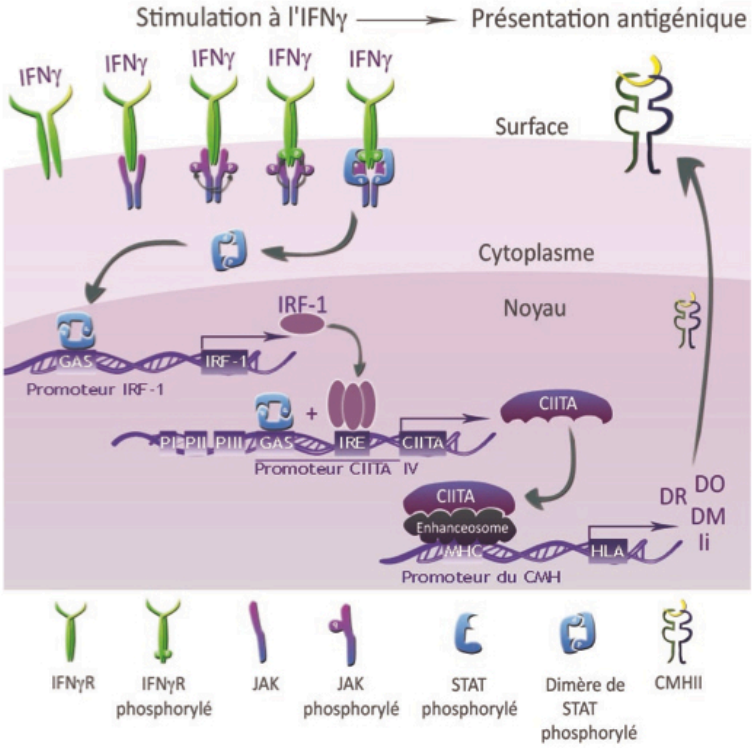

Figure 1.4. Représentation schématique de la transcription des gènes de CMH II en réponse à l'IFNγ.

1.4.1.2. Stimulation à l'IFNγ

Suite à une stimulation à l'IFNγ, quatre différents facteurs de transcription, constituant l'enhanceosome, se lient à un module situé de 150 à 300 paires de bases en amont du premier gène du regroupement des gènes de CMH II: le module SXY (voir Figure 1.4.) [79].

L'enhanceosome protège contre la méthylation, ce qui améliore l'accessibilité aux gènes, et est responsable du recrutement de CIITA [14].

D'un autre côté, un autre facteur de transcription nommé STAT1 se lie à la séquence activée par l'IFNγ (*gamma interferon activating sequence*: GAS) et à la séquence E-box [77]. Cette liaison permet d'augmenter l'accessibilité à la chromatine via l'acétylation des histones, ce qui facilite la transcription des gènes.

La transcription de CIITA dépend de quatre différents promoteurs. Son isoforme ''pI'' est la plus efficace, mais c'est l'isoforme ''pIV' qui est induite par l'IFNγ.

L'expression induite par l'IFNγ peut être inhibée par différentes cytokines telles que le facteur de croissance des tumeurs β (*tumor growth factor β*: TGFβ), l'IFNα et l'IL-4 [79].

1.4.1.3. Les fonctions de CIITA

De son côté, CIITA a plusieurs rôles. Il recrute des facteurs de transcription ainsi que des co-activateurs et phosphoryle l'ARN polymérase II, améliorant ainsi l'accessibilité aux gènes [77]. Il permet sa propre transcription et celles du groupe des gènes de classe II, comprenant HLA-DR, -DP, -DQ, -DM, -DO, la chaîne invariante, TAP1, TAP2, PSMB8 et PSMB9 (voir Figure 1.4.) [14,79]. Les gènes TAP1 et TAP2, impliqués dans le transport de peptides du cytoplasme au RE, sont des transporteurs essentiels à la présentation antigénique par le CMH I. De leur côté, PSMB8 et PSMB9 permettent la dégradation de protéines ubiquitinées et donc la génération de peptides [14]. Ce sont des sous-unités de l'immunoprotéasome, abondantes chez les CPAs [80]. En plus de favoriser la production de peptides propices à la présentation par les molécules du CMH, l'immunoprotéasome possède une activité protéolytique augmentée par rapport aux protéasomes réguliers, ce qui prévient l'accumulation d'agrégats protéiques et favorise le maintient de l'homéostasie des protéines [80,81].

Finalement, la régulation de HLA-DO par le CIITA demeure controversée. Certains groupes prétendent que CIITA induit l'expression de la chaîne α de HLA-DO mais non de la chaîne β alors que d'autres affirment qu'il active la transcription des deux chaînes de la molécule [77,82,83].

1.4.2. Régulation post-traductionnelle

1.4.2.1. Régulation de CIITA

Plusieurs mécanismes de régulation post-traductionnels ciblent directement CIITA [84–86]. Par exemple, la dégradation de CIITA entraînera la diminution de la transcription des gènes de classe II. Une méthode alternative pour contrer l'activité transcriptionnelle de CIITA est la désacéthylation des histones [79].

1.4.2.2. Inhibition du chargement peptidique

Un autre mécanisme de prévention de la présentation antigénique est l'induction de HLA-DO. La présence de HLA-DO aura pour effet d'inhiber l'échange peptidique et ainsi empêcher la présentation d'une variété d'antigènes [60].

La régulation du pH est également un facteur important. Ainsi, dans les CDs immatures, le niveau de pH trop élevé ne permet pas la dégradation de la chaîne invariante et donc la formation de complexes CMH-peptide [33]. De la même manière, la régulation négative des cathepsines S et L prévient la dégradation de la chaîne invariante [33].

1.4.2.3. L'interleukine-10

De nombreuses cytokines influencent la présentation antigénique. Parmi celles-ci figure l'interleukine-10 (IL-10), une cytokine immunosuppressive et anti-inflammatoire [75]. L'IL-10 est importante pour la terminaison des réponses immunitaires et inflammatoires [87]. Elle est sécrétée par les LTs activés, les LBs et les monocytes [75]. Chez les monocytes humains, elle cause une inhibition de la sécrétion des cytokines inflammatoires induites par le LPS ainsi qu'une diminution des molécules de CMH II, d'adhésion et de co-stimulation (CD80 et CD86) à la membrane plasmique. L'IL-10 engendre une accumulation du CMH II dans les MIICs sans en affecter la transcription, la synthèse, l'assemblage ou le chargement peptidique [75,88].

1.4.2.4. La régulation du CMH II chez les cellules dendritiques

Chez les CDs immatures, les complexes CMH II-peptides sont rapidement endocytés et dégradés [89,90]. La demi-vie des molécules de CMH II est donc réduite chez les CDs immatures. Suite à leur maturation, le CMH II ainsi que les molécules de co-stimulation CD80 et CD86 sont redistribuées à la surface cellulaire afin de permettre la présentation antigénique et l'activation des LTs CD4+ [91].

Néanmoins, les mécanismes permettant la régulation différentielle de l'expression de surface du CMH II selon le stade de maturation chez les CDs étaient controversés. Une étude a démontré la présence des molécules de CMH II dans les vésicules internes des MVBs chez les CDs immatures. Selon cette étude, c'est la rétrofusion des vésicules internes avec la membrane limitante du compartiment suite aux signaux de maturation qui rendrait possible la présentation de surface. Selon ce modèle, la réorganisation des MVBs serait le mécanisme de régulation des CDs [92].

Un autre modèle voulait que les complexes CMH II-chaîne invariante ne maturent pas chez les CDs immatures en raison de la présence de l'inhibiteur de protéases cystatin. Ainsi, la prévention de la dégradation de la chaîne invariante empêchait l'apparition des complexes CMH II-peptide et donc, la présentation antigénique. Néanmoins, de nombreux groupes ont par la suite démontré que la demi-vie de la chaîne invariante était semblable chez les CDs immatures et matures, ce qui invalida ce modèle [91].

Les mécanismes de régulation de la présentation antigénique des CDs n'étaient alors pas caractérisés. Certains groupes proposèrent que la protéine AP-2 serait responsable de l'endocytose rapide des complexes de CMH II observée chez les CDs au stade immature. Suite à la maturation de ces dernières, AP-2 serait clivé, ce qui bloquerait l'endocytose des molécules de CMH II et favoriserait leur accumulation à la surface cellulaire. Néanmoins, cette hypothèse demeure controversée [37].

1.4.2.4. L'ubiquitination du CMH II

En 2006, l'ubiquitination du CMH II fut identifiée comme étant responsable des divergences observées au niveau de la régulation de la molécule aux différents stades de maturation des CDs [37,93]. Dans une étude menée par le groupe de Mellman, il fut démontré que l'activation au LPS de CDs immatures mettait fin à l'ubiquitination de la molécule. C'est l'ubiquitination de la queue cytoplasmique de la chaîne β qui serait responsable de ce changement de régulation [94].

Par la suite, MARCH1 et MARCH8 furent identifiés comme régulateurs de l'expression de surface du CMH II chez les LBs [95,96]. Les travaux de Gatti ont démontré que l'ubiquitination du CMH II des CDs immatures était médiée par MARCH1 [97]. Ils ont étudié la régulation du gène de MARCH1 chez les CDs et ont démontré son induction au stade mature seulement.

Par la suite, un groupe a montré que les différents types de CDs régulaient différemment MARCH1. Ainsi, les CDs plasmacytoïdes induisent toujours l'expression de MARCH1 suite à leur maturation. Cette propriété leur permettrait, contrairement aux CDs conventionnelles de présenter un répertoire peptidique continuellement renouvelé et serait à la base des fonctions distinctes de ces deux sous-types de CDs [98].

De nombreux groupes ont étudié la régulation du CMH II par MARCH1. La lysine 225 de la chaîne β de HLA-DR fut identifiée comme étant la cible de l'ubiquitination [93,95,97]. Néanmoins, il semblerait que la lysine 219 de la chaîne α de la molécule puisse également être ubiquitinée à de moindres niveaux [99].

1.5. L'ubiquitination

L'ubiquitine est une protéine de 76 acides aminés qui, lorsque conjuguée à sa cible, agit comme étiquette moléculaire [100]. Elle est exprimée chez toutes les cellules eucaryotes [101]. Les différents effets de cette modification protéique sont déterminés par les domaines spécialisés pouvant lier l'ubiquitine: les motifs de liaison à l'ubiquitine (*ubiquitin binding motifs*: UBM) aussi nommés domaines de liaison à l'ubiquitine (*ubiquitin binding domain*:

UBD) [100,102]. Il existe plus de 20 types d'UBMs dont: le motif d'interaction avec l'ubiquitine (*ubiquitin interacting motif*: UIM), le domaine associé à l'ubiquitine (*ubiquitin-associated domain*: UBA) et le domaine de conjugaison à l'ubiquitine (*ubiquitin-conjugating domain*: UBC) [103,104].

Cette modification protéique est impliquée dans de nombreux processus dont la régulation des réponses immunitaires innées et adaptatives [105]. De plus, la machinerie d'ubiquitination cellulaire est souvent utilisée par les microorganismes pathogènes pour échapper aux mécanismes de défense du système immunitaire [103,106]. Parmi ceux-ci, de nombreux virus encodent des protéines qui leur permettent d'exploiter à leur avantage le système ubiquitine de leur hôte.

1.5.1. Les types d'ubiquitination

On retrouve différents types d'ubiquitination; la mono-, la multi- et la poly-ubiquitination [107]. Il existe également plusieurs sous-types de poly-ubiquitination. Chaque type et sous-type adopte une structure spatiale différente, recrute ainsi un UBM particulier et engendre alors différents effets dans la cellule (voir Tableau 1.1) [104,107].

Type	Sous-type	Fonction
Monoubiquitination	-	Endocytose des protéines membranaires
		Reconnaissance protéique
		Réparation de l'ADN/transcription
		Envoi au TGN des protéines naissantes
		Bourgeonnement viral
Multiubiquitination	-	Endocytose des protéines membranaires
		Reconnaissance protéique
		Réparation de l'ADN/transcription
Polyubiquitination	K-6	Dégradation proteasomale
	K-11	Dégradation proteasomale, ERAD
		Endocytose des protéines membranaires
	K-27	Dégradation proteasomale, ERAD
	K-29	Dégradation lysosomale
		Dégradation proteasomale
	K-33	Dégradation proteasomale
	K-48	Dégradation proteasomale
	K-63	Endocytose des protéines membranaires
		Reconnaissance protéique
		Réparation de l'ADN/transcription
		Dégradation lysosomale
		Dégradation proteasomale
Chaîne mixte	-	Inconnu

Tableau 1.1: Effets des différents types et sous-types d'ubiquitination.

1.5.1.1. La mono-ubiquitination

La mono-ubiquitination est la conjugaison d'une seule ubiquitine à une cible [103]. Tous les

types d'ubiquitination débutent par la mono-ubiquitination de la cible. Dans le cas de la formation de polychaînes, c'est l'étape limitante de la réaction [108].

La mono-ubiquitination d'une protéine donne lieu à des signaux non protéolytiques (voir Tableau 1.1). Parmi ceux-ci se retrouvent le trafic des protéines membranaires vers les compartiments endocytaires, la reconnaissance protéique et la réparation de l'ADN [108–110].

L'internalisation des récepteurs mono-ubiquitinés est une étape cruciale pour la désensibilisation en réponse à plusieurs signaux. Par exemple, le récepteur du facteur de croissance épidermique (*epidermal growth factor*: EGF) [111].

1.5.1.2. La multi-mono-ubiquitination

La multi-mono-ubiquitination ou multi-ubiquitination est la conjugaison d'une seule ubiquitine à différents résidus d'une molécule. Ses effets sont les mêmes que ceux de la mono-ubiquitination (voir Tableau 1.1.) [109]. Néanmoins, la multi-ubiquitination augmente la liaison des protéines contenant les UBM et donc, l'efficacité de la reconnaissance [112].

1.5.1.3. La poly-ubiquitination

La poly-ubiquitination est la conjugaison d'une chaîne d'ubiquitine à un substrat [103]. L'ubiquitine contient 7 lysines qui peuvent elles-mêmes être la cible d'une autre ubiquitine [113]. Les poly-chaînes ainsi formées sont de type K-6, K-11, K-27, K-29, K-33, K-48 ou K-63 dépendent de la lysine utilisée [103,107].

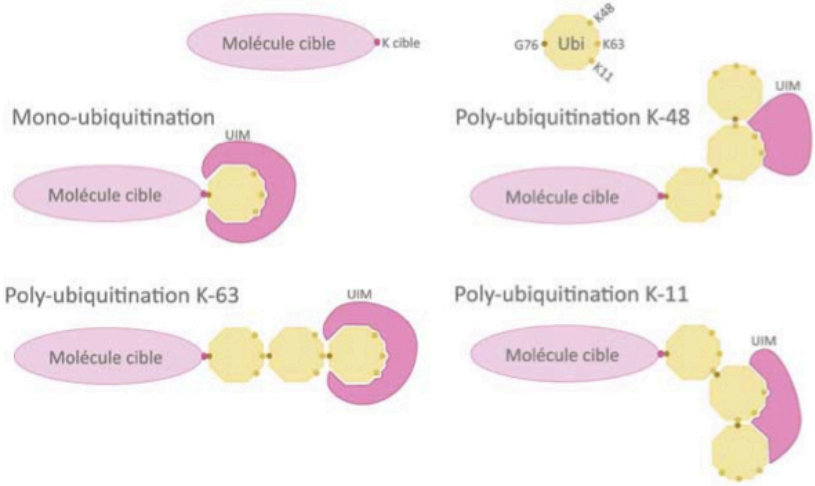

Figure 1.5. Schéma représentant différentes conformations adoptées par les différents types de chaînes de polyubiquitines. Les UIMs interagissent spécifiquement avec les différents types de chaîne.

L'addition d'ubiquitines pour former des poly-chaînes à une protéine mono-ubiquitinée se nomme l'élongation. Cette étape est de 5 à 30 fois plus rapide que la mono-ubiquitination [108]. L'élongation de la chaîne peut se faire une ubiquitine à la fois ou en bloc.

Les effets de la poly-ubiquitination sont nombreux et dépendent du sous-type de poly-chaînes [113]. On retrouve principalement des poly-chaînes de types K-48 et K-63, mais également des K-11 et K-29. Les autres types de poly-chaînes sont beaucoup moins fréquents.

Différents UBD reconnaîtront les différents types de chaînes, ayant ainsi différents effets (voir Tableau 1.1) [100,104]. Par exemple, le protéasome 19S reconnait les poly-chaînes de type K-48 composées d'un minimum de quatre ubiquitines [109,114,115]. D'un autre côté, les chaînes de type K-63 ressemblent à une chaîne d'ubiquitines linéaires [116]. Pour cette raison, ce type d'ubiquitination mène aux mêmes effets que la mono-ubiquitination. Les effets sont tels que la redirection vers les endosomes tardifs et la transduction de signaux [103,117]. Néanmoins, de récentes évidences portent à croire que les poly-chaînes de type K-63 peuvent également causer une dégradation protéosomale [118].

1.5.1.4. Les chaînes de type mixte

Il a été décrit que des chaînes mixtes peuvent être formées. Ces chaînes peuvent comprendre plus d'une branche ou encore une bifurcation. Néanmoins, la fonction de ce type de chaînes est inconnue à ce jour [107].

1.5.2. Le processus d'ubiquitination

L'ubiquitination est une réaction enzymatique séquentielle qui implique trois enzymes: l'enzyme d'activation de l'ubiquitine (E1), l'enzyme de conjugaison de l'ubiquitine (E2) et l'ubiquitine ligase (E3) [108,114].

Dans un premier temps, la E1 active l'ubiquitine libre dans le cytoplasme par la formation d'un lien thioesther entre la cystéine de son site actif et la glycine 76 de l'ubiquitine. Ce processus nécessite la consommation d'énergie sous forme d'ATP. L'ubiquitine activée peut ensuite former un lien avec une cystéine du site actif d'une E2. Par la suite, le complexe E2-ubiquitine se lie à la E3 qui détermine le substrat et permet le transfert de l'ubiquitine à la molécule cible (voir Figure 1.6.) [114].

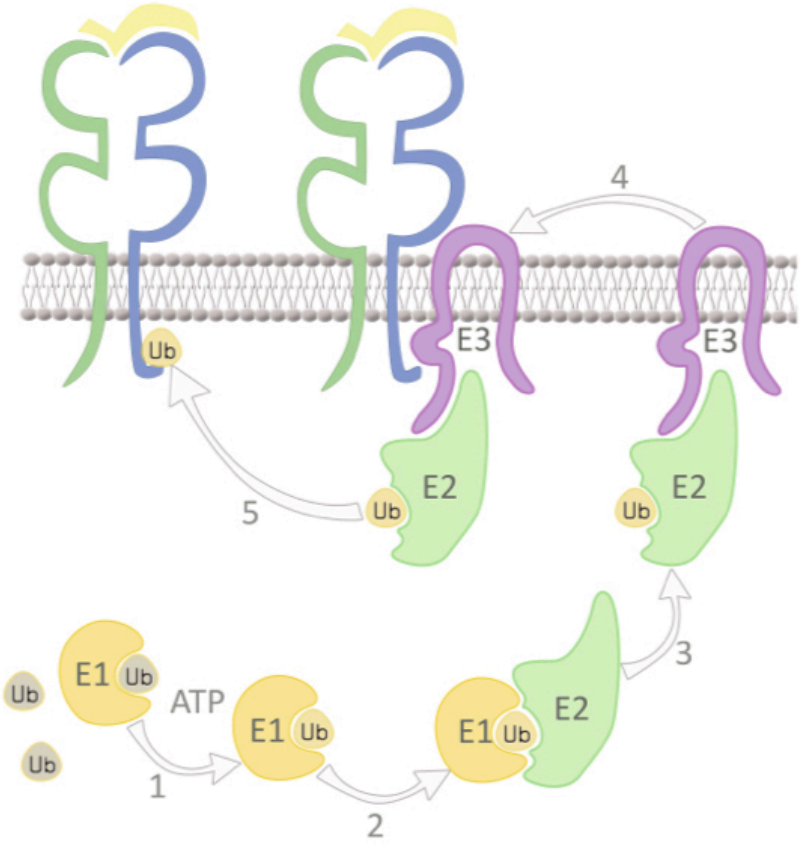

Figure 1.6. Représentation schématique du processus d'ubiquitination.

La liaison de l'ubiquitine à son substrat se fait par un lien covalent entre la glycine C-terminale de l'ubiquitine et une lysine [113,119]. Le choix du résidu à ubiquitiner se fait en fonction de l'accessibilité et non de la séquence primaire [108,120].

En 2005, le groupe de Coscoy a démontré pour la première fois qu'un autre acide aminé que la lysine pouvait lier l'ubiquitine [121]. Nous savons désormais que l'ubiquitine peut également être conjuguée aux sérines, aux thréonines et aux cystéines [120,122].

1.5.3. Les joueurs

1.5.3.1. Les enzymes d'activation E1

Jusqu'à tout récemment, une seule E1 avait été identifiée. À ce jour, deux E1s ont été identifiées chez les mammifères. La découverte de la deuxième date de 2007 [123].

1.5.3.2. Les enzymes de conjugaison E2

À l'heure actuelle, il y a plus de 40 E2s de répertoriées [114,124]. Ce sont les régulateurs clés de l'assemblage des chaînes. En plus de déterminer la topologie de la chaîne, elles font le changement de l'étape d'initiation à celle d'élongation [114]. Chaque E2 se spécialise dans un certain type d'ubiquitination, mais plusieurs ont des fonctions redondantes [125]. Par exemple, Ubc13 est la seule E2 à permettre la formation de poly-chaînes de type K-63, UBE2S et UBE2C forment des courtes chaînes de type K-11 alors que Ubc13 et Ubc5 permettent la conjugaison de la première ubiquitine [126].

L'affinité des E2s pour les E1s augmente lorsque ces dernières portent une ubiquitine. En effet, la E1 subit un changement conformationnel qui permet alors une meilleure exposition du site de liaison à la E2 [114]. Les E2s ont des domaines de liaison à la E1 et à la E3 qui se chevauchent, empêchant ainsi la liaison simultanée aux deux enzymes, ce qui aurait pour conséquence le ralentissement du processus. Il y a donc liaison séquentielle de la E2 à la E1, puis à la E3. La E2 se relie ensuite à la E1 pour se recharger et le processus recommence [108].

Chaque E2 peut interagir avec plusieurs E3s et les sites de liaison pour les différentes E3s ne sont pas nécessairement les mêmes [114]. De plus, les E2 peuvent lier des cofacteurs qui influenceront leur localisation et leur spécificité envers la E3 [114]. Aussi, les E2s peuvent elles-mêmes être membranaires. UbcH6 par exemple, permet une accessibilité plus facile à certaines E3 [127].

1.5.3.3 Les E3 ubiquitine ligases

Il y a entre 600 et 1000 E3s répertoriées chez les mammifères et ces chiffres ne cessent d'augmenter [108,114]. Les E3s, de par leur variété en terme de localisation ainsi que de par leur capacité à interagir directement avec le substrat, sont les molécules permettant

la spécificité de la réaction. Pour cette raison, ce sont les plus régulées du processus [109]. La dérégulation des E3s, par exemple MDM2, est d'ailleurs associée à plusieurs cancers [121,128].

Certains microorganismes pathogènes tels que *Shigella* et *Salmonella* injectent des E3s afin de permettre la dégradation de molécules comme les MAPKs, ce qui favorise leur évasion du système immunitaire [99,129]. D'autres microorganismes pathogènes manipulent les systèmes d'ubiquitination de leur hôte afin de bloquer des réactions qui ne leur sont pas favorables [106].

Il y a deux catégories de E3 ubiquitine ligases: les homologues du C-terminal de E6-AP (*homologous to E6-AP carboxy terminus*: HECT) et les nouveaux gènes très intéressants (*really interesting new gene*: RING).

1.5.3.3.1 Les E3 ubiquitin ligases de type HECT

Il existe environ 60 familles de E3s de type HECT [114]. Ces E3s forment un lien thioester avec une cystéine conservée des E2s. Contrairement aux E3s de type RING, elles sont catalytiquement actives et ce sont elles qui médient le transfert de l'ubiquitine à la cible [119].

1.5.3.3.2 Les E3 ubiquitin ligases de type RING

La plus grande majorité des E3 ligases sont de type RING et la plupart d'entre elles sont cytoplasmiques ou nucléaires [130]. Elles servent de pont entre la E2 et la molécule cible [121,130]. C'est la liaison avec le RING qui permet l'activation complète de la E2 et l'ubiquitination du substrat [114]. Ces E3s peuvent agir seules ou encore nécessiter la présence d'autres protéines pour lier leur substrat et alors agir sous forme de complexe.

1.6. Le domaine RING

Ce domaine était d'abord considéré comme étant impliqué dans la réparation de l'ADN. Plusieurs années plus tard, un premier lien a été établit avec l'ubiquitination. C'est seulement en 1999 que la fonction de E3 ubiquitine ligase a été confirmée [108].

Les RINGs classiques sont longs de 100 acides aminés [125,130]. D'un point de vue structurel, ils permettent la coordination de 2 atomes de zinc [108,131]. Ils font partie des motifs doigt de zinc caractérisés par une séquence conservée de cystéines et d'histidines. Les cystéines et les histidines des doigts sont les résidus qui lient le zinc. Les RINGs classiques comprennent dans l'ordre 3 cystéines, une histidine et 4 cystéines: C3HC4 [132].

Un autre type de RING est le C4HC3 nommé RING-CH ou RINg-v (''RING variant'') [133]. Ces RINGs sont plus petits et font approximativement 60 acides aminés de long [125]. Ils sont fortement apparentés aux homéodomaines de plantes (*plant homeodomaines*: PHD) et aux domaines de la protéine associée à la leucémie (*leukemia-associated protein domain*: LAP). Les poxvirus et les herpesvirus possèdent des E3 ligases avec un RING de type RING-CH [133].

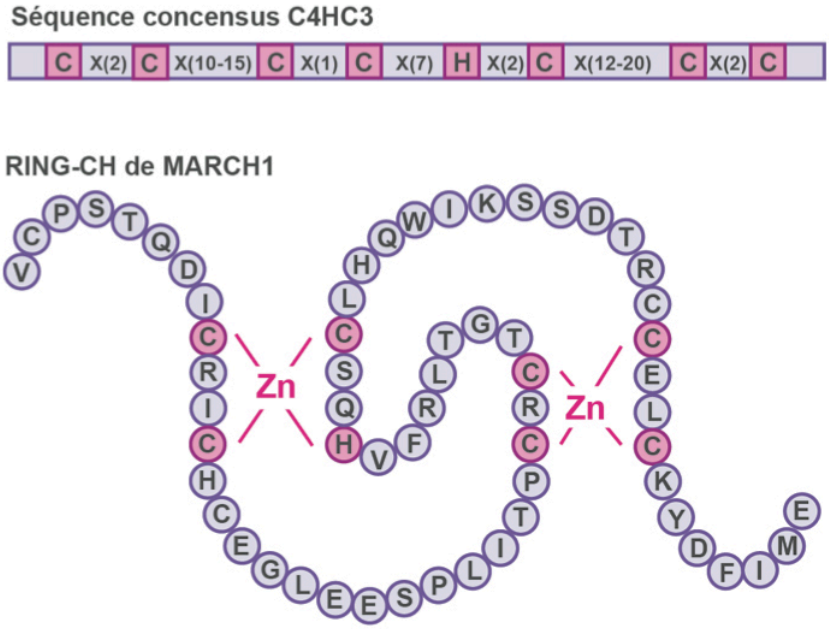

Figure 1.7. Représentation schématique du RING-CH des MARCHs. A) Séquence consensus des RINGs C4HC3 montrant les espacements entre les cystéines et l'histidine qui coordonnent les deux ions zinc. Les espacements ont été déterminés par la comparaison entre les différents RINGs des MARCHs cellulaires et viraux. B) Représentation schématique du RING de MARCH1.

1.6.1. Ses fonctions

Le RING est responsable de l'activité ligase, mais également de l'interaction avec d'autres protéines [134]. En effet, les RINGs sont globulaires et rigides, ce qui constitue une plateforme de choix pour les interactions protéiques. Pour cette raison, il est souvent

responsable de la reconnaissance du substrat. De plus, la formation de dimères via les RINGs est fréquemment observée [108,135]. Tel que mentionné précédemment, c'est également lui qui permet la liaison à la E2 [136]. Néanmoins, cette liaison est souvent très faible et nécessite la fortification de l'interaction par d'autres régions [108].

1.6.2. Les RINGs inactifs

Certaines protéines possèdent des RINGs qui n'ont pas d'activité E3, par exemple, Bard1, Bmi1 et MdmX. Dans tous les cas, ils hétérodimérisent avec des protéines possédant un RING actif, ce qui a pour effet d'augmenter l'activité ligase de ces dernières [108,134,135].

1.6.3. L'inactivation des RINGs

Dans l'étude des E3 ligases, le meilleur contrôle est souvent la molécule étudiée pour laquelle on inactive le RING par mutation. Une manière efficace d'inactiver un RING est d'en muter les cystéines, détruisant par contre sa structure générale. La mutation des résidus isoleucine et tryptophane permet de modifier la polarité et la charge du RING. Ces acides aminés sont conservés et ce sont les tryptophanes du RING qui lient les E2s. Ces modifications engendrent une inactivation du domaine sans trop en affecter la structure générale [108].

1.6.4 Régulation des RINGs.

La phosphorylation est souvent requise pour la régulation des E3s [108]. Par exemple, la phosphorylation de MDMx mène à sa dégradation [135]. De plus, la fonction et l'expression de plusieurs protéines de type RING sont régulées par autoubiquitination [137]. D'autres, comme MDMx et MDM2 forment des dimères, permettant ainsi la trans-ubiquitination [135]. Une autre modification connue du RING est la neddylation qui consiste en l'ajout de molécules de NEDD8 par un processus similaire à celui de l'ubiquitination. Celle-ci stimule l'interaction avec le E2, ce qui a pour effet d'augmenter l'ubiquitination de la molécule cible [109].

1.7. Les molécules de la famille MARCH

MARCH signifie RING-CH associé à la membrane (*membrane-associated RING-CH*). Les membres de la famille MARCH sont parfois nommés MIR ce qui signifie modulateur de la reconnaissance immunitaire (*modulator of immune recognition*) [133]. La famille comporte les MIR viraux et les MARCHs cellulaires [138]. Le membre original est mK3, encodé par un herpesvirus et a été démontré en 2000 comme étant impliqué dans l'évasion virale [139].

La caractéristique principale des membres de cette famille est la présence d'un RING de type C4H3C. De plus, la majorité sont des protéines de type III, c'est-à-dire qu'elles possèdent 2 domaines cytoplasmiques [121,130].

1.7.1. Les homologues viraux

1.7.1.1. Les gènes viraux acquis des eucaryotes

Les virus acquièrent plusieurs de leurs gènes chez les hôtes qu'ils infectent. Les gènes sélectionnés sont ceux qui sont favorables à l'établissement d'une infection ou alors à l'évasion du système immunitaire. C'est le cas de l'herpesvirus associé au sarcome de Karposi (*Karposi's associated herpes Virus*: KSHV), pour qui de nombreux gènes ont été acquis des mammifères qu'ils infectent. Parmi ceux-ci, KSHV possède un homologue viral de l'IL-6, dont la séquence comporte 24.7% d'identité avec la cytokine humaine. Ce faible pourcentage est néanmoins suffisant à l'obtention d'une structure tridimensionnelle similaire entre les deux molécules. L'IL-6 a été démontrée comme favorisant les tumeurs chez plusieurs myélomes via un mécanisme de suppression de l'apoptose. De plus, les sarcomes de Karposi expriment de hauts niveaux du récepteur de l'IL-6 et il a été démontré que l'IL-6 viral encodée par KSHV favoriserait la croissance de ces tumeurs [140].

De la même manière, les MARCHs viraux auraient été acquis des vertébrés. Leurs cibles étant des joueurs de la reconnaissance immunitaire, ils favorisent l'établissement

d'une infection latente en prévenant la présentation antigénique et donc l'élimination des cellules infectées par le système immunitaire [126,130].

1.7.1.2. La nomenclature

Les homologues viraux sont produits par poxvirus et les gamma-herpesvirus. Le gamma-herpesvirus murin encode le gène K3. La protéine se nomme MIR1 ou alors mK3 pour murin-K3. De son côté, KSHV encode également un gène nommé K3, mais aussi un second gène fortement similaire nommé K5. Les protéines issues de ces gènes se nomment MIR1 et MIR2, ou encore kK3 et kK5 pour KSHV-K3 ou -K5 [130,138].

L'appellation MIR est favorisée, néanmoins, dans un objectif de simplification et pour différencier le MIR1 du virus murin et humain, mK3, kK3 et kK5 seront ici utilisés pour désigner les différentes protéines.

1.7.1.3. Leurs cibles

mK3 cible le CMH I, la molécule d'adhésion ICAM-1, le co-récepteur CD86 ainsi que CD1d [130,133]. Il se lie à la chaîne lourde du CMH I au RE et permet la formation de poly-chaînes d'ubiquitine de type K-48. Cette ubiquitination, en collaboration avec la voie de dégradation associée au RE ERAD (*ER-associated degradation*), voie qui permet la rétro-translocation des protéines mal repliées du RE au cytoplasme, cause la dégradation protéosomale du CMH I [141,142].

De leur côté, kK3 et kK5 ciblent également le CMH I, ICAM-1, CD86 et CD1d. De plus, kK5 cible aussi MICA et MICB, PECAM, ALCAM, le récepteur de l'IFN-γ et AICL [143]. Ils sont retrouvés dans le RE, mais contrairement à mK3, ils agiraient plutôt depuis la membrane plasmique. Leur interaction avec le CMH I permet la génération d'une polychaîne de type K-63, ce qui a pour effet l'endocytose des molécules et leur dégradation de manière lysosome-dépendante [121,130,144].

D'autres virus de la famille herpes possèdent un gène K3. Par exemple, le virus rhésus de fibromatose produit la protéine rfK3 qui touche également ICAM1, mais non B7.2 [145].

1.7.1.4 Les domaines et motifs

1.7.1.4.1. Les domaines transmembranaires

Les portions transmembranaires (TM) de kK3 et kK5 sont requises pour la reconnaissance de la cible [126,146–148]. De plus, les TMs sont requis pour dimériser en plus de jouer un rôle dans la compartimentalisation.

1.7.1.4.2. Les motifs tyrosine

Ces E3 virales possèdent un motif de localisation lysosomal à base de tyrosine qui est requis pour leurs fonctions [121]. En effet, la mutagénèse dirigée de ces motifs inactive les molécules [149].

1.7.1.4.3. Le domaine DIRT

DIRT signifie domaine entre le RING-CH et le premier TM (*domain in-between the RING-CH and the first TM*). Il a été démontré pour mK3 que cette portion de la molécule était requise à la reconnaissance des substrats à ubiquitiner [120].

C'est par une recherche d'homologie de séquence que les MARCHs cellulaires ont été découverts [150].

1.7.2. Les MARCHs cellulaires

La plupart des MARCHs ciblent des molécules jouant d'importants rôles dans les réponses immunitaires tels que le CMH I, le CMH II et CD4 (voir Tableau 1.2) [121].

Il y a 11 membres chez les eucaryotes [151]. À l'exception de MARCH7 et MARCH10, qui sont cytoplasmiques, ils sont tous membranaires (voir schéma de MARCH1: Figure 1.8.) [121]. Les cibles sont regroupées dans le Tableau 1.2.

1.7.2.1. Les paires de MARCHs

Il existe trois paires de MARCHs fortement reliés: MARCH2 et MARCH3, MARCH1 et MARCH8, et MARCH4 et MARCH9 [133].

Molécule	Cible	Inhibition
MARCH1	CMHII	Présentation d'antigènes protéiques
	CD86	Co-stimulation des LTs
	Tfr	Incorporation du Fer
	Fas	Cytotoxicité des LTs
	HLA-DM	Chargement peptidique
MARCH2	Syntaxine-6	Trafic des endosomes au TGN
	Tfr	Incorporation du Fer
	CD86	Co-stimulation des LTs
MARCH3	hDLG1	Jonction cellulaire épithéliales
	Syntaxine-6	Trafic des endosomes au TGN
MARCH4	CMHI	Présentation d'antigènes protéiques
	CD4	Présentation antigénique
	Mult1	Activation des cellules NK
	CD44	Interactions cellulaires
	CD81	Transduction de signaux
MARCH5	SOD1	Fission mitochondriale
	FIS1	Fission mitochondriale
	MFN1	Fission mitochondriale
	DRP1	Fission mitochondriale
	TANK	Activation NF-KB, réponse TLR7
MARCH6	DIO2	ERAD
MARCH7	LIF	Blocage transcriptionnel
	FoxP3	Blocage transcriptionnel
	SOCS3	Dérégulation réponse aux cytokines
MARCH8	CMHII	Présentation d'antigènes protéiques
	CD86	Co-stimulation des LT
	Tfr	Incorporation du Fer
	Fas	Cytotoxicité des cellules Ts
	HLA-DM	Chargement peptidique
	CD44	Interactions cellulaires
	CD81	Transduction de signaux
MARCH9	CMHI	Présentation d'antigènes protéiques
	CD4	Présentation antigénique
	ICAM-1	Synapse immunologique
	Mult1	Activation des cellules NK
	HLA-DM	Chargement peptidique
MARCH10	N/D	élongation flagelles
MARCH11	CD4	Présentation antigénique
	AP-1	Trafic entre TGN et endosomes

Tableau 1.2. Cibles des différents MARCHs.

MARCH2 et MARCH3 sont exprimés de manière ubiquitaire et se localisent dans les endosomes [133]. Ils ciblent certains SNARES et influencent le trafic entre le réseau trans-golgien et les endosomes. MARCH2 a également été démontré comme régulant le récepteur à la transferrine (Tfr) et HLA-DM [152,153].

MARCH4 et MARCH9 ciblent le CMH I et CD4 et ne peuvent ubiquitiner que les lysines. MARCH9 touche également ICAM1 et se localise aux lysosomes [121,154]. De son côté, MARCH4 est retrouvé au RE [133].

La troisième paire, MARCH1 et MARCH8, concerne directement cette étude et sera discutée dans la prochaine section.

1.7.2.2. Les autres membres de la famille

Les autres membres de la famille étant moins connus, seulement quelques particularités seront présentées. MARCH5 se localise dans les mitochondries alors que MARCH11 est abondant dans les spermatozoïdes en développement [121,155,156]. MARCH6 possède 13 domaines TMs et est localisé dans le RE. Il médie une dégradation de type protéosomale en collaboration avec la voie ERAD [121,157]. MARCH7 est exprimé dans le cerveau, le thymus, les muscles et les reins. Il est impliqué dans le développement thymique en ciblant LIF et FoxP3 [158]. Il joue

également un rôle important dans le développement neuronal [151]. De plus, il possède la capacité de s'autoubiquitiner [121,151].

1.7.3. MARCH8

MARCH8 a été le premier MARCH cellulaire à être caractérisé [95]. C'est en 2006 que le groupe du Dr. Ishido a identifié MARCH8 comme ciblant le CMH II. Il s'agissait de la première démonstration d'une E3 ubiquitine ligase qui cible cette molécule [95].

MARCH8 n'était pas encore classé parmi les MARCHs et portait alors le nom de c-MIR (*cellular-MIR*: MIR-cellulaire). Son RING était alors considéré comme un domaine de type BKS-PHD et proposé comme étant un nouveau type de RING [159].

1.7.3.1 La souris transgénique MARCH8

La génération d'une souris transgénique exprimant MARCH8 sous le contrôle du promoteur de la chaîne invariante a permis l'expression de la protéine dans les LBs, les macrophages et certaines CDs. Son expression forcée engendre une diminution de l'expression de CD86, du Tfr et de Fas. De plus, les souris transgéniques MARCH8 présentaient plus de CMH II ubiquitiné que les souris sauvages [95].

1.7.3.2. Mécanisme d'action

Il a été proposé que MARCH8 interagirait avec sa cible à la surface cellulaire [160]. Cela permettrait l'ubiquitination depuis la surface cellulaire pour ainsi causer l'endocytose et la dégradation lysosomale de ses molécules cibles [160].

1.7.3.3. La paire MARCH8-MARCH1

Les régions catalytiques et TMs de MARCH8 et MARCH1 comportent 80% d'identité de séquence [96]. Il s'agit de la paire la mieux caractérisée. Ils ont pour cibles communes CD86, Fas, le Tfr, le CMH II et HLA-DM [133,153,161,162].

1.8. MARCH1

Il a d'abord été démontré que MARCH1 permettait la régulation du CMH II chez les LBs [96]. Cette régulation se fait via l'ubiquitination de la lysine 225 de la queue cytoplasmique de la chaîne β du CMH II [93,96]. Dans une publication subséquente, notre groupe a démontré l'ubiquitination du CMH II en présence de MARCH1 ainsi que l'interaction directe entre les deux molécules (voir Annexe I) [163].

MARCH1 permet également l'ubiquitination de CD86 sur plusieurs lysines, mais préférentiellement celle proximales aux domaines TMs [161]. L'ubiquitination de HLA-DM cible l'unique lysine présente sur la chaîne α de la molécule [162].

Finalement, la présence ou l'absence d'ubiquitination du CMH II a été démontrée comme jouant un rôle clé dans les différentes propriétés de présentation des différents sous-types de CDs [98].

1.8.1. La souris MARCH1 KO

L'équipe du Dr. Ishido a généré une souris déficiente pour l'expression de MARCH1 [96]. Ils ont démontré qu'il y avait plus de CMH II et que la demi-vie de la protéine était prolongée chez cette souris par rapport à la souris sauvage. Aussi, des travaux par d'autres groupes ont démontré une présence augmentée de CD86 à la surface cellulaire pour la souris MARCH1 KO [153,161].

1.8.2. L'expression

Des expériences de PCR quantitatif en temps réel ont révélé que l'expression de MARCH1 est restreinte aux organes lymphoïdes secondaires [96,133]. Cette E3 est retrouvée chez les CDs immatures seulement [96,97]. MARCH1 est également exprimé chez les LBs, particulièrement ceux de type folliculaires [96,164]. Finalement, on le retrouve aussi chez les macrophages et à de très faibles niveaux chez les LTs [96].

Malgré la détection de l'ARN messager de MARCH1, la protéine endogène n'est pas détectable [165]. De plus, sa demi-vie est très courte, soit de 30 minutes chez les CDs murines primaires, ce qui laisse croire à la présence de mécanismes de régulation post-traductionnels [165]. Les mécanismes de régulation de MARCH1 sont encore peu caractérisés.

1.8.3. Mécanismes de régulation

1.8.3.1. Régulation transcriptionnelle

Tel que mentionné plus haut, la transcription de MARCH1 est réprimée suite à la maturation des CDs induite par une stimulation au LPS [97].

De plus, nous avons démontré que la transcription de MARCH1 est induite suite à une stimulation de monocytes primaires humains à l'IL-10. C'est d'ailleurs le seul membre de la famille MARCH à être induit par l'IL-10 dans ces cellules. De plus, nos résultats montrent qu'en absence de MARCH1, il y a inhibition de la rétention intracellulaire du CMH II en réponse à l'IL-10 [163]. Aussi, de récents travaux démontrent l'induction de MARCH1 par l'IL-10 dans les CDs [166]. Finalement, une autre étude démontre que MARCH1 est responsable de la rétention intracellulaire de CD86 en réponse à l'IL-10 chez ces cellules [153]. Globalement, ces résultats font de MARCH1 une molécule clef dans la réponse anti-inflammatoire à l'IL-10.

1.8.3.2. Régulation post-traductionnelle

Le groupe de Lybarger a observé qu'un mutant inactif de la molécule murine était exprimé à de plus forts niveaux, permettant ainsi de suspecter l'ubiquitination comme mécanisme d'autorégulation. Néanmoins, ils n'ont pas été en mesure de détecter l'ubiquitination de la molécule [165]. De plus, un autre groupe rapporte qu'aucune différence en termes de stabilité n'a pu être observée entre MARCH1 et un mutant au RING inactif [138].

La dégradation de la protéine murine se fait de manière lysosomale et requiert les cystéines protéases, tout particulièrement la cathepsine L [165]. Néanmoins, le mécanisme et les signaux demeurent inconnus.

1.8.4. Régulation de l'activité ligase

Une étude récente a démontré que la molécule CD83 prévenait l'activité ligase de MARCH1 à l'égard du CMH II et de CD86. En effet, les résultats de Goodnow et de son équipe ont démontré qu'en présence du domaine TM de CD83, l'association entre MARCH1 et le CMH II est prévenue, empêchant ainsi l'ubiquitination de la molécule [166]. Il s'agit de la première démonstration d'une protéine pouvant bloquer l'effet de MARCH1 envers sa cible.

1.8.5. Localisation

Deux motifs tyrosine de localisation endosomale ont été identifiés dans la portion cytoplasmique C-terminale de la molécule murine et humaine (Figure 1.8.). La mutation de ces motifs inactive la molécule. Il semblerait aussi que d'importants éléments de localisation soient présents dans la portion cytoplasmique N-terminale, étant donné que la suppression d'une importante portion de ce domaine délocalise la protéine [165].

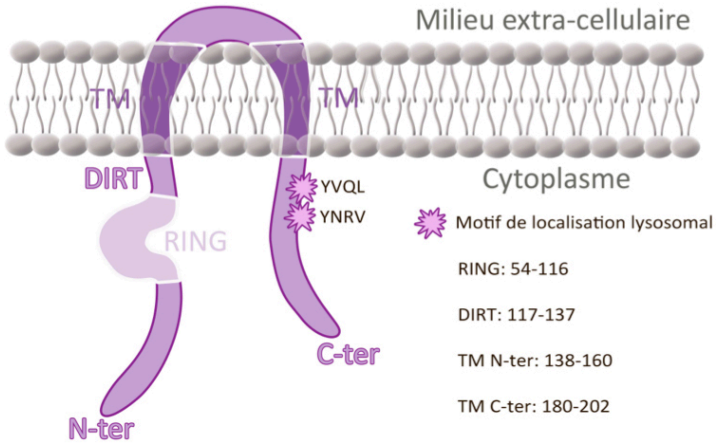

Figure 1.8. Représentation schématique des différents domaines et motifs de MARCH1.

Le groupe de Lybarger a démontré que MARCH1 murin se situe dans les endosomes tardifs de type lysosomes de par sa colocalisation avec la protéine LAMP-1 [165]. Puisque MARCH1 dirige le trafic aux endosomes tardifs du CMH II, pour en causer une dégradation rapide, il est possible que la molécule voyage avec son substrat jusqu'à cette destination finale [97].

Néanmoins une colocalisation avec le marqueur Furine du réseau trans-golgien a également été rapportée. Ceci n'exclut pas la possibilité que MARCH1 chemine via le golgi vers les endosomes tardifs, mais démontre que sa localisation n'est pas restreinte aux endosomes [165].

1.9 Objectifs et hypothèses de travail

1.9.1 Importance des travaux

MARCH1 est un régulateur clef de l'expression de surface du CMH II. La présentation antigénique étant une étape cruciale dans le développement de la réponse immunitaire adaptative, la compréhension des mécanismes de régulation de MARCH1 est essentielle à sa manipulation. Ainsi, une meilleure caractérisation de la molécule permettrait son utilisation éventuelle dans divers traitements. Par exemple, l'efficacité de la vaccination à base de CDs repose sur la présentation antigénique efficace de ces cellules [167,168]. Dans cette situation par exemple, l'activité de MARCH1 n'est pas souhaitable.

D'un autre côté, de nombreuses maladies auto-immunes sont causées par la reconnaissance d'auto-antigènes présentés et reconnus par les LTs. Ainsi, la sclérose en plaque et l'encéphalomyélite allergique expérimentale (EAE) chez la souris sont causées par la reconnaissance et la destruction des antigènes de la myéline. La disparition de la gaine protectrice expose alors le nerf, ce qui en diminue l'efficacité de transduction de signal [169,170]. Un autre exemple est le diabète. Dans ce cas, les cellules β des ilots de Langerhans du pancréas présentent des antigènes cellulaires qui entraînent leur destruction par les LTs et l'apparition de la maladie [13]. Dans le cas de ces maladies, l'induction ou l'activation de MARCH1 ou d'un autre membre de la famille dans les cellules productrices de myéline ou les cellules bêta préviendrait la présentation d'auto-antigènes et le développement de ces troubles auto-immuns.

Néanmoins, la manipulation efficace de MARCH1 requiert une compréhension en profondeur de ses mécanismes d'action, de ciblage et de régulation. À l'heure actuelle, les

connaissances concernant MARCH1 sont limitées et la majorité des informations connues concernent la molécule murine. Les travaux présentés dans cette étude portent sur la structure-fonction de MARCH1 humain dans le contexte de la régulation de l'expression de surface des molécules de CMH II.

1.9.2. Hypothèses

1.9.2.1. Autorégulation de MARCH1

Étant donné l'impossibilité de détecter la forme protéique de MARCH1, et ce malgré une importante induction au niveau transcriptionnel, nous croyons que la molécule s'autorégule par ubiquitination.

Hypothèse 1: MARCH1 s'autoubiquitine.

1.9.2.2. Formation de dimères

En raison des nombreuses E3 ubiquitine ligases formant des homo- et des hétéro-dimères et l'observation de ce phénomène chez les homologues viraux des MARCHs, nous croyons que MARCH1 possède la capacité de dimériser.

Hypothèse 2: MARCH1 dimérise.

1.9.2.3. Importance des domaines transmembranaires

Puisque les TMs sont souvent impliqués dans les interactions protéiques et que plusieurs MARCHs ont été démontrés comme interagissant avec leurs cibles par ces régions, nous pensons qu'ils sont impliqués dans la reconnaissance et l'interaction avec le CMH II.

Hypothèse 3: Les domaines transmembranaires de MARCH1 reconnaissent et interagissent avec la cible.

1.9.2.4. Nécessité des motifs de localisation pour la fonction

Étant donné la démonstration de la nécessité des motifs tyrosine de localisation endosomale de la queue cytoplasmique C-terminale de MARCH8 humain et de MARCH1

murin, nous émettons l'hypothèse que ces motifs soient également requis pour la fonction de MARCH1 humain. Ainsi, la localisation de MARCH1 dans les endosomes tardifs serait nécessaire à la régulation de surface du CMH II par celui-ci.

Hypothèse 4: La localisation endosomale de MARCH1 est médiée par ses motifs tyrosine et est requise pour sa fonction.

1.9.3 Objectif

L'objectif de ce projet est de caractériser les éléments requis au fonctionnement de MARCH1. Nous désirons identifier les motifs et les portions de la molécule jouant un rôle dans la régulation de la présentation antigénique. Ainsi, les motifs identifiés au niveau de la séquence primaire, de même que les pré-requis soupçonnés en matière de complexe protéique, de structure et de localisation seront étudiés. La principale approche utilisée est la mutagénèse dirigée des divers éléments connus ou soupçonnés d'avoir un rôle dans la fonction ou la régulation de la molécule. La génération de molécules chimériques et la délétion de portions entières de MARCH1 ont également servi d'outils pour cette étude.

CHAPITRE 2: Article 1

Autoregulation of MARCH1 expression by dimerization and autoubiquitination

Préface

Le premier article comprend la caractérisation des mécanismes de régulation de MARCH1. Les techniques employées sont l'immunoprécipitation, l'immunoblot, la microscopie confocale, le marquage radioactif, la cytométrie en flux et le BRET. Dans cet article, nous démontrons l'autouiquitination de MARCH1 ainsi que sa capacité à former des homo- et des hétérodimères avec d'autres membres de la famille. De plus, nous identifions les domaines transmembranaires comme étant essentiels à la dimerization ainsi qu'à l'interaction avec le substrat.

Le projet est né d'une expérience de BRET, effectuée par curiosité, alors que je m'entraînais à maîtriser la technique. Les résultats ont révélé la formation de dimères de MARCH1. La suite logique du projet a été élaborée par le Dr. Jacques Thibodeau et moi-même en réaction à chacun des résultats obtenus.

Référence de l'article 1

Marie-Claude Bourgeois-Daigneault and Jacques Thibodeau, **Autoregulation of MARCH1 expression by dimerization and autoubiquitination**, Journal of Immunology 2012.

Published April 16, 2012, doi:10.4049/jimmunol.1102708

Autoregulation of MARCH1 Expression by Dimerization and Autoubiquitination

Marie-Claude Bourgeois-Daigneault and Jacques Thibodeau

Some members of the membrane-associated RING-CH family of E3 ubiquitin ligases (MARCHs) are membrane-bound and target major players of the immune response. MARCH1 ubiquitinates and downregulates MHC class II expression in APCs. It is induced by IL-10 and despite a strong increase in mRNA expression in human primary monocytes, the protein remains hardly detectable. To gain insights into the posttranslational regulation of MARCH1, we investigated whether its expression is itself regulated by ubiquitination. Our results demonstrate that MARCH1 is ubiquitinated in transfected human cell lines. Polyubiquitin chain-specific Abs revealed the presence of K48-linked polyubiquitin chains. A mutant devoid ofl ysine residues in the N- and C-terminal regions was less ubiquitinated and had a prolonged half-life. Reduced ubiquitination was also observed for an inactive mutated form of the molecule (M1WI), suggesting that MARCH1 is capable of autoubiquitination. Immunoprecipitation and energy transfer experiments demonstrated that MARCH1 homodimerizes and also forms heterodimers with others family members. Coexpression of MARCH1 decreased the protein levels of the inactive M1WI, suggesting a transubiquitination process. Taken together, our results suggest that MARCH1 may regulate its own expression through dimerization and autoubiquitination. The Journal of Immunology, 2012, 188: 000–000.

Ubiquitination is a multistep process involving three enzymes called E1, E2, and E3 (1). In eukaryotes, only two E1s are responsible for the ATP-dependent activation of ubiquitin (2). In a typical reaction, one of the at least 38 E2 enzymes interacts with activated ubiquitin and one of a near thousand E3 enzymes. The E3 then recognizes the target molecule and catalyzes the transfer of ubiquitin on a lysine or, less frequently, serine, cysteine, or threonine residues (1, 3–5). The E2/E3 combination and the localization of the complex confer the specificity to the reaction toward a given substrate (1, 6). Addition of ubiquitin moieties can lead to lysosomal or proteasomal degradation of the target, to a change in subcellular localization, or to modulation ofi ts interactions with different partners. The fate of ubiquitinated molecules will depend on the type of modification, which can be mono-, multi-, or polyubiquitination (1). There are seven lysines in ubiquitin and each can be modified by the addition of another ubiquitin moiety. The best characterized polyubiquitin chains are K48- and K63-linked polymers, which lead to different spatial conformations and recruitment of different adaptor proteins through their ubiquitin-binding motifs (7). There are also some mixed polyubiquitin chains in which branching occurs following the modification of different lysines in the ubiquitin chain. There is no specific function attributed yet to these chains and only few examples have been described (6, 8). Of these, the ubiquitin-driven endocytosis of MHC class I (MHC I) mediated by K5 was shown to require branched K11 and K63 polyubiquitin chains (9).

The membrane-associated RING-CH (MARCH) proteins are part of a family of RING-v type E3 ubiquitin ligases containing 11 known members, some of which target important players of the immune response (10). Most MARCH proteins have two transmembrane regions and possess a catalytic RING domain in their N-terminal cytoplasmic portion (10). They are homologs of poxviruses modulator ofi mmune recogn tion (MIR)1 and MIR2 proteins, which downregulate MHC I to escape the immune response during the establishment ofl atent infections (11). Two close members of the family, MARCH1 and MARCH8, downregulate the cell surface expression of MHC class II (MHC II) molecules, CD95, B7.2, and Tfr (10–13).

Although MARCH8 appears to be expressed in many different cell types, MARCH1 is mainly found in secondary lymphoid organs, more specifically in the endocytic pathway of dendritic cells (DCs) and B cells (11–15). MARCH1 reduces the half-life of peptide/MHC II complexes by causing their redistribution from recycling endosomes to lysosomes (12, 16). MARCH1 is highly expressed in conventional immature DCs, and its downregulation by LPS stabilizes cell surface peptide/MHC II complexes (15). In plasmacytoid DCs, Villadangos and colleagues (17) have shown that MARCH1 was still highly expressed after activation, allowing a rapid turnover of MHC II and efficient presentation of viral Ags. The differential regulation of MARCH1 expression might confer specific functions to conventional DCs and plasmacytoid DCs.

Recently, we showed that MARCH1 is induced by IL-10 in human primary monocytes and ubiquitinates MHC II molecules, leading to their intracellular retention and reduced Ag presentation (18). Several studies reported that the endogenous MARCH1 protein is hardly detectable even after an upregulation in mRNA expression (12, 18, 19). Jabbour et al. (19) recently estimated that the half-life of MARCH1 is ≈30 min.

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Received for publication September 19, 2011. Accepted for publication March 13, 2012.

This work was supported by Canadian Institutes for Health Research Grant 36355. M.-C.B.-D. received a student scholarship from the Cole Foundation.

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Abbreviations used in this article: BRET, bioluminescence resonance energy transfer; DC, dendritic cell; DIRT, domain in between the RING-CH type and the first transmembrane region; $EGFP_2$, enhanced GFP2; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; MARCH, membrane-associated RING-CH; MHC I, MHC class I; MHC II, MHC class II; MIR, modulator ofi mmune recognition; Rluc, Renilla luciferase; TM, transmembrane; YFP, yellow fluorescent protein.

The turnover of several E3 enzymes is regulated by homodimerization and transubiquitination (20). For instance, Itch, an E3 ubiquitin ligase involved in apoptosis and T cell differentiation, regulates its own expression by self-ubiquitination (21). Other examples include TRAF6, MDM2/MDMX, and RAF1, which were shown to form homodimers by interacting through the RING domains (2, 22–24). Interestingly, MARCH9 is capable of autoubiquitination and dimerizes with a RING-less splice variant, confirming the possible RING-independent dimer formation of ubiquitin ligases (25). Alternatively, the viral MIR1 and MIR2 also homodimerize but most likely via their transmembrane domains (26–28). In this study, we investigated the possible role of autoubiquitination in the posttranslational regulation of MARCH1 expression. We show that MARCH1 is capable of autoubiquitination, thus regulating its own expression.

Materials and Methods

Antibodies

The following Abs were used: rabbit polyclonal anti-GFP that recognizes both GFP and yellow fluorescent protein (YFP; Invitrogen, Laval, QC, Canada); mouse IgG_{2a} anti–HLA-DR (L243) (29); mouse IgG_{2a} anti-MHC I (W6-32) (30); mouse IgG_1 anti-ubiquitin (P4D1) (Fisher Scientific, Ottawa, ON, Canada); human anti-K48– and -K63–linked polyubiquitin Abs Apu2.07 and Apu3.A8, respectively (obtained from Genentech, South San Francisco, CA) (31); mouse IgG_1 anti-myc (9e10) (BioLegend, San Diego, CA); mouse anti-MARCH1 (H1) (clone 2G9.2.2.2; a gift from Dr. K. Fruh, University of Oregon); mouse anti-CD63 (H5C6 from Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, University of Iowa, Ames, IA); and Alexa Fluor 594-coupled goat-anti mouse Ab (Invitrogen).

Reagents

Polyethylenimine (2.5 kDa linear) was from Polysciences (Warrington, PA). Cycloheximide, MG132, N-ethylmaleimide, leupeptin, chloroquine, bafilomycin A1, and epoxomicin were purchased from Sigma-Aldrich (Oakville, ON, Canada) and used at final concentrations of 100 mg/ml, 10 mM, 0.2 mM, 50 mg/ml, 100 mM, 20 mM, and 1mM, respectively.

A mix of ^{35}S-labeled cysteine and methionine was purchased from PerkinElmer (Vaudreuil-Dorion, QC, Canada), and benzyl coelenterazine was from Nanolight Technology (Pinetop, AZ).

Plasmids and mutagenesis

The myc, Renilla luciferase (Rluc), enhanced GFP2 ($EGFP_2$), and enhanced YFP (EYFP) tags were fused by PCR overlap to the N terminus of MARCH or TAP1 molecules using pcDNA3.1_myc_MCS, pcDNA3.1_Rluc_MCS, pcDNA3.1_EYFP_MCS, or pcDNA3.1_$EGFP_2$_MCS constructs obtained from Dr. Daniel Lamarre (University of Montreal) (32). The MARCH1–9 chimeric molecules were generated by the PCR overlap and cloned in the BsiWI and NotI restriction sites of pcDNA3.1_EYFP_MCS. GFP-ubiquitin constructs were obtained from Addgene (Cambridge, MA). The pEGFP-N1-MARCH9 construct was a gift from Dr. Evelina Gatti (Centre d'Immunologie de Marseille-Luminy, Marseille, France). pBI_K3 and pUHD10.1_K5 plasmids were obtained from Dr. Klaus Fruh (Oregon Health and Science University). MARCH1WI was obtained by mutating the I65 and W97 residues for alanines. The MARCH1K-0 mutant was generated in multiple steps. First, the N-terminal 30 aas were deleted. Then, position K203 was mutated to an arginine and residues K213, K229, K230, K233, and K244 were substituted for alanines. MARCH1ΔRING was made by deleting the first 117 aas. MARCH1DCter was obtained by introducing a stop codon and deleting on the cDNA the coding sequence corresponding to the last 70 aas. MARCH1 DCytos was obtained by deleting the first 138 and last 69 aas coding regions of the cDNA. For the YFP-M1K203A, lysine 203 was substituted for an alanine. For the different MARCH1–9 chimeric molecules, we established the different domains to be the following: on MARCH1, RING is from residue 54 to 116, domain in between the RING-CH type and the first transmembrane region (DIRT) (33) is from 117 to 137, and N- and C-terminal transmembrane regions are from 138 to 160 and from 180 to 202, respectively. On MARCH9, the RING is from residue 109 to 156, DIRT is from 157 to 182, and N- and C-terminal transmembrane regions are from 183 to 205 and from 219 to 239, respectively.

Immunofluorescence

HeLa cells (6.25×10^4 per well) were plated on coverslips in 24-well plates 24 h prior to transfection. Cells were transfected as described below and incubated for 48 h before staining. Cells were fixed, permeabilized, and stained directly on coverslips as described below. Hoechst 33342 (Invitrogen) was used to stain the nucleus. Images were taken using an LSM 510 Meta Zeiss confocal microscope. Cells were excited at 405 nm with a 420 nm longpass filter for Hoechst staining, at 405 nm with a 470–500 nm bandpass filter for the GFP, at 488 nm with a 505–530 nm bandpass filter (for cells that were also stained for CD63) or with a 460 nm longpass filter (for cells that coexpressed GFP-MARCH1) for YFP, and the goat anti-mouse Alexa Fluor 594 Ab was scanned at 543 nm with a 560 nm longpass filter.

Cell culture and transfections

HeLa, HEK 293T, and HEK 293E CIITA (a gift from Dr. Viktor Steimle, Sherbrooke University) cells were cultured in DMEM supplemented with 5% FBS (Wisent, Saint-Jean-Baptiste, QC, Canada).

For transient transfections, 10^6 HeLa cells were plated in 10-cm petri dishes. After 24 h, cells were transfected using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's protocol. For HEK 293T and HEK 293E CIITA, 1.5×10^6 cells were plated 24 h prior to transfection. Cells were transfected using 3 ng polyethylenimine/ng DNA (Polysciences).

The HEK 293T myc-MARCH1 stable cell line was generated by transfecting the pcDNA3.1_myc-MARCH1 plasmid in polyethylenimine and by selecting cells resistant to hygromycin B (Wisent).

Pulse-chase experiments

HeLa cells were transiently transfected with YFP, YFP-MARCH1, YFP-MARCH1K-0, or YFP-MARCH1 ΔRING. After 24 h, cells were distributed into six-well plates. The day after, cells were starved for 45 min in methionine- and cysteine-free media (Wisent) at 37°C and pulsed for 1 h with the same media supplemented with [^{35}S]cysteine and [^{35}S]methionine radiolabeling mix at 0.25 mCi/ml. Cells were then washed with PBS at 37°C and incubated for the different times in warm DMEM supplemented with 5% FBS. At each time point, cells were scrapped and frozen. Samples were lysed and immunoprecipitated as described below. Proteins were separated by SDS-PAGE (12%), transferred to nylon membranes, and analyzed using a Typhoon Trio Phosphorimager (Amersham Biosciences).

Bioluminescence resonance energy transfer

The pcDNA3.1_Rluc-M1 (20 ng) was cotransfected in 1.25×10^5 HEK 293T cells with 0–500 ng pcDNA3.1_YFP-MARCH1, pcDNA3.1_YFP-MARCH8, or pcDNA3.1_YFP-TAP1 expression vectors, resulting in different fluorescence/luminescence ratios (34). Cells were harvested after 48 h and washed. For each sample, 3×10^5 cells were plated in duplicate into a 96-well plate. The background values of fluorescence were determined on a Mithras LB940 spectrofluorometer before the addition of coelenterazine by measuring the fluorescence emissions at 538 nm after an excitation at 485 nm. After the addition of coelenterazine at a final concentration of 5mM, the luminescence and fluorescence emission in the 460–500 and 510–550 nm windows, respectively, were measured on a Mithras LB940 multidetector plate reader. The bioluminescence resonance energy transfer (BRET) ratio on the y-axis was calculated by dividing the acceptor-emitted fluorescence by the donor-emitted luminescence. BRET ratios were normalized by subtracting the background signal from cells transfected without YFP. The fluorescence over luminescence ratio on the x-axis is the ratio between the fluorescence of acceptor (YFP-YFP0, where YFP0 is the fluorescence value of cells expressing the BRET donor alone) and the luminescence of the acceptor.

Immunoprecipitation and Western blot analysis

Cells were lysed in 1% Triton X-100 lysis buffer supplemented with complete protease inhibitor mixture (Roche, Laval, QC, Canada). For ubiquitination experiments, lysis buffer was supplemented with MG132 and N-ethylmaleimide (18). Cells were lysed on ice and centrifuged. The postnuclear supernatants were precleared for 1 h with protein G-coated Sepharose beads (GE Healthcare, Mississauga, ON, Canada) and specific proteins were immunoprecipitated overnight using protein G-Sepharose beads precoated with the selected Ab. The beads were washed and the samples were analyzed by SDS-PAGE (10%). For immunoprecipitations using the K48- and K63-specific Abs, cells were lysed at room temperature in lysis buffer containing 8 M urea (supplemented with MG132, N-ethylmaleimide, and complete protease inhibitors as described above). Samples were centrifuged and postnuclear supernatants were diluted to 4 M

urea before performing the immunoprecipitations. For Western blotting, proteins were transferred to Hybond ECL membrane (Amersham Biosciences) and analyzed with specific mAbs. Goat anti-mouse and anti-rabbit Abs coupled to peroxidase (Bio/Can Scientific) were used as secondary Abs and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate [peroxidase]; Roche).

For pulse-chase experiments, cells were lysed in radioimmunoprecipitation assay lysis buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8]). Lysates were precleared first for 2 h with beads and then for 2 h with mouse serum-coated beads before immunoprecipitation with the selected Abs.

For signal quantification, the films were scanned and analyzed using Photoshop CS4. Briefly, the colors were inverted and the mean intensity of the signal in a blank area was subtracted from that of different portions of the same size.

Results

MARCH1 is polyubiquitinated

The endogenous MARCH1 is barely detectable in primary human monocytes and DCs as well as in mouse B cells (12, 14, 18). Because the half-life of MARCH1 is short and many ubiquitin ligases appear to be themselves ubiquitinated (35), we sought to determine whether MARCH1 was ubquitinated. To address this issue, we overexpressed MARCH1 in HEK 293T cells, but again the expression remained barely detectable by flow cytometry and on immunoblots using a MARCH1-specific mAb (data not shown). In an effort to increase the yield of MARCH1 in cell lines and to facilitate the detection of ubiquitinated forms on immunoblots, we fused YFP to the N-terminal part of the ubiquitin ligase (YFP-M1). HEK 293T cells were transiently transfected with YFP-M1 and ubiquitination of MARCH1 was assessed following immunoprecipitation with a YFP-specific mAb. Controls included cells transfected with the YFP or a MARCH1 mutant lacking all of the extra-RING cytoplasmic lysines (YFP-M1K-0) (Fig. 1A, 1B). The YFP-M1 fusion protein has a predicted molecular mass of 57 kDa, and a smear of ubiquitinated proteins was detected for YFP-M1 starting at ≈80 kDa. No such smear was observed for YFP-MARCH1K-0 even though the YFP blot revealed similar amounts of immunoprecipitated MARCH1 proteins (Fig. 1B, right panel). Although we cannot rule out that some lysine residues on YFP have been modified in the context of the fusion protein, the strength of the signal detected for the wild-type YFP-M1 as compared with the control YFP-M1K-0 confirms that MARCH1 is ubiquitinated. These results do not discriminate between ubiquitination of different lysines on YFP-M1 and the addition of a polyubiquitin chain to a single specific residue.

To investigate the type of ubiquitination taking place in these conditions, we coexpressed MARCH1 and a GFP-tagged lysineless ubiquitin (GFP-ubiK-0) that prevents further elongation of a chain once linked to the substrate (36, 37). As a control, we used an inactive ubiquitin molecule (GFP-ubiG76) that cannot be conjugated to any target (36). Because these ubiquitin mutants are tagged with GFP, the experiments were conducted with wild-type MARCH1. The ligase was immunoprecipitated using an Ab (H1) specific to the C-terminal end of MARCH1, and the presence of ubiquitin was monitored on immunoblots using a GFP-specific mAb (Fig. 1C). Because the molecular masses of MARCH1 and the GFP-ubi fusion protein are 25 and 33 kDa, respectively, the presence of one discrete band at 58 kDa and a smear of high molecular mass indicated the presence of a monoubiquitinated as well as polyubiquitinated MARCH1 species. A similar pattern was obtained using the GFP-ubiK-0 mutant that prevents further chain

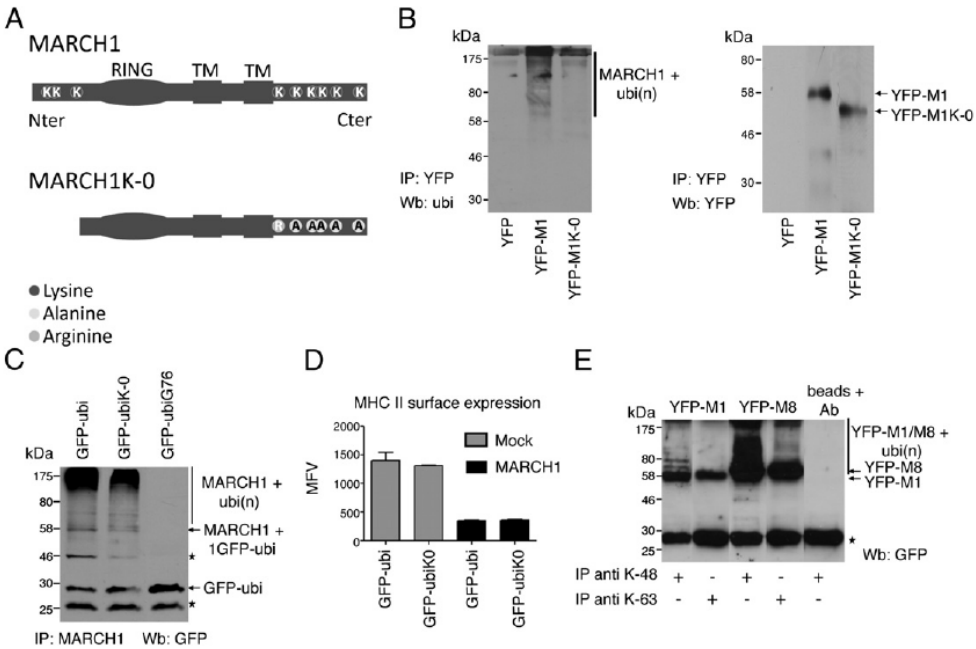

FIGURE 1. MARCH1 is polyubiquitinated. (A) Schematic representation of the mutations introduced into the M1K-0 mutant. (B) HEK 293T cells were transfected with YFP, YFP-M1, or YFP-M1K-0 and lysed after 48 h. MARCH1 was immunoprecipitated with a YFP-specific Ab and ubiquitin was revealed by Western blotting. The right panel shows the same samples probed for the presence of YFP-MARCH1. (C) HEK 293T cells stably expressing MARCH1 were transfected with GFP-ubi, GFP-ubiK-0, or GFP-ubiG76, lysed, immunoprecipitated for MARCH1, and blotted with a GFP-specific Ab. (D) Flow cytometry analysis of MHC II surface expression in HEK 293E CIITA cells transfected or not with MARCH1 and GFP-ubi or GFP-ubiK-0. Error bars represent SD obtained for two different transfections. (E) HEK 293T cells were transfected with YFP-M1 or YFP-M8, lysed, immunoprecipitated with a K48 or K63 polyubiquitin-specific Ab and blotted for YFP. Data are representative of three (B), (C), and (E) or five (D) independent experiments. *Immunoprecipitating Abs.

elongation. This resulted in a smear of stronger intensity at the top of the gel. Collectively, these results confirm that MARCH1 is polyubiquitinated.

A band off ree GFP-ubiquitin was also present in all conditions. Because the GFP-ubiG76 cannot form the first thioester bond with the E1, a step that is also called the activation of ubiquitin, it is likely that these free ubiquitins were bound to other molecules coprecipitating with MARCH1.

To determine whether the reduced polyubiquitination of MARCH1 caused by the coexpression of GFP-ubiK-0 affects the activity of the molecule, we coexpressed MARCH1 and GFP-ubi or GFP-ubiK-0 in HEK 293E CIITA cells and measured the cell surface expression of MHC II (Fig. 1D). MARCH1 triggered an important decrease in MHC II expression at the cell surface in the presence of GFP-ubi, showing that ubiquitin fusion proteins are functional. The MHC II mean fluorescence values were similar for MARCH1-positive cells independent of the cotransfection of GFP-ubi or GFP-ubiK-0, suggesting that long polyubiquitin chains are not required for either the function of MARCH1 or the intracellular retention of MHC II.

To determine the type of polyubiquitin chains attached to MARCH1, we performed immunoprecipitations with Abs specific for K48- or K63-linked polyubiquitin chains and looked for MARCH proteins on immunoblots (Fig. 1E). The presence of multiple bands around 58, 66, and 72 kDa for samples from YFP-MARCH1–transfected cells and immunoprecipitated with the K48-specific mAb suggests that this type of linkage is predominant. The intense smear observed in the YFP-M8 samples confirms the presence of K48 polyubiquitin chains. Unexpectedly, we detected bands at 58 kDa for YFP-M1 and at 63 kDa for YFP-MARCH8 samples, which correspond to unmodified forms of the molecules. The presence of nonubiquitinated TRAF6 molecules was also observed following immunoprecipitations with these Abs in HEK 293T cells (31).

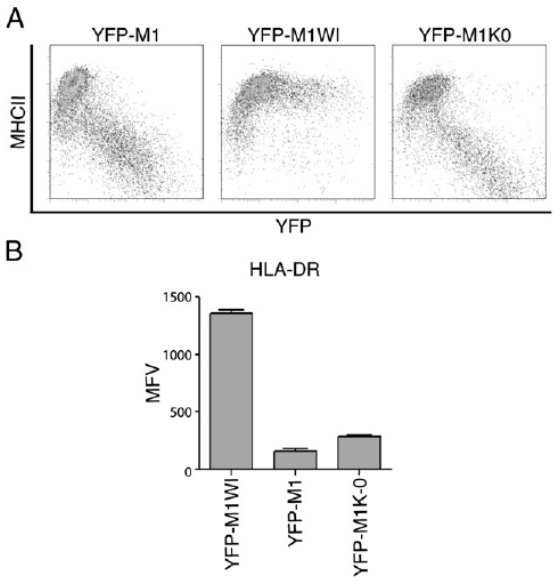

FIGURE 2. Ubiquitination of MARCH1 is not required for its function. (A) Flow cytometry analysis showing the MHC II surface expression of HEK 293E CIITA cells transfected with YFP-M1, YFP-M1WI, or YFP-M1K-0. (B) Flow cytometry analysis of HLA-DR surface expression in HEK 293E CIITA cells transfected with YFP-M1WI, YFP-M1, or YFP-M1K-0. Error bars represent SD obtained for two different transfections. Data are representative of six independent experiments.

Ubiquitination of MARCH1 is not required for its function

The activity of RING-type E3 ubiquitin ligases such as Chfr and TRAF6 has been shown to be modulated by ubiquitination (35, 38, 39). We tested whether site-directed mutagenesis of the extra-RING lysines and the ensuing reduced ubiquitination would impair the capacity of MARCH1 to downregulate cell surface expression of MHC II molecules. As a negative control, we used an inactive mutant, M1WI, which contains two mutations in the RING domain that prevent the binding of E2s (40). Fig. 2A shows that M1K-0 is very efficient at downregulating MHC II from the plasma membrane. The mean fluorescence values corresponding to the MHC II surface staining in YFP-positive cells confirm that M1K-0 is as potent as MARCH1 (Fig. 2B) This demonstrates that ubiquitination of MARCH1 on the N- or C-terminal lysines is not required for its activity (Fig. 2B).

Ubiquitination of MARCH1 regulates its turnover

Recently, Lybarger and colleagues (19) have demonstrated that mouse MARCH1 is the subject of a tight posttranslational regulation through lysosomal degradation. To determine whether this was also the case in our system, YFP-M1–transfected cells were treated with proteasome or lysosome inhibitors. The effect on YFP-M1 protein levels was assessed by flow cytometry (Fig. 3A). Our results showed an increase in YFP-M1 expression with proteasome and lysosome inhibitors, except for leupeptin. Still, the important augmentation of YFP-M1 expression in the presence of the acidification inhibitors chloroquine and bafilomycin confirms the role of endocytic compartments in the degradation of MARCH1. Interestingly, the two proteasome inhibitors also increased the expression of YFP-M1. Epoxomicin is more specific than MG132, which can also inhibit lysosomal calpains and cysteine proteases (41, 42). The augmentation of YFP-M1 expression following epoxomicin treatment shows a role of the proteasome in the turnover of MARCH1, in line with the presence of K48-linked polyubiquitin chains (Fig. 1E).

To rule out the possibility that some posttranslational modifications on the YFP tag affected the degradation of the fusion protein and to validate our results obtained by flow cytometry, we carried out an experiment with untagged MARCH1. To do so, we stably expressed MARCH1 in HEK 293T cells and treated the cells with cycloheximide, to inhibit the protein synthesis, or with MG132 or bafilomycin (Fig. 3B). Western blot analysis using the 1H mAb revealed a faint 30-kDa band in mock-treated (DMSO, EtOH) MARCH1-transfected cells that was absent from control HEK 293T cells. Inhibition of protein synthesis with cycloheximide reduced MARCH1 levels whereas both MG132 and bafilomycin caused an increase in the amount of MARCH1 proteins. The samples were blotted with an actin-specific Ab to allow quantification of the signals. The data revealed a 2-fold increase in MARCH1 protein levels for the cells treated with MG132 or bafilomycin (Fig. 3B, right panel). Collectively, the results obtained by Western blotting for the untagged protein and by flow cytometry using the YFP-tagged construct suggest that the degradation of MARCH1 implicates both the proteasome and endosomes.

We then assessed the importance of MARCH1 ubiquitination in its turnover. Interestingly, transfection experiments in HEK 293T (Fig. 3C) and HeLa cells (data not shown) consistently showed stronger expression of the YFP-M1K-0 mutant as compared with YFP-M1, suggesting that removal of ubiquitin acceptor lysines may stabilize the protein. To test this hypothesis, we compared the stability of M1 and M1K-0. Pulse-chase experiments were per-

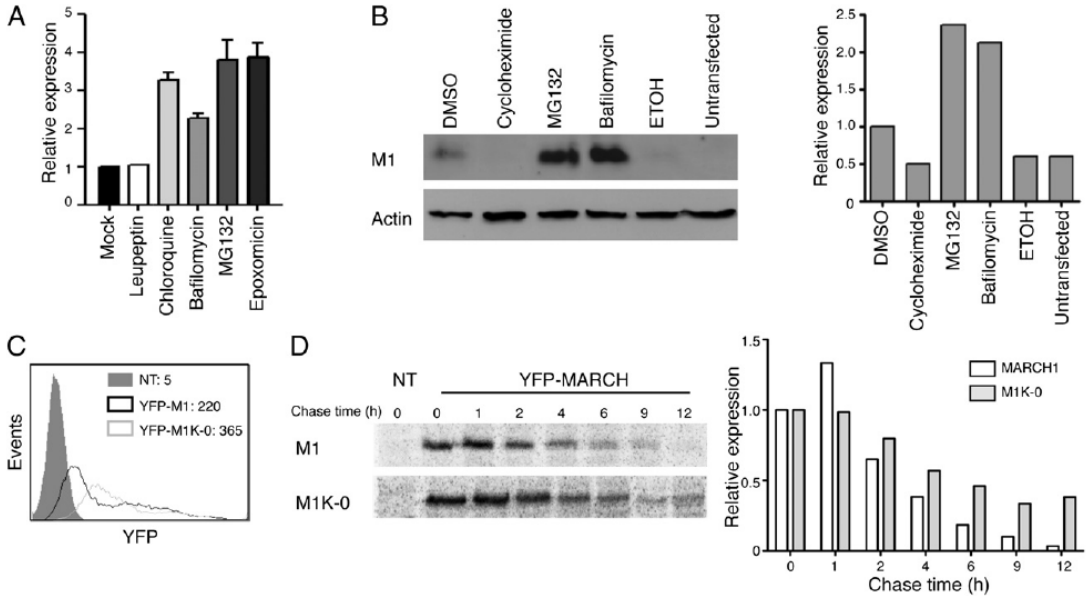

FIGURE 3. The lysine-less mutant of MARCH1 has a prolonged half-life. (A) HeLa cells were transfected with YFP-M1, harvested after 24 h, and plated in 24-well plates. Cells were incubated for another 16 h in the presence of pharmacological inhibitors of various pathways. Cells were analyzed by flow cytometry. The mean fluorescence value obtained for control untreated cells was set as 1. Error bars represent SD obtained for three different transfections. (B) HEK 293T cells stably expressing MARCH1 were treated for 16 h with pharmacological inhibitors of various pathways. Cells were lysed and analyzed by Western blotting using Abs against MARCH1 and actin. The right panel shows the densitometric quantification of MARCH1 bands over the signal of the corresponding actin bands. The results were expressed as relative to the values of untreated cells. The value obtained for control untreated cells was set as 1. (C) Flow cytometry analysis showing the YFP expression of HEK 293E CIITA cells transfected with YFP-M1 or YFP-M1K-0. Numbers indicate the mean fluorescence values. (D) HeLa cells were transfected with YFP-M1 or YFP-M1K-0, pulsed for 45 min with radiolabeled amino acids, and chased for different periods of time. The cells were lysed, immunoprecipitated with a YFP-specific Ab, and analyzed by SDS-PAGE. The radioactive bands were detected using a Phosphorimager. The signals of the bands were quantified and normalized to the one obtained at time 0 h for each condition. Data are representative of 6 (A), 2 (B), 10 (C), or 3 (D) independent experiments.

formed on transiently transfected HeLa cells (Fig. 3D). The results showed that the YFP-tagged MARCH1 has a short half-life of ~4 h whereas the K-0 variant was much more stable and was still detectable after 12 h chase (Fig. 3D). Taken together, these results demonstrate that ubiquitination of MARCH1 affects its stability and that the protein is going through endosomal and proteasomal degradation.

Autoubiquitination of MARCH1

As automodification has been demonstrated for several E3 ubiquitin ligases (2, 20, 22–24), we compared the levels of ubiquitin bound to YFP-M1 versus the inactive mutant YFP-M1WI expressed transiently in HeLa cells. MARCH proteins were immunoprecipitated and analyzed on immunoblots for the presence of higher molecular mass, ubiquitinated species (Fig. 4A). Because the YFP-M1 fusion protein has a molecular mass of 57 kDa, ubiquitinated forms are expected to migrate at 65 kDa or more. The intensity of the smear was reduced for M1WI compared to the wild-type molecule (Fig. 4A), in line with the capacity of MARCH1 to ubiquitinate itself. The YFP immunoblot revealed that similar amounts of MARCH1 were immunoprecipitated in these conditions. Interestingly, ubiquitination of the inactive YFP-M1WI mutant was not totally abolished, suggesting that MARCH1 is also the substrate of another endogenous E3 ubiquitin ligase.

To determine whether MARCH1 could regulate its expression through ubiquitination, we performed a pulse-chase experiment and compared the degradation kinetics of MARCH1 and the inactive mutant. Fig. 4B shows that MARCH1 has a short half-life of ~3 h whereas the inactive variant M1WI was found to be very stable, even after 6 h chase (Fig. 4B, bottom panel). Collectively, these results are in line with a model where MARCH1 undergoes autoubiquitination and regulates its own half-life.

MARCH1 forms homo- and heterodimers

Many E3 ubiquitin ligases were shown to form dimers capable of transubiquitination (2, 20, 23, 24, 43). The above-described experiments suggest that MARCH1 is involved in homo- and heterodimeric interactions. Thus, we carried out energy transfer (BRET) experiments between MARCH1 molecules or between MARCH1 and MARCH8. The dimerization with MARCH8 was investigated because the two proteins show ~80% sequence homology and are close family members (12). TAP1 is a transmembrane endoplasmic reticulum (ER) resident and was used as negative control (44). BRET is a very sensitive assay that allows the detection in living cells of interactions between two protein partners, one tagged with Rluc and one tagged with YFP (34, 45, 46). The nonradiative luminescence emitted by Rluc upon the addition of its substrate, coelenterazine, can excite the YFP if the two molecules are at a distance of 100 Å or less. The fluorescence emitted upon the addition of the substrate is called the BRET signal. Random interactions of the two partners will create a nonsaturating signal that increases along with the amount of proteins. Alternatively, the BRET signal obtained from the specific interaction of two partners should reach a plateau since the energy donor will become saturated in conditions where expression of the acceptor molecule is increasing. We transfected in HEK 293T cells the Rluc-tagged versions of MARCH1 or TAP1

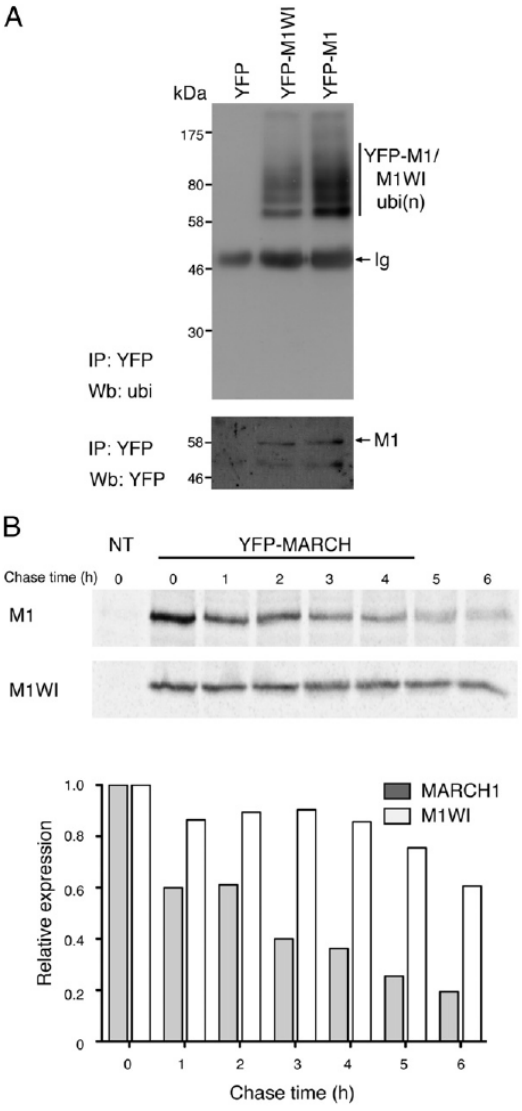

FIGURE 4. Autoubiquitination of MARCH1. (A) HeLa cells were transfected with YFP, YFP-M1, and YFP-M1WI, lysed, and proteins were immunoprecipitated with a YFP-specific Ab. Ubiquitin was detected by Western blotting. The bottom panel shows the same samples probed for the presence of YFP-MARCH1. (B) HeLa cells were transfected with YFP-M1 or YFP-M1WI, pulsed for 45 min with radioactive-labeled cysteine and methionine, and chased for different periods of time. The cells were lysed and MARCH1 was immunoprecipitated with a YFP-specific Ab. Radioactive material was detected using a Phosphorimager. The signals of the bands were quantified and normalized to the one obtained at time 0 h for each condition. Data are representative of at least four (A) or three (B) independent experiments.

together with increasing amounts of YFP-M1 or YFP-M8. Saturating BRET signals were obtained, showing the homodimerization of MARCH1 and its heterodimerization with MARCH8 (Fig. 5A, left and middle panels). Alternatively, a linear curve was obtained between TAP1 and MARCH1, demonstrating that these two molecules do not specifically interact (Fig. 5A, right panel).

Homodimerization of MARCH1 was confirmed by coimmunoprecipitation of transiently transfected MARCH molecules. Fig. 5B shows that YFP-MARCH1, but not the control YFP, coimmunoprecipitated with myc-MARCH1. We also performed immunoprecipitations between MARCH1 and MARCH8 and investigated the homodimerization of MARCH8 (Fig. 5C). The presence of a band corresponding to YFP-MARCH1 following the immunoprecipitation of myc-MARCH8 confirmed the formation of heterodimers, as deduced from the BRET experiment described above. Also, a band corresponding to YFP-MARCH8 was detected in the same conditions, demonstrating homodimerization of the molecule. We investigated also the heterodimerization of MARCH1 with a more distant family member. MARCH9 protein sequence displays 12% similarity to MARCH1 and was recently shown to form homodimers (25). Myc-tagged MARCH1 was immunoprecipitated and the presence of associated MARCH9-GFP was assessed on immunoblots (Fig. 5D). MARCH9 is slightly larger than MARCH1 and the results demonstrate that the two molecules interact with one another.

Many RING-type E3 ligases dimerize via their RING domains (24, 45, 47, 48). We expressed MARCH1 with a RING-less variant and performed coimmunoprecipitation experiments (Fig. 5E). The presence of a band at 45 kDa that corresponds to YFP-MARCH1 DRING upon the immunoprecipitation of myc-MARCH1 demonstrated that the RING domain is not essential for dimerization. In the same experiment, we tested a MARCH1 variant lacking its C-terminal cytoplasmic region (YFP-M1DCter) and another variant lacking both ofi ts cytoplasmic domains, except for the RING (YFP-M1 DCytos). The results revealed that the two constructs coimmunoprecipitated with myc-M1. Thus, dimerization appears to be independent of the cytoplasmic domains of MARCH1.

To further support the finding that MARCH1 dimerizes, we made use of a misfolded and mislocalized mutant. The mutation of the cytoplasmic lysine 203 for an alanine (M1K203A) eliminates the charge lining the C-terminal transmembrane domain and produces an almost inactive MARCH1 mutant (Fig. 6A). The mutation abolishes MARCH1 trafficking to endosomal compartments, presumably by preventing ER egress. Confocal microscopy experiments on HeLa cells transfected with YFP-M1 or YFP-M1K203A showed partial colocalization with the lysosomal marker CD63 for YFP-M1 whereas the K203A mutant rather displayed a diffuse staining reminiscent of the ER (Fig. 6B). We reasoned that dimer formation between this YFP-M1K203A and a wild-type MARCH1 might affect the trafficking of one or the other partner. To test this hypothesis, a GFP_2-tagged MARCH1 was coexpressed in HeLa cells either with the YFP-M1K203A mutant or a control YFP-M1 (Fig. 6C). As expected, there is a perfect colocalization between YFP-M1 and GFP_2-M1 (Fig. 6C, top row). Interestingly, we observed a redistribution of the K203A mutant and colocalization with GFP_2-MARCH1 in peripheral vesicles, suggesting that dimerization occurred between the two molecules and that the wild-type MARCH1 assisted the folding and/or trafficking of the mutant (Fig. 6C). To test whether this redistribution would translate in a gain of activity, we coexpressed YFP-M1K203A with the inactive MARCH1WI and looked at cell surface expression of MHC II by flow cytometry (Fig. 6D). The results show that YFP-M1K203A is more active in cells coexpressing MARCH1WI, in line with a model where MARCH1 forms dimers in the ER. Collectively, these results demonstrate the capacity of MARCH1 to homo- and heterodimerize.

Dimerization of MARCH1 depends on the transmembrane domains

To map functional domains in MARCH1 (Table I), we generated a series of chimeric molecules between MARCH1 and MARCH9, two ubiquitin ligases with different target specificities. Whereas

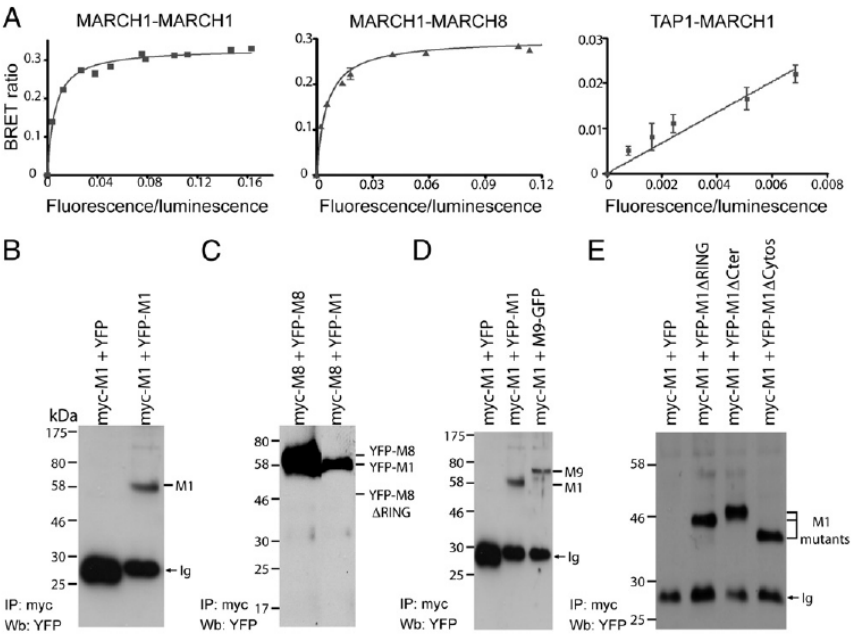

FIGURE 5. MARCH1 forms homo- and heterodimers. (A) HEK 293T cells were transfected with Rluc-M1 or TAP1-Rluc and increasing amounts of YFP-M1 or YFP-M8. The BRET ratio was calculated by dividing the fluorescence with substrate, subtracted from the fluorescence without substrate, by the luminescence. Error bars represent SD obtained for two different transfections. (B–E) Western blot analysis of myc-specific immunoprecipitations of HEK 293T cells expressing myc-M1 or -M8 and YFP, YFP-M1, YFP-M8, M9-GFP, YFP-M1 ΔRING, YFP-M1 ΔCter, or YFP-M1 ΔCytos. The gels were all in reducing condition except for (C). Data are representative of six (A) or at least three (B–E) independent experiments.

MARCH1 downregulates all isotypes of MHC II but not MHC I, MARCH9 was shown by other groups to act on MHC I and only on the DQ isotype of MHC II (25, 49). In our overexpression system, however, we found that MARCH9 was also active on HLA-DR. We reasoned that by introducing portions of MARCH1 ofi ncreasing length into MARCH9, one should be able to delineate functional regions. Starting at the N-terminal region of MARCH9, five chimeric molecules were generated and tested for their capacity to downregulate MHC I molecules in transiently transfected HEK 293E CIITA cells (Fig. 7A, 7B). Results show that the first 54 (MARCH1–9RING) or 105 (MARCH1–9DIRT) amino acids of MARCH1 transplanted into MARCH9 did not affect its ability to downregulate MHC I. The MARCH1–9DIRT bears the RING domain of MARCH1 fused to the DIRT region of MARCH9. The DIRT domain was previously identified in K3 and shown to be important for target recognition (33). However, introducing a larger fragment of MARCH1 encompassing the entire N-terminal cytoplasmic region (M1–9TMNter) strongly reduced functionality. Whereas the nature of the RING does not appear to be important, these results demonstrate that the DIRT domain included between amino acids 106 and 137 is important for the activity of M9. Accordingly, fusion proteins with larger parts of MARCH1 showed a dramatic reduction in their capacity to downregulate MHC I. The same constructs were tested for their activity on MHC II molecules. Importantly, both MARCH1 and MARCH9 act on HLA-DR in this assay but with very different efficiencies. A complete downregulation of MHC II surface expression is observed at low levels of YFP-MARCH1 whereas MARCH9 requires that a stronger expression is achieved before downregulation of MHC II can be measured (Fig.7B). Interestingly, whereas M1–9RING was still fully active, the M1–9DIRT mutant was almost completely inactive (Fig. 7B). This is in sharp contrast with the results obtained on MHC I. Extending the MARCH1 portion up to the N-terminal transmembrane region (M1–9TMNter) abolished the activity, suggesting that this mutant may not interact with MHC II molecules. To test this hypothesis, we expressed the various mutants in MHC II cells and verified the interaction by coimmunoprecipitation. Fig. 7C shows that just like M1 and M9, the M1–9TMNter mutant associates with DR (Fig. 7C, lane 6). Interestingly, extending the M1 sequence to include the N-terminal transmembrane (TM) domain (M1–9.5) prevented both MHC II binding and downregulation (Fig. 7B, 7C). These mutants are properly folded as judged by their efficient trafficking to the endocytic pathway (data not shown). Extending further the M1 sequence to include the second TM region (M1–9Cter) rescued MHC II binding and, marginally, downregulation. MARCH1 and its substrates interact through their TM regions (13). Our results suggest that important interactions between the two TM regions are needed for interacting with MHC II molecules while the enzymatic activity requires that both N- and C-terminal cytoplasmic regions be from the same molecule. We further investigated the importance of the TM regions in MHC II binding by creating new chimeric molecules bearing only short segments of MARCH9. These are the N-terminal transmembrane and luminal domains (M1–9-1Cter) or a longer version including both transmembrane regions (M1–9-1Cter) of MARCH9 (Fig. 7A). Both mutants coimmunoprecipitated with HLA-DR, showing that the N-terminal TM domain of MARCH1 specifically requires the corresponding C-terminal TM region whereas the N-terminal TM domain of M9 can accommodate both MARCH1 and MARCH9 C-terminal TM regions (Fig. 7C, lanes 7 and 8). It is intriguing that these two mutants remain inactive, but these findings suggest a complex interplay between multiple functional domains and motifs inside MARCH proteins.

Transmembrane domains have been involved in the dimerization of K3 and K5 (28). In this context, we tested the capacity of our YFP-tagged mutants to dimerize with myc-MARCH1. Following coexpression in HEK 293T cells, MARCH1 was immunoprecipitated and the presence of the mutants was assessed on immunoblots using a YFP-specific Ab (Fig. 7D). Interestingly, the same chimeric molecule that did not bind HLA-DR (M1–9.5) was unable to dimerize with MARCH1. These results suggest that dimerization, substrate binding, and catalytic activity may all be structurally linked. Still, the existence of nonfunctional chimeric molecules capable of dimerization and of binding to HLA-DR suggests the existence of multiple functional domains.

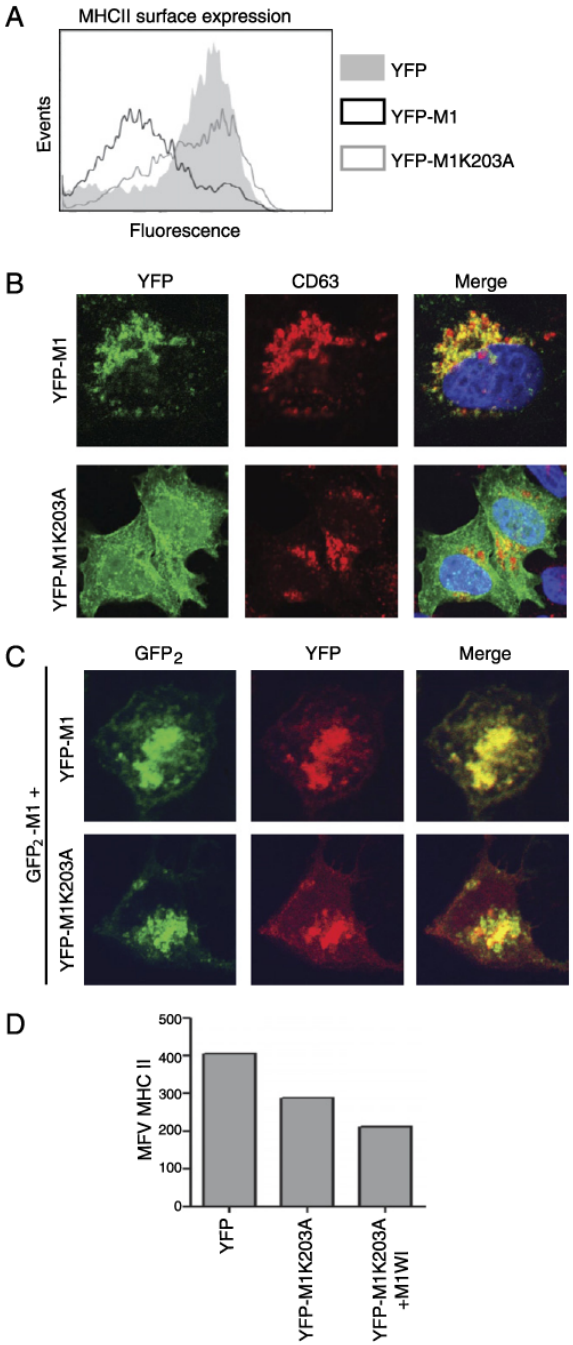

FIGURE 6. Dimer formation rescues the localization and function of a mislocalized mutant of MARCH1. (A) Flow cytometry analysis showing the MHC II surface expression of HEK 293E CIITA cells transfected with YFP, YFP-M1, or YFP-M1K203A. (B) Confocal microscopy images of HeLa cells transfected with YFP-M1 or YFP-M1K203A and stained with Hoechst as well as for CD63. Original magnification3 400. (C) Confocal microscopy images of HeLa cells transfected with GFP2-M1 and YFP-M1 or YFP-M1K203A. Cells were transfected with GFP $_2$ and YFP fluorescent proteins. Original magnification3 600. (D) Flow cytometry analysis showing the MHC II surface expression of HEK 293E CIITA cells transfected with YFP or YFP-M1K203A with or without M1WI. Data are representative of seven (A) or three (B–D) independent experiments.

Dimer formation regulates the stability of MARCH1

The above-described results demonstrated that MARCH1 is ubiquitinated and forms dimers. Next, we investigated the possibility that dimerization modulates the stability of the ligase. An inactive MARCH1 might dimerize and increase the expression of a coexpressed wild-type MARCH1. To test this hypothesis, we stably transfected HEK 293T cells with untagged MARCH1 and transiently supertransfected cells with either YFP or YFP-M1WI (Fig. 8A). Cell lysates were analyzed on immunoblots using a MARCH1-specific Ab. Wild-type MARCH1 migrates at a molecular mass of 24 kDa, whereas YFP-M1 is detected at 58 kDa. We observed a dramatic increase in MARCH1 expression when cotransfected with YFP-M1WI as compared with the control YFP (Fig. 8A, left panel). The band at 24 kDa is not observed in cells that do not express the wild-type MARCH1 and thus does not correspond to a mere degradation product of the YFP-M1 fusion proteins (Fig. 8A, right panel).

The same observation should hold true for YFP-M1 in the presence of M1WI, even though the former is somehow stabilized by the YFP fusion. To test this hypothesis, we coexpressed YFP-M1 with MARCH1 or M1WI and compared the expression of the fusion proteins by flow cytometry (Fig. 8B, left panel). Interestingly, the levels of YFP-M1 increased in the presence of M1WI, suggesting that dimerization with an inactive form increased stability. Alternatively, when coexpressed with wild-type MARCH1, the expression of YFP-M1WI was reduced (Fig. 8B, right panel). These results suggest that MARCH1 proteins dimerize and regulate their own expression. Together with the results of Fig. 4B showing that MARCH1 is capable of autoubiquitination, it is likely that transubiquitination is involved and that the stability of the complex decreases as more ubiquitin moieties are added to either partner.

Discussion

MARCH1 plays a critical role in the regulation of adaptive immunity. Although CIITA was defined as the master regulator of MHC II gene transcription, MARCH1 appears to play a similar role but at the posttranslational level. In mouse and human immature conventional DCs, MARCH1 is responsible for the reduced display of MHC II molecules. Upon maturation following TLR stimulation, MARCH1 expression is shut down to favor Ag presentation (14, 18, 50). Alternatively, the immunosuppressive cytokine IL-10 upregulates MARCH1 gene expression to minimize the display of MHC II molecules (18, 51).

The mechanistic basis for the target specificity of MARCH1 is unknown, and overexpression may cause downregulation of other nonphysiological substrates. For example, our data show that MARCH9 can downregulate MHC II molecules in transfected cells (Fig. 7B). Also, Bartee et al. (11) reported that MARCH9 downregulates surface expression of MHC I molecules. Still, MARCH9-deficient animals showed no phenotype concerning the expression levels of either MHC I or II molecules (5). These observations emphasize the importance for the cell to tightly regulate MARCH expression levels. Moreover, MARCH1 is extremely active as judged by the strong downregulation of MHC II surface expression in barely fluorescent YFP-M1–transfected cells (see Fig. 2A). Thus, it may not be so surprising that the MARCH1 protein is hardly detectable in primary cells using Abs that otherwise are highly efficient on transfected cells expressing a YFP-stabilized MARCH1 (Ref. 18 and data not shown). The exact mechanism by which GFP fusion proteins get stabilized remains nebulous, but the same effect was obtained upon fusion of the GFP at the N or C terminus of MARCH1 (data not shown).

Table I. Summary of the structural and functional characteristics of MARCH1, MARCH9, and the different mutants used in this study.

	Activity on				
	MHC I	MHC II	MHC II Binding	Endocytic Localization	Dimer Formation with MARCH1
M1	2	+	+	+	+
M1K-0	NS +	+	+	+	NS +
M1WI	NS +	2	+	+	NS +
M1K203A	NS +	+/ 2	+	2	NS +
M9	+	+	+	+	+
M1–9RING	+	+	+	+	+
M1–9DIRT	+	2	+	+	+
M1–9TMNter	2	2	+	+	+
M1–9.5	2	2	2	+	2
M1–9Cter	2	2	+	+	+
M1–9-1TMCter	2	2	+	+	2
M1–9-1Cter	2	2	+	+	+

NS, Data not shown.

Lybarger and colleagues (19) have recently shown that MARCH1 turns over with rapid kinetics in APCs. In the present study, we tested the hypothesis that MARCH1 undergoes autoubiquitination and regulates its own half-life. We showed in transfected cells that MARCH1 is polyubiquitinated in a K48 manner on at least one of its several lysines. Interestingly, expression of the GFP-ubiK-0 did not affect the ability of MARCH1 to downregulate MHC II molecules. However, in the same experimental conditions, we have not been able to inhibit the downregulation of MHC I molecules by the control K5, as described previously (9). This is most likely due to the fact that we work in transiently cotransfected cells where the levels of the ubiquitin ligase and the GFP-ubiK-0 increase proportionally, preventing saturation of the system with the mutant ubiquitin.

Pharmacological inhibitors of acidification increased MARCH1 levels, in line with a role of lysosomes in MARCH1 degradation. The fact that leupeptin prevented degradation of mouse but not human MARCH1 points to differences in the protein structure or experimental systems used (19). For example, the HeLa cells tested in this study are deficient in cathepsin S, a major target of

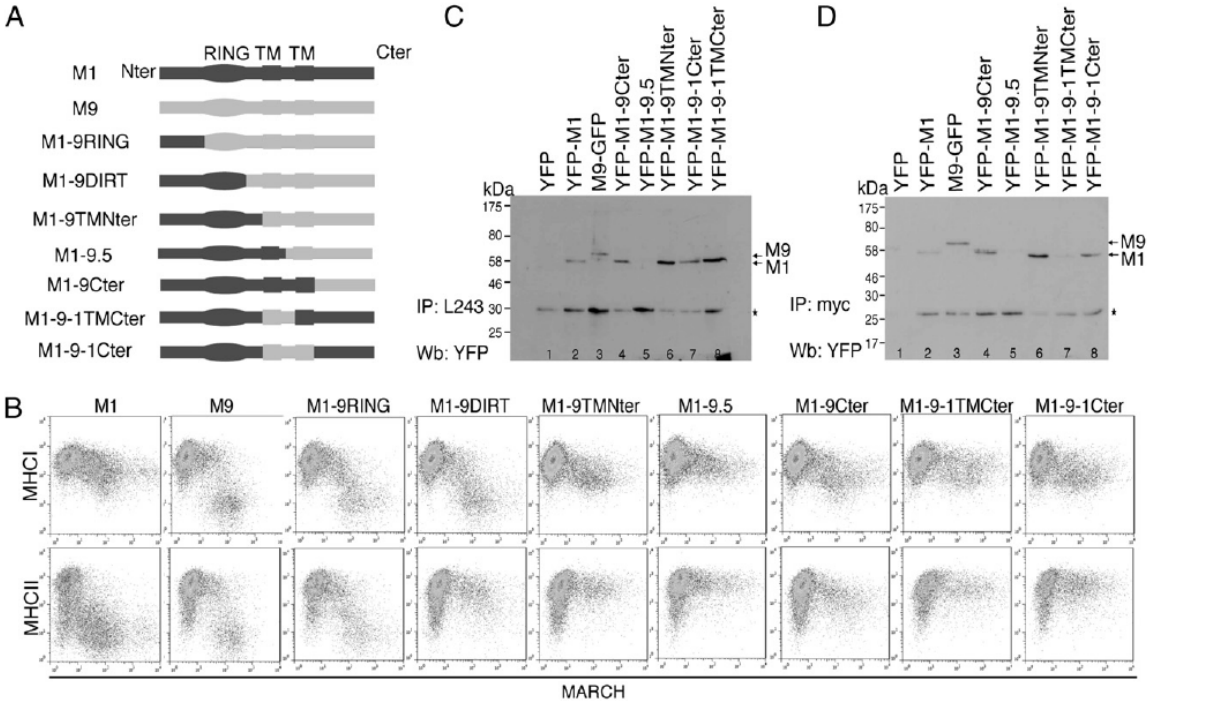

FIGURE 7. MARCH1 forms dimers via its transmembrane domains. (A) Schematic representation of the different M1–9 mutants used in this study. (B) Flow cytometry analysis showing MHC I and II surface expression in HEK 293E CIITA cells transfected with YFP-M1, M9-GFP, or the different chimeric constructs. (C) Western blot analysis of YFP, YFP-M1, M9-GFP, or the different YFP-MARCH chimeric molecules coimmunoprecipitated with MHC II from HEK 293E CIITA cells using L243. (D) Western blot analysis of YFP, YFP-M1, M9-GFP, or the different YFP-MARCH chimeric molecules coimmunoprecipitated from transfected HEK 293T cells with myc-MARCH1 using a myc-specific Ab. The asterisk marks the Abs. Data are representative of at least five (B) or three (C and D) independent experiments.

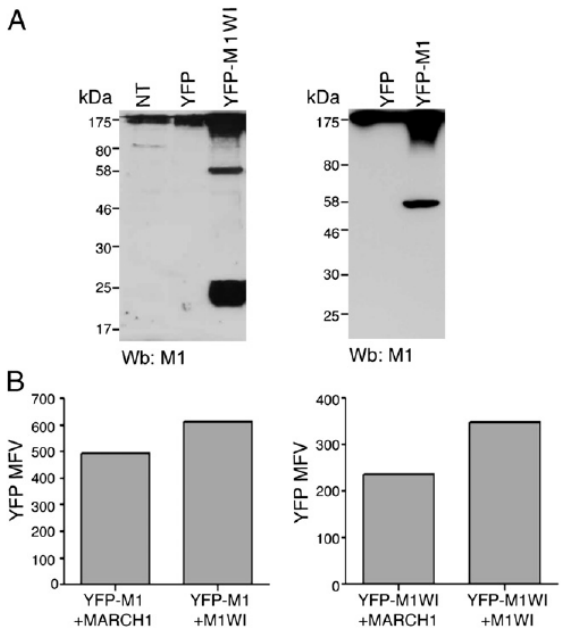

FIGURE 8. MARCH1 is stabilized by the coexpression of an inactive mutant. (A) Western blot analysis of HEK 293T cells stably expressing myc-MARCH1 and transfected with YFP or YFP-M1WI (left panel) or of HEK 293T cells transfected with YFP or YFP-M1 (right panel) using a MARCH1-specific Ab. (B) Flow cytometry analysis of YFP expression in HEK 293T cells transfected with YFP-M1 or YFP-M1WI together with MARCH1 or M1WI. Data are representative of at least two independent experiments.

leupeptin in immune cells (52). Still, the protective effect of chloroquine indicates that multiple types of proteases may be involved. Although the relationship between the extent of ubiquitination and the kinetics ofl ysosomal degradation is well established for cell surface hormone receptors (53, 54), it was rather surprising to find that proteasome inhibitors protected MARCH1 from degradation (Fig. 3A). Our results confirmed those of Lybarger and colleagues (19), who showed that mouse MARCH1 expression was affected by MG132 in DC2.4 cells. Proteasomes mainly degrade cytosolic, nuclear, and ER-dislocated proteins, but the expression of some integral membrane proteins can be influenced by the inhibition of the proteasome (55, 56). As described for the epidermal growth factor receptor, proteasomal activity could be required for deubiquitination of MARCH1 prior to its lysosomal degradation (57).

The MARCH1K-0 mutant is truncated just before the RING domain, and deletion of this region was shown to increase the stability of the mouse MARCH1 (19). Fig. 2B shows that M1K-0 behaves similar to the wild-type molecule in regard to its capacity to downregulate MHC II from cell surface. The fact that the lysine-less mutant, while stabilized, does not downregulate its target more efficiently than does MARCH1 is explainable by the small amount of the protein sufficient to fully eradicate MHC II molecules from the cell surface. This is in line with the weak in vivo expression of the endogenous protein.

We have addressed the role of autoubiquitination in the regulation of the half-life ofMARCH1. Many other RING-type E3s are known to form dimers and to transregulate their own expression and/or function (22–24, 58). Our results demonstrate the capacity of MARCH1 to form homo- as well as heterodimers with other members of the MARCH family. The proportion of MARCH1 molecules engaged in a dimer at any given time is difficult to evaluate. Interestingly, a misfolded MARCH1 mutant retained in the ER but that is still capable off orming dimers with wild-type MARCH1 did not act as a dominant negative (data not shown). This suggests that dimers are labile. In fact, none of the inactive mutants showed dominant-negative properties, in line with a model where the dimerization partner interacts transiently and possibly repeatedly with the ligase. This implies also that dimers composed of active and inactive forms of MARCH1 would be functional. Recently, MARCH9 was shown to form heterodimers with a RING-less natural splice variant that did not demonstrate dominant-negative activity (25).

Whether MHC II molecules interact with MARCH1 monomers or dimers remains to be determined, but the fact that a nondimerizing inactive mutant (M1–9.5) is unable to bind MHC II suggests that it might be the case. However, we cannot formally rule out that this particular mutant is somewhat misfolded. The fact that inactive mutants can dimerize but do not act as dominant-negative suggests that the interface implicated in MARCH/MARCH interactions is different from the one involved in substrate binding. Much effort will be needed to decipher at the molecular level the conformation of MARCH1 dimers and how autoubiquitination occurs.

Most of the RING-type E3s that form dimers do so through their RING domain (23). However, MIR1 and MIR2 have been proposed to dimerize by their transmembrane domains mainly because of the presence on immunoblots of bands of high molecular weights that were absent when analyzing mutants devoid of their TM regions (27, 28). The fact that MARCH9 forms homodimers as well as heterodimers with a RING-less natural splice variant demonstrated that the RING is not necessary for dimer formation (25). Our results show for another member of the family that the RING domain is indeed dispensable for dimer formation (Fig. 5E). The only MARCH mutant (M1–9.5) that was unable to dimerize with wild-type MARCH1 is the one in which the N-terminal TM domain of MARCH1 is next to the TM domain of MARCH9. As mentioned above, this molecule may be misfolded, as the luminal region where the fusion takes place is very different in M1 and M9 molecules. However, we cannot rule out that some of the chimeric molecules formed dimers with MARCH1 but are unable to homodimerize. Still, our results suggest that tertiary interactions between the two transmembrane domains allow the contact with another monomer or serve to properly position functional domains. For example, truncation of the C-terminal cytoplasmic domain of mouse MARCH1 revealed the existence of a critical region that may be implicated in the activity of the ligase through interactions with the N-terminal tail (19).

The N-terminal TM domain of MARCH1 cannot be substituted for the one of M9. As documented for the viral homologs, the replacement of the RING by the one of another E3s does not affect the function of MIR1 (59). Interestingly, MARCH9 retained full activity against MHC I molecules in the context of the MARCH1 RING domain (M1–9DIRT) whereas the chimeric molecules has lost most ofi ts activity against MHC II molecules (Fig. 7). This suggests that MARCH1 and MARCH9 interact differently with MHC II molecules and that the RING domain of MARCH1 needs to interact specifically with other portions of the molecule. Extending the MARCH1 portion to include the DIRT domain was not sufficient to restore the activity. Future experiments will investigate the interplay between the N-terminal and C-terminal cytoplasmic tail, as Jabbour and colleagues (19) have demonstrated the presence of key functional residues following the second, C-terminal TM region.

In conclusion, our results demonstrated the capacity of MARCH1 to dimerize and heterodimerize, leading to ubiq-

uitination and degradation. Knowing that many members of that family have overlapping functions, that some are upregulated following different stimuli, and that the localization of E3s is important for their function, we can postulate that in certain circumstances heterodimerization could modify the localization and target specificity of the complex. For example, we do not know at present whether MARCH proteins dimerize with K3 or K5 and how this could affect their respective functions. Future experiments will further characterize the structural aspects of dimer formation and will address their biological impact.

Acknowledgments

We thank Genentech, Inc. for providing the Apu2.07 and Apu3.A8 Abs, Dr. Klaus Fruh for the H1 Ab and K3 and K5 expression plasmids, Marie-Ève Racine for the pcDNA3.1_TAP1-Rluc expression plasmid, and Dr. Daniel Lamarre for the myc-, GFP_2-, YFP-, and Rluc-cloning plasmids.

Disclosures

The authors have no financial conflicts of interest.

References

1. d'Azzo, A., A. Bongiovanni, and T. Nastasi. 2005. E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. Traffic 6: 429–441.
2. Deshaies, R. J., and C. A. Joazeiro. 2009. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 78: 399–434.
3. Cadwell, K., and L. Coscoy. 2005. Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. Science 309: 127–130.
4. Wang, X., R. A. Herr, W. J. Chua, L. Lybarger, E. J. Wiertz, and T. H. Hansen. 2007. Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. J. Cell Biol. 177: 613–624.
5. Ishido, S., E. Goto, Y. Matsuki, and M. Ohmura-Hoshino. 2009. E3 ubiquitin ligases for MHC molecules. Curr. Opin. Immunol. 21: 78–83.
6. Ikeda, F., and I. Dikic. 2008. Atypical ubiquitin chains: new molecular signals: "Protein Modifications: Beyond the Usual Suspects" review series. EMBO Rep. 9: 536–542.
7. Hicke, L., H. L. Schubert, and C. P. Hill. 2005. Ubiquitin-binding domains. Nat. Rev. Mol. Cell Biol. 6: 610–621.
8. Ye, Y., and M. Rape. 2009. Building ubiquitin chains: E2 enzymes at work. Nat. Rev. Mol. Cell Biol. 10: 755–764.
9. Boname, J. M., M. Thomas, H. R. Stagg, P. Xu, J. Peng, and P. J. Lehner. 2010. Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. Traffic 11: 210–220.
10. Ohmura-Hoshino, M., E. Goto, Y. Matsuki, M. Aoki, M. Mito, M. Uematsu, H. Hotta, and S. Ishido. 2006. A novel family of membrane-bound E3 ubiquitin ligases. J. Biochem. 140: 147–154.
11. Bartee, E., M. Mansouri, B. T. Hovey Nerenberg, K. Gouveia, and K. Früh. 2004. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J. Virol. 78: 1109–1120.
12. Matsuki, Y., M. Ohmura-Hoshino, E. Goto, M. Aoki, M. Mito-Yoshida, M. Uematsu, T. Hasegawa, H. Koseki, O. Ohara, M. Nakayama, et al. 2007. Novel regulation of MHC class II function in B cells. EMBO J. 26: 846–854.
13. Corcoran, K., M. Jabbour, C. Bhagwandin, M. J. Deymier, D. L. Theisen, and L. Lybarger. 2011. Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). J. Biol. Chem. 286: 37168–37180.
14. De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre, and E. Gatti. 2008. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc. Natl. Acad. Sci. USA 105: 3491–3496.
15. Baravalle, G., H. Park, M. McSweeney, M. Ohmura-Hoshino, Y. Matsuki, S. Ishido, and J. S. Shin. 2011. Ubiquitination of CD86 is a key mechanism in regulating antigen presentation by dendritic cells. J. Immunol. 187: 2966–2973.
16. Berger, A. C., and P. A. Roche. 2009. MHC class II transport at a glance. J. Cell Sci. 122: 1–4.
17. Young, L. J., N. S. Wilson, P. Schnorrer, A. Proietto, T. ten Broeke, Y. Matsuki, A. M. Mount, G. T. Belz, M. O'Keeffe, M. Ohmura-Hoshino, et al. 2008. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. Nat. Immunol. 9: 1244–1252.
18. Thibodeau, J., M. C. Bourgeois-Daigneault, G. Huppé, J. Tremblay, A. Aumont, M. Houde, E. Bartee, A. Brunet, M. E. Gauvreau, A. de Gassart, et al. 2008. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. Eur. J. Immunol. 38: 1225–1230.
19. Jabbour, M., E. M. Campbell, H. Fares, and L. Lybarger. 2009. Discrete domains of MARCH1 mediate its localization, functional interactions, and posttranscriptional control of expression. J. Immunol. 183: 6500–6512.
20. de Bie, P., and A. Ciechanover. 2011. Ubiquitination of E3 ligases: self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. Cell Death Differ. 18: 1393–1402.
21. Scialpi, F., M. Malatesta, A. Peschiaroli, M. Rossi, G. Melino, and F. Bernassola. 2008. Itch self-polyubiquitylation occurs through lysine-63 linkages. Biochem. Pharmacol. 76: 1515–1521.
22. Fang, S., J. P. Jensen, R. L. Ludwig, K. H. Vousden, and A. M. Weissman. 2000. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. 275: 8945–8951.
23. Kawai, H., V. Lopez-Pajares, M. M. Kim, D. Wiederschain, and Z. M. Yuan. 2007. RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. Cancer Res. 67: 6026–6030.
24. Linke, K., P. D. Mace, C. A. Smith, D. L. Vaux, J. Silke, and C. L. Day. 2008. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. Cell Death Differ. 15: 841–848.
25. Hoer, S., L. Smith, and P. J. Lehner. 2007. MARCH-IX mediates ubiquitination and downregulation of ICAM-1. FEBS Lett. 581: 45–51.
26. Haque, M., J. Chen, K. Ueda, Y. Mori, K. Nakano, Y. Hirata, S. Kanamori, Y. Uchiyama, R. Inagi, T. Okuno, and K. Yamanishi. 2000. Identification and analysis of the K5 gene of Kaposi's sarcoma-associated herpesvirus. J. Virol. 74: 2867–2875.
27. Sanchez, D. J., L. Coscoy, and D. Ganem. 2002. Functional organization of MIR2, a novel viral regulator of selective endocytosis. J. Biol. Chem. 277: 6124–6130.
28. Lehner, P. J., S. Hoer, R. Dodd, and L. M. Duncan. 2005. Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. Immunol. Rev. 207: 112–125.
29. Lampson, L. A., and R. Levy. 1980. Two populations of Ia-like molecules on a human B cell line. J. Immunol. 125: 293–299.
30. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. Cell 14: 9–20.
31. Newton, K., M. L. Matsumoto, I. E. Wertz, D. S. Kirkpatrick, J. R. Lill, J. Tan, D. Dugger, N. Gordon, S. S. Sidhu, F. A. Fellouse, et al. 2008. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell 134: 668–678.
32. Baril, M., M. E. Racine, F. Penin, and D. Lamarre. 2009. MAVS dimer is a crucial signaling component of innate immunity and the target of hepatitis C virus NS3/4A protease. J. Virol. 83: 1299–1311.
33. Herr, R. A., J. Harris, S. Fang, X. Wang, and T. H. Hansen. 2009. Role of the RING-CH domain of viral ligase mK3 in ubiquitination of non-lysine and lysine MHC I residues. Traffic 10: 1301–1317.
34. Perroy, J., S. Pontier, P. G. Charest, M. Aubry, and M. Bouvier. 2004. Real-time monitoring of ubiquitination in living cells by BRET. Nat. Methods 1: 203–208.
35. Weissman, A. M., N. Shabek, and A. Ciechanover. 2011. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. Nat. Rev. Mol. Cell Biol. 12: 605–620.
36. Bergink, S., F. A. Salomons, D. Hoogstraten, T. A. Groothuis, H. de Waard, J. Wu, L. Yuan, E. Citterio, A. B. Houtsmuller, J. Neefjes, et al. 2006. DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A. Genes Dev. 20: 1343–1352.
37. Duncan, L. M., S. Piper, R. B. Dodd, M. K. Saville, C. M. Sanderson, J. P. Luzio, and P. J. Lehner. 2006. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. EMBO J. 25: 1635–1645.
38. Plechanovová, A., E. G. Jaffray, S. A. McMahon, K. A. Johnson, I. Navrátilová, J. H. Naismith, and R. T. Hay. 2011. Mechanism of ubiquitylation by dimeric RING ligase RNF4. Nat. Struct. Mol. Biol. 18: 1052–1059.
39. Oh, Y. M., S. J. Yoo, and J. H. Seol. 2007. Deubiquitination of Chfr, a checkpoint protein, by USP7/HAUSP regulates its stability and activity. Biochem. Biophys. Res. Commun. 357: 615–619.
40. Hewitt, E. W., L. Duncan, D. Mufti, J. Baker, P. G. Stevenson, and P. J. Lehner. 2002. Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation. EMBO J. 21: 2418–2429.
41. Meng, L., R. Mohan, B. H. Kwok, M. Elofsson, N. Sin, and C. M. Crews. 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proc. Natl. Acad. Sci. USA 96: 10403–10408.
42. Gelman, M. S., E. S. Kannegaard, and R. R. Kopito. 2002. A principal role for the proteasome in endoplasmic reticulum-associated degradation of misfolded intracellular cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. 277: 11709–11714.
43. Fang, S., K. L. Lorick, J. P. Jensen, and A. M. Weissman. 2003. RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer. Semin. Cancer Biol. 13: 5–14.
44. Grommé, M., and J. Neefjes. 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol. Immunol. 39: 181–202.
45. Xu, Y., D. W. Piston, and C. H. Johnson. 1999. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc. Natl. Acad. Sci. USA 96: 151–156.
46. Pfleger, K. D., R. M. Seeber, and K. A. Eidne. 2006. Bioluminescence resonance energy transfer (BRET) for the real-time detection of protein-protein interactions. Nat. Protoc. 1: 337–345.
47. Liew, C. W., H. Sun, T. Hunter, and C. L. Day. 2010. RING domain dimerization is essential for RNF4 function. Biochem. J. 431: 23–29.
48. Brzovic, P. S., P. Rajagopal, D. W. Hoyt, M. C. King, and R. E. Klevit. 2001. Structure of a BRCA1-BARD1 heterodimeric RING-RING complex. Nat. Struct. Biol. 8: 833–837.

49. Bartee, E., A. McCormack, and K. Fru¨h. 2006. Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. PLoS Pathog. 2: e107.
50. Walseng, E., K. Furuta, B. Bosch, K. A. Weih, Y. Matsuki, O. Bakke, S. Ishido, and P. A. Roche. 2010. Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. Proc. Natl. Acad. Sci. USA 107: 20465–20470.
51. Tze, L. E., K. Horikawa, H. Domaschenz, D. R. Howard, C. M. Roots, R. J. Rigby, D. A. Way, M. Ohmura-Hoshino, S. Ishido, C. E. Andoniou, et al. 2011. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. J. Exp. Med. 208: 149–165.
52. Bania, J., E. Gatti, H. Lelouard, A. David, F. Cappello, E. Weber, V. Camosseto, and P. Pierre. 2003. Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. Proc. Natl. Acad. Sci. USA 100: 6664–6669.
53. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19: 141–172.
54. Vembar, S. S., and J. L. Brodsky. 2008. One step at a time: endoplasmic reticulum-associated degradation. Nat. Rev. Mol. Cell Biol. 9: 944–957.
55. Ward, C. L., S. Omura, and R. R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. Cell 83: 121–127.
56. Bonifacino, J. S., and L. M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. 72: 395–447.
57. Alwan, H. A., E. J. van Zoelen, and J. E. van Leeuwen. 2003. Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. J. Biol. Chem. 278: 35781–35790.
58. Peschard, P., G. Kozlov, T. Lin, I. A. Mirza, A. M. Berghuis, S. Lipkowitz, M. Park, and K. Gehring. 2007. Structural basis for ubiquitin-mediated dimerization and activation of the ubiquitin protein ligase Cbl-b. Mol. Cell 27: 474–485.
59. Goto, E., S. Ishido, Y. Sato, S. Ohgimoto, K. Ohgimoto, M. Nagano-Fujii, and H. Hotta. 2003. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. J. Biol. Chem. 278: 14657–14668.

CHAPITRE 3: Article 2

Identification of a novel motif that affects the conformation and activity of the MARCH1 E3 ubiquitin ligase

Préface

Le deuxième article porte sur l'investigation de l'importance de la localisation de MARCH1 pour sa fonction ainsi que l'identification d'un nouveau motif. De nombreux mutants de MARCH1 ont été générés, par substitution et par délétion, ainsi que des chimères entre MARCH1 et le CMH II ou la chaîne invariante. Nous avons eu recours aux techniques de FRET, de microscopie confocale, de cytométrie en flux, d'analyse d'exosomes, de western blot et de BRET pour ce projet. Dans cet article, nous démontrons que les motifs de ciblage aux endosomes compris dans la queue cytoplasmique C-terminale de MARCH1 sont fonctionnels, mais non requis à l'activité de la molécule. Nos résultats révèlent également la présence d'éléments de ciblage dans la portion cytoplasmique N-terminale de MARCH1. Finalement, nous identifions la valine 221 comme faisant partie d'un motif essentiel au fonctionnement optimal de MARCH1 sans lequel l'orientation spatiale des queues cytoplasmiques se voit altérée.

Le premier article nous a permis d'approfondir nos connaissances en matière de régulation de MARCH1. Nous nous sommes ensuite intéressé à l'importance de la localisation et des motifs à base de tyrosine pour la fonctionnalité de la molécule. L'identification du motif VQNC dans la portion cytoplasmique C-terminale de MARCH1 était inattendue.

Référence

Marie-Claude Bourgeois-Daigneault and Jacques Thibodeau, **Identification of a novel motif in MARCH1 that affects the conformation and the E3 ubiquitin ligase activity**, Soumis à Molecular and Cellular Biology.

Identification of a novel motif that affects the conformation and activity of the MARCH1 E3 ubiquitin ligase

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MARCH1, a member of the membrane-associated RING-CH family of E3 ubiquitin ligases, regulates antigen presentation by down-regulating the cell surface expression of MHC class II and CD86 molecules. MARCH-1 is a transmembrane protein that exposes both its N- and C-terminus to the cytoplasm. Here, we have conducted a structure-function analysis of its two cytoplasmic tails to gain insights into the trafficking of MARCH1 in the endocytic pathway. Fusion of the N-terminal portion of MARCH1 to a type II transmembrane reporter molecule revealed that this cytoplasmic tail contains endosomal sorting motifs. The C-terminal domain also appears to contain intracellular sorting signals as it reduced surface expression of a type I transmembrane reporter molecule. Mutation of the two putative C-terminal tyrosine-based sorting signals did not affect the activity of human MARCH1. However, it reduced its incorporation into exosomes. Moreover, site-directed mutagenesis pointed to a functional C-terminal $_{221}$VQNC$_{224}$ sequence that affects the spatial organization of the two cytoplasmic regions. This motif is also found in other RING-type E3 ubiquitin ligases, such as parkin. Altogether, these findings highlight the complex regulation of MARCH1 trafficking in the endocytic pathway as well as the intricate interactions between its cytoplasmic tails.

The intracellular trafficking of MHC class II molecules (MHC II) has been extensively studied in various species and types of APCs. Synthesized in the ER, MHC II heterodimers assemble with the invariant chain (Ii) and cross the Golgi apparatus before ultimately reaching the endosomes (5, 37). It is believed that a fraction of the newly synthesized MHC II/Ii pool associates with the clathrin adaptor AP-1 and reaches early endosomes directly from the trans-Golgi network (10, 52). However, most complexes appear to first reach the plasma membrane through the default secretory pathway. At the cell surface, the leucine-based cytoplasmic motifs of Ii are recognized by AP-2 and the cargo is internalized through clathrin-coated vesicles (20, 44). Once in the endocytic pathway, the MHC II/Ii complex ultimately reaches acidic compartments where Ii is degraded (16). A variety of non-cysteine proteases, including AEP, are capable of cleaving the luminal part of Ii to generate a 22 KDa fragment (p22) (41). Then, cysteine proteases sequentially generate p10 and the class II-associated invariant chain peptide (CLIP), which remains associated to MHC II (30). The exchange of CLIP for an antigenic peptide is mediated by the non-classical MHC II molecule HLA-DM and terminates the association of Ii and MHC II (18). The peptide-MHC II complex is then free to go to the cell surface (37). After being displayed at the plasma membrane, peptide-loaded MHC II molecules are endocytosed in sorting endosomes through an ARF6-dependent, clathrin-independent, mechanism and most complexes are returned to the cell surface via recycling tubular endosomes (59).

Recently, it has been shown that ubiquitination participates in the intracellular trafficking of MHC II, especially in resting cells or under immunosuppressive conditions (17, 43, 53, 56, 66). Ubiquitination is a well known post-translational protein modification that leads to many different effects, from endocytosis and vesicular trafficking to degradation and regulation of the cell cycle (1). The attachment of ubiquitin on a substrate involves three players: First, the activating enzyme (E1) activates free ubiquitins. Second, the activated moiety interacts with an ubiquitin-conjugating enzyme (E2) (62). Then, the E2s associate with ubiquitin ligases (E3), which recognize and modify the appropriate targets (19).

The membrane-associated-RING-CH (MARCH) protein family includes nearly a dozen E3 ubiquitin ligases that target important players of the immune response (31). Most of the RING-type E3 ubiquitin ligases are cytoplasmic or nuclear proteins (38). With the exception of MARCH7 and MARCH10, MARCH family members are multi-pass transmembrane proteins (46, 61). MARCH1 shares

many targets with MARCH8, its closest family member. MHC II, CD86, transferrin receptor, HLA-DM and Fas were all reported to be ubiquitinated and down-regulated from cell surface by MARCH1 and MARCH8 (4, 33, 43, 48). While MARCH1 expression is restricted to secondary lymphoid organs (4), it can be induced or repressed by different stimuli. For example, we have shown that IL-10 modulates its expression in human primary monocytes and mouse B cells (9, 24). In addition, many groups have demonstrated that LPS-induced maturation of dendritic cells repressed MARCH1 expression, allowing the cell surface expression of newly synthesized MHC II molecules (17, 48, 58, 60, 64).

While the overall structure of MARCH1 and the function of its RING domain are well defined, the other parts of the molecule remain to be fully characterized. The RING domain, localized in the cytoplasmic N-terminal region participates in the ubiquitin transfer from the E2 to its substrate (19). A study about the viral homologue mK3 has highlighted the importance of the DIRT domain, *i.e.* the domain localized between the RING and the first transmembrane, which is involved in substrate recognition (13). Also, the group of Lybarger reported the presence of C-terminal cytoplasmic endosomal sorting motifs in murine MARCH1 (mMARCH1) (32). Other key regions in MARCH proteins include their transmembrane domains, which are implicated in target recognition and dimer formation (9, 15, 38). We have recently demonstrated that dimerization allows autoregulation of MARCH1 through ubiquitination (9).

The fine details of the spatiotemporal interaction between MARCH1/8 and MHC II remain to be characterized. Early studies suggested that MARCH8 ubiquitinates its targets at the plasma membrane and induces their rapid endocytosis (48). In mouse primary B cells, it appears that ubiquitinated MHC II molecules can be immunoprecipitated from the plasma membrane. Also, in MARCH1-deficient B cells, the cell-surface MHC II molecules were shown to be stabilized (43). These results contrast with those obtained in DCs where no difference in the kinetics or mode of MHC II internalization was observed between MARCH1-deficient and -proficient mouse cells (32, 43). A recent study by Eyster *et al*. also demonstrated that ubiquitination of different targets by MARCH1 and 8 would affect their trafficking at the level of recycling endocytic structures, preventing their return to the plasma membrane (22).

The expression of MARCH1 is closely regulated and very low levels of the protein are detectable in primary cells (43). MARCH1 can ubiquitinate a variety of unrelated cytoplasmic domains and part of the specificity most likely arise from the tight compartimentalization of the enzyme. To clarify the mechanisms by which MARCH1 mediates the intracellular retention of MHC II, we further characterized the structure-function properties of the two MARCH1 cytoplasmic domains using reporter molecules and site-directed mutagenesis. Our results highlighted the presence of a short functional C-terminal amino acid sequence (VQNC) that is conserved in other E3 ubiquitin ligases and which appears to interact with the N-terminal cytoplasmic tail, close to the RING domain.

MATERIALS AND METHODS

Antibodies. Mouse IgG_1 XD5 (recognizes the DRβ chain), mouse IgG_{2a} L243 (recognizes the HLA-DRα chain in the context of the heterodimer) and mouse IgG2b ISCR3 (recognizes the HLA-DR heterodimer) have been described previously (35). Mouse IgG_1 BU45 (extracytoplasmic domain of Ii), mouse monoclonal IgG_1 CerCLIP (specific to the CLIP region: amino acids 103 to 117) and a rabbit polyclonal antibody against CLIP (generously provided by Dr P. Cresswell, Yale University). For the immunofluorescence microscopy experiments, we used the anti-Lamp-1 (H5C6; mouse IgG_1) and anti-CD63 from the Developmental Studies Hybridoma Bank (NICHD, University of Iowa, IA). The anti-calnexine and Alexa^{594}-coupled goat-anti-mouse antibodies were purchased from Invitrogen (Laval, Canada).

Reagents. Polyethyleimine (2.5 KDa linear) was obtained from Polysciences Inc. (Warrington, PA, USA). Lipofectamine, Plus reagent and Hoechst 33342 were purchased from Invitrogen (Laval, Canada). A complete protease inhibitors cocktail (Roche, Laval, QC, Canada), Endoglycosidase H (New England Biolabs, Ontario, Canada) and benzyl coelenterazine (Nanolight Technology, Pinetop, AZ, USA) were used according to the manufacturer's recommendations.

Plasmids and mutagenesis. $EGFP_2$-MARCH1 and EYFP-MARCH1 plasmids were obtained by fusing $EGFP_2$ or EYFP to the N-terminus of MARCH1 by the PCR overlapping method using the pcDNA3.1_ $EGFP_2$_MCS or the pcDNA3.1_EYFP_MCS (9). The DRβ-EYFP was obtained by fusing the EYFP to the C-terminus of DRβ by the same method using the pcDNA3.1_MCS_EYFP plasmid. The DRβ-M1 was constructed by fusing DRβ (methionine 1 to arginine 251) to human MARCH1 (glutamine 201 to valine

272). The M1-Ii chimeric molecule was constructed by fusing the MARCH1 methionine 1 to lysine 137 sequence to the glycine 50 of Iip33. The chimeric molecules were generated by PCR overlap and cloned in the *BsiW1* and *Not1* restriction sites of pcDNA3.1_MCS. The M1Y205F, M1Y215F, M1YY (Y205F and Y215F), M1V221A, M1V221E, M1Q222A, M1N223A and M1C224A were obtained by mutating the corresponding residues by PCR overlap. The $M1_{KKxx}$, M1Δ42, M1Δ48, M1Δ51 and M1ΔCter were obtained by the addition of a stop codon after the triplets coding for glutamic acid 232, lysine 230, cystein 224, valine 221 or cysteine 202, respectively. The EYFP-MARCH1-Rluc and EYFP-M1V221E-Rluc were obtained by fusing EYFP-MARCH1 or EYFP-M1V221E to the N-terminus of Rluc by PCR overlap using the pcDNA3.1_MCS_Rluc.

Immunofluorescence microscopy. HeLa cells were plated on coverslips 24 hours prior to transfection. Cells were transfected and incubated for 48 hours. Cells were then fixed, permeabilized and stained on cover slips. Samples were analyzed on a LSM 510 Meta Zeiss confocal microscope. For FRET experiments, 5 x 10^5 cells were plated and transfected on 35mm glass bottom dishes (MatTek, Ashland, MA, USA) 48 hours prior to the experiment. Images of living cells were taken using a LSM Meta Zeiss confocal microscope. For $EGFP_2$, the cells were excited at a 405nm wavelength and the signal was detected by using a 470-500nm band-pass filter. For YFP, cells were scanned at 488nm with a 560nm long-pass filter and the FRET signal was detected at 530 to 560nm upon excitation at 405nm.

Exosomes purification and analysis. Ten 10 cm petri dishes per condition were transfected and the media was changed for exosome-free media after 24 hours. Cells were cultured for 24h and the media was collected. Exosomes were purified by ultracentrifugation and coupled to latex beads for flow cytometry analysis, as described previously (25).

Cell culture and transfections. HEK293T, HEK 293E CIITA, HeLa and HeLa DR1 cells were cultured in DMEM supplemented with 5% FBS (Wisent, Saint-Jean Baptiste, QC, Canada). HEK 293T cells were transfected using 3ug of polyethyleimine per ug of DNA, as described previously (9). HeLa cells were transfected using lipofectamine and Plus reagent, according to the manufacturer's protocol (Invitrogen, Laval, Canada).

Bioluminescence Resonance Energy Transfert (BRET). HEK 293T cells were transfected with YFP-MARCH1-Rluc or YFP-M1V221E-Rluc 48h prior to the experiment. Cells were harvested, washed and 10^5 cells per condition were plated in duplicate in a 96-well plate. The background fluorescence was determined before the addition of coelenterazine at a concentration of 5uM. The luminescence and fluorescence intensities were determined using a Mithras LB940 spectrofluorometer (Berthold technologies, Bad Wildbad, Germany) and multiplate reader with the settings described previously (9). The BRET ratio was calculated by dividing the acceptor-emitted fluorescence by the donor-emitted luminescence. BRET ratios were normalized by subtracting the background signal from cells transfected without YFP.

Western blot analysis. Cells were lysed in 1% Triton X-100 lysis buffer complemented with complete protease inhibitor cocktail. Post-nuclear supernatants were analyzed by SDS-PAGE in reducing conditions. Proteins were transferred to Hybond ECL membrane (GE Healthcare, Mississauga, ON, Canada) and analysed with the rabbit anti-CLIP antibody. Goat anti-rabbit coupled to peroxydase antibody (Bio/Can Scientific) was used and detection was done using a chemiluminescence blotting substrate (POD) from Roche (Laval, Quebec, Canada).

RESULTS

MARCH1 is not active in the biosynthetic pathway. Some members of the MARCH-like family of ubiquitin ligases, such as mK3 and MARCH6, modify their targets in the ER (7, 36). Indeed, mK3 inhibits antigen presentation by ubiquitinating MHC class I molecules and causing their degradation through the ERAD (ER-associated degradation) pathway (7). Also, MARCH2, 3, 4 and 9 appear to be located along the biosynthetic and secretory pathways, including the trans-Golgi network (4). We have addressed the possibility that ubiquitination of MHC II in the ER might play a role in the down-regulation of surface expression. We generated an ER-retained MARCH1 mutant by deleting the last 40 amino acids of the C-terminal cytoplasmic tail (Fig. 1A, B). This MARCH1Δ40 variant (called $M1_{KKxx}$) ends with the sequence KKLE, creating a classical di-lysine (KKxx) ER-retention motif (47). Indeed, immunofluorescence microscopy showed that the $M1_{KKxx}$ mutant colocalized with calnexin and was confined to the ER in transfected HeLa cells (Fig. 1C). The MARCH1 constructs used in this study are tagged at their N-terminus with YFP to facilitate their detection. The expression of the untagged MARCH1

is weak and the fusion with the YFP stabilizes the molecule without affecting the function (9). To test the impact of mislocalization, $M1_{KKxx}$ was transiently transfected in CIITA-expressing cells and the surface expression of MHC II was monitored by flow cytometry. A control MARCH1 lacking 2 additional residues (M1Δ42) was generated in order to inactivate the ER retention motif (Fig. 1A, B). Figure 1D shows that, as compared to wild-type MARCH1 (YFP-MARCH1) or M1Δ42, which both efficiently down-regulate MHC II from the cell surface, the ER-retained $M1_{KKxx}$ was totally inactive. As it has been suggested previously that MARCH1 does not target MHC II/Ii complexes (53), the possibility remained that Ii created steric hindrance, thereby preventing ubiquitination of MHC II cytoplasmic domains in the ER. Thus, we compared the activity of the deleted MARCH1 molecule in cells stably expressing only HLA-DR1. Figure 1E shows that, as compared to cells expressing the control YFP (left panel), only the WT MARCH1 was able to down-regulate cell surface MHC II expression. Altogether, these results show that MARCH1 does not act from the ER.

MARCH1 and MHC II interact in the endocytic pathway. Recent results have shown that MARCH1 does not affect the kinetics of MHC II endocytosis from the plasma membrane (59). Coupled to the fact that clathrin-independent endocytosis of specific cargo proteins is not affected by the expression of MARCH1, these results suggest that ubiquitination occurs in endocytic vesicles (22). To gather further evidence for an interaction between MHC II and MARCH1 in vesicles, we have used immunfluorescence microscopy on live HeLa cells to detect FRET between transiently transfected GFP_2-MARCH1 and DRα/β-YFP. Figure 2 shows two series of images of the same cells, one preceding (upper panels) and the other following (bottom panels) bleaching of the selected areas. Prior to bleaching, a weak signal is observed for GFP_2-MARCH1, which colocalizes with the strong signal observed for DRβ-YFP. The FRET signal represents the detection of the fluorescence emitted by the YFP after laser excitation of the GFP_2. This signal reflects the transfer of energy that occurs when the two molecules are close one to the other. Instead of being released as fluorescence, the energy emitted by the GFP_2 upon stimulation is absorbed and quenched by the YFP in conditions where the two molecules are nearby. To release this fluorescence, selected areas that show a strong YFP and FRET signals were bleached (white squares). In these conditions, the YFP cannot accept or emit energy. Subsequent to the bleach, the images showed an important augmentation of the fluorescence emitted by GFP_2, a complete abolition of the YFP signal and an important diminution of the FRET signal in the bleached areas. The release of the GFP_2 fluorescence confirms the close proximity of the two molecules. Thus, our results showing a MARCH1-MHC II interaction in vesicles are in line with a model where internalized cargo proteins are ubiquitinated in sorting vesicles and redirected deeper into the endocytic pathway (22).

The N-terminal cytoplasmic tail of MARCH1 includes sorting signals. Jabbour *et al*. have recently demonstrated the presence of endosomal sorting motifs in mouse MARCH1 (32). The above-described interaction of human MARCH1 with DR in cytoplasmic vesicles is in line with the presence of such motifs. We further investigated the mechanisms regulating the trafficking of human MARCH1. We first looked at the N-terminal cytoplasmic domain using Ii as a reporter molecule. Ii is a type II protein, allowing replacement of its cytoplamic tail with the N-terminal domain of MARCH1 (M1-Ii) (Fig. 3A). HEK 293T cells were transfected with M1-Ii in the presence or absence of DR and analyzed by flow cytometry (Fig. 3B). Our results showed that the reporter molecule was present at cell surface (left panel). When expressed with DR, CLIP was detected at the plasma membrane, demonstrating that the complex reached the endosomes where M1-Ii was processed (right panel).

We compared by flow cytometry the surface expression of transiently transfected M1-Ii, Iip33 and Iip35, the latter isoform bearing an ER retention motif which prevents egress in the absence of DR (40). We also analyzed the total expression of the various molecules by staining cells after membrane permeabilization. Figure 3C shows, as expected, that Iip33 but not Iip35 was present on the plasma membrane (left panel). The surface over total MFV ratio allows for the normalization of expression and as shown for the control Iip35, a low value indicates that the transfected protein is well expressed but resides mainly in intracellular compartments (right panel). Interestingly, the ratio measured for M1-Ii was lower than for Iip33, suggesting that the chimeric molecule is somewhat retained inside the cell. Similar results were obtained upon co-expression of MHC II molecules (Fig. 3D).

Once in the endocytic pathway, Ii is sequentially degraded to generate CLIP. Indeed, discrete cleavage products can be observed in Ii-transfected HEK 293T cells. Analysis of cell lysates on immunoblots with a CLIP-specific polyclonal antibody showed the presence of full length Iip33 as well as the classical

20-22 KDa (p22) and 10-12 KDa (p10) products (Fig. 3E) (2, 37). As M1-Ii includes a longer cytoplasmic tail than Ii, the expected corresponding fragments would be larger: *i)* 45 KDa for the entire M1-Ii, *ii)* 34 KDa (here called M1-Iip22 by analogy with Ii) and *iii)* 22 KDa (here called M1-Iip10). To investigate the presence of these cleaved forms, HEK 293T cells were transfected with M1-Ii and analyzed on immunoblots (Figure 3E, right lane). Interestingly, only two major forms were observed for M1-Ii as the smaller M1-Iip10 was absent, even after overexposure of the membrane (not shown). These results suggest that the cytoplasmic tail of Iip33 and the N-terminal region of MARCH1 contain sorting signals that are not redundant.

Endosomal sorting elements in the C-terminal cytoplasmic domains

A recent study reported the presence of two tyrosine-based endosomal sorting motifs in the C-terminal cytoplasmic domain of mouse MARCH1 and human MARCH8 (6, 26, 32). In line with the presence of these motifs within the molecule, we and others have shown that human MARCH1 is localized into CD63 and LAMP-1 positive endosomal vesicles (4, 9). To further investigate the presence of functional sorting motifs in the C-terminal cytoplasmic portion of human MARCH1, we used DR as a reporter molecule and replaced the DRβ cytoplasmic tail with the C-terminal domain of MARCH1 (Fig. 4A). This DRβ-M1 chimeric chain was transiently transfected in HEK 293T along with DRα (DR-M1) and the expression of DR was analyzed by flow cytometry. Figure 4B reveals that, as compared to wild-type DR, the DR-M1 heterodimer is not efficiently expressed at the cell surface (left panels). The surface over total expression ratio suggests that the reporter molecule is somewhat retained intracellularly (Fig. 4B, right panel).

Differential role of the tyrosine-based motifs in mouse and human MARCH1.

The previous experiments are in line with the presence of sorting elements within the C-terminal cytoplasmic domain of human MARCH1. Knowing the importance of the tyrosine-based endosomal sorting signals for the function of human MARCH8 and mouse MARCH1, we investigated the functionality of these conserved motifs in human MARCH1. The two key tyrosine residues were changed for phenylalanines by site-directed mutagenesis (M1Y205F and M1Y215F) (Fig. 5A). A mutant with both tyrosines mutated (M1YY) was also generated. The activity of these YFP-tagged MARCH1 variants was tested in HEK 293T CIITA cells for the down-regulation of surface MHC II. Our results confirmed the importance of tyrosine 222 for mouse MARCH1 (data not shown) and human MARCH8 (Fig. 5B). Cells expressing M8Y222F showed very little MHC II down-regulation as compared to cells transfected with the control MARCH1. Goto *et al*. had shown previously that deletion of either tyrosine motif affected the function of MARCH8 (26). However, our results demonstrate that only the N-terminal motif is critical. This discrepancy is probably due to an indirect effect caused by the deletion of the four residues constituting the putative C-terminal motif in MARCH8 (26). Interestingly, human MARCH1 was insensitive to the loss of either motif (Fig. 5C). Thus, our results highlight some structure-function differences between highly conserved MARCH family members.

The tyrosine-based endosomal sorting motifs of MARCH1 affect its trafficking. Our above-described analysis suggested that the tyrosine motifs are not required for the down-regulation of MHC II. Also, no difference was observed by immunofluorescence microscopy in the co-localization of CD63, a marker for endocytic compartment, with either the YFP-M1, YFP-M1Y205F or YFP-M1Y215F molecules (Fig. S1).

Tyrosine-based sorting signals are responsible for the incorporation of many endosomal proteins into exosomes (57). We investigated the presence of YFP, YFP-MARCH1 and the YFP-M1YY proteins into the microvesicles shedded from transfected cells. HEK 293T cells-derived exosomes were purified from the culture media by ultracentrifugation. The cells and the exosomes, the latter coupled to latex beads, were analyzed by flow cytometry for YFP and CD9 expression. CD9 has previously been shown to incorporate exosomes and was used as an internal control to determine the amount of exosomes (25). Our results showed a complete absence of YFP in exosomes even though the protein is expressed at high levels upon transient transfection (Fig. 6, upper panel). In contrast, YFP-MARCH1 and YFP-M1YY were both found in exosomes. To compare the sorting efficiency of the various molecules, we calculated the ratio of fluorescence in the exosomes over the cells. These ratios were corrected for the values obtained with CD9, which should be identical for all transfections. The analysis corroborated the absence of YFP from exosomes, but revealed an important reduction in the incorporation of YFP-M1YY as compared to YFP-MARCH1 (Fig. 6, bottom panel). These data suggest that the tyrosine motifs are functional and finely tune the trafficking of MARCH1.

A short sequence in the C-terminal cytoplasmic portion of MARCH1 is important for its activity. As the tyrosine motifs are dispensable for the function of human MARCH1 in our experimental system, we asked whether the N-terminal cytoplasmic tail bearing the RING domain would be sufficient to down-regulate MHC II molecules. To test this possibility, we truncated the entire cytoplasmic C-terminal portion (ΔCter; Fig. 7A). Flow cytometry experiments in HEK 293E CIITA cells showed that this mutant was inactive in down-regulating surface DR (Figure 7B). To better define the region needed to complement the RING domain and knowing that the Δ42 construct was fully active (Fig. 1D), we generated two truncated molecules that shortened the C-terminal of another 6 (Δ48) or 9 (Δ51) residues (Fig. 1A and 7B). Interestingly, the Δ51 mutant showed a dramatic reduction in activity as compared to Δ48, suggesting that this region contains key functional elements. These results confirm those of Jabbour *et al.* who showed that deletion of the last 50 C-terminal amino acids abrogated the activity of mMARCH1 (32).

The valine 221 is important for the function of MARCH1. To better characterize the region comprised between the Δ51 and Δ48 deletion mutants, we made alanine substitutions at positions Q222, N223 and C224. Interestingly, none of these mutants was impaired in its ability to down-regulate cell surface MHC II molecules in transfected HEK 293E CIITA cells (Fig. 8A). The weaker expression of the N223A mutant was not observed in independent transfections (data not shown). These results suggest that the important residues are most likely located in the vicinity of the C-terminus in the Δ51 mutant. Alternatively, the secondary structure of this short 222-224 region may be more important than the nature of the specific amino acids. To investigate these possibilities, we tested a triple mutant (M1VQN-AAA) and one in which only the V221 position was modified (M1V221A). Interestingly, mutation of the valine 221 was sufficient to reduce the activity of the enzyme (Fig. 8B). The triple VQN-AAA mutation and the Δ51 deletion (Fig. 7B) appeared even more deleterious than the single V221A mutation, suggesting that the whole $_{221}$VQNC$_{224}$ stretch is important.

Mutation of valine 221 affects conformation of the cytoplasmic tails. A blast of the human genome revealed the presence of VQNC motifs in other proteins, including the RING-type E3 ubiquitin ligases arkadia and parkin (45, 50). Parkin is a 465 amino acid protein with an ubiquitin-like (UBL) domain at its N-terminus and two RING finger domains (50). Of note, substitution of the valine for a glutamic acid in the VQNC motif of parkin is associated with the development of Parkinson disease (29). Replacement of V221 in MARCH1 by a glutamate (M1V221E) had the same effect as the alanine substitution (data not shown). The role of the VQNC stretch in parkin has not been investigated at the molecular level but it appears that the pathogenic V56E mutation might affect the half-life of the protein (28). In MARCH1, we noted that the V221 residue is located 19 amino acids away from the end of the second TM region (Fig. 1). Although the ternary structure of the cytoplasmic domains is unknown, it is possible that the V221 interacts with the RING domain, which starts 21 residues from the plasma membrane on the juxtaposed N-terminal cytoplasmic region. To investigate whether the V221E change could modify the relative orientation of the two cytoplasmic tails, we performed a BRET experiment between two different tags attached at each extremity of the MARCH1 molecule (Fig. 9A). YFP was attached to the N-terminus of MARCH1 while Rluc was linked to the C-terminus of the same molecule. The efficiency of energy transfer between the luciferase substrate and YFP was compared between HEK 293T cells transfected with the tagged MARCH1 versus the tagged M1V221E (Fig 9B). We found that the BRET ratio was reduced by half for the construct bearing the V221E mutation. This result demonstrates that the spatial orientation of the two cytoplasmic tails is modified and that the two tags are further apart in the MARCH1 mutant.

DISCUSSION

MARCH1 is found at extremely low levels in primary cells, suggesting that its deregulated expression or activity can be detrimental to the cell. To further characterize the mode of action of MARCH1, we first looked for the site of interaction with MHC II. Our FRET experiments demonstrated the colocalization of MARCH1 and MHC II in cytoplasmic vesicles (Fig. 2). Although Eyster *et al.* were able to detect some MARCH1 at the plasma membrane, it is well established that MARCH1 is mainly localized in the endocytic pathway (4, 22). Thus, our results are in line with a model in which MARCH1 would not affect the endocytosis of the target molecules but would rather regulate trafficking inside the endocytic pathway (22).

The use of invariant chain as a chimeric reporter molecule highlighted the existence of N-terminal sorting elements. The fact that M1-Ii is somewhat retained intracellularly suggested that its trafficking is regulated along its journey to the endosomes.

Also, the N-terminal tail of MARCH1 in M1-Ii prevented the production of the p10-like byproduct, highlighting the complexity of MARCH1 trafficking. This was surprising as co-localization experiments by many different groups suggested that MARCH1 resides in lysosomal-like, Lamp-1^+ compartments (4, 32). One possibility is that the p22-like fragment does not gain access to cathepsin S^+ compartments, preventing further degradation. However, there was no obvious accumulation of the p22-like fragment. Another possibility is that the chimeric molecule gains access more rapidly to lysosomal-like compartments, thereby accelerating the degradation of p10. Such a scenario would be in line with the presence of CLIP at the plasma membrane. Jabbour *et al*. have previously suggested that the N-terminal 66 amino acids of mMARCH1 might affect transport pathways (32). The N-terminal tail of MARCH1 contains a putative tyrosine-based motif and a typical ExxxLI motif for endosomal sorting (8). However, as these motifs are located in or close to the RING region and at the proximity of the plasma membrane, it is not clear if they would be functional. Of note, MARCH1 contains two FL motifs in its first 36 amino acids. Such a leucine-based sequence has been implicated in the basolateral sorting and internalization of MHC II molecules (55, 63). Interestingly, it has been reported previously that the FL and LL motifs are not redundant (39), perhaps explaining the different patterns of proteolytic fragments detected on immunoblots between Ii and M1-Ii.

The fine tuning of MARCH1 trafficking may necessitate signals in both its cytoplasmic tails. Indeed, two studies demonstrated the presence of functional tyrosine-based motifs in the C-terminal region of murine MARCH1 and human MARCH8 (26, 32). These signals do not appear to be so critical for human MARCH1, at least when testing for the modulation of MHC II expression (Fig. 5). Interestingly, we have shown that MARCH1 was incorporated into exosomes, a process that was less efficient when the tyrosine motifs were mutated (Figure 6). Tyrosine-based endosomal sorting motifs are known to trigger the incorporation of many cargo proteins into exosomes, including the transferrin receptor (27). We cannot rule out the possibility that the tyrosine motifs exert an indirect effect by regulating the capacity of MARCH1 to get ubiquitinated (9). This post-translational modification is known to modulate the incorporation of many proteins into exosomes (12). There is an increasing number of studies reporting the inter-cellular transport of material via exosomes (21, 42, 54). If MARCH1 from IL-10-responsive DCs can be transferred in exosomes to regulate the cell surface display of specific targets in distant APCs remains to be determined (49).

The search for functional domains allowed the identification of a critical amino acid sequence close to the C-terminal tyrosine motifs. The VQNC stretch, including the critical V221 residue, appears to be in close proximity of the RING domain located on the other cytoplasmic tail. Structural alterations in this region might affect the binding site for important proteins in the ubiquitination complex, such as an E2. However, the V221 point mutants are still partially active and preliminary mass spectrometry analyses following pull-down experiments did not reveal qualitative differences in the co-immunoprecipitated material (data not shown). Our BRET analysis rather suggested that the V221 is somewhat buried into the 3D structure of the enzyme and that its mutation affects the overall conformation of the cytoplasmic tails. Although the sequence "VQNC" can be found in many proteins, it is interesting to note that it is present in the E3 ligase parkin. This cytoplasmic protein has two RING-finger motifs separated by an IBR (in-between RING fingers) region (50). Its N-terminal region contains a UBL domain, which includes the $_{56}$VQNC$_{59}$ motif. Most importantly, the V56 residue is changed for a glutamate in a family with recessive inheritance of Parkinson disease (29, 34). The V56 residue lines the short α2 helical region composed of four residues, including the QNC stretch (51). The V56 side chain points toward the interior of the domain and its substitution probably affects the orientation of the exposed α2 helix. There is no structural homology between the C-terminal cytoplasmic tail of MARCH1 and UBLs according to the Structure Prediction Meta Server (11). Still, the possibility remains that some short structural features, such as the VQNC sequence, act like specific sub-regions of UBL domains. Indeed, the N-terminal UBL domain of parkin has been ascribed many different functions. For example, it binds the S5a proteasomal subunit to facilitate the degradation of parkin's substrates (51). Also, the UBL domain interacts with Eps15, a multifunctional adaptor which binds ubiquitin and regulates endocytosis as well as intracellular trafficking of various cargo proteins (23, 65). Future experiments will address the capacity of MARCH1 to interact with such ubiquitin-interacting motif (UIM) proteins. Another possible role for the MARCH1 VQNC motif could be in the autoregulation of the enzymatic activity. The UBL domain of parkin was proposed to fold close to the RING domain and to inhibit autoubiquitination (14). If mutation of V221 affects the enzymatic activity and/or autoubiquitination of MARCH1 remains to be determined. However, our BRET analysis showing

the altered conformation of MARCH1 is in line with an interaction between the two cytoplasmic tails.

MARCH1 is a relatively small protein but as a multipass integral membrane protein, its tertiary structure promises to be difficult to resolve. Our results and those of *Jabbour et al*. (32) showed the presence of multiple functional domains, highlighting the complexity of using ubiquitin as a regulatory mechanism of protein expression and trafficking. Moreover, the list of MARCH1 targets is likely to expand in the near future and new functions for MARCH1 may emerge (3). Further studies are warranted to fully characterize the structure-function aspects of ubiquitination by MARCH proteins.

ACKNOWLEDGEMENTS

This work was supported by grant #36355 from the Canadian Institutes of Health Research (CIHR). MCBD was supported by scholarships from the Cole foundation and the University of Montreal.

We thank Jonathan Boulais for helping with bioinformatic analyses. Edward Fon and Jean-François Trempe for helpful discussions. We also thank Peter Cresswell for antibodies and the Developmental Studies Hybridoma Bank for the CD63 (under the auspices of the NICHD, National Institutes of Health, and is maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242).

REFERENCES

1. **Adhikari, A., and Z. J. Chen**. 2009. Diversity of polyubiquitin chains. Developmental cell. Elsevier Inc. **16**:485-6.
2. **Bania, J., E. Gatti, H. Lelouard, A. David, F. Cappello, E. Weber, V. Camosseto, and P. Pierre**. 2003. Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. Proceedings of the National Academy of Sciences of the United States of America **100**:6664-9.
3. **Bartee, E., C. a Eyster, K. Viswanathan, M. Mansouri, J. G. Donaldson, and K. Früh**. 2010. Membrane-Associated RING-CH proteins associate with Bap31 and target CD81 and CD44 to lysosomes. PloS one **5**:e15132.
4. **Bartee, E., M. Mansouri, B. T. Hovey Nerenberg, K. Gouveia, K. Früh, B. T. H. Nerenberg, K. Fru, and J. V. Irol**. 2004. Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins. Society **78**:1109-1120.
5. **Berger, A. C., and P. A. Roche**. 2009. MHC class II transport at a glance. Journal of cell science **122**:1-4.
6. **Biochem, J., M. Ohmura-hoshino, E. Goto, Y. Matsuki, M. Aoki, and M. Mito**. 2006. JB Minireview — Membrane Traffic in Physiology and Pathology A Novel Family of Membrane-Bound E3 Ubiquitin Ligases. Genome **154**:147-154.
7. **Boname, J. M., B. D. de Lima, P. J. Lehner, and P. G. Stevenson**. 2004. Viral Degradation of the MHC Class I Peptide Loading Complex. Immunity **20**:305-317.
8. **Bonifacino, J. S., and L. M. Traub**. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annual review of biochemistry. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA **72**:395-447.
9. **Bourgeois-Daigneault, M.-C., and J. Thibodeau**. 2012. Autoregulation of MARCH1 Expression by Dimerization and Autoubiquitination. Journal of immunology **188**:jimmunol.1102708-.
10. **Brachet, V., G. Pehau-Arnaudet, C. Desaymard, G. Raposo, and S. Amigorena**. 1999. Early Endosomes Are Required for Major Histocompatiblity Complex Class II Transport to Peptide-loading Compartments. Mol. Biol. Cell **10**:2891-2904.
11. **Bujnicki, J. M., A. Elofsson, D. Fischer, and L. Rychlewski**. 2001. Structure prediction meta server. Bioinformatics **17**:750-751.
12. **Buschow, S. I., J. M. P. Liefhebber, R. Wubbolts, and W. Stoorvogel**. 2005. Exosomes contain ubiquitinated proteins. Blood cells, molecules & diseases **35**:398-403.
13. **Cadwell, K., and L. Coscoy**. 2008. The specificities of Kaposi's sarcoma-associated herpesvirus-encoded E3 ubiquitin ligases are determined by the positions of lysine or cysteine residues within the intracytoplasmic domains of their targets. Journal of virology **82**:4184-9.
14. **Chaugule, V. K., L. Burchell, K. R. Barber, A. Sidhu, S. J. Leslie, G. S. Shaw, and H. Walden**. 2011. Autoregulation of Parkin activity through its ubiquitin-like domain. The EMBO journal. European Molecular Biology Organization **30**:2853-67.
15. **Corcoran, K., M. Jabbour, C. Bhagwandin, M. J. Deymier, D. L. Theisen, and L. Lybarger**. 2011. Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). The Journal of biological chemistry **286**:37168-80.
16. **Cresswell, P.** 1996. Invariant Chain Structure and MHC Class II Function. Cell **84**:505-507.
17. **De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre, and E. Gatti**. 2008. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proceedings of the National Academy of Sciences of the United States of America **105**:3491-6.
18. **Denzin, L. K., and P. Cresswell**. 1995. HLA-DM induces clip dissociation from MHC class II αβ dimers and facilitates peptide loading. Cell **82**:155-165.
19. **Deshaies, R. J., and C. A. P. Joazeiro**. 2009. RING domain E3 ubiquitin ligases. Annual review of biochemistry. Annual Reviews **78**:399-434.
20. **Dugast, M., H. Toussaint, C. Dousset, and P. Benaroch**. 2005. AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. The Journal of biological chemistry **280**:19656-64.
21. **Escrevente, C., S. Keller, P. Altevogt, and J. Costa**. 2011. Interaction and uptake of exosomes by ovarian cancer cells. BMC cancer **11**:108.
22. **Eyster, C. A., N. B. Cole, S. Petersen, K. Viswanathan, K. Früh, and J. G. Donaldson**. 2011. MARCH ubiquitin ligases alter the itinerary of clathrin-independent cargo from recycling to degradation. Molecular biology of the cell **22**:3218-30.
23. **Fallon, L., C. M. L. Bélanger, A. T. Corera, M. Kontogiannea, E. Regan-Klapisz, F. Moreau, J. Voortman, M. Haber, G. Rouleau, T. Thorarinsdottir, A. Brice, P. M. P. van Bergen En Henegouwen, and E. A. Fon**. 2006. A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. Nature cell biology. Nature Publishing Group **8**:834-42.
24. **Galbas, T., V. Steimle, R. Lapointe, S. Ishido, and J. Thibodeau**. 2012. MARCH1 down-regulation in IL-10-activated B cells increases MHC class II expression. Cytokine.
25. **Gauvreau, M.-E., M.-H. Côté, M.-C. Bourgeois-Daigneault, L.-david Rivard, F. Xiu, A. Brunet, A. Shaw, V. Steimle, J. Thibodeau, and M.- Elaine**. 2009. Sorting of MHC class II molecules into exosomes through a ubiquitin-independent pathway. Traffic **10**:1518-27.
26. **Goto, E., M. Mito-Yoshida, M. Uematsu, M. Aoki, Y. Matsuki, M. Ohmura-Hoshino, H. Hotta, M. Miyagishi, and S. Ishido**. 2008. An excellent monitoring system for surface ubiquitination-induced internalization in mammals. PloS one **3**:e1490.
27. **Géminard, C., A. De Gassart, L. Blanc, and M. Vidal**. 2004. Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. Traffic **5**:181-93.
28. **Henn, I. H., J. M. Gostner, P. Lackner, J. Tatzelt, and K. F. Winklhofer**. 2005. Pathogenic mutations inactivate parkin by distinct mechanisms. Journal of neurochemistry **92**:114-22.
29. **Hoenicka, J.** 2002. Molecular Findings in Familial Parkinson Disease in Spain. Archives of Neurology **59**:966-970.
30. **Hsing, L. C., and A. Y. Rudensky**. 2005. The lysosomal cysteine proteases in MHC class II antigen presentation. Immunological reviews **207**:229-41.
31. **Ishido, S., E. Goto, Y. Matsuki, and M. Ohmura-Hoshino**. 2009. E3 ubiquitin ligases for MHC molecules. Current opinion in immunology **21**:78-83.
32. **Jabbour, M., E. M. Campbell, H. Fares, and L. Lybarger**. 2009. Discrete domains of MARCH1 mediate its localization, functional interactions, and posttranscriptional control of expression. Journal of immunology **183**:6500-12.
33. **Jahnke, M., J. Trowsdale, and A. P. Kelly**. 2012. Ubiquitination of Human Leukocyte Antigen (HLA)-DM by Different Membrane-

associated RING-CH (MARCH) Protein Family E3 Ligases Targets Different Endocytic Pathways.Jahnke, M., Trowsdale, J., & Kelly, A. P. (2012). Ubiquitination of Human Leukocyte Antigen The Journal of biological chemistry **287**:7256-64.

34. **Kahle, P. J., and C. Haass**. 2004. How does parkin ligate ubiquitin to Parkinson's disease? EMBO reports. European Molecular Biology Organization **5**:681-5.
35. **Khalil, H., A. Brunet, and J. Thibodeau**. 2005. A three-amino-acid-long HLA-DRbeta cytoplasmic tail is sufficient to overcome ER retention of invariant-chain p35. Journal of cell science **118**:4679-87.
36. **Kreft, S. G., L. Wang, and M. Hochstrasser**. 2006. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). The Journal of biological chemistry **281**:4646-53.
37. **Landsverk, O. J. B., O. Bakke, and T. F. Gregers**. 2009. MHC II and the endocytic pathway: regulation by invariant chain. Scandinavian journal of immunology **70**:184-93.
38. **Lehner, P. J., S. Hoer, R. Dodd, and L. M. Duncan**. 2005. Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. Immunological reviews **207**:112-25.
39. **Li, H. C., E. Y. Li, L. Neumeier, L. Conforti, and M. Soleimani**. 2007. Identification of a novel signal in the cytoplasmic tail of the Na+:HCO3- cotransporter NBC1 that mediates basolateral targeting. American journal of physiology. Renal physiology **292**:F1245-55.
40. **Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S. L. Schmid, V. Quaranta, and P. A. Peterson**. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. Nature **348**:600-5.
41. **Manoury, B., D. Mazzeo, D. N. Li, J. Billson, K. Loak, P. Benaroch, and C. Watts**. 2003. Asparagine Endopeptidase Can Initiate the Removal of the MHC Class II Invariant Chain Chaperone. Immunity **18**:489-498.
42. **Mathivanan, S., H. Ji, and R. J. Simpson**. 2010. Exosomes: extracellular organelles important in intercellular communication. Journal of proteomics **73**:1907-20.
43. **Matsuki, Y., M. Ohmura-Hoshino, E. Goto, M. Aoki, M. Mito-Yoshida, M. Uematsu, T. Hasegawa, H. Koseki, O. Ohara, M. Nakayama, K. Toyooka, K. Matsuoka, H. Hotta, A. Yamamoto, and S. Ishido**. 2007. Novel regulation of MHC class II function in B cells. The EMBO journal **26**:846-54.
44. **McCormick, P. J., J. A. Martina, and J. S. Bonifacino**. 2005. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. Proceedings of the National Academy of Sciences of the United States of America **102**:7910-5.
45. **Miyazono, K., and D. Koinuma**. 2011. Arkadia--beyond the TGF-β pathway. Journal of biochemistry **149**:1-3.
46. **Nathan, J. a, and P. J. Lehner**. 2009. The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. Experimental cell research. Elsevier Inc. **315**:1593-600.
47. **Nilsson, T., M. Jackson, and P. A. Peterson**. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell **58**:707-718.
48. **Ohmura-Hoshino, M., E. Goto, Y. Matsuki, M. Aoki, M. Mito, M. Uematsu, H. Hotta, and S. Ishido**. 2006. A novel family of membrane-bound E3 ubiquitin ligases. Journal of biochemistry **140**:147-54.
49. **Quah, B. J. C., and H. C. O'Neill**. 2005. The immunogenicity of dendritic cell-derived exosomes. Blood cells, molecules & diseases **35**:94-110.
50. **Rankin, C. A., A. Roy, Y. Zhang, and M. Richter**. 2011. Parkin, A Top Level Manager in the Cell's Sanitation Department. The open biochemistry journal **5**:9-26.
51. **Sakata, E., Y. Yamaguchi, E. Kurimoto, J. Kikuchi, S. Yokoyama, S. Yamada, H. Kawahara, H. Yokosawa, N. Hattori, Y. Mizuno, K. Tanaka, and K. Kato**. 2003. Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. EMBO reports **4**:301-6.
52. **Salamero, J., R. Le Borgne, C. Saudrais, B. Goud, and B. Hoflack**. 1996. Expression of major histocompatibility complex class II molecules in HeLa cells promotes the recruitment of AP-1 Golgi-specific assembly proteins on Golgi membranes. The Journal of biological chemistry **271**:30318-21.
53. **Shin, J.-S., M. Ebersold, M. Pypaert, L. Delamarre, A. Hartley, and I. Mellman**. 2006. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature **444**:115-8.
54. **Simons, M., and G. Raposo**. 2009. Exosomes--vesicular carriers for intercellular communication. Current opinion in cell biology **21**:575-81.
55. **Simonsen, A., K. W. Pedersen, T. W. Nordeng, A. von der Lippe, E. Stang, E. O. Long, and O. Bakke**. 1999. Polarized transport of MHC class II molecules in Madin-Darby canine kidney cells is directed by a leucine-based signal in the cytoplasmic tail of the beta-chain. Journal of immunology **163**:2540-8.
56. **Thibodeau, J., M.-C. Bourgeois-Daigneault, G. Huppé, J. Tremblay, A. Aumont, M. Houde, E. Bartee, A. Brunet, M.-E. Gauvreau, A. de Gassart, E. Gatti, M. Baril, M. Cloutier, S. Bontron, K. Früh, D. Lamarre, and V. Steimle**. 2008. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. European journal of immunology. NIH Public Access **38**:1225-30.
57. **Théry, C., L. Zitvogel, and S. Amigorena**. 2002. Exosomes: composition, biogenesis and function. Nature reviews. Immunology **2**:569-79.
58. **Tze, L. E., K. Horikawa, H. Domaschenz, D. R. Howard, C. M. Roots, R. J. Rigby, D. A. Way, M. Ohmura-Hoshino, S. Ishido, C. E. Andoniou, M. A. Degli-Esposti, and C. C. Goodnow**. 2011. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. The Journal of experimental medicine **208**:149-65.
59. **Walseng, E., O. Bakke, and P. A. Roche**. 2008. Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. The Journal of biological chemistry **283**:14717-27.
60. **Walseng, E., K. Furuta, B. Bosch, K. A. Weih, Y. Matsuki, O. Bakke, S. Ishido, and P. A. Roche**. 2010. Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. Proceedings of the National Academy of Sciences of the United States of America **107**:20465-70.
61. **Wang, X., R. a Herr, and T. Hansen**. 2008. Viral and cellular MARCH ubiquitin ligases and cancer. Seminars in cancer biology **18**:441-50.
62. **Ye, Y., and M. Rape**. 2009. Building ubiquitin chains: E2 enzymes at work. Nature reviews. Molecular cell biology **10**:755-64.
63. **Zhong, G., P. Romagnoli, and R. N. Germain**. 1997. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. Journal of Experimental Medicine **185**:429-38.
64. **ten Broeke, T., G. van Niel, M. H. M. Wauben, R. Wubbolts, and W. Stoorvogel**. 2011. Endosomally stored MHC class II does not contribute to antigen presentation by dendritic cells at inflammatory conditions. Traffic **12**:1025-36.
65. **van Bergen En Henegouwen, P. M.** 2009. Eps15: a multifunctional adaptor protein regulating intracellular trafficking. Cell communication and signaling : CCS **7**:24.
66. **van Niel, G., R. Wubbolts, T. Ten Broeke, S. I. Buschow, F. A. Ossendorp, C. J. Melief, G. Raposo, B. W. van Balkom, and W. Stoorvogel**. 2006. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. Immunity **25**:885-94.

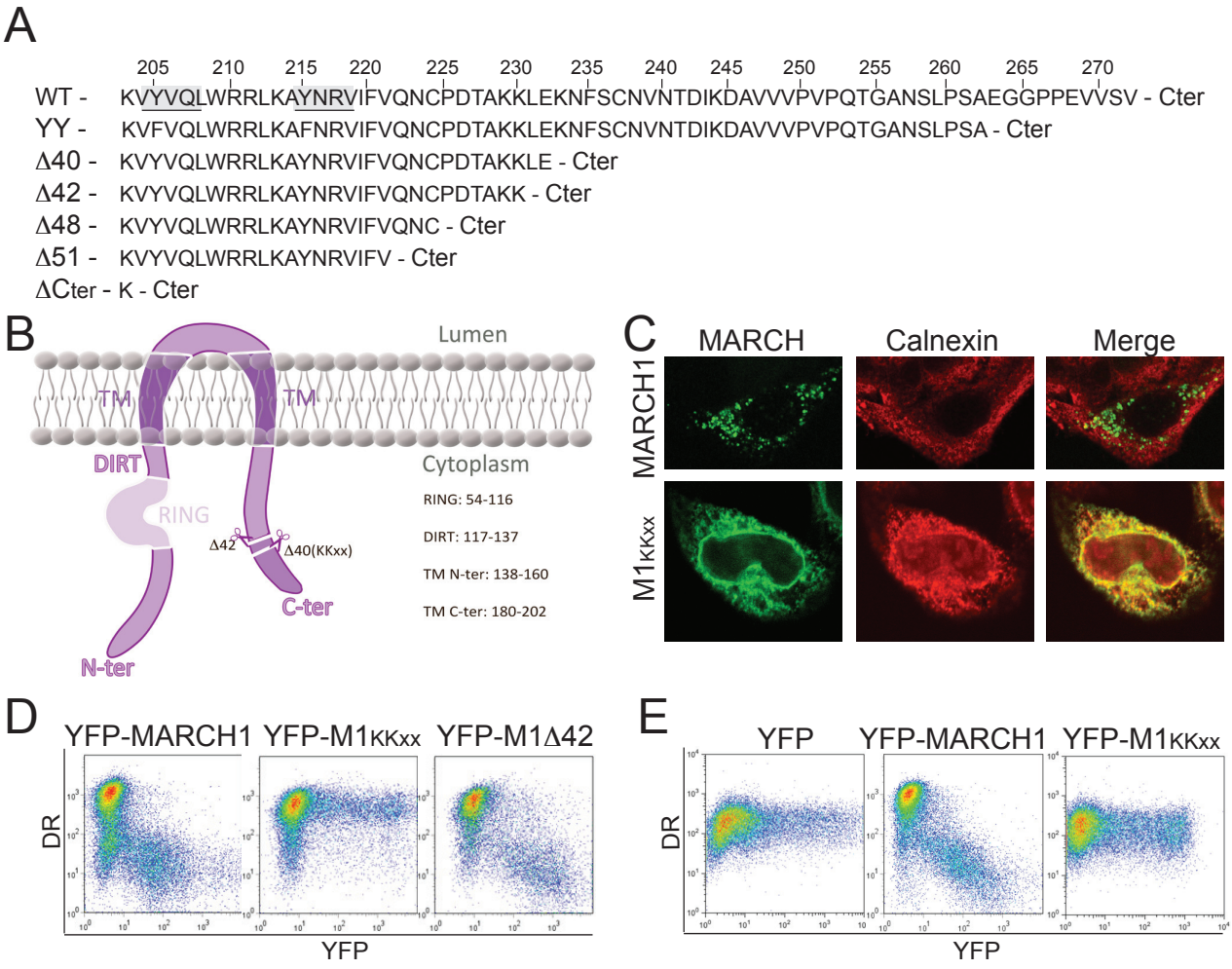

FIG 1 MARCH1 does not prevent the cell surface expression of MHC II in the endoplasmic reticulum. (A) Sequence alignment of the C-terminal cytoplasmic portions of the MARCH1 mutants used in this study. The underlined shaded sequences indicate the predicted tyrosine-based sorting motifs. (B) Schematic representation of M1Δ42 and M1KKxx. (C) Confocal microscopy analysis of HeLa cells transfected with YFP-M1KKxx or YFP-MARCH1 and stained for calnexin. (D) HEK 293T CIITA cells were transfected with YFP-MARCH1, YFP-M1KKxx or YFP-M1Δ42 and analyzed by flow cytometry for the surface expression of DR. (E) HeLa DR1 cells were transfected with YFP, YFP-MARCH1 or YFP-M1KKxx and stained for the cell surface expression of DR. Data are representative of at least two experiments.

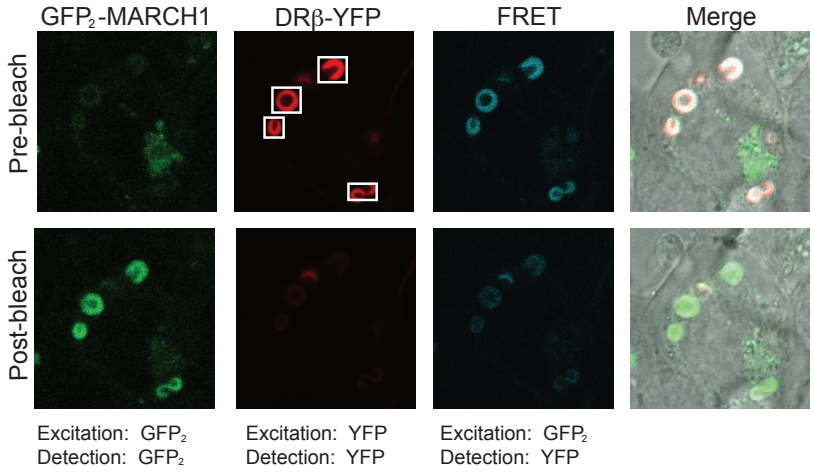

FIG 2 MARCH1 and HLA-DR interact in intracellular vesicles. HeLa cells were transfected on coverslips with GFP2-MARCH1, HLA-DRα and HLA-DRβ-YFP. After 48 hours, confocal microscopy images of live cells were taken. A first series of images (upper panels) were taken before bleaching of defined areas (white squares). Then, a second series of images were taken (bottom panels). The experiment is representative of three independent transfections.

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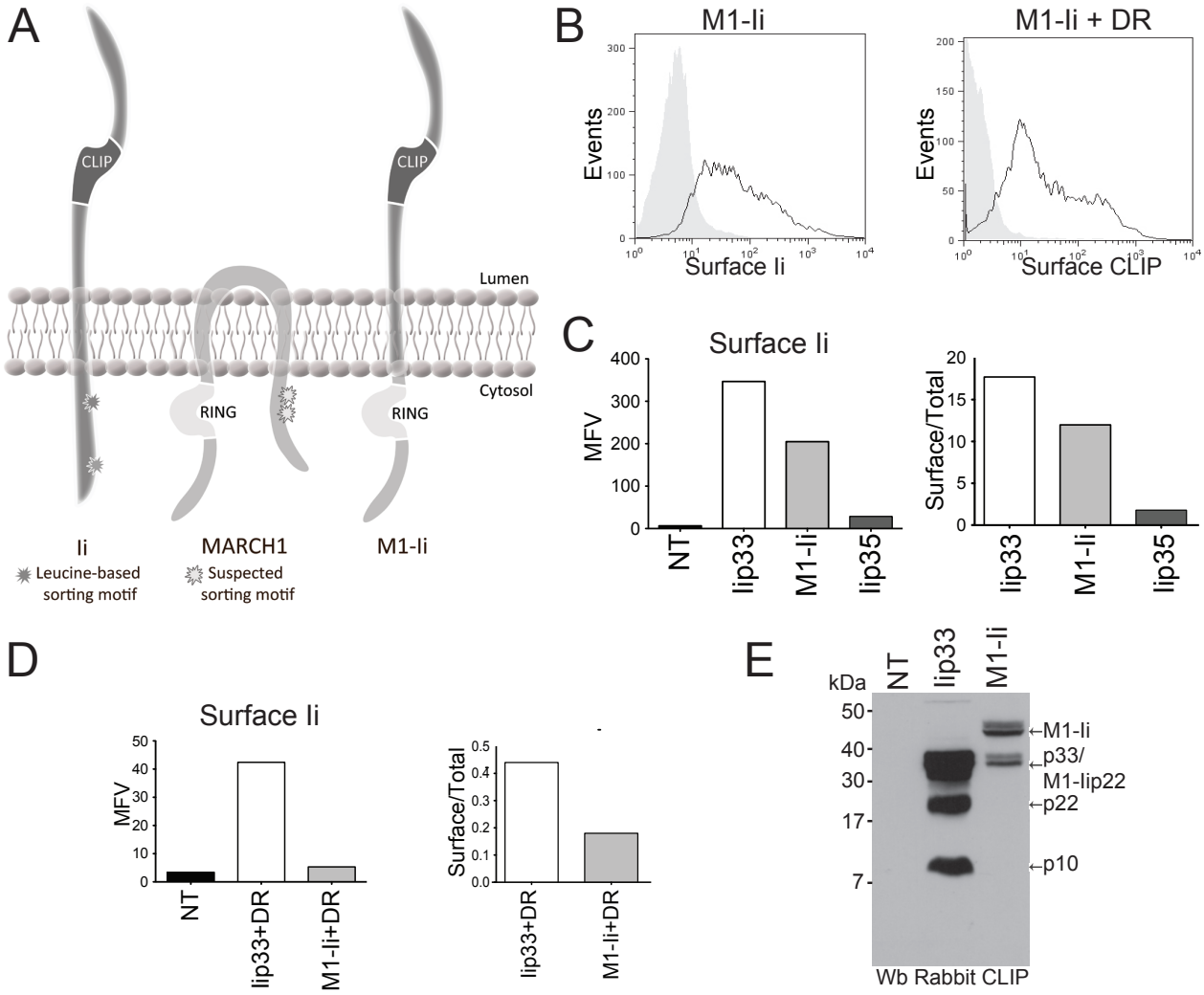

FIG 3 The MARCH1 N-terminal cytoplasmic portion includes sorting signals. (A) Schematic representations of Ii, MARCH1 and the M1-Ii chimera used in this study. (B) Flow cytometry analysis of HEK 293T cells transfected with M1-Ii with or without DR. Cells were stained for the Ii luminal region (left panel) or DR-CLIP complexes (right panel). The shaded curves represent the cells stained with the secondary antibody alone. (C) HEK 293T cells were transfected or not with Iip33, M1-Ii or Iip35. Cells were analyzed by flow cytometry and the MFVs were plotted as bar charts (left panel). Cells were also permeabilized and stained to measure total cellular expression. A ratio of the surface over total expression was plotted (right panel). NT indicates untransfected cells. (D) HEK 293T cells were analyzed as in panel C but after co-transfection of DR. (E) HEK 293T cells were transfected with Iip33 or M1-Ii. NT indicates untransfected cells. After 48 h, cells were lysed and proteins were analyzed on immunoblots using a CLIP-specific rabbit polyclonal antibody. Data are representative of at least three (B, C and D) or two (E) independent experiments.

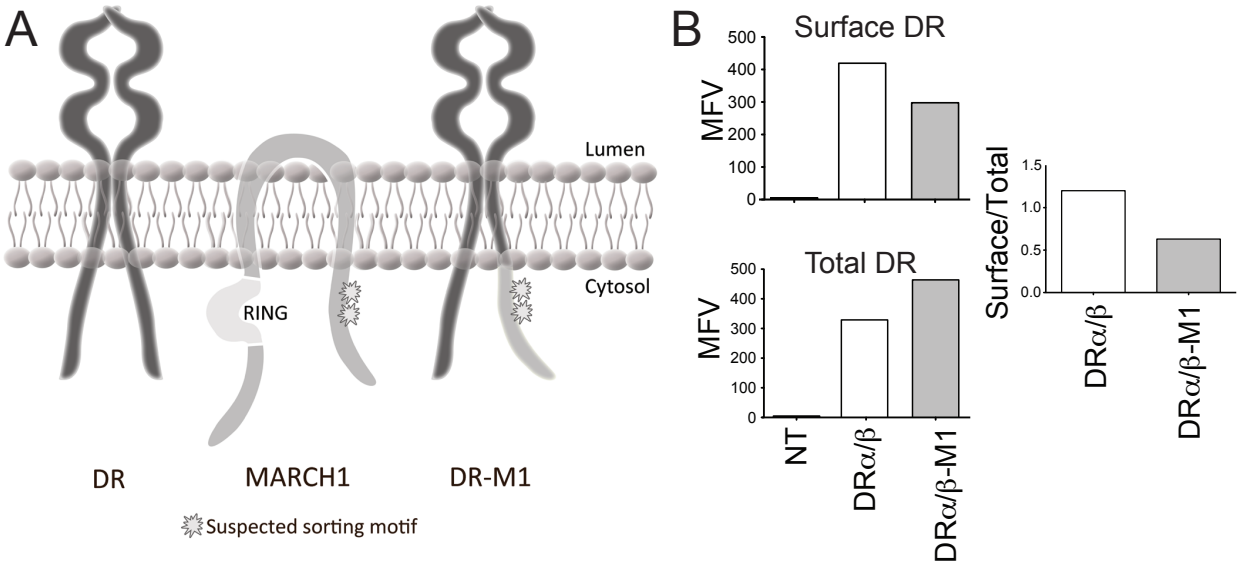

FIG 4 The C-terminal cytoplasmic domain of MARCH1 encodes functional sorting elements. (A) Schematic representations of DR, MARCH1 and the DR-M1 chimeric molecule used in this study. (B) HEK 293T cells transfected or not (NT) with DRα and with either DRβ or DRβ-M1. After 48h, cells were analyzed by flow cytometry for DR expression before (surface) or after (total) membrane permeabilization. The MFVs were plotted as bar charts. Data are representative of four different experiments.

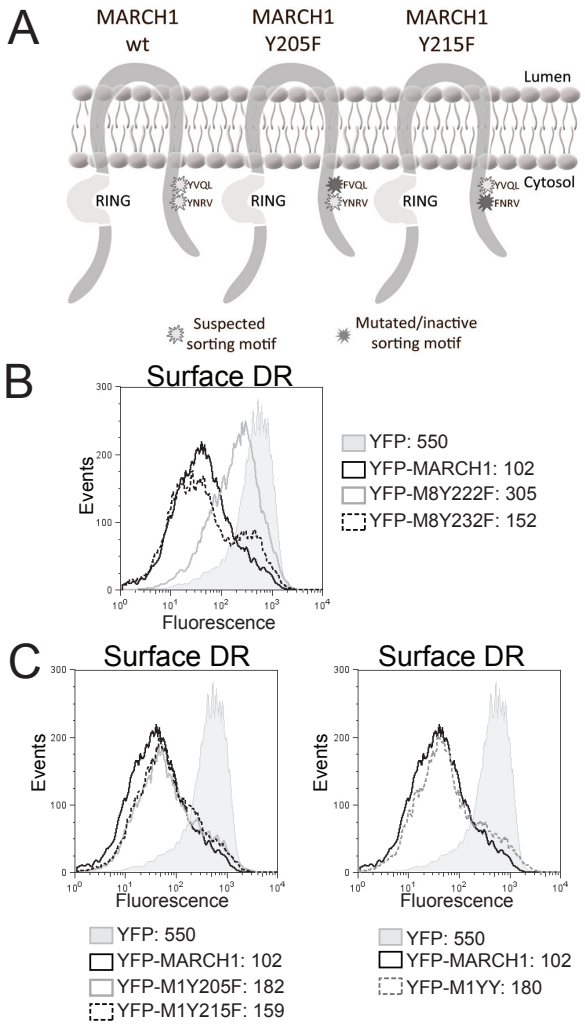

FIG 5 The tyrosine sorting motifs of human MARCH1 are dispensable for the down-regulation of MHC II. (A) Schematic representations of MARCH1 and mutants in which substitutions were introduced. (B) Flow cytometry analysis showing the MHC II expression at the surface of YFP-positive HEK 293E CIITA cells transfected with YFP, YFP-MARCH1, YFP-M8Y222F or YFP-M8Y232F, or (C) YFP, YFP-MARCH1, YFP-M1Y205F or YFP-M1Y215F (left panel) or YFP, YFP-MARCH1 or YFP-M1YY (right panel). The numbers in the legends indicate the MFVs. Data are representative of at least four different experiments.

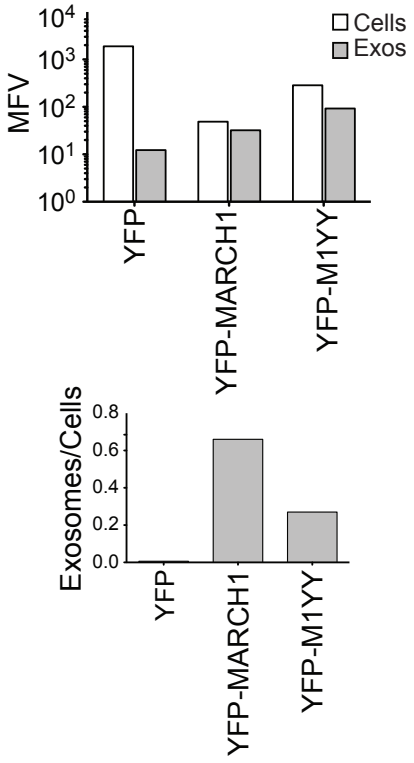

FIG 6 The tyrosine sorting motifs of human MARCH1 are functional. HEK 293T cells were transfected with YFP, YFP-MARCH1 or YFP-M1YY. After 48h, the cells were analyzed by flow cytometry. The secreted exosomes were collected, coupled to beads, stained for CD9 and analyzed by flow cytometry. The upper panel represents the MFV obtained for YFP in cells and the exosomes in each condition. The bottom panel represents a ratio of the YFP MFVs and normalized for CD9 expression. The experiment was performed twice.

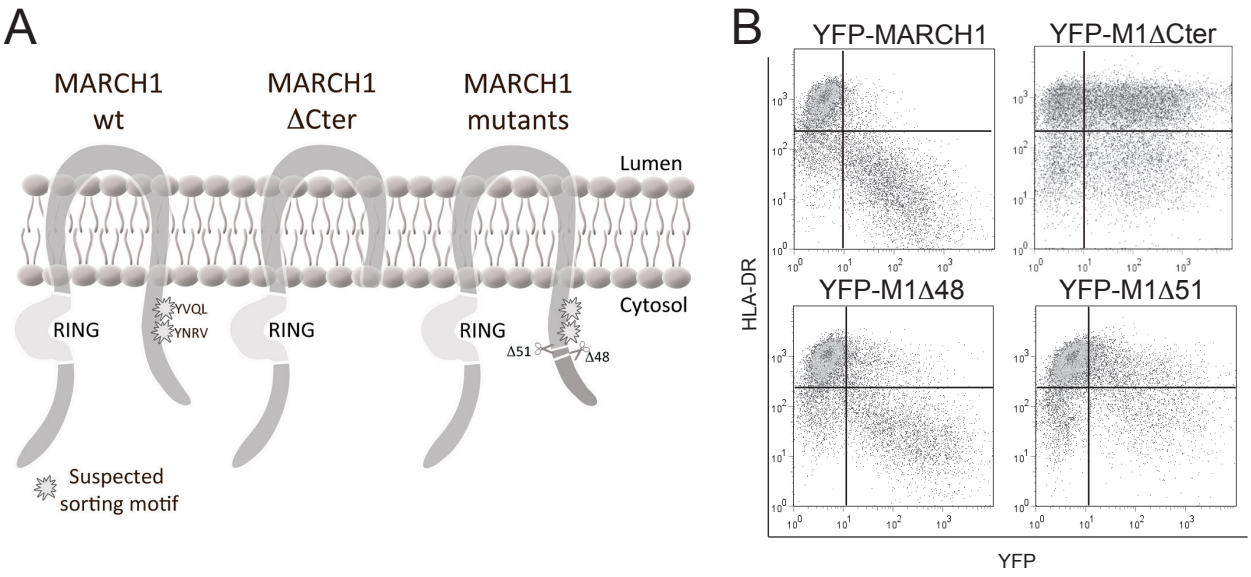

FIG 7 The C-terminal cytoplasmic portion of MARCH1 contains a short sequence that is essential for its function toward MHC II. (A) Schematic representations of MARCH1 and the truncated mutants. (B) HEK 293T cells were transfected with YFP-MARCH1, YFP-M1ΔCter, YFP-M1Δ48 or YFP-M1Δ51 and analyzed by flow cytometry. The dot plots show the surface expression of DR relative to YFP fluorescence. Data are representative of at least four different experiments.

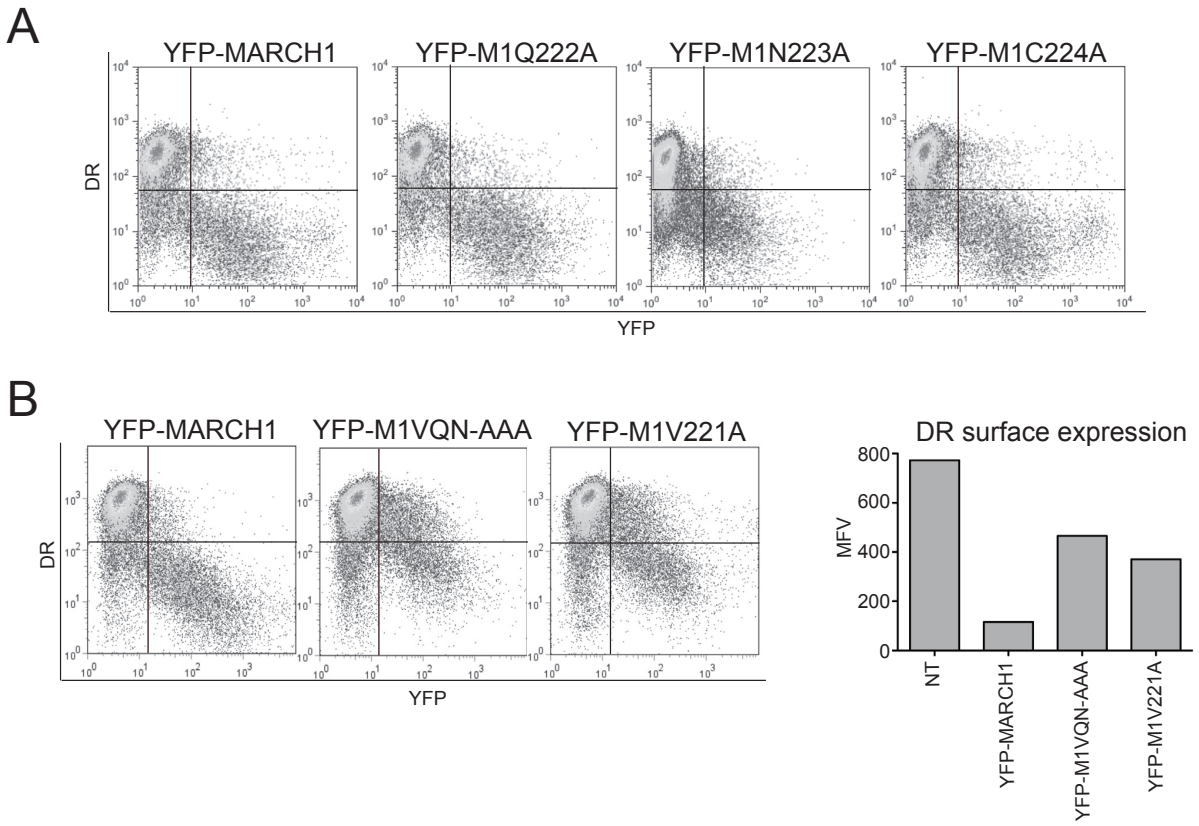

FIG 8 The valine 221 is important for the function of MARCH1. (A) HEK 293E CIITA cells were transfected with YFP-MARCH1, YFP-M1Q222A, YFP-M1N223A or YFP-M1C224A and analyzed by flow cytometry. The dot plots show the surface expression of DR relative to YFP expression. (B) HEK 293E CIITA cells were transfected with YFP-MARCH1, YFP-M1VQN-AAA or YFP-M1V221A and analyzed by flow cytometry. The dot plots show the surface expression of DR relative to YFP expression. The MFV for DR expression on YFP+ cells was plotted (right panel). Data are representative of two (A) and three (B) different experiments.

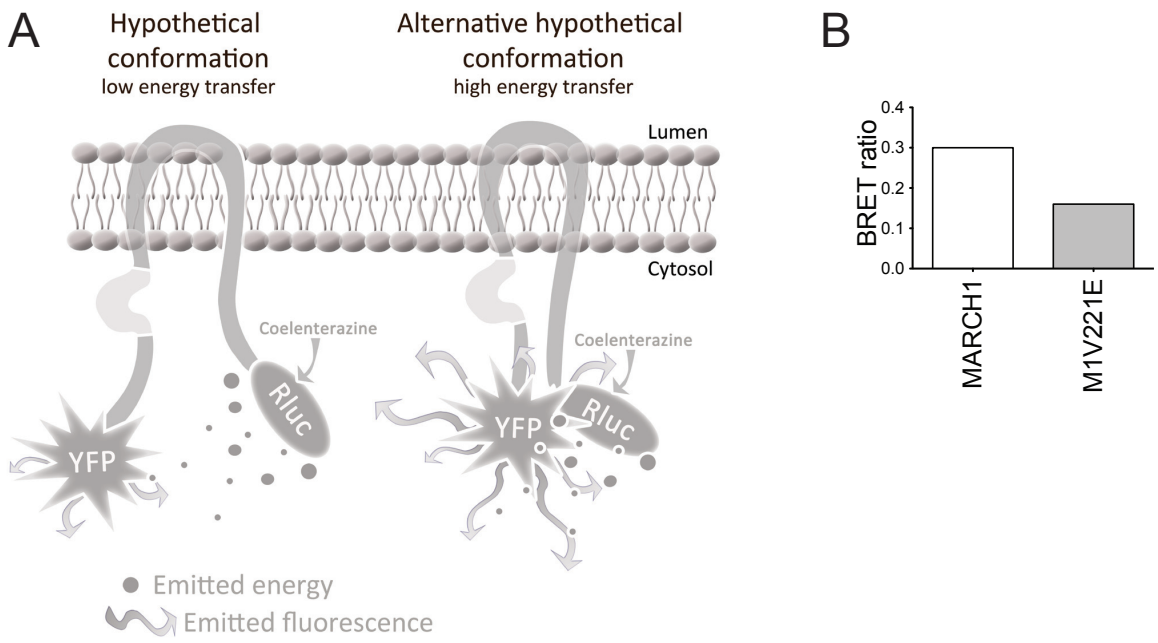

FIG 9 The MARCH1 V221 residue influences the spatial orientation of the cytoplasmic tails. (A) Schematic representations of YFP-MARCH1-Rluc in different hypothetical conformations that would influence the strength of the BRET signal. (B) HEK 293T cells were transfected with YFP-MARCH1-Rluc or YFP-M1V221E-Rluc. The fluorescence and the luminescence emitted by the cells were measured before and after the addition of coelenterazine H. The BRET ratio was calculated by dividing the fluorescence with substrate (subtracted from the fluorescence without substrate) by the luminescence values. The results were represented as a bar chart. This experiment was repeated with an independent transfection.

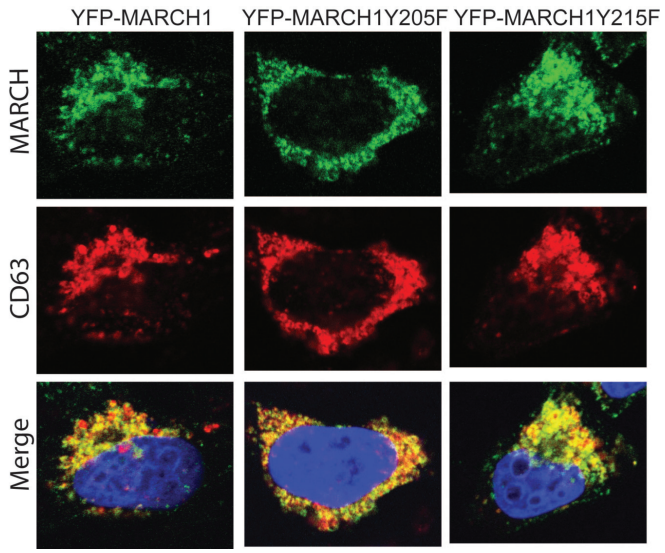

FIG S1 Mutation of the tyrosine motifs in MARCH1 does not affect its subcellular localization. Confocal microscopy analysis of HeLa cells transfected with YFP-MARCH1, YFP-M1Y205F or YFP-M1Y215F and stained with Hoechst as well as for CD63. The experiment was repeated with an independent transfection.

Chapitre 4 : Discussion

4.1. Conditions expérimentales

Étant donné l'impossibilité de détecter la protéine endogène, les expériences incluses dans cet ouvrage ont été effectuées en système de surexpression. De plus, la difficulté d'obtenir de hauts niveaux d'expression de la protéine nous a mené à l'utilisation de formes fusionnées à la YFP. La YFP, ayant une demi-vie d'environ 13 heures, stabilise MARCH1 dont la demi-vie est de 30 minutes pour la molécule murine [171]. Ainsi, nous avons obtenu une molécule dont l'expression est plus stable et la détection facilitée. Nous évaluons la demi-vie de YFP-M1 comme étant d'un peu moins de 4 heures en cellules HeLa (Figure 2.3.D). De plus, nos résultats ont montré que YFP-M1 était actif contre ses substrats le CMH II et Tfr et ce, à de très faibles concentrations (Figure 2.2.A et résultats non publiés). Aussi, nos images de microscopie témoignent d'une localisation endosomale de la molécule, ce qui est en accord avec la littérature de MARCH1 (voir section 1.8.5.). La fusion de la YFP à MARCH1 ne semble donc pas avoir affecté la molécule en ce qui concerne son ciblage et l'interaction avec ses ligands, mais possède l'avantage d'avoir stabilisé son expression, ce qui a grandement simplifié cette étude [172].

4.2. Autorégulation de l'expression de MARCH1

Malgré une importante augmentation de la transcription de MARCH1 en monocytes primaires humains et l'utilisation d'un anticorps reconnaissant la protéine en systèmes de surexpression, nous n'avons jamais été en mesure de détecter la protéine endogène. De plus, la transfection de MARCH1 non fusionné à la YFP montre des niveaux d'expression très bas. Les difficultés rencontrées par notre groupe ont également été rapportées par d'autres. Cette problématique laissait présager la présence d'un système de régulation au niveau post-traductionnel (voir section 1.8.3.). De plus, de nombreux exemples sont répertoriés d'E3 ubiquitine ligases dont l'expression est régulée par autoubiquitination (voir section 1.6.4.). Ce sont ces éléments qui ont conduit à l'établissement de la première hypothèse voulant que MARCH1 s'autoubiquitine (voir section 1.9.2.1.).

4.2.1. Différentes hypothèses

Il y a toutefois des alternatives à ce modèle à garder en tête. En effet, il est possible que MARCH1 possède des motifs de ciblage ou interagisse avec des partenaires le ciblant vers les lysosomes, où la molécule serait rapidement dégradée. Cette possibilité sera discutée plus loin. Une seconde option serait que MARCH1 nécessite l'activité d'une chaperonne qui serait régulée seulement suite à certains stimulis pour le stabiliser et permettre l'augmentation de sa demi-vie. Cette possibilité est renforcée par la découverte de l'association de MARCH1 avec la chaperonne Bap31 du RE. Cette protéine est exprimée de manière ubiquitaire, néanmoins il serait intéressant de vérifier ses niveaux d'expression dans les monocytes primaires humains et les différentes lignées utilisées dans cette étude. Dans le cas où celui-ci serait faiblement exprimé, il se peut que l'induction massive de MARCH1 via l'IL-10 en monocytes primaires humains ou alors l'importante transcription de la molécule causée par la transfection de plasmides qui l'encode sature la capacité chaperonne de Bap31. Afin de vérifier cette hypothèse, une approche serait alors de comparer la demi-vie de MARCH1 avec et sans la co-transfection de Bap31.

4.2.2. Différence de régulation chez les molécules murine et humaine

Alors que nous caractérisions la potentielle ubiquitination de MARCH1, le groupe de Lybarger a publié une étude dans laquelle ils ont tenté de détecter des formes ubiquitinées de MARCH1 murin [165]. Leurs résultats furent négatifs. À l'opposé, nos résultats démontrent clairement l'ubiquitination de la molécule humaine (Figure 2.1.B). Ceci porte à croire qu'il y aurait une différence entre les modes de régulation des formes murine et humaine de MARCH1. Néanmoins, il se peut également que les différences observées soient liées au système expérimental utilisé. Afin d'invalider cette possibilité, il faudrait tenter de détecter l'ubiquitination de la forme murine de MARCH1 dans notre système. Néanmoins, la détection de l'ubiquitination de la molécule murine en cellules humaines introduirait un artéfact. Il serait alors difficile de croire en la relevance physiologique de ces résultats et, même dans le cas ou un résultat positif serait observé,

nous ne pourrions pas affirmer que la molécule murine peut être ubiquitinée chez la souris. À l'inverse, nous pourrions transfecter la forme humaine dans leur système et tenter de détecter la forme ubiquitinée de MARCH1 humain et murin en parallèle. L'expression de la forme humaine de MARCH1 en cellules de souris introduirait également un biais. Par contre, ayant déjà démontré que MARCH1 humain était ubiquitiné en cellules humaines, l'obtention d'un résultat positif en cellules de souris confirmerait la présence de tous les éléments nécessaires à l'ubiquitination de la molécule dans ce modèle et révèlerait un mode de régulation différent des molécules murine et humaine. D'un autre côté, le résultat inverse ne permettrait pas de tirer aucune conclusion. En effet, il est possible que la molécule humaine, bien que fonctionnelle dans le contexte des cellules de souris, nécessite des éléments absents de chez ces dernières en ce qui concerne sa régulation protéique.

4.2.3. Facteurs favorisant l'ubiquitination de MARCH1

Nos travaux ont démontré l'ubiquitination de MARCH1. Néanmoins, les signaux menant à cette modification protéique n'ont pas été étudiés. Ainsi, il se peut que la molécule soit ubiquitinée seulement dans certaines conditions. Par exemple, lorsque les molécules s'accumulent et s'expriment à de forts niveaux comme ce serait le cas lors d'une stimulation prolongée à l'IL-10. Afin de répondre à cette question, il serait d'abord intéressant d'évaluer la proportion de MARCH1 ubiquitiné en fonction des niveaux d'expression de la protéine. Ainsi, différentes quantités de MARCH1 seraient transfectées et la proportion de la molécule modifiée pourrait alors être comparée par immunoblot.

D'un autre côté, c'est peut-être la localisation de MARCH1 qui permet son ubiquitination. En effet, la E2 responsable de cette modification n'est peut-être accessible que dans certains compartiments. Par exemple, UbcH6 est une E2 membranaire et sa localisation se restreint au RE. Dans le cas où cette E2 serait responsable de l'ubiquitination de MARCH1, celle-ci aurait lieu tôt après sa synthèse. Nous pourrions tester l'effet d'un inhibiteur du trafic vésiculaire sur l'ubiquitination de MARCH1. La bréfeldine A et le monensin bloquent la sortie du RE. Ainsi, les molécules seraient alors confinées au RE et n'accèderaient pas aux compartiments endosomaux. Ensuite, de la même manière que celle

utilisée dans notre article 1, ces cellules seraient lysées et les extraits protéiques seraient immunoprécipités avec un anticorps spécifique à MARCH1. Les échantillons seraient ensuite étudiés pour la présence d'ubiquitine par immunoblot et les conditions en présence et en absence d'inhibiteurs pourraient être comparées.

4.2.4. Trans-régulation de MARCH1

Le plus haut degré d'ubiquitination de la molécule sauvage en comparaison avec un mutant inactif (M1WI), ainsi que la demi-vie prolongée de ce dernier pointent vers un mécanisme d'autorégulation (Figure 2.4.A et B). Tel que mentionné dans l'article 1, il est à noter que le mutant inactif présentait également un certain niveau d'ubiquitination, ainsi une autre E3 ligase se lie à MARCH1 pour y permettre l'ajout d'ubiquitines. De plus, de nombreuses études portent sur la dimérisation et l'ubiquitination des E3 ligases (voir section 1.6.1.). À la lumière de ces informations, nous avons posé la seconde hypothèse: MARCH1 dimérise.

4.3. MARCH1 forme des homo- et des hétérodimères

Plusieurs groupes avaient préalablement démontré ou avancé la possibilité que des membres de la famille MARCH forment des dimères (voir section 1.7.). Néanmoins, aucune étude n'avait établi de rôle physiologique à cette multimérisation.

Nos résultats de BRET et d'immunoprécipitations ont révélé la formation d'homodimères de MARCH1, ainsi que d'hétérodimères avec MARCH8 et MARCH9 (Figure 2.5. A à D). De plus, la majorité des E3 ubiquitine ligases qui forment des dimères le font via le domaine RING (voir section 1.6.1.) et l'utilisation de nombreux mutants dont certaines portions avaient été supprimées nous a permis d'affirmer que le RING, ainsi que les portions cytoplasmiques N- et C-terminales n'étaient pas impliqués dans ce processus (Figure 2.5. E). En effet, ces résultats, en combinaison avec ceux obtenus par des chimères entre MARCH1 et MARCH9, ont révélé l'importance des domaines TMs dans la formation de dimères par MARCH1. Les MARCHs sont des E3 ligases membranaires, ce qui est

exceptionnel pour ce type de molécule. De plus, les TMs sont fréquemment impliqués dans les interactions protéiques. Cette caractéristique des MARCHs serait donc responsable de leur différence avec les autres E3s décrites dans la littérature.

La construction des chimères M1-9 nous a également permis de répondre à la troisième hypothèse: les domaines transmembranaires de MARCH1 reconnaissent et interagissent avec la cible.

4.4 L'importance des TMs de MARCH1 dans son interaction avec le CMH II

De nombreux travaux rapportent l'importance des TMs des MARCHs pour l'interaction avec le substrat (voir section 1.7.). Nous avons donc testé l'importance des domaines TMs de MARCH1 pour son interaction avec le CMH II. Les résultats ont démontré qu'ils étaient requis.

Les TMs de MARCH1 sont donc requis pour la formation de dimères ainsi que pour son interaction avec sa cible. Néanmoins, il nous est impossible d'affirmer, à la lumière de nos résultats, si la dimérisation est nécessaire à l'association avec la cible et à l'ubiquitination. Une récente publication a étudié la question de l'ubiquitination par les E3s de type RING formant des dimères en prenant pour modèle RNF4 [173]. Leurs résultats ont montré que bien que la E2 puisse lier la E3 sous forme de monomère, le dimère est requis à la catalyse du lien thioester entre la E2 et l'ubiquitine et donc, à l'ubiquitination de la cible. Cette possibilité avait également été précédemment avancée par un autre groupe [174]. Tel que discuté dans l'article 1, d'autre E3s comme MDM2 et MDMx forment des dimères pour permettre l'ubiquitination en trans de la molécule cible. Dans ces cas, la dimérisation est requise à la fonction.

4.5 L'importance du dimère pour l'interaction avec la cible

4.5.1. Premier modèle: les dimères ne sont pas requis

Dans un premier modèle, il y aurait formation de dimères via l'association des domaines TMs, ce qui permettrait l'ubiquitination de la molécule et la régulation de son expression. Le dimère ne serait pas requis à l'association avec la cible. La faible expression de MARCH1 diminuerait alors les probabilités de formation de dimères et favoriserait donc l'interaction avec le substrat. À l'opposé, la forte expression de MARCH1 favoriserait sa dimérisation et la molécule serait plus rapidement autoubiquitinée et dégradée. La demi-vie de MARCH1 étant diminuée, les interactions avec le CMH II seraient alors moins fréquentes. Dans ce cas, MARCH1 n'accomplirait plus sa fonction de modulateur de la présentation antigénique. Par exemple, en présence d'une stimulation prolongée à l'IL-10, l'accumulation des molécules de MARCH1 empêcherait celui-ci d'accomplir certains aspects de sa fonction.

4.5.2. Deuxième modèle: les dimères sont requis

Dans un deuxième modèle, la dimérisation serait un pré requis à l'association avec la cible et donc à la fonction de MARCH1. Ainsi, MARCH1 dimériserait pour ensuite lier sa cible et l'ubiquitiner. Selon ce modèle, la dimérisation de MARCH1 pourrait avoir lieu rapidement après sa synthèse et les molécules demeureraient possiblement associées jusqu'à leur dégradation.

Il serait intéressant de discriminer entre ces deux possibilités. Une approche serait d'effectuer une immunoprécipitation du CMH II suivie d'un immunoblot envers MARCH1 en conditions natives. Ainsi, la taille du complexe observé pourrait nous renseigner sur le nombre de sous-unités présentes dans celui-ci. Dans un même type d'expérience, une seconde approche serait de co-exprimer MARCH1 et MARCH8 et évaluer la présence de ces deux molécules, qui ont une taille légèrement différente, dans un complexe ayant le même poids moléculaire. Cette dernière option pourrait révéler l'interaction d'un

hétérodimère avec le CMH II. Une autre approche pourrait être le fractionnement d'extraits protéiques sur gradient de sucrose. Les fractions seraient par la suite testées pour la présence de MARCH1 et du CMH II et la taille des complexes serait évaluée en comparaison avec la présence de molécules de poids moléculaire connu. Finalement, dans le but de tester la fonctionnalité d'un dimère de MARCH1, il serait intéressant de créer un mutant double MARCH1. Les deux molécules reliées entre-elles par une courte séquence flexible d'acides aminés auraient la possibilité de s'assembler tel un dimère forcé de MARCH1 et nous pourrions en évaluer la fonctionnalité et la demi-vie.

4.5.3. Prévention de la formation de dimères par CD83

Une récente publication démontre que le domaine TM de CD83 bloque l'activité de MARCH1 envers CD86 [166]. En effet, en présence de CD83, l'association de MARCH1 à CD86, ainsi que l'ubiquitination de ce dernier n'ont pas lieu. Dans cette étude, l'équipe du Dr. Goodnow a également démontré l'interaction de MARCH1 avec CD83. C'est cette association qui prévient l'interaction avec la cible. Il serait intéressant d'étudier l'effet de la présence de CD83 sur la formation du dimère de MARCH1. Dans le cas où la formation de dimères serait requise à l'association de MARCH1 à sa cible, CD83 pourrait agir à ce niveau plutôt que de simplement bloquer l'association à CD86 comme le proposent les auteurs.

4.5.4. Importance de radeaux lipidiques dans la fonction de MARCH1

Nos travaux ont révélé l'importance des domaines TMs de MARCH1 pour sa fonction. De plus, une étude a démontré l'implication des TMs de plusieurs MARCHs, dont MARCH1, dans l'interaction avec la chaperonne Bap31. Cette interaction permettrait le contrôle du transport intracellulaire des MARCHs, indiquant ainsi un autre rôle des TMs de MARCH1 [175]. Les domaines TMs ont un rôle bien établi dans l'incorporation des protéines membranaires aux radeaux lipidiques [176]. De plus, le CMH II, CD86, HLA-DM et Fas, quatre des cinq substrats connus de MARCH1, se retrouvent dans ces microdomaines [177–179]. Il serait donc possible que la nécessité des TMs de MARCH1 pour son interaction avec

le CMH II soit partiellement dépendante de leur fonction dans l'incorporation de celui-ci aux microdomaines membranaires appropriés. Les radeaux lipidiques sont le plus souvent localisés au niveau de la membrane plasmique [180]. Ces microdomaines sont formés à la surface cellulaire d'où ils peuvent être endocytés puis acheminés aux endosomes tardifs ou encore retourner à la surface [181]. Certaines études démontrent même la présence de ceux-ci dans les exosomes [182]. La localisation de MARCH1 aux endosomes tardifs, son potentiel transit par la membrane cellulaire et son incorporation dans les exosomes (discuté en section 4.7.) ne vont donc pas à l'encontre de sa possible incorporation à ces microdomaines.

Dans un premier temps, il serait intéressant de déterminer la présence ou l'absence de MARCH1 dans ces microdomaines. Pour ce faire, des cellules exprimant MARCH1 seraient d'abord lysées en tampon MES-Triton, puis les extraits protéiques seraient fractionnés par ultracentrifugation sur gradient de sucrose allant de 5 à 30%. Par la suite, les fractions correspondantes aux radeaux lipidiques seraient analysées pour la présence de la molécule. Une autre approche impliquerait l'utilisation de methyl-β-cyclo-dextrin, afin de détruire les radeaux lipidiques, suivie d'immunoprécipitations et de l'analyse des interactions MARCH1-CMH II. Cette technique pourrait révéler l'importance de ces microdomaines dans l'interaction de MARCH1 avec son substrat.

D'un autre côté, Tfr, le cinquième substrat connu de MARCH1, n'est pas retrouvé dans les radeaux lipidiques et aucune étude n'a investigué l'importance des TMs pour l'interaction entre les deux molécules [183]. Une seconde approche serait donc d'étudier l'importance des domaines TMs dans l'association de MARCH1 avec Tfr.

4.6. L'implication de la localisation de MARCH1 pour son activité

La première publication de notre groupe concernant MARCH1 date de 2008 (voir Annexe I) [163]. Nous nous étions intéressés à l'implication de MARCH1 dans la réponse à l'IL-10. Dans cette étude, nous avons révélé la présence d'une interaction entre MARCH1

et HLA-DR par immunoprécipitation. Dans le cadre de ces travaux, une expérience de FRET avait clairement démontré l'interaction entre ces deux molécules dans des vésicules intracellulaires. Néanmoins, cet aspect de l'interaction MARCH1-CMH II ne fut pas étudié en détails et n'a pas été inclus dans nos travaux sur l'IL-10.

Quelques années plus tard, Jabbour et ses collègues se sont penchés sur l'implication des motifs de ciblage à la voie endosomale compris dans la portion cytoplasmique C-terminale de MARCH1 murin [165]. Leurs résultats ont démontré la nécessité de ces motifs pour la fonction de la molécule. De la même manière, des travaux par le groupe du Dr. Ishido ont démontré la nécessité des motifs tyrosine de MARCH8 pour son activité [160]. Ces évidences nous ont mené à poser la quatrième hypothèse: les motifs de ciblage de la queue cytoplasmique C-terminale de MARCH1 sont requis pour sa fonction (voir section 1.9.2.4.).

À notre grande surprise, la mutation des deux motifs tyrosine de MARCH1 n'eut aucun effet sur la fonction de celle-ci à l'encontre de l'expression de surface du CMH II. De plus, il a été démontré que la molécule murine était stabilisée par un traitement à la leupeptine, mais nos résultats ont montré le contraire chez l'humain (Figure 2.3.A) [165]. Il a également été observé que la molécule murine s'exprimait à de plus forts niveaux lorsque ses motifs tyrosine étaient mutés. Encore une fois, nos résultats indiquaient le contraire chez la molécule humaine. Il a été proposé que la molécule serait rapidement dégradée lorsqu'elle attendrait les compartiments lysosomaux et que ses motifs tyrosine seraient responsables de cette localisation. Nos résultats et ceux publiés pour la molécule murine divergeaient donc en de nombreux points concernant les motifs de ciblage de ceux-ci. Nous avons donc validé les résultats obtenus dans notre système en reproduisant les mutations correspondantes à celles publiées pour MARCH1 murin et MARCH8 humain en guise de contrôles. Nos résultats confirment ceux précédemment publiés voulant que ces motifs de ciblage soient requis à la fonction de MARCH1 murin et de MARCH8. Notre système est donc approprié pour l'étude des motifs de ciblage et il existe bel et bien une différence au niveau de la nécessité de ceux-ci chez la molécule humaine et murine.

Nous nous sommes alors questionné(s) sur la fonctionnalité de ces motifs chez la molécule humaine. La génération d'un double mutant de MARCH1 pour ses motifs tyrosine (MARCH1YY) est à la base de notre étude de ceux-ci. La différence observée entre MARCH1 humain et murin en ce qui concerne la nécessité des motifs tyrosine pour leur fonction n'est pas imputable aux motifs comme tels, à leurs composition ou encore leurs distances de la membrane. D'ailleurs, nos résultats ont démontré que ces motifs étaient fonctionnels en ce qui concerne la localisation de la molécule. La Figure 4.1 montre un alignement des séquences protéiques de MARCH1 humain et murin.

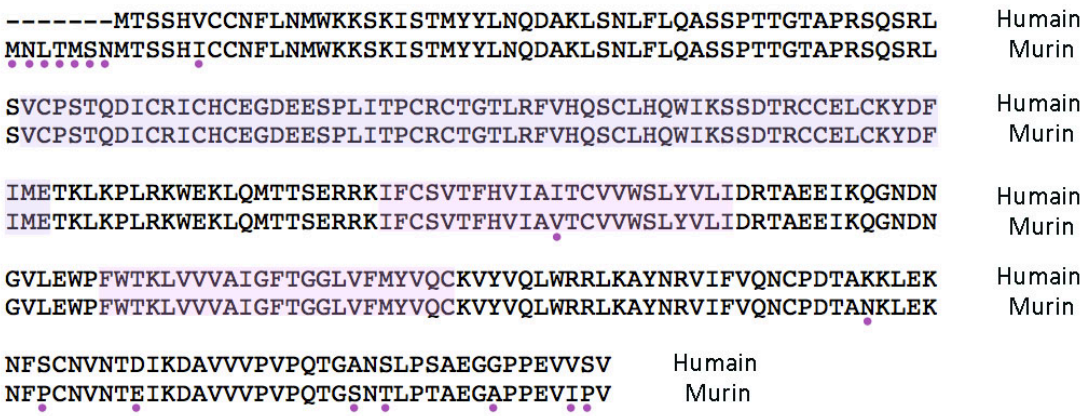

Figure 4.1. Alignement des séquences protéiques de MARCH1 humain et murin. La région en mauve représente le RING et les régions en rose représentent les TMs. Les points mauves indiquent les endroits où les séquences divergent.

Dans les deux cas, les séquences sont identiques et à égale distance de la membrane. En effet, le motif le plus près de la membrane en est séparé d'un seul acide aminé et la séquence est YVQL. L'autre motif est YNRV et débute dix acides aminés après le début du premier. Les motifs sont donc intacts, de même que les résidus environnants. Bien que les séquences des molécules complètes soient identiques à 96%, l'extrémité C-terminale comporte à elle seule 6 différents acides-aminés. Il est possible que cette région soit impliquée dans des intéractions avec différentes protéines adaptatrices nécessaires à la relocalisation de la molécule murine, alors que la molécule humaine ne nécessiterait pas ces adapteurs. Néanmoins, un mutant de MARCH1 murin délété pour ses 20 derniers résidus a

été démontré comme étant toujours actif, montrant ainsi que cette portion de la molécule n'est pas requise à la fonction de celui-ci [165].

Nos difficultés à visualiser une différence de localisation en microscopie, probablement causées par la surexpression des molécules, nous ont dirigés vers une autre approche. Dans une étude précédente, alors que nous nous intéressions à l'importance de l'ubiquitination du CMH II pour son incorporation dans les exosomes, nous avons remarqué la présence de MARCH1 et MARCH8 dans ceux-ci (voir annexe II) [184]. Nous n'avons toutefois pas inclus ces observations dans notre article et pour l'instant, aucun autre groupe n'a rapporté la présence d'un membre de la famille MARCH dans les exosomes.

4.7. MARCH1 dans les exosomes

4.7.1. Implication de l'ubiquitination de MARCH1 dans son incorporation exosomale

Les motifs tyrosine sont connus pour leur implication dans l'incorporation exosomale. D'un autre côté, l'ubiquitination est également reconnue pour son implication dans ce processus et l'article 1 démontre clairement l'ubiquitination de MARCH1 [172,185]. L'étude de l'incorporation exosomale du double mutant tyrosine a donc permis dans un premier temps de confirmer la fonctionnalité des motifs et dans un deuxième temps d'établir leur implication dans le processus. Toutefois, malgré la plus faible présence du mutant YY dans les exosomes, celui-ci est toujours incorporé. Il est possible que son ciblage aux microvésicules soit également partiellement médié par son ubiquitination. Une approche intéressante afin de répondre à cette question serait de vérifier l'incorporation du mutant M1K-0 dans les exosomes.

4.7.2. Transfert exosomal inter-cellulaire de MARCH1

De nombreuses études portent sur la communication intercellulaire via les exosomes [186]. Par exemple, les cellules nerveuses échangent des ARNs à la synapse cellulaire par

transfert d'exosomes [187]. Étant donné l'implication de MARCH1 dans la réponse anti-inflammatoire à l'IL-10 et le nombre restreint de cellules exprimant celle-ci, il pourrait être avantageux pour l'organisme de propager MARCH1. Ainsi, l'incorporation exosomale de MARCH1 pourrait conduire à sa présence dans les cellules environnantes sans pour autant nécessiter l'activation de sa transcription chez celles-ci. Pour l'instant, le seul signal connu permettant d'activer la transcription de MARCH1 est la stimulation à l'IL-10, or de nombreuses cellules ne possèdent pas le récepteur et ne répondent donc pas à celle-ci [188]. La présence de MARCH1 dans les exosomes serait alors un moyen efficace de permettre l'action de celui-ci dans ces cellules qui ne l'expriment pas. Pour répondre à cette question, il serait intéressant de purifier des exosomes exprimant MARCH1 pour ensuite les faire incorporer par d'autres cellules. Afin de purifier les exosomes, nous utiliserions la même méthode que dans notre deuxième article. Nous transfecterions donc des HEK 293T avec YFP-MARCH1 et un contrôle inactif et 48 heures plus tard, nous isolerions les microvésicules par ultracentrifugation. Les exosomes purifiés seraient par la suite incubés 2h avec des cellules exprimant le CMH II mais non transfectées avec MARCH1. Les cellules qui seraient positives pour la YFP auraient incorporé les exosomes. Par cytométrie en flux, il serait alors possible d'observer l'expression de surface du CMH II. Une diminution de celui-ci témoignerait alors de la possibilité de transférer MARCH1 d'une cellule à l'autre via les exosomes.

Certains groupes proposent que les exosomes pourraient être utilisés dans différentes thérapies. Ainsi, ils seraient utilisés comme vecteurs, par exemples de micro ARNs, et permettraient le transfert de matériel aux cellules de l'organisme [189]. Un autre groupe propose que le ciblage d'antigènes associés aux cancers dans les exosomes serait une avenue de choix pour augmenter l'efficacité des vaccins contre les tumeurs [190]. L'utilisation des exosomes semble donc être une future et intéressante avenue thérapeutique.

4.8. Éléments régulateurs en N-terminal de MARCH1

Notre deuxième article a également permis de démontrer la présence d'éléments de ciblage dans la portion cytoplasmique N-terminale de MARCH1 (voir Figure 3.2.). Ceci avait été démontré pour la molécule murine et nos résultats confirment ces observations précédentes [165]. Néanmoins, la suppression de cette portion de MARCH1 n'a aucun effet sur sa fonction envers le CMH II. De plus, contrairement à la molécule murine, un mutant dépourvu de la portion cytoplasmique extra-RING N-terminale n'est pas exprimé à de plus forts niveaux que la molécule sauvage [165]. L'analyse informatique de la séquence primaire de cette portion de la molécule à l'aide de la base de données EMBL n'a révélé la présence d'aucun motif de ciblage connu.

Dans un premier temps, il serait donc intéressant de générer une série de mutants dont la portion cytoplasmique N-terminale serait de plus en plus courte afin de déterminer les régions importantes de celle-ci. De la même manière que nos mutants ont conduit à l'identification du motif VQNC de la queue cytoplasmique C-terminale, ces mutants de la portion N-terminale seraient étudiés par cytométrie en flux pour leur influence sur l'expression de surface du CMH II. Il demeure possible que ce soit l'association par cette région à d'autres molécules et non la présence de motifs de ciblage qui permette l'envoi de la molécule à certains compartiments. Dans ce cas, des expériences de spectrométrie de masse lors desquels des immunoprécipitations de MARCH1 versus MARCH1ΔNter seraient comparées pourraient permettre l'identification des partenaires impliqués dans cette relocalisation. Une première série d'expériences pourrait être conduite en HEK 293T en raison de la facilité à obtenir de forts niveaux de la molécule dans ces cellules. Aussi, dans la majorité de nos expériences, nous utilisons une fusion de MARCH1 avec la YFP situé en N-terminal de la molécule (YFP-M1). Nous possédons également une fusion où la protéine fluorescence se retrouve en C-terminal (M1-YFP). Afin de minimiser l'impact de la fusion sur les résultats obtenus, il serait important d'utiliser M1-YFP plutôt que YFP-M1.

4.9. Le motif VQNC

Toujours dans le but de caractériser les éléments structuraux requis à la fonction de MARCH1, nous avons généré une série de mutants pour lesquels la queue cytoplasmique C-terminale était de plus en plus courte (voir Figure 3.7.). Ces constructions ont mené à l'identification du motif VQNC et de sa valine comme étant primordiaux pour la fonction de la molécule. Nos résultats de BRET indiquent une modification de l'organisation spatiale des queues cytoplasmiques de MARCH1 lorsque la valine est mutée.

4.9.1. Rôle du VQNC dans la liaison avec le motif doigt de zinc

Le motif VQNC se retrouve également chez EEA1 et ASAP1, deux protéines contenant un domaine doigt de zinc. Ce domaine est structurellement semblable au RING puisqu'il s'agit d'une structure rigide stabilisée par la liaison à un atome de zinc et fréquemment impliquée dans les interactions protéique et à la liaison à l'ADN [191]. Ainsi, le VQNC est potentiellement impliqué dans la liaison aux structures en doigt de zinc et non seulement aux RINGs. Dans le cas où cette hypothèse serait véridique, le motif VQNC aurait alors un rôle plus général. Cette possibilité n'invalide toutefois pas notre modèle selon lequel la liaison du VQNC de MARCH1 à son RING soit requise à l'activité de celui-ci. En effet, la structure du RING est nécessaire à l'activité ligase et un élément qui ne serait pas favorable à la coordination du zinc aurait des effets similaires sur la structure du RING et des motifs doigt de zinc. Nos conclusions concernant l'implication du motif VQNC dans la fonction du RING pourraient alors s'appliquer également aux motifs doigt de zinc en général et ainsi toucher une plus vaste sélection de protéines.

4.10. Mode d'action de MARCH1 et MARCH8

4.10.1. Interaction transitoire avec le substrat

Les résultats présentés ici amènent à se questionner sur les mécanismes sous-jacents à l'activité de MARCH1. En effet, nos résultats ont montré que le CMH II, MARCH1 et

MARCH8 sont présents dans les exosomes, mais que la présence des MARCHs n'affecte pas celle du CMH II dans ceux-ci (voir annexe II). L'association de deux molécules comportant des signaux d'incorporation dans les exosomes devrait normalement avoir un effet positif sur la présence de chacune d'elles dans ceux-ci. L'absence de cette influence permet de supposer que les molécules se dissocient suite à leur interaction et sont incorporées de manière indépendante aux exosomes. L'interaction entre MARCH1 ou MARCH8 et le CMH II serait alors transitoire.

4.10.2. Le site d'ubiquitination

Une étude propose que l'action de MARCH8 ciblerait des composantes de la voie endocytaire alors que MARCH1 agirait directement sur ses substrats [162]. Mis à part leurs différents patrons d'expression, il s'agit d'une première différence notée entre les deux molécules.

Nos résultats ont permis de révéler deux autres différences dans le fonctionnement de MARCH1 et MARCH8. En effet, une étude a montré que MARCH8 se lie à sa cible et l'ubiquitine depuis la surface cellulaire [160]. Au contraire, nos résultats de FRET montrent une association de MARCH1 et HLA-DR dans des vésicules intracellulaires (Figure 3.1). La seconde différence révélée par nos travaux concerne l'implication des signaux tyrosine qui ne seraient pas requis à la fonction de MARCH1, contrairement à MARCH8 (voir section 4.6.). Il est possible que la présence des motifs tyrosine soit requise à l'endocytose du complexe MARCH8-substrat à la membrane cellulaire et alors témoigne des différents modes d'action des molécules. Néanmoins, tel que mentionné plus haut (voir section 4.6.), les motifs de ciblage des deux molécules sont identiques. Un modèle possible pourrait impliquer l'interaction de MARCH8 avec des protéines adaptatrices permettant l'endocytose de celui-ci en complexe avec sa cible. Ainsi, les motifs de ciblage de MARCH8 seraient requis à sa fonction.

4.11. Les particularités de MARCH1 murin et humain

4.11.1 Importance de l'étude de MARCH1 humain

Avant nos travaux, il n'y avait aucune différence de connue entre les formes humaine et murine de MARCH1. Les différences que nous avons révélées pour la forme humaine sont: l'auto-ubiquitination (voir section 4.2.2.) et l'implication des motifs de ciblage (voir section 4.6.). Ces différences sont importantes puisque les mécanismes d'action et de régulation divergent pour ces deux molécules. Dans un but ultime de manipuler MARCH1 dans le traitement de maladies auto-immunes, l'étude de la molécule humaine au détriment de la forme murine est mise de l'avant par ce que nous avons découvert.

4.11.2. Une souris transgénique MARCH1

Les souris transgéniques sont presque indispensables à l'étude des molécules dans un système immunitaire complet. Notre laboratoire a récemment fait l'acquisition d'une lignée de souris KO pour leur expression de MARCH1. Bien que celles-ci soient un outil puissant pour l'étude de l'expression de MARCH1, les résultats ci-inclus démontrent que l'extrapolation des résultats obtenus chez la molécule murine à la molécule humaine est questionnable. Par exemple, les motifs tyrosine de MARCH1 murin sont requis pour sa fonction envers le CMH II dans les cellules humaines alors qu'ils sont dispensables à MARCH1 humain. Ainsi, l'étude de la molécule murine, même dans un contexte humain, montre d'importantes différences. Afin de contrer ce problème et étant donné que nous sommes intéressés à la molécule humaine et non murine, il serait intéressant d'utiliser la souris MARCH1 KO pour générer une souris transgénique exprimant le gène humain. Nous savons que la molécule humaine est fonctionnelle en cellules de souris. Ainsi, MARCH1 humain possède la capacité de réguler négativement le CMH II murin, ce qui rendrait possible l'utilisation de la molécule humaine en souris. Cette souris serait fort utile pour toutes les facettes de l'étude de la forme protéique de MARCH1. Sa régulation, sa

localisation, ses partenaires d'interaction, ses modifications protéiques et ses mécanismes d'action pourraient alors être étudiés. Nous aurions alors les nombreux avantages de l'analyse d'une molécule dans le contexte d'un organisme multicellulaire sans avoir le désavantage d'étudier la forme murine de MARCH1 que nous savons différente de la forme humaine en de nombreux points.

4.12. MARCH1 est un gène suppresseur de tumeurs

L'utilisation de MARCH1 dans le traitement des cancers est une avenue envisageable. Cette idée est d'ailleurs renforcée par une étude parue en 2009. Dans cet article, le groupe du Dr. Largaespada identifia MARCH1 comme étant un gène suppresseur de tumeurs. Ils utilisèrent un modèle de mutagenèse par transposons ciblés dans le foie chez la souris, ce qui leur permit l'identification de 19 gènes [192]. Les gènes identifiés comportaient souvent des insertions chez les souris ayant développé des carcinomes hépatocellulaires. Dans la même étude, des échantillons de tumeurs hépatiques humaines ont confirmé ce résultat.

Ainsi, un défaut de l'induction de MARCH1 dans ces tissus favorise le développement de tumeurs. À l'inverse, la présence de MARCH1 dans ceux-ci pourrait alors permettre la régression ou, du moins, de freiner le développement des cellules cancéreuses. Ces travaux montrent donc l'implication de MARCH1 dans la tumorogénèse. Cette découverte peut sembler contradictoire avec le rôle de MARCH1 dans la régulation de la présentation antigénique. Néanmoins, MARCH1 possède d'autres fonctions dans les réponses immunitaires. Parmi celles-ci, il bloque la glycolyse (résultats non publiés et brièvement discutés en 4.13), un processus essentiel au développement de nombreuses tumeurs. En interférant avec la glycolyse, MARCH1 empêche la génération d'énergie nécessaire à la prolifération rapide des cellules et agit donc comme agent suppresseur de tumeur. L'étude de l'implication de MARCH1 dans la glycolyse est l'un des projets de recherche actuels de notre équipe.

4.13 Les différentes fonctions de MARCH1

Notre étude des éléments structuraux requis à la fonction de MARCH1 concernait la régulation de surface du CMH II. Néanmoins, nous n'avons pas étudié l'importance des différentes portions et domaines de MARCH1 dans l'expression de surface de ses autres cibles connues. De plus, une expérience de spectrométrie de masse nous a permis d'identifier de nouveaux partenaires d'interaction de MARCH1. Parmi ceux-ci se retrouvaient de nombreux gènes impliqués dans la glycolyse. Nos résultats préliminaires montrent un effet négatif de la présence de MARCH1 envers l'activité glycolytique de cellules HeLa et HEK 293T. Il serait donc intéressant de tester les différents éléments étudiés dans l'article 2, tels que l'implication des motifs de ciblage et la localisation de MARCH1, dans l'activité antiglycolytique de la molécule.

Conclusion

L'ubiquitination est une modification protéique permettant un changement rapide au sein de la cellule. Ainsi, les molécules peuvent être modifiées rapidement en réponse aux changements environnementaux. La machinerie d'ubiquitination est si efficace que les virus en ont intégré certains gènes afin d'augmenter leur virulence.

Les MARCHs sont une famille regroupant une dizaine d'E3 ubiquitine ligases membranaires. Ces molécules sont des joueurs clés des réponses immunitaires. De plus en plus d'études portent sur MARCH1, soit le plus caractérisé de la famille. Son implication dans la réponse à anti-inflammatoire à l'IL-10 est primordial tant au niveau de la régulation de la présentation antigénique que de la molécule de co-stimulation CD86. Avant d'entreprendre nos travaux, nous connaissions peu de pré-requis structuraux à la fonction de MARCH1 envers le CMH II. Notre étude a mis en évidence de nombreux éléments dont l'importance était insoupçonnée.

Les deux articles présentés en chapitre 2 et 3 ont répondu à de nombreuses questions concernant le mécanisme par lequel MARCH1 prévient l'expression de surface du CMH II. Ils ont démontré la formation de dimères de MARCH1 et d'hétérodimères avec MARCH8 et MARCH9, l'autoubiquitination de MARCH1 et sa dégradation protéosomale et lysosomale, l'implication des TMs dans la formation de dimères et l'interaction avec le substrat, la fonctionnalité mais la non nécessité des motifs de ciblage de la queue cytoplasmique C-terminale de MARCH1 pour sa fonction, l'interaction de MARCH1 avec son substrat dans des compartiments intracellulaires et la présence d'un motif VQNC dont la valine est responsable de l'orientation spatiale des queues cytoplasmiques. Néanmoins, de nombreux points restent à approfondir. Tels que mentionnés en discussion, l'implication du dimère dans l'interaction MARCH1-CMH II, la présence de MARCH1 dans les radeaux lipidiques et le potentiel transfert cellule-cellule de MARCH1 par voie exosomale sont plusieurs questions qui méritent d'être explorées.

Nous commençons seulement à comprendre MARCH1, ses rôles, ses mécanismes d'action et sa régulation. Les perspectives proposées ne sont que quelques avenues à explorer. Malgré la progression importante des connaissances apportées par notre étude, nous sommes encore loin de comprendre toutes les facettes de MARCH1.

Bibliographie

1. Pancer, Z. & Cooper, M. D. The evolution of adaptive immunity. *Annual review of immunology* **24**, 497–518 (2006).
2. Uthaisangsook, S., Day, N. K., Bahna, S. L., Good, R. A. & Haraguchi, S. Innate immunity and its role against infections. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **88**, 253–64; quiz 265–6, 318 (2002).
3. Le Bon, A. & Tough, D. F. Links between innate and adaptive immunity via type I interferon. *Current Opinion in Immunology* **14**, 432–436 (2002).
4. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–50 (2011).
5. Medzhitov, R. & Janeway, C. A. An ancient system of host defense. *Current Opinion in Immunology* **10**, 12–15 (1998).
6. Medzhitov, R. & Janeway, C. A. Innate Immunity: The Virtues of a Nonclonal System of Recognition. *Cell* **91**, 295–298 (1997).
7. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
8. Luster, A. D. The role of chemokines in linking innate and adaptive immunity. *Current Opinion in Immunology* **14**, 129–135 (2002).
9. Hoebe, K., Janssen, E. & Beutler, B. The interface between innate and adaptive immunity. *Nature immunology* **5**, 971–4 (2004).
10. Neefjes, J., Jongsma, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews. Immunology* **11**, 823–36 (2011).
11. Gallucci, S. & Matzinger, P. Danger signals: SOS to the immune system. *Current Opinion in Immunology* **13**, 114–119 (2001).
12. Matzinger, P. The danger model: a renewed sense of self. *Science (New York, N.Y.)* **296**, 301–5 (2002).
13. Calderon, B. & Unanue, E. R. Antigen presentation events in autoimmune diabetes. *Current opinion in immunology* **24**, 119–28 (2012).
14. Handunnetthi, L., Ramagopalan, S. V., Ebers, G. C. & Knight, J. C. Regulation of major histocompatibility complex class II gene expression, genetic variation and disease. *Genes and immunity* **11**, 99–112 (2010).
15. Edwards, L. J. & Evavold, B. D. T cell recognition of weak ligands: roles of signaling, receptor number, and affinity. *Immunologic research* **50**, 39–48 (2011).
16. Ravichandran, K. S. & Burakoff, S. J. Evidence for differential intracellular signaling via CD4 and CD8 molecules. *The Journal of experimental medicine* **179**, 727–32 (1994).
17. Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annual review of immunology* **20**, 621–67 (2002).
18. Itano, A. A. & Jenkins, M. K. Antigen presentation to naive CD4 T cells in the lymph node. *Nature immunology* **4**, 733–9 (2003).
19. Barry, M. & Bleackley, R. C. Cytotoxic T lymphocytes: all roads lead to death. *Nature reviews. Immunology* **2**, 401–9 (2002).
20. Romagnani, S. T-cell subsets (Th1 versus Th2). *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **85**, 9–18; quiz 18, 21 (2000).
21. Zhu, J. & Paul, W. E. Heterogeneity and plasticity of T helper cells. *Cell research* **20**, 4–12 (2010).
22. King, C. New insights into the differentiation and function of T follicular helper cells. *Nature reviews. Immunology* **9**, 757–66 (2009).
23. Landsverk, O. J. B., Bakke, O. & Gregers, T. F. MHC II and the endocytic pathway: regulation by invariant chain. *Scandinavian journal of immunology* **70**, 184–93 (2009).
24. Ackerman, A. L., Giodini, A. & Cresswell, P. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* **25**, 607–17 (2006).

25. Strawbridge, A. B. & Blum, J. S. Autophagy in MHC class II antigen processing. *Current opinion in immunology* **19**, 87–92 (2007).
26. Lengauer, C., Kinzler, K. W. & Vogelstein, B. Genetic instabilities in human cancers. *Nature* **396**, 643–9 (1998).
27. Houghton, A. N. Cancer antigens: immune recognition of self and altered self. *The Journal of experimental medicine* **180**, 1–4 (1994).
28. Maggioni, C., Braakman, I. & Marsh, M. *Membrane Trafficking in Viral Replication*. **285**, 175–198 (Springer-Verlag: Berlin/Heidelberg, 2005).
29. Rocha, N. & Neefjes, J. MHC class II molecules on the move for successful antigen presentation. *The EMBO journal* **27**, 1–5 (2008).
30. Bouillon, M. *et al*. Lipid raft-dependent and -independent signaling through HLA-DR molecules. *The Journal of biological chemistry* **278**, 7099–107 (2003).
31. Al-Daccak, R., Mooney, N. & Charron, D. MHC class II signaling in antigen-presenting cells. *Current Opinion in Immunology* **16**, 108–113 (2004).
32. CHARRON, D., BRICK-GHANNAM, S., RAMIREZ, R. & MOONEY, N. HLA class-II-mediated B-lymphocyte activation : signal transduction and physiologic consequences. *Research in immunology* **142**, 467–474 (1991).
33. Berger, A. C. & Roche, P. A. MHC class II transport at a glance. *Journal of cell science* **122**, 1–4 (2009).
34. Trombetta, E. S. & Mellman, I. Cell biology of antigen processing in vitro and in vivo. *Annual review of immunology* **23**, 975–1028 (2005).
35. Stockinger, B. Capacity of antigen uptake by B cells, fibroblasts or macrophages determines efficiency of presentation of a soluble self antigen (C5) to T lymphocytes. *European journal of immunology* **22**, 1271–8 (1992).
36. Vyas, J. M., Van der Veen, A. G. & Ploegh, H. L. The known unknowns of antigen processing and presentation. *Nature reviews. Immunology* **8**, 607–18 (2008).
37. Santambrogio, L. & Strominger, J. L. The ins and outs of MHC class II proteins in dendritic cells. *Immunity* **25**, 857–9 (2006).
38. Cresswell, P. Assembly, transport, and function of MHC class II molecules. *Annual review of immunology* **12**, 259–93 (1994).
39. Zhong, G., Romagnoli, P. & Germain, R. N. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *Journal of Experimental Medicine* **185**, 429–38 (1997).
40. Cresswell Invariant Chain Structure and MHC Class II Function. *Cell* **84**, 505–507 (1996).
41. Strubin, M., Berte, C. & Mach, B. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *The EMBO journal* **5**, 3483–8 (1986).
42. Lotteau, V. *et al*. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600–5 (1990).
43. Khalil, H., Brunet, A. & Thibodeau, J. A three-amino-acid-long HLA-DRbeta cytoplasmic tail is sufficient to overcome ER retention of invariant-chain p35. *Journal of cell science* **118**, 4679–87 (2005).
44. Roche, P. A. & Cresswell, P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* **345**, 615–8 (1990).
45. Stumptner, P. & Benaroch, P. Interaction of MHC class II molecules with the invariant chain: role of the invariant chain (81-90) region. *The EMBO journal* **16**, 5807–18 (1997).
46. Anderson, M. S. & Miller, J. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 2282–6 (1992).
47. Pieters, J., Bakke, O. & Dobberstein, B. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* **106**, 831–846 (1993).
48. Sanderson, F. *et al*. Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science* **266**, 1566–1569 (1994).

49. Liljedahl, M. *et al*. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *The EMBO journal* **15**, 4817–24 (1996).
50. Kropshofer, H. *et al*. A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *The EMBO journal* **17**, 2971–81 (1998).
51. Doebele, R. C., Busch, R., Scott, H. M., Pashine, A. & Mellins, E. D. Determination of the HLA-DM Interaction Site on HLA-DR Molecules. *Immunity* **13**, 517–527 (2000).
52. Narayan, K., Su, K. W., Chou, C.-L., Khoruzhenko, S. & Sadegh-Nasseri, S. HLA-DM mediates peptide exchange by interacting transiently and repeatedly with HLA-DR1. *Molecular immunology* **46**, 3157–62 (2009).
53. Marks, M. S. *et al*. A lysosomal targeting signal in the cytoplasmic tail of the beta chain directs HLA-DM to MHC class II compartments. *The Journal of cell biology* **131**, 351–69 (1995).
54. Lindstedt, R., Liljedahl, M., Péléraux, A., Peterson, P. A. & Karlsson, L. The MHC class II molecule H2-M is targeted to an endosomal compartment by a tyrosine-based targeting motif. *Immunity* **3**, 561–572 (1995).
55. Copier, J. *et al*. Targeting signal and subcellular compartments involved in the intracellular trafficking of HLA-DMB. *J. Immunol.* **157**, 1017–1027 (1996).
56. Potter, P. K. *et al*. Accurate intracellular localization of HLA-DM requires correct spacing of a cytoplasmic YTPL targeting motif relative to the transmembrane domain. *European journal of immunology* **29**, 3936–44 (1999).
57. Bonifacino, J. S. & Dell'Angelica, E. C. Molecular bases for the recognition of tyrosine-based sorting signals. *The Journal of cell biology* **145**, 923–6 (1999).
58. Rohrer, J., Schweizer, A., Russell, D. & Kornfeld, S. The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *The Journal of cell biology* **132**, 565–76 (1996).
59. Bonifacino, J. S. & Traub, L. M. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annual review of biochemistry* **72**, 395–447 (2003).
60. Denzin, L. K., Fallas, J. L., Prendes, M. & Yi, W. Right place, right time, right peptide: DO keeps DM focused. *Immunological reviews* **207**, 279–92 (2005).
61. Brunet, A., Samaan, A., Deshaies, F., Kindt, T. J. & Thibodeau, J. Functional characterization of a lysosomal sorting motif in the cytoplasmic tail of HLA-DObeta. *The Journal of biological chemistry* **275**, 37062–71 (2000).
62. Rodionov, D. G. Medium Chains of Adaptor Complexes AP-1 and AP-2 Recognize Leucine-based Sorting Signals from the Invariant Chain. *Journal of Biological Chemistry* **273**, 6005–6008 (1998).
63. Saftig, P. & Klumperman, J. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nature reviews. Molecular cell biology* **10**, 623–35 (2009).
64. Stern, L. J., Potolicchio, I. & Santambrogio, L. MHC class II compartment subtypes: structure and function. *Current opinion in immunology* **18**, 64–9 (2006).
65. Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. & Geuze, H. J. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349**, 669–76 (1991).
66. Pierre, P. *et al*. HLA-DM Is Localized to Conventional and Unconventional MHC Class II–Containing Endocytic Compartments. *Immunity* **4**, 229–239 (1996).
67. Hsing, L. C. & Rudensky, A. Y. The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunological reviews* **207**, 229–41 (2005).
68. Riese, R. J. & Chapman, H. A. Cathepsins and compartmentalization in antigen presentation. *Current Opinion in Immunology* **12**, 107–113 (2000).
69. Villadangos, J. A., Riese, R. J., Peters, C., Chapman, H. A. & Ploegh, H. L. Degradation of mouse invariant chain: roles of cathepsins S and D and the influence of major histocompatibility complex polymorphism. *The Journal of experimental medicine* **186**, 549–60 (1997).
70. Münz, C. Antigen processing via autophagy--not only for MHC class II presentation anymore? *Current opinion in immunology* **22**, 89–93 (2010).
71. Schmid, D. & Münz, C. Innate and adaptive immunity through autophagy. *Immunity* **27**, 11–21 (2007).

72. Denzin, L. K. & Cresswell, P. HLA-DM induces clip dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell* **82**, 155–165 (1995).
73. Liljedahl, M. *et al*. Altered Antigen Presentation in Mice Lacking H2-O. *Immunity* **8**, 233–243 (1998).
74. van Ham, M. *et al*. Modulation of the Major Histocompatibility Complex Class II-Associated Peptide Repertoire by Human Histocompatibility Leukocyte Antigen (Hla)-Do. *Journal of Experimental Medicine* **191**, 1127–1136 (2000).
75. Koppelman, B., Neefjes, J. J., de Vries, J. E. & de Waal Malefyt, R. Interleukin-10 Down-Regulates MHC Class II αβ Peptide Complexes at the Plasma Membrane of Monocytes by Affecting Arrival and Recycling. *Immunity* **7**, 861–871 (1997).
76. Grant, B. D. & Donaldson, J. G. Pathways and mechanisms of endocytic recycling. *Nature reviews. Molecular cell biology* **10**, 597–608 (2009).
77. Reith, W., LeibundGut-Landmann, S. & Waldburger, J.-M. Regulation of MHC class II gene expression by the class II transactivator. *Nature reviews. Immunology* **5**, 793–806 (2005).
78. Mach, B., Steimle, V., Martinez-Soria, E. & Reith, W. Regulation of MHC class II genes: lessons from a disease. *Annual review of immunology* **14**, 301–31 (1996).
79. LeibundGut-Landmann, S. *et al*. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *European journal of immunology* **34**, 1513–25 (2004).
80. van Deventer, S. & Neefjes, J. The Immunoproteasome Cleans up after Inflammation. *Cell* **142**, 517–8 (2010).
81. Seifert, U. *et al*. Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell* **142**, 613–24 (2010).
82. Khalil, H. *et al*. Class II transactivator-induced expression of HLA-DObeta in HeLa cells. *Tissue Antigens* **60**, 372–382 (2002).
83. Nagarajan, U. M. *et al*. Class II Transactivator Is Required for Maximal Expression of HLA-DOB in B Cells. *J. Immunol.* **168**, 1780–1786 (2002).
84. Ting, J. P.-Y. & Trowsdale, J. Genetic Control of MHC Class II Expression. *Cell* **109**, S21–S33 (2002).
85. Boss, J. M. & Jensen, P. E. Transcriptional regulation of the MHC class II antigen presentation pathway. *Current Opinion in Immunology* **15**, 105–111 (2003).
86. Del Pozzo, G., Ciullo, M. & Guardiola, J. Regulation of HLA class II gene expression: the case for posttranscriptional control levels. *Microbes and Infection* **1**, 943–948 (1999).
87. de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *The Journal of experimental medicine* **174**, 1209–20 (1991).
88. Moore, K. W., O'Garra, A., de Waal Malefyt, R., Vieira, P. & Mosmann, T. R. Interleukin-10. *Annual review of immunology* **11**, 165–90 (1993).
89. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. **388**, 782–787 (1997).
90. Pierre, P. *et al*. Developmental regulation of MHC class II transport in mouse dendritic cells. **388**, 787–792 (1997).
91. Villadangos, J. A., Schnorrer, P. & Wilson, N. S. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunological reviews* **207**, 191–205 (2005).
92. Kleijmeer, M. *et al*. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *The Journal of cell biology* **155**, 53–63 (2001).
93. van Niel, G. *et al*. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity* **25**, 885–94 (2006).
94. Shin, J.-S. *et al*. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature* **444**, 115–8 (2006).
95. Ohmura-Hoshino, M. *et al*. Inhibition of MHC Class II Expression and Immune Responses by c-MIR. *J. Immunol.* **177**, 341–354 (2006).

96. Matsuki, Y. *et al*. Novel regulation of MHC class II function in B cells. *The EMBO journal* **26**, 846–54 (2007).
97. De Gassart, A. *et al*. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3491–6 (2008).
98. Young, L. J. *et al*. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nature immunology* **9**, 1244–52 (2008).
99. Lapaque, N. *et al*. Salmonella regulates polyubiquitination and surface expression of MHC class II antigens. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 14052–7 (2009).
100. Chen, Z. J. & Sun, L. J. Nonproteolytic functions of ubiquitin in cell signaling. *Molecular cell* **33**, 275–86 (2009).
101. Bonifacino, J. S. & Weissman, A. M. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annual review of cell and developmental biology* **14**, 19–57 (1998).
102. Haglund, K. & Stenmark, H. Working out coupled monoubiquitination. *Nature cell biology* **8**, 1218–9 (2006).
103. Jiang, X. & Chen, Z. J. The role of ubiquitylation in immune defence and pathogen evasion. *Nature reviews. Immunology* **12**, 35–48 (2012).
104. Hurley, J. H. *et al*. Ubiquitin-binding domains. *The Biochemical journal* **399**, 361–72 (2006).
105. Bhoj, V. G. & Chen, Z. J. Ubiquitylation in innate and adaptive immunity. *Nature* **458**, 430–7 (2009).
106. Zhang, L., Villa, N. Y. & McFadden, G. Interplay between poxviruses and the cellular ubiquitin/ubiquitin-like pathways. *FEBS letters* **583**, 607–14 (2009).
107. Ikeda, F. & Dikic, I. Atypical ubiquitin chains: new molecular signals. "Protein Modifications: Beyond the Usual Suspects" review series. *EMBO reports* **9**, 536–42 (2008).
108. Deshaies, R. J. & Joazeiro, C. A. P. RING domain E3 ubiquitin ligases. *Annual review of biochemistry* **78**, 399–434 (2009).
109. d' Azzo, A., Bongiovanni, A. & Nastasi, T. E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. *Traffic (Copenhagen, Denmark)* **6**, 429–41 (2005).
110. Duncan, L. M. *et al*. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *The EMBO journal* **25**, 1635–45 (2006).
111. Huangfu, W.-C. & Fuchs, S. Y. Ubiquitination-dependent regulation of signaling receptors in cancer. *Genes & cancer* **1**, 725–34 (2010).
112. Haglund, K. Distinct monoubiquitin signals in receptor endocytosis. *Trends in Biochemical Sciences* **28**, 598–604 (2003).
113. Adhikari, A. & Chen, Z. J. Diversity of polyubiquitin chains. *Developmental cell* **16**, 485–6 (2009).
114. Ye, Y. & Rape, M. Building ubiquitin chains: E2 enzymes at work. *Nature reviews. Molecular cell biology* **10**, 755–64 (2009).
115. Thrower, J. S., Hoffman, L., Rechsteiner, M. & Pickart, C. M. Recognition of the polyubiquitin proteolytic signal. *The EMBO journal* **19**, 94–102 (2000).
116. Varadan, R. *et al*. Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. *The Journal of biological chemistry* **279**, 7055–63 (2004).
117. Piper, R. C. & Lehner, P. J. Endosomal transport via ubiquitination. *Trends in cell biology* **21**, 647–55 (2011).
118. Saeki, Y. *et al*. Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome. *EMBO Journal* **28**, 359–371 (2009).
119. Gao, M. & Karin, M. Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. *Molecular cell* **19**, 581–93 (2005).
120. Cadwell, K. & Coscoy, L. The specificities of Kaposi's sarcoma-associated herpesvirus-encoded E3 ubiquitin ligases are determined by the positions of lysine or cysteine residues within the intracytoplasmic domains of their targets. *Journal of virology* **82**, 4184–9 (2008).

121. Wang, Herr, R. a & Hansen, T. Viral and cellular MARCH ubiquitin ligases and cancer. *Seminars in cancer biology* **18**, 441–50 (2008).
122. Cadwell, K. & Coscoy, L. Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science (New York, N.Y.)* **309**, 127–30 (2005).
123. Jin, J., Li, X., Gygi, S. P. & Harper, J. W. Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. *Nature* **447**, 1135–8 (2007).
124. Boname, J. M. *et al*. Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. *Traffic (Copenhagen, Denmark)* **11**, 210–20 (2010).
125. Liu, Y.-C. Ubiquitin ligases and the immune response. *Annual review of immunology* **22**, 81–127 (2004).
126. Nathan, J. a & Lehner, P. J. The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. *Experimental cell research* **315**, 1593–600 (2009).
127. Boname, J. M., de Lima, B. D., Lehner, P. J. & Stevenson, P. G. Viral Degradation of the MHC Class I Peptide Loading Complex. *Immunity* **20**, 305–317 (2004).
128. Zhang, H.-G., Wang, J., Yang, X., Hsu, H.-C. & Mountz, J. D. Regulation of apoptosis proteins in cancer cells by ubiquitin. *Oncogene* **23**, 2009–15 (2004).
129. Wilson, J. E., Katkere, B. & Drake, J. R. Francisella tularensis induces ubiquitin-dependent major histocompatibility complex class II degradation in activated macrophages. *Infection and immunity* **77**, 4953–65 (2009).
130. Lehner, P. J., Hoer, S., Dodd, R. & Duncan, L. M. Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. *Immunological reviews* **207**, 112–25 (2005).
131. Lipkowitz, S. & Weissman, A. M. RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nature reviews. Cancer* **11**, 629–43 (2011).
132. Borden, K. L. & Freemont, P. S. The RING finger domain: a recent example of a sequence—structure family. *Current Opinion in Structural Biology* **6**, 395–401 (1996).
133. Bartee, E. *et al*. Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins. *Journal of virology* **78**, 1109–1120 (2004).
134. Kawai, H., Lopez-Pajares, V., Kim, M. M., Wiederschain, D. & Yuan, Z.-M. RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer research* **67**, 6026–30 (2007).
135. Linke, K. *et al*. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell death and differentiation* **15**, 841–8 (2008).
136. Bazirgan, O. a, Garza, R. M. & Hampton, R. Y. Determinants of RING-E2 fidelity for Hrd1p, a membrane-anchored ubiquitin ligase. *The Journal of biological chemistry* **281**, 38989–9001 (2006).
137. Canning, M., Boutell, C., Parkinson, J. & Everett, R. D. A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. *The Journal of biological chemistry* **279**, 38160–8 (2004).
138. Ishido, S., Goto, E., Matsuki, Y. & Ohmura-Hoshino, M. E3 ubiquitin ligases for MHC molecules. *Current opinion in immunology* **21**, 78–83 (2009).
139. Stevenson, P. G., Efstathiou, S., Doherty, P. C. & Lehner, P. J. Inhibition of MHC class I-restricted antigen presentation by gamma 2-herpesviruses. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8455–60 (2000).
140. Neipel, F., Albrecht, J. C. & Fleckenstein, B. Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? *Journal of virology* **71**, 4187–92 (1997).
141. Mehnert, M., Sommer, T. & Jarosch, E. ERAD ubiquitin ligases: multifunctional tools for protein quality control and waste disposal in the endoplasmic reticulum. *BioEssays : news and reviews in molecular, cellular and developmental biology* **32**, 905–13 (2010).
142. Tamura, T., Sunryd, J. C. & Hebert, D. N. Sorting things out through endoplasmic reticulum quality control. (2010).at <http://informahealthcare.com/doi/abs/10.3109/09687688.2010.495354>
143. Thomas, M. *et al*. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 1656–61 (2008).

144. Coscoy, L. & Ganem, D. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8051–6 (2000).
145. Harris, S., Lang, S. M. & Means, R. E. Characterization of the rhesus fibromatosis herpesvirus MARCH family member rfK3. *Virology* **398**, 214–23 (2010).
146. Ishido, S., Wang, C., Lee, B. S., Cohen, G. B. & Jung, J. U. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *Journal of virology* **74**, 5300–9 (2000).
147. Sanchez, D. J., Coscoy, L. & Ganem, D. Functional organization of MIR2, a novel viral regulator of selective endocytosis. *The Journal of biological chemistry* **277**, 6124–30 (2002).
148. Ishido, S. *et al*. Inhibition of Natural Killer Cell–Mediated Cytotoxicity by Kaposi's Sarcoma–Associated Herpesvirus K5 Protein. *Immunity* **13**, 365–374 (2000).
149. Means, R. E., Ishido, S., Alvarez, X. & Jung, J. U. Multiple endocytic trafficking pathways of MHC class I molecules induced by a Herpesvirus protein. *The EMBO journal* **21**, 1638–49 (2002).
150. Ohmura-hoshino, M., Goto, E., Matsuki, Y., Aoki, M. & Mito, M. JB Minireview — Membrane Traffic in Physiology and Pathology A Novel Family of Membrane-Bound E3 Ubiquitin Ligases. *Genome* **154**, 147–154 (2006).
151. Nathan, J. A. *et al*. The ubiquitin E3 ligase MARCH7 is differentially regulated by the deubiquitylating enzymes USP7 and USP9X. *Traffic (Copenhagen, Denmark)* **9**, 1130–45 (2008).
152. Nakamura, N., Fukuda, H., Kato, A. & Hirose, S. MARCH-II is a syntaxin-6-binding protein involved in endosomal trafficking. *Molecular biology of the cell* **16**, 1696–710 (2005).
153. Baravalle, G. *et al*. Ubiquitination of CD86 is a key mechanism in regulating antigen presentation by dendritic cells. *Journal of immunology (Baltimore, Md. : 1950)* **187**, 2966–73 (2011).
154. Hoer, S., Smith, L. & Lehner, P. J. MARCH-IX mediates ubiquitination and downregulation of ICAM-1. *FEBS letters* **581**, 45–51 (2007).
155. Nakamura, N., Kimura, Y., Tokuda, M., Honda, S. & Hirose, S. MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. *EMBO reports* **7**, 1019–22 (2006).
156. Morokuma, Y. *et al*. MARCH-XI, a novel transmembrane ubiquitin ligase implicated in ubiquitin-dependent protein sorting in developing spermatids. *The Journal of biological chemistry* **282**, 24806–15 (2007).
157. Kreft, S. G., Wang, L. & Hochstrasser, M. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *The Journal of biological chemistry* **281**, 4646–53 (2006).
158. Muthukumarana, P. A. D. S. *et al*. Evidence for functional inter-relationships between FOXP3, leukaemia inhibitory factor, and axotrophin/MARCH-7 in transplantation tolerance. *International immunopharmacology* **6**, 1993–2001 (2006).
159. Goto, E. *et al*. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. *The Journal of biological chemistry* **278**, 14657–68 (2003).
160. Goto, E. *et al*. An excellent monitoring system for surface ubiquitination-induced internalization in mammals. *PloS one* **3**, e1490 (2008).
161. Corcoran, K. *et al*. Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). *The Journal of biological chemistry* **286**, 37168–80 (2011).
162. Jahnke, M., Trowsdale, J. & Kelly, A. P. Ubiquitination of Human Leukocyte Antigen (HLA)-DM by Different Membrane-associated RING-CH (MARCH) Protein Family E3 Ligases Targets Different Endocytic Pathways.Jahnke, M., Trowsdale, J., & Kelly, A. P. (2012). Ubiquitination of Human Leukocyte Antigen . *The Journal of biological chemistry* **287**, 7256–64 (2012).
163. Thibodeau, J. *et al*. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *European journal of immunology* **38**, 1225–30 (2008).
164. Galbas, T., Steimle, V., Lapointe, R., Ishido, S. & Thibodeau, J. MARCH1 down-regulation in IL-10-activated B cells increases MHC class II expression. *Cytokine* (2012).doi:10.1016/j.cyto.2012.03.015

165. Jabbour, M., Campbell, E. M., Fares, H. & Lybarger, L. Discrete domains of MARCH1 mediate its localization, functional interactions, and posttranscriptional control of expression. *Journal of immunology* **183**, 6500–12 (2009).
166. Tze, L. E. *et al*. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *The Journal of experimental medicine* **208**, 149–65 (2011).
167. Gilboa, E. DC-based cancer vaccines. *The Journal of clinical investigation* **117**, 1195–203 (2007).
168. Evel-Kabler, K. & Chen, S.-Y. Dendritic cell-based tumor vaccines and antigen presentation attenuators. *Molecular therapy : the journal of the American Society of Gene Therapy* **13**, 850–8 (2006).
169. He, X. *et al*. Structural Snapshot of Aberrant Antigen Presentation Linked to Autoimmunity. *Immunity* **17**, 83–94 (2002).
170. Ramagopalan, S. V. & Ebers, G. C. Multiple sclerosis: major histocompatibility complexity and antigen presentation. *Genome medicine* **1**, 105 (2009).
171. Chabot, J. R., Pedraza, J. M., Luitel, P. & van Oudenaarden, A. Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock. *Nature* **450**, 1249–52 (2007).
172. Bourgeois-Daigneault, M.-C. & Thibodeau, J. Autoregulation of MARCH1 Expression by Dimerization and Autoubiquitination. *Journal of immunology* **188**, jimmunol.1102708– (2012).
173. Plechanovová, A. *et al*. Mechanism of ubiquitylation by dimeric RING ligase RNF4. *Nature structural & molecular biology* **18**, 1052–9 (2011).
174. Liew, C. W., Sun, H., Hunter, T. & Day, C. L. RING domain dimerization is essential for RNF4 function. *The Biochemical journal* **431**, 23–9 (2010).
175. Bartee, E. *et al*. Membrane-Associated RING-CH proteins associate with Bap31 and target CD81 and CD44 to lysosomes. *PloS one* **5**, e15132 (2010).
176. Staubach, S. & Hanisch, F.-G. Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert review of proteomics* **8**, 263–77 (2011).
177. Huby, R. D. J. Intracellular Phosphotyrosine Induction by Major Histocompatibility Complex Class II Requires Co-aggregation with Membrane Rafts. *Journal of Biological Chemistry* **274**, 22591–22596 (1999).
178. Meyer zum Bueschenfelde, C. O., Unternaehrer, J., Mellman, I. & Bottomly, K. Regulated Recruitment of MHC Class II and Costimulatory Molecules to Lipid Rafts in Dendritic Cells. *J. Immunol.* **173**, 6119–6124 (2004).
179. Muppidi, J. R. & Siegel, R. M. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nature immunology* **5**, 182–9 (2004).
180. Lingwood, D. & Simons, K. Lipid rafts as a membrane-organizing principle. *Science (New York, N.Y.)* **327**, 46–50 (2010).
181. Simons, K. Jamming the endosomal system: lipid rafts and lysosomal storage diseases. *Trends in Cell Biology* **10**, 459–462 (2000).
182. de Gassart, A., Geminard, C., Fevrier, B., Raposo, G. & Vidal, M. Lipid raft-associated protein sorting in exosomes. *Blood* **102**, 4336–44 (2003).
183. Harder, T., Scheiffele, P., Verkade, P. & Simons, K. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *The Journal of cell biology* **141**, 929–42 (1998).
184. Gauvreau, M.-E. *et al*. Sorting of MHC class II molecules into exosomes through a ubiquitin-independent pathway. *Traffic* **10**, 1518–27 (2009).
185. Buschow, S. I., Liefhebber, J. M. P., Wubbolts, R. & Stoorvogel, W. Exosomes contain ubiquitinated proteins. *Blood cells, molecules & diseases* **35**, 398–403 (2005).
186. Rechavi, O., Goldstein, I. & Kloog, Y. Intercellular exchange of proteins: the immune cell habit of sharing. *FEBS letters* **583**, 1792–9 (2009).
187. Smalheiser, N. R. Exosomal transfer of proteins and RNAs at synapses in the nervous system. *Biology direct* **2**, 35 (2007).
188. Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* **19**, 683–765 (2001).

189. Lässer, C. Exosomal RNA as biomarkers and the therapeutic potential of exosome vectors. *Expert opinion on biological therapy* (2012).doi:10.1517/14712598.2012.680018
190. Hartman, Z. C. *et al*. Increasing vaccine potency through exosome antigen targeting. *Vaccine* **29**, 9361–7 (2011).
191. Matthews, J. M. & Sunde, M. Zinc fingers--folds for many occasions. *IUBMB life* **54**, 351–5 (2002).
192. Keng, V. W. *et al*. A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma. *Nature biotechnology* **27**, 264–74 (2009).

Annexe I

Published in final edited form as:
Eur J Immunol. 2008 May ; 38(5): 1225–1230. doi:10.1002/eji.200737902.

Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes

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Abstract

IL-10 is a potent anti-inflammatory cytokine interfering with antigen presentation by inducing the intracellular sequestration of MHC class II (MHC-II) molecules. Here we studied the contribution of membrane-associated RING-CH (MARCH) ubiquitin ligase family members to the IL-10-induced down-regulation of MHC-II molecules. We found that MARCH1 and MARCH8 proteins are the most potent family members for the down-regulation of MHC-II surface expression in transfected cells, but only MARCH1 mRNA expression is strongly induced by IL-10 in human primary monocytes. We detected mono-and poly-ubiquitinated forms of MHC-II molecules both in IL-10-treated monocytes and in cells transfected with MARCH1. We also show direct interaction between MHC-II and MARCH1 molecules in co-immunoprecipitation assays. Finally, we found that siRNA-mediated knockdown of MARCH1 reverses IL-10-induced MHC-II down-regulation in primary monocytes. Thus, the immunosuppressive effect of IL-10 on antigen presentation is mediated through induced expression of MARCH1.

Keywords

Antigen-presenting cells; Cytokines; Immune regulation; Inflammation; MHC

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Supporting Information for this article is available at www.wiley-vch.de/contents/jc_2040/2008/37902_s.pdf

Conflict of interest
The authors declare no financial or commercial conflict of interest.

Introduction

MHC class II (MHC-II) molecules play an essential role during the cellular immune response by presenting pathogen-derived peptides to $CD4^+$ T helper cells. MHC-II genes and molecules show complex and regulated expression patterns controlled both at the transcriptional and post-translational levels. Constitutive and IFN-γ-inducible MHC-II gene expression are largely under the control of the MHC-II transactivator CIITA [1,2].

Several examples of post-translational control of MHC-II transport have been described, especially in the myeloid lineages of cells. In monocyte-derived immature DC, MHC-II molecules are mainly sequestered in intracellular compartments and DC maturation leads to re-localization of MHC-II molecules at the cell surface, while at the same time MHC-II gene transcription is shut off [3–5]. Interestingly, it has been shown recently that intracellular localization of MHC-II molecules in immature DC coincides with ubiquitination of the cytoplasmic tail of MHC-II β-chains by an as yet unidentified ubiquitin ligase, and that MHC-II ubiquitination is lost during DC maturation [6,7].

In monocytes, the capacity to stimulate CD4 Tcell responses is strongly inhibited by the immunosuppressive activity of IL-10. This pleiotropic anti-inflammatory cytokine interferes with antigen presentation by down-regulating both MHC-II and the costimulatory molecules CD80 and CD86 [8–10]. In human primary monocytes, IL-10 did not affect MHC-II gene transcription, protein synthesis or loading of the MHC-II αβ complexes with antigenic peptides. Rather, IL-10-treatment caused the intracellular accumulation of mature MHC-II complexes and prevented their display at the plasma membrane by an unidentified mechanism [9].

Membrane-associated RING-CH (MARCH) proteins are cellular homologs of viral transmembrane E3-ubiquitin ligases, such as the KSHV8 K3 (MIR 1) and K5 (MIR 2) molecules, which have been shown to ubiquitinate the cytoplasmic tails of MHC-I molecules [11,12]. Nine human MARCH protein family members have been described [13]. MARCH8 (c-MIR) was shown to down-regulate CD86 [14], an activity that was shared by MARCH1 and MARCH2 [13]. More recently, transgenic overexpression of MARCH8 led to reduced MHC-II cell surface expression in mice [15], while B cells from MARCH1-deficient mice showed increased expression of MHC-II at the plasma membrane [16].

Thus, IL-10-induced down-regulation of cell surface MHC-II and CD86 in monocytes was reminiscent of the activities of MARCH8 and MARCH1. Here, we identify MARCH1 as the mediator of the IL-10-induced immunosuppressive effect on the MHC-II antigen-presentation pathway.

Results and discussion

IL-10 induces the expression of MARCH1

As expected, IL-10 strongly inhibited HLA-DR and CD86 cell surface expression in $CD14^+$ primary monocytes (Fig. 1A) and MHC-II molecules accumulated in internal vesicles in these cells (Supporting Information Fig. S1). We first compared the capacity of MARCH protein family members with a similar membrane topology to MARCH1 and −8 [13] to down-regulate MHC-II cell surface expression. We found that the highly homologous MARCH1 and −8 proteins were the most potent in reducing the CIITA-induced cell surface expression of HLA-DR in transiently transfected 293EBNA cells, but other MARCH proteins also had a certain inhibitory effect under these conditions (Fig. 1B). MARCH4 expression negatively affected cell survival and thus seemed to have a more general effect (data not shown). MARCH1 and −8 efficiently down-regulated all three human MHC-II isotypes; HLA-DR, -DQ, and -DP (Supporting Information Fig. S2).

Next, we analyzed *via* quantitative RT-PCR the mRNA expression levels of MARCH family members in cells treated with IL-10 (Fig. 1C). In untreated primary monocytes, the MARCH1, −2 and −8 mRNA were at comparable low levels, which were not affected by treatment with IFN-γ (Fig. 1C, lanes 1 and 2). However, addition of IL-10 for 16 h increased MARCH1 mRNA expression over 40-fold for the representative donor shown in Fig. 1C (lane 4), while MARCH2 and −8 levels increased only 2–3-fold. IL-10-induced expression of MARCH1 was independent of the presence of IFN-γ (data not shown). The lack of sensitivity of MARCH-specific antibodies precluded the analysis of protein expression in these cells. MARCH3, −4 and −9 mRNA expression levels were undetectable or very low and not responsive to IL-10 or IFN-γ in monocytes (data not shown). These results suggested that IL-10-induced MARCH1 expression may be important for the observed immunosuppressive effects in monocytes.

MARCH1 ubiquitinates HLA-DR molecules

Ubiquitination of mouse MHC-II β-chains by MARCH proteins has been described recently [15,16].We thus looked for ubiquitinated forms of HLA-DR in HeLa/CIITA cells expressing MARCH1. HLA-DR molecules were immunoprecipitated with mAb L243 and analyzed by western blotting using the ubiquitin-specific mAb P4D1 (Fig. 2A, upper panel). HLA-DR molecules showed the presence of a band of 40–42 kDa, which is compatible with the size of an HLA-DR β-chain (28–30 kDa) bearing one ubiquitin moiety (arrowhead), only in MARCH1-positive and not in vector-transfected cells (Fig. 2A). A band of similar size corresponding to mono-ubiquitinated MHC-II I-Aβ was observed in immature mouse DC [6]. Interestingly, a typical ladder of ubiquitinated material (bracket) was detected at higher molecular weights, suggesting that the β-chain is also poly-ubiquitinated in MARCH1-positive cells. L243 recognizes peptide-loaded MHC-II [17], indicating that MARCH1 can ubiquitinate mature HLA-DR molecules.

IL-10 induces ubiquitination of MHC-II in monocytes

We next immunoprecipitated HLA-DR molecules from IL-10-treated monocytes. western blotting for ubiquitin revealed the presence of a major band of about 40 kDa and a smear of higher molecular weight in the sample treated with IL-10 (Fig. 2B, left panel). This is very similar to the pattern observed in HeLa/CIITA cells transfected with MARCH1 (Fig. 2A), suggesting that IL-10 induces both mono- and poly-ubiquitination of MHC-II molecules. Bands corresponding to ubiquitinated HLA-DRβ chains could not be revealed when the immunoprecipitated material was re-blotted with XD5.117, probably due to their low abundance (Fig. 2B, right panel). These results clearly indicate that IL-10 induces ubiquitination of HLA-DR molecules in monocytes.

MARCH1 interacts with HLA-DR

The presence of a RING-CH E3-type ubiquitin ligase domain in MARCH1 suggests that the latter comes close to MHC-II molecules in a multimolecular ubiquitination complex. We tested for direct interaction between HLA-DR molecules and MARCH1 by co-immunoprecipitation. 293T cells were transiently transfected with HLA-DR and an enhanced yellow fluorescent protein (EYFP)-MARCH1 fusion protein either alone or in combination. EYFPMARCH1 was fully functional for the down-regulation of MHC-II molecules (Supporting Information Fig. S3). HLA-DR molecules were immunoprecipitated with mAb L243 and analyzed by western blotting (Fig. 2C). A GFP-specific antiserum revealed co-immunoprecipitated EYFP-MARCH1 only in cells co-expressing HLA-DR and EYFP-MARCH1 (Fig. 2C, top panel, lane 3). We also detected *in vivo* interactions between HLA-DR and MARCH1 in intracellular vesicles by fluorescence energy transfer (FRET) microscopy (data not shown). These results suggest that HLA-DRβ is a direct substrate for the MARCH1 E3 ubiquitin ligase activity.

Knockdown of MARCH1 reverses IL-10-induced down-regulation of MHC-II in monocytes

We transfected IFN-γ-stimulated primary monocytes with fluorescent siRNA in the presence or absence of IL-10. Control siRNA had no effect on MHC-II expression under any condition tested, nor did MARCH1-specific siRNA affect the levels of cell surface MHC-II expression in the absence of IL-10 (Fig. 3A). In contrast, knockdown of MARCH1 strongly inhibited IL-10-dependent down-regulation of cell surface HLA-DR (Fig. 3A, compare green and red profiles). MARCH1 protein levels could not be analyzed due to lack of antibody sensitivity, but MARCH1 mRNA levels were reduced by 70% in cells treated with MARCH1-directed siRNA (Fig. 3B). These results demonstrate that MARCH1 is necessary for the IL-10-induced MHC-II down-regulation in monocytes.

Concluding remarks

The results presented here provide strong evidence that negative modulation of antigen-presentation function in monocytes by IL-10 is mediated by the induction of MARCH1. Our finding of ubiquitinated HLA-DR molecules in MARCH1-transfected cells and in IL-10-treated monocytes is the first demonstration of this modification in human MHC-II molecules. Down-regulation of murine MHC-II proteins by murine MARCH1 and −8 has been found to be dependent on the single lysine residue in the cytoplasmic tail of the β-chain [15,16]. As expected, we found that the corresponding lysine residue in the cytoplasmic tail of HLA-DRβ is also important for MARCH1-induced down-regulation of human HLA-DR molecules (Supporting Information Fig. S3). Co-immunoprecipitation indicates that MARCH1 and HLA-DR molecules interact directly. We detected a relatively important fraction of HLA-DR molecules, both in MARCH-transfected cells and in IL-10-treated monocytes, that appeared to be mono-ubiquitinated in addition to poly-ubiquitinated. Mono- and oligo-ubiquitination have been shown to be important sorting signals for membrane proteins, inducing mostly sorting to endosomal and lysosomal vesicles [18]. Accordingly, studies in mouse DC showed that ubiquitination prevents recycling and increases the targeting of MHC-II into luminal vesicles of multivesicular bodies [7,16].

While MARCH1 and −8 have very similar effects in down-regulating MHC-II, they appear to be expressed quite independently. MARCH8 expression appears to be broadly distributed with highest levels in lung and pancreas [13]. On the other hand, the highest levels of human MARCH1 mRNA expression was found in lymph node and splenic tissues [13], and MARCH1 is important for the regulation of MHC-II in murine B cells [16]. In addition, MARCH1 is expressed in immature human DC and is down-regulated during DC maturation [19]. Our results show that IL-10 modulates mainly the expression of MARCH1 and this is the first demonstration of the regulation of MARCH gene expression by cytokines and its impact under physiological conditions. Analysis of the contribution of MARCH gene regulation to immunopathology will be of great future interest.

Materials and methods

Plasmids

Expression vectors for the different MARCH protein, EBS-CIITA and EBS-enhanced GFP (EGFP) constructs have been described previously [13,20]. An N-terminal fusion between EYFP (Clontech) and MARCH1 was generated by PCR and cloned into the cDNA expression vector pcDNA3.1 (Invitrogen).

Antibodies

The HLA-DR-specific mAb L243 and XD5.117 have been described previously [21]. L243-allophycocyanin and CD86-PE (IT2.2) were from BD Biosciences. HLA-DR specific HK14-

QantumRed and FLAG-specific M2 mAb were from Sigma. P4D1 is specific for ubiquitin (Covance). The GFP-specific rabbit antiserum was from Invitrogen. Rat anti-human IL-10 was from Serotec.

Cell culture

Experiments with human primary monocytes were approved by institutional ethics committees. Human monocytes were isolated by positive selection of $CD14^+$ cells (Miltenyi Biotech) from PBMC obtained by leukapheresis. Cells were incubated for 16 h at 37°C with human recombinant IFN-γ and IL-10 (Peprotech) at 167 and 0.1 ng/mL, respectively. siRNA experiments were performed on fresh monocytes using non-targeting control or MARCH1-specific cocktails of siRNA from Qiagen (1.3 nM; coupled to Alexa 488) and Dharmacon (ON-TARGETplus SMARTpool; 0.6 nM). Cells were electroporated prior to the addition of IFN-γ and IL-10 using an Amaxa Nucleofector II and the Amaxa Monocyte Nucleofection kit. Transfection efficiency was between 20 and 40% and only transfected fluorescent cells were found to be responsive to IL-10. Flow cytometry analysis was performed on live, Alexa 488-positive cells.

Immunoprecipitation and western blotting

HeLa/CIITA cells and primary monocytes were lysed in buffer containing 1% Triton X-100, 25 mM *N*-ethylmaleimide, 5 μM MG132 (all from Sigma) and protease inhibitors (Roche). MHC-II molecules were immunoprecipitated with L243 and heated at 95°C for 5 min in gel-loading buffer. Samples were resolved by SDS-PAGE and analyzed by western blotting. For the co-immunoprecipitation experiment, 293T cells were lysed in buffer containing 1% CHAPS.

Quantitative RT-PCR analysis

Total RNA from 10^6 frozen monocytes was isolated (Absolutely RNA kit; Stratagene) and first-strand cDNA generated (Expand RT, Roche). Real-time PCR was performed using SYBR Green reagents (Invitrogen) on a Stratagene MX3000P instrument. Samples were normalized for HPRT expression. Quantification was carried out *via* standard dilution curves of linearized plasmid DNA. All primer pairs except those for MARCH2 are located in different exons or span intron/exon boundaries. All PCR experiments were repeated at least twice with highly comparable results. Primer sequences are shown in the Supporting Information Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Rafick Sékaly for cDNA and vectors, and Philippe Pierre for helpful discussions. This work was supported by NIH grants R01 CA/AI094011–04 and R21 CA109674–02, and the Medical Research Foundation to K.F. E.B. is supported by an institutional training grant for molecular pathogenesis (T32 AI007472). J. Thibodeau, A.B. and V.S. are scholars of the Fonds de la recherche en santé du Québec (FRSQ). J. Thibodeau is supported by a Canadian Institutes for Health Research (CIHR)-INSERM International Scientific Exchanges fellowship. D.L. holds the Novartis / Canadian Liver Foundation Hepatology Research Chair. J. Thibodeau and D.L. are supported by the CIHR and INSERM. V.S. is supported by the National Science and Engineering Research Council of Canada (NSERC, grant 262845–03) and the Canadian Foundation for Innovation (CFI).

References

1. Steimle V, Otten LA, Zufferey M, Mach B. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell 1993;75:135–146. [PubMed: 8402893]

2. LeibundGut-Landmann S, Waldburger JM, Krawczyk M, Otten LA, Suter T, Fontana A, Acha-Orbea H, Reith W. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. Eur. J. Immunol 2004;34:1513–1525. [PubMed: 15162420]
3. Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature 1997;388:782–787. [PubMed: 9285591]
4. Pierre P, Turley SJ, Gatti E, Hull M, Meltzer J, Mirza A, Inaba K, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. Nature 1997;388:787–792. [PubMed: 9285592]
5. Landmann S, Muhlethaler-Mottet A, Bernasconi L, Suter T, Waldburger JM, Masternak K, Arrighi JF, et al. Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. J. Exp. Med 2001;194:379–391. [PubMed: 11514596]
6. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature 2006;444:115–118. [PubMed: 17051151]
7. van Niel G, Wubbolts R, Ten Broeke T, Buschow SI, Ossendorp FA, Melief CJ, Raposo G, et al. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. Immunity 2006;25:885–894. [PubMed: 17174123]
8. Willems F, Marchant A, Delville JP, Gerard C, Delvaux A, Velu T, de Boer M, Goldman M. Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. Eur. J. Immunol 1994;24:1007–1009. [PubMed: 7512027]
9. Koppelman B, Neefjes JJ, de Vries JE, de Waal Malefyt R. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. Immunity 1997;7:861–871. [PubMed: 9430231]
10. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol 2001;19:683–765. [PubMed: 11244051]
11. Lehner PJ, Hoer S, Dodd R, Duncan LM. Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. Immunol. Rev 2005;207:112–125. [PubMed: 16181331]
12. Ohmura-Hoshino M, Goto E, Matsuki Y, Aoki M, Mito M, Uematsu M, Hotta H, Ishido S. A novel family of membrane-bound e3 ubiquitin ligases. J. Biochem. (Tokyo) 2006;140:147–154. [PubMed: 16954532]
13. Bartee E, Mansouri M, Hovey Nerenberg BT, Gouveia K, Fruh K. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J. Virol 2004;78:1109–1120. [PubMed: 14722266]
14. Goto E, Ishido S, Sato Y, Ohgimoto S, Ohgimoto K, Nagano-Fujii M, Hotta H. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. J. Biol. Chem 2003;278:14657–14668. [PubMed: 12582153]
15. Ohmura-Hoshino M, Matsuki Y, Aoki M, Goto E, Mito M, Uematsu M, Kakiuchi T, et al. Inhibition of MHC class II expression and immune responses by c-MIR. J. Immunol 2006;177:341–354. [PubMed: 16785530]
16. Matsuki Y, Ohmura-Hoshino M, Goto E, Aoki M, Mito-Yoshida M, Uematsu M, Hasegawa T, et al. Novel regulation of MHC class II function in B cells. EMBO J 2007;26:846–854. [PubMed: 17255932]
17. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature 1990;345:615–618. [PubMed: 2190094]
18. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multivesicular-body sorting. Nat. Rev. Mol. Cell. Biol 2002;3:893–905. [PubMed: 12461556]
19. de Gassart A, Camossetto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, Gatti E. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc. Natl. Acad. Sci. USA 2008;105:3491–3496. [PubMed: 18305173]
20. Hake SB, Masternak K, Kammerbauer C, Janzen C, Reith W, Steimle V. CIITA leucine rich repeats control nuclear localisation, *in vivo* recruitment to the MHC class II enhanceosome, and MHC class II gene transactivation. Mol. Cell. Biol 2000;20:7716–7725. [PubMed: 11003667]
21. Khalil H, Brunet A, Saba I, Terra R, Sekaly RP, Thibodeau J. The MHC class II beta chain cytoplasmic tail overcomes the invariant chain p35-encoded endoplasmic reticulum retention signal. Int. Immunol 2003;15:1249–1263. [PubMed: 13679394]

Abbreviations

MARCH, membrane-associated RING-CH; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein.

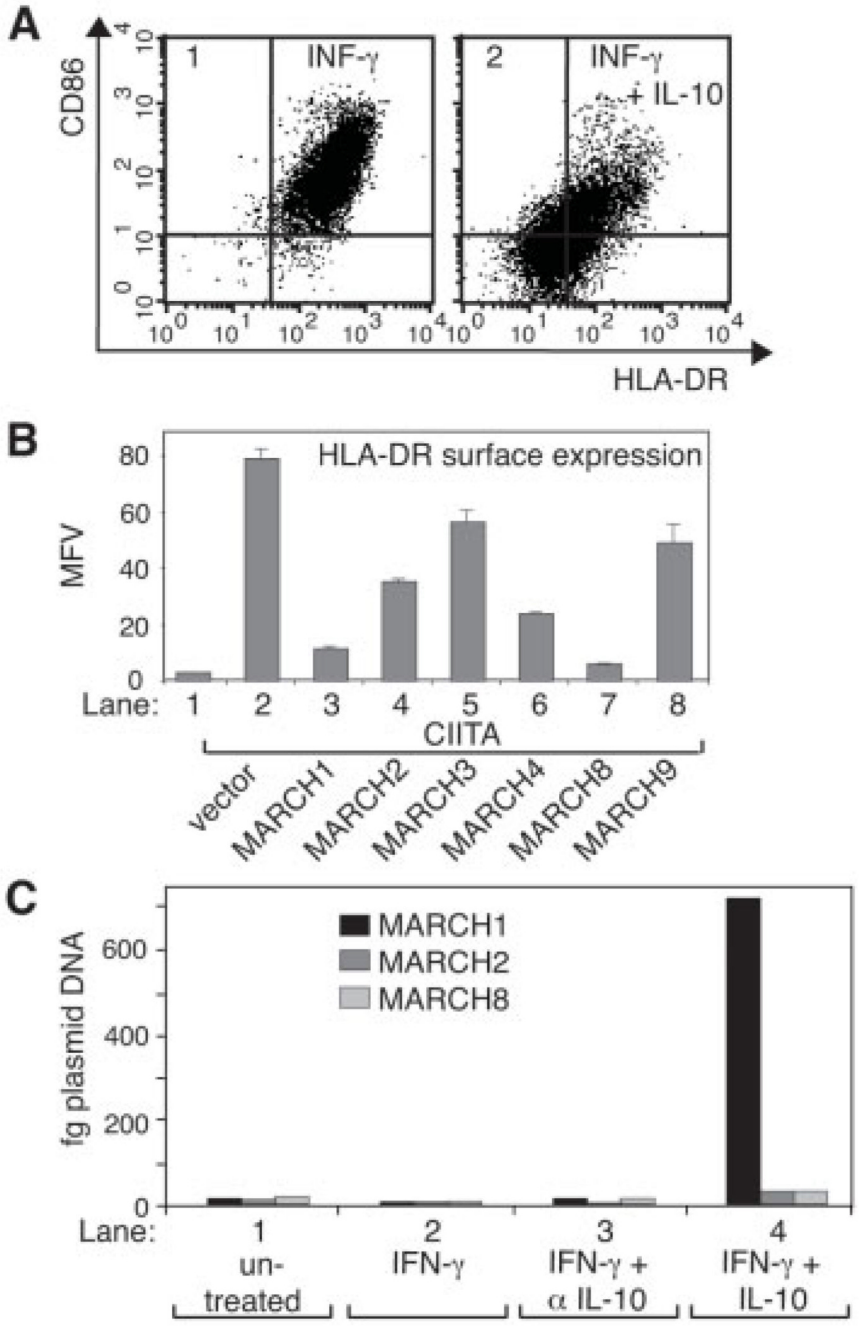

Figure 1.

IL-10 induces MARCH1 expression. (A) Primary human monocytes were incubated with IFN-γ in the absence (panel 1) or presence (panel 2) of IL-10 for 16 h and analyzed by flow cytometry for HLA-DR and CD86 expression. (B) 293EBNA cells were transiently co-transfected with EGFP (lanes 1–8), CIITA (lanes 2–8), and with MARCH cDNA (lanes 3–8) and analyzed by flow cytometry after 48 h. Shown are mean fluorescence values (MFV) of cell surface HLA-DR expression gated on EGFP-positive cells. Values and SD are derived from duplicates of independent transfections. (C) MARCH1, −2, and −8 mRNA expression in primary human monocytes was analyzed by real-time RT-PCR. Cells were left either untreated (lane 1), or incubated for 16 h in the presence of IFN-γ (lane 2), IFN-γ and an anti-IL-10mAb (20 μg/mL;

lane 3) or IFN-γ and IL-10 (lane 4). Samples were normalized for HPRT expression and results are expressed as the amount of equivalent linearized plasmid DNA standard for each MARCH. Shown is a representative example from three independent donors.

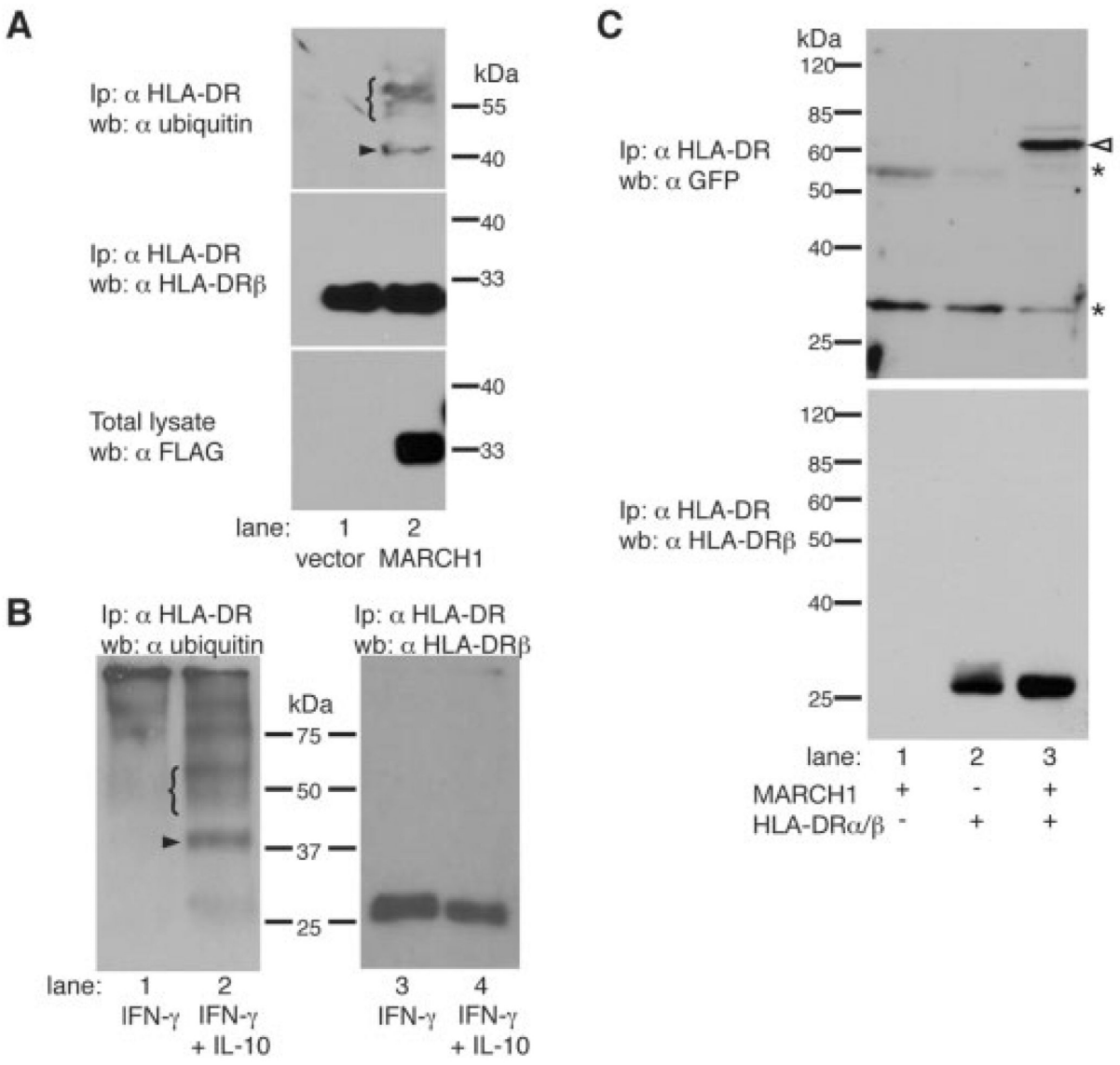

Figure 2.

HLA-DR is ubiquitinated in MARCH1-transfected cells as well as in IL-10 treated monocytes, and interacts with MARCH1. (A) HeLa/CIITA cells were transfected with empty vector (lane 1) or an expression plasmid for FLAG-tagged MARCH1 (lane 2). At 24 h post transfection, cells were lysed and HLA-DR was immunoprecipitated using L243. Samples were separated by SDS-PAGE and analyzed on immunoblots using the ubiquitin-specific mAb P4D1 (top panel) and HLA-DRβ-specific mAb XD5.117 (middle panel). Total lysates were analyzed by immunoblotting with FLAG-specific mAb M2 (bottom panel). The arrowhead indicates the presence of mono-ubiquitinated HLA-DRβ, the bracket that of polyubiquitinated molecules. (B) Primary human monocytes were cultured for 16 h in the presence of IFN-γ (lanes 1, 3) or IFN-γ plus IL-10 (lanes 2, 4). Cells were lysed and HLA-DR molecules were immunoprecipitated with L243. Non-reduced samples were separated by SDS-PAGE and

analyzed for ubiquitin by immunoblotting using P4D1 (left panel). The membrane was stripped and incubated with the HLA-DRβ-specific mAb XD5.117 (right panel). Similar results were obtained using cells from a second donor (data not shown). (C) 293T cells were transiently transfected with EYFP-MARCH1 alone (lane 1),with HLA-DRα/β (lane 2), or co-transfected with HLA-DRα/β and EYFP-MARCH1 (lane 3). At 24 h post transfection, cells were lysed and HLA-DR molecules were immunoprecipitatedwith mAb L243. Samples were separated by SDS-PAGE and analyzed on an immunoblot with a GFP-specific antiserum (top panel). The arrowhead indicates the presence of the EYFP-MARCH1 fusion protein (predicted molecular mass 58.4 kDa) and the asterisks indicate the Ig heavy and light chains. EYFP-MARCH1 expression in samples from lanes 1 and 3 was verified by flow cytometry (data not shown). The membrane was stripped and incubated with the HLA-DRβ specific mAb XD5.117 (bottom panel).

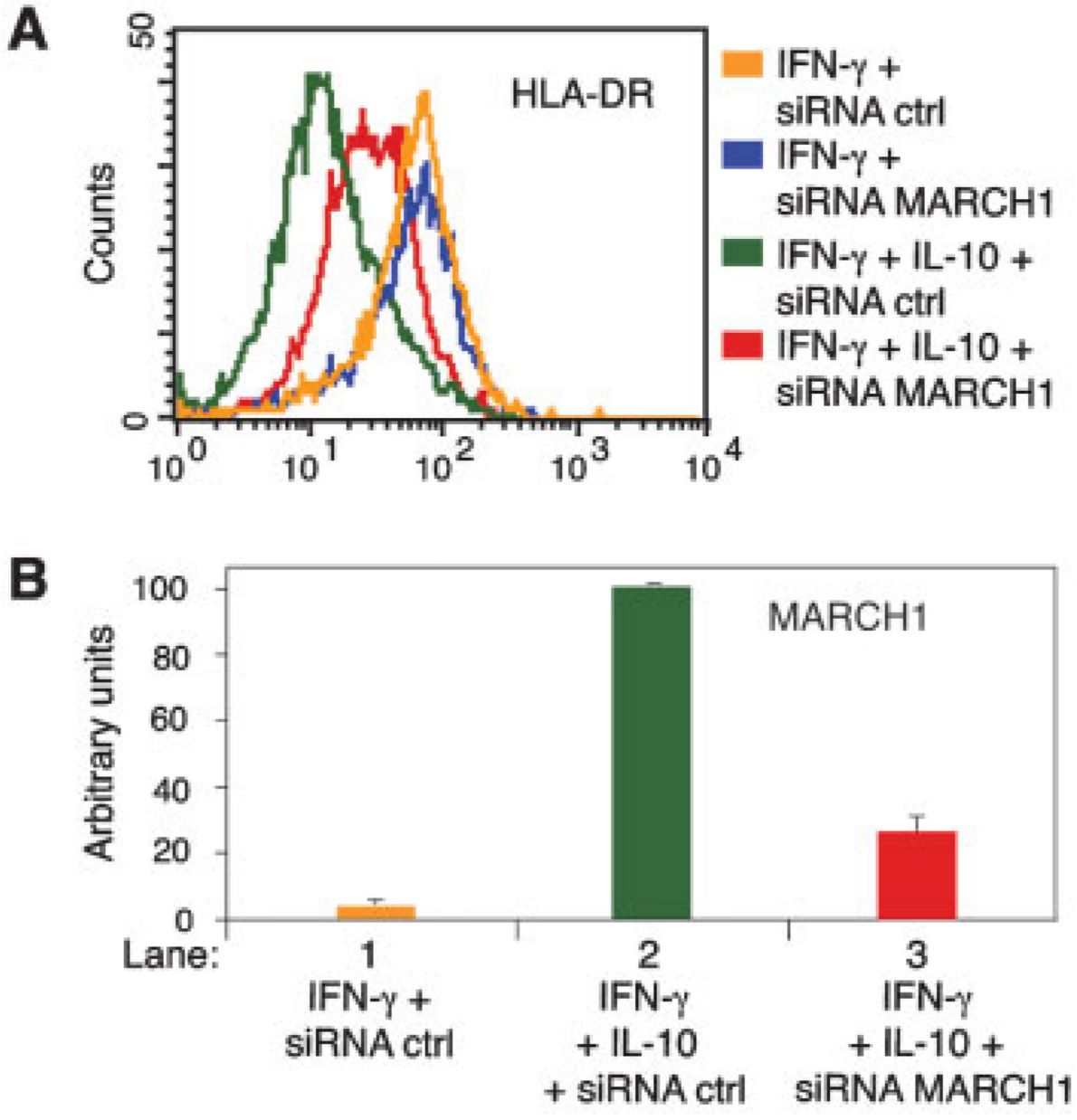

Figure 3.

IL-10 induced down-regulation of MHC-II is dependent on MARCH1. Primary human monocytes were transfected with fluorescent-labeled (Alexa 488) control or MARCH1-specific siRNA. Cells were cultured for 16 h in the presence of IFN-γ or IFN-γ plus IL-10. (A) HLA-DR cell surface expression of siRNA-transfected cells was analyzed by flow cytometry. (B) MARCH1 mRNA expression of the cells shown in (A) was analyzed by real-time RT-PCR. Samples were normalized for HPRT expression and MARCH1 expression of the sample in lane 2 was arbitrarily set at 100. Values and SD are from duplicate PCR. Similar results were obtained on another donor.

Annexe II

Traffic 2009; 10: 1518–1527

doi: 10.1111/j.1600-0854.2009.00948.x

Sorting of MHC Class II Molecules into Exosomes through a Ubiquitin-Independent Pathway

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Major histocompatibility complex class II (MHC-II) molecules accumulate in exocytic vesicles, called exosomes, which are secreted by antigen presenting cells. These vesicles are released following the fusion of multivesicular bodies (MVBs) with the plasma membrane. The molecular mechanisms regulating cargo selection remain to be fully characterized. As ubiquitination of the MHC-II β-chain cytoplasmic tail has recently been demonstrated in various cell types, we sought to determine if this post-translational modification is required for the incorporation of MHC-II molecules into exosomes. First, we stably transfected HeLa cells with a chimeric HLA-DR molecule in which the β-chain cytoplasmic tail is replaced by ubiquitin. Western blot analysis did not indicate preferential shedding of these chimeric molecules into exosomes. Next, we forced the ubiquitination of MHC-II in class II transactivator (CIITA)-expressing HeLa and HEK293 cells by transfecting the MARCH8 E3 ubiquitin ligase. Despite the almost complete downregulation of MHC-II from the plasma membrane, these molecules were not enriched in exosomes. Finally, site-directed mutagenesis of all cytoplasmic lysine residues on HLA-DR did not prevent inclusion into these vesicles. Taken together, these results demonstrate that ubiquitination of MHC-II is not a prerequisite for incorporation into exosomes.

Key words: exosomes, HLA-DR, MARCH-protein ubiquitin ligases, MHC, MVBs, ubiquitin

Received 12 September 2008, revised and accepted for publication 19 May 2009, uncorrected manuscript published online 27 May 2009, published online 30 June 2009

Human leukocyte antigens (HLAs) such as HLA-DR (DR) are major histocompatibility complex class II (MHC-II) proteins required for peptide presentation to $CD4^+$ T cells (1). DR molecules are formed by the association of α- and β-chains in the endoplasmic reticulum (ER) (2). Three αβ heterodimers form a nonameric complex following binding to a trimer of invariant chains (Ii) (3). The complex is transported into endosomes, where Ii is degraded (2). In transfected cell lines expressing MHC-II molecules in the absence of Ii, the MHC-II molecules are loaded with endogenous polypeptides in the ER and are efficiently transported to the plasma membrane (4,5).

Depending on the cell type, MHC-II rich compartments (MIICs) contain different types of endocytic vesicles, of which multivesicular bodies (MVBs) are the most prominent (6). Internal luminal vesicles (ILVs) in MVBs are generated through the inward budding of endosomal membrane microdomains (7) and these structures have been proposed to play a crucial role in the loading of MHC-II molecules with peptides (8). Indeed, Neefjes and collaborators have recently provided evidence that DR molecules acquire foreign peptides on the internal membranes of MVBs (9).

In addition to their specialized function in antigen presentation, MVBs allow molecular turnover of proteins through fusion with lysosomes [reviewed in (10)]. MVBs can also fuse with the plasma membrane, leading to the release of microvesicles into the extracellular environment. These so-called exosomes have been originally described in reticulocytes, which eliminate obsolete molecules, such as the transferrin receptor (TfR) during maturation (11,12). Most cell types secrete exosomes in the form of 50–90 nm vesicles, which are enriched in a variety of lipids and proteins in a cell-type specific manner [reviewed in (13,14)].

Exosomes from antigen presenting cells (APCs) such as dendritic cells (DCs) and B lymphocytes are enriched in classical MHC-I and -II as well as adhesion and co-stimulatory molecules (15–19). It is believed that these vesicles represent stable antigen presentation entities, capable of propagation through biological fluid to disseminate antigens and establish communications between immune cells (14,20). Exosomes derived from APCs show immunomodulatory properties, mainly after adsorption on DCs (13,14,21). They may also be involved in the pathogenesis of various infectious diseases or cancers (14,18,22). Exosomes are therefore of potential clinical relevance as diagnostic tools or vaccines (23,24).

The mechanisms by which various types of cargos are incorporated into exosomal membranes are beginning to be unraveled. The best characterized mode of

incorporation into ILVs relies on ubiquitination of cargo proteins and their interaction with various endosomal sorting complex required for transport (ESCRT) complexes at the surface of MVBs (25,26). This was shown in yeast and metazoans for a variety of molecules (G-protein-coupled receptors, receptor tyrosine kinases, transporters, channels, etc.), involving a growing number of ubiquitin ligases (10,27,28). The ubiquitination process is initiated through formation of a covalent isopeptide bond between the C-terminal glycine of a first ubiquitin moiety and, generally, a lysine residue within the target protein [reviewed in (29)]. Polyubiquitination through the lysine-48 residue (Ubi-K ^{48}) usually tags the target for degradation. On the other hand, monoubiquitination on one or several residues, or Ubi-K ^{63} branching chains can act as a signal for the endocytosis and trafficking of transmembrane proteins (30–32). Incorporation of cargo into ILVs is accompanied by its deubiquitination as a necessary step to avoid retention at the limiting membrane (33–38). Ubiquitin-independent pathways of cargo entry into ILVs have also been reported; however, the underlying mechanisms are not well understood (39–45).

The mechanisms by which MHC molecules reach ILVs remain ill defined. Recently, in DCs, mono- and poly-ubiquitination of mouse MHC-II molecules has been reported (46,47). Interestingly, mutation of the unique β-chain cytoplasmic lysine residue prevented ubiquitination and decreased the delivery of I-A to the internal membranes of MVBs (46–48). MARCH1 (membrane-associated RING-CH) and MARCH8 proteins have now been identified as key players in the trafficking of MHC-II, both in human and mouse cells. MARCH1 and MARCH8 E3 ubiquitin ligases downregulate MHC-II from the cell surface, preventing their recycling and causing them to accumulate in late vesicles (49). While MARCH1 expression is limited to lymphoid cells and regulated by external stimuli, MARCH8 expression appears to be more widespread (50–54). These recent findings on the regulation of MHC-II trafficking suggested to us that ubiquitination of MHC-II might explain their presence in exosomes.

Results and Discussion

A HLA-DR β-ubiquitin fusion protein does not preferentially accumulate into exosomes

To assess the potential impact of MHC-II ubiquitination on cell surface expression and incorporation into exosomes, we generated chimeric DR β-ubiquitin fusion proteins (Figure 1). This approach has been tested in a number of systems to induce internalization of stable plasma membrane proteins (27,30). We first used the HeLa cell line, because it can be easily transfected to express MHC-II variants and has been used extensively for MHC-II antigen presentation studies and the characterization of endocytic transport (55–58). Although exosomes have been purified from a variety of cell types including

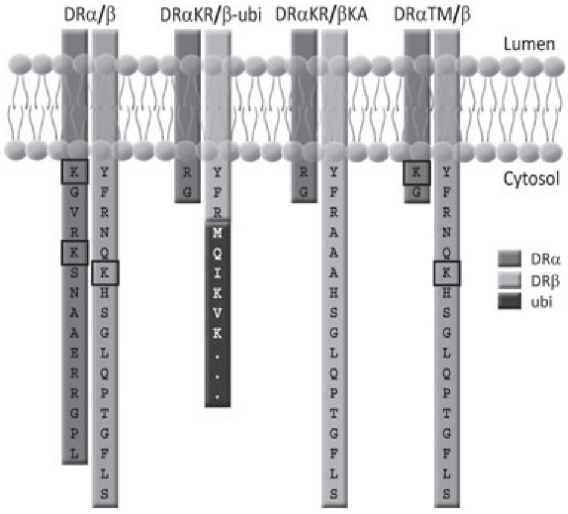

Figure 1: Schematic representation of the DR molecules used in this study. The primary amino acid sequence of the cytoplasmic tails is shown. Lysine residues in the DR cytoplasmic segments are squared. Only the N-t erminal region of the ubiquitin chain is shown in the β-ubi fusion protein.

epithelial cells (59,60), we ascertained by immunoelectron microscopy that HeLa cells do secrete exosomes. We collected the culture medium and concentrated exosomes by ultracentrifugation before purification on a sucrose gradient. Fractions corresponding to a density of 1.08–1.18 g/mL were pooled, and the exosomes were collected by centrifugation (15). The samples were stained for CD9 or CD63, two markers of exosomes (61) (Figure 2A). The presence of vesicles, their morphology and size (50–100 nm) as well as the detection of embedded molecules such as CD9 and CD63 indicate that HeLa cells produce exosomes.

We expressed the DR β-ubi fusion protein in HeLa cells together with a truncated DR α chain devoid of any cytoplasmic lysine residues (DR αKR/DR β-ubi) (Figure 1). We also used a mutant form of the ubiquitin polypeptide in which lysine 48 was changed for an arginine (DR β-ubiKR). This construct prevents polyubiquitination through the 'classical' K48 pathway. However, formation of K63-branched polyubiquitin chains involved in intracellular trafficking is still possible (62). Stable transfectants of HeLa cells expressing the transfected DR-ubi fusion proteins (DR αKR/DR β-ubi, DR αKR/DR β-ubiK48R) were sorted on magnetic beads using a DR-specific monoclonal antibody (mAb). Cell surface expression for both types of chimeric molecules was reduced compared to HeLa cells expressing wild-type DR molecules, and flow cytometric analysis of permeabilized cells showed that total protein levels were also reduced (Figure 2B). Confocal microscopy revealed that the intracellular distribution of the chimeric molecules was very similar to that observed for wild-type DR inasmuch as both accumulate in CD63 $^{+}$ punctate

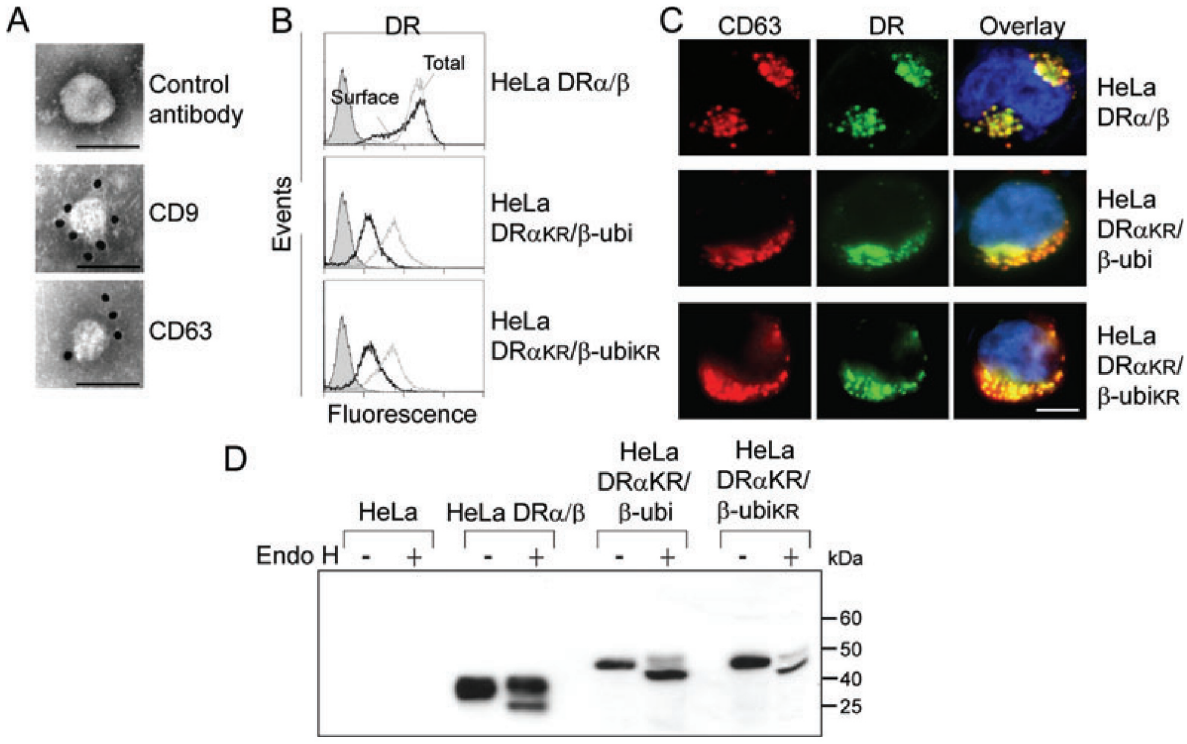

Figure 2: HeLa cells produce exosomes and sort transfected MHC-II molecules into late endocytic vesicles. A) Transmission electron microscopy of immunogold-labeled whole-mount exosomes from HeLa CIITA cells. Scale bar is 100 nm. B) Flow cytometry analysis of stably transfected HeLa cells expressing HLA-DRα/β, DRαKR/β-ubi or DRαKR/β-ubiKR. Cells were stained with the DR-specific L243 mAb before (surface, thick black line) or after permeabilization (total, thin gray line). Shaded curves represent the fluorescence of non-permeabilized cells stained with the secondary antibody alone. C) Cells grown on coverslips were permeabilized, stained for CD63 and HLA-DR (L243) and analyzed by fluorescence microscopy. The nucleus is stained in blue. Scale bar is 10 μm. D) Cell lysates were prepared, treated as indicated with Endo H and analyzed on immunoblots using the DR β-specific mAb XD5.117.

vesicles of the endocytic pathway (Figure 2C). However, some co-localization was also observed with calnexin, an ER marker (data not shown). In agreement with this finding, a substantial amount of the chimeric DR–β-ubi chains remained endoglycosidase H (Endo H)-sensitive in these cells, suggesting that a fraction of the molecules were misfolded (Figure 2D). As expected, the MHC-II β-chain fused to ubiquitin migrated at a molecular weight of about 42 KDa on western blots.

We reasoned that the chimeric MHC-II molecules that found their way to the MVBs were possibly efficiently shed as part of exosomes, thereby explaining the low levels of surface expression and reduced levels of cell-associated Endo H-resistant molecules. After ultracentrifugation of the culture medium, pellets containing exosomes were analyzed on immunoblots (Figure 3A). While CD9 and CD63 tetraspanins were found in exosomes from all cell lines, the DR-ubiquitin fusion proteins were not enriched as compared to the wild-type DR. The MHC-II molecules in all exosome samples were Endo H-resistant, suggesting their passage through the Golgi network (Figure 3B). To ascertain that these molecules were indeed derived from exosomes, the culture supernatants were centrifuged and the pellets were applied to sucrose gradients. Fractions were collected and their density was measured before proteins were precipitated. Figure 3C shows that the wild-type and DR-ubiquitin β-chains are found in the exosomal fractions, which correspond to densities between 1.08 and 1.22 g/mL (15). The presence of CD9 in the same fractions of the gradient confirms the integrity of the preparations (17). These results suggest that the weak cellular expression of the chimeric DR-ubiquitin molecule cannot be explained by selective shedding into exosomes. A similar construct based on mouse I-A was found to enter MVBs efficiently; however, its shedding into exosomes was not analyzed (47). The possibility remains that because of misfolding or ubiquitin-driven sorting, the bulk of our DR-ubi fusion protein may be rapidly degraded in HeLa cells. Of note, in contrast to the construct used by Mellman and collaborators, most of the cytoplasmic tail of the MHC-II β-chain was omitted in our fusion protein (47). Also, a recombinant ubiquitin fusion protein may be efficiently internalized and yet be unable to function as an endosomal trafficking signal (30,32). These caveats

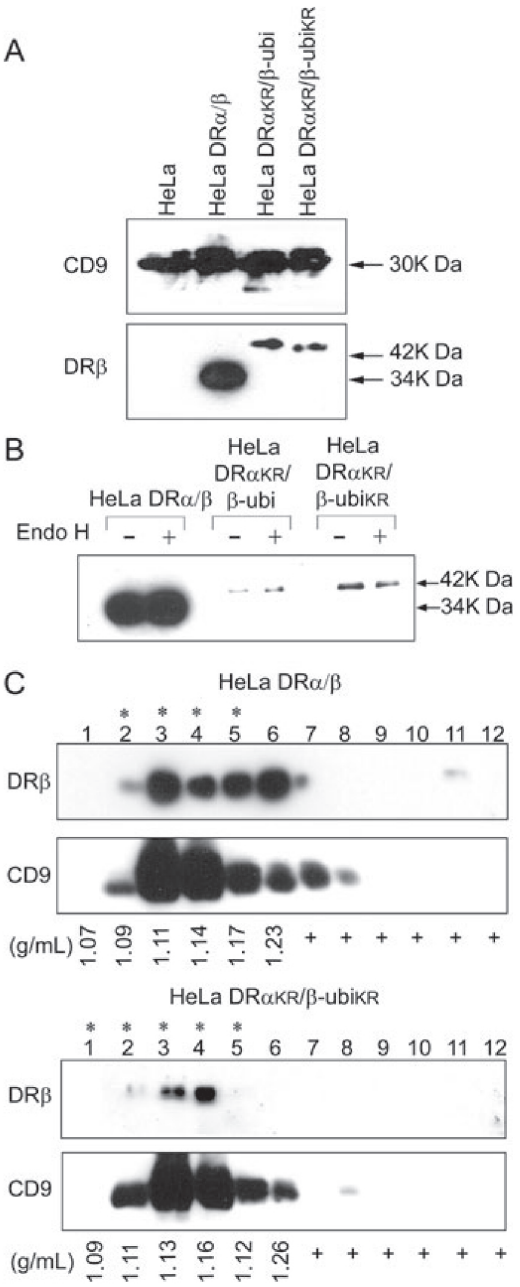

Figure 3: DR-ubiquitin constructs are not preferentially shed into exosomes. A) Exosomes were puri fied by differential centrifugation and pellets were analyzed by immunoblotting for the presence of CD63, CD9 and DR β (XD5.117). B) Proteins were detergent extracted from exosome pellets, treated with Endo H as indicated and analyzed for the presence of DR β using XD5.117 mAb. C) Exosomes obtained by ultracentrifugation were purified on a continuous sucr ose gradient (0.25–2 M sucrose). Fractions were collected, separated by SDS–PAGE and blotted for expression of DR β (XD5.117) and CD9. Gradient fractions with densities between 1.08 and 1.22 g/mL, where exosomes normally float, are indicated by asterisks (*).

prompted us to study the role of MHC-II ubiquitination using a more physiological approach.

Forced ubiquitination of HLA-DR by MARCH8 does not increase shedding into exosomes

It has recently been shown that MARCH1 and MARCH8 ubiquitinate MHC-II molecules, preventing their recycling to the cell surface (51–54,63). Moreover, the half-life of I-A molecules decreased in a proteasome-independent manner in MARCH-expressing cells, a finding compatible not only with lysosomal degradation but also with increased exosomal shedding (51,52). To test the latter possibility, enhanced yellow fluorescent protein (EYFP)-tagged MARCH8 was transiently expressed in HeLa class II transactivator (CIITA) cells and the expression of MHC-II was monitored after 48 h. Figure 4A shows that MARCH8 expression downregulates cell surface DR expression very efficiently (upper left panel). The loss of cell surface MHC-II is accompanied by a reduction in the total protein level of DR, as determined by the staining of permeabilized cells (Figure 4A, upper right). As expected, and as we have shown for MARCH1 (54), DR molecules in MARCH8-expressing HeLa and HEK293T cells are mono- and polyubiquitinated [data not shown; see also (63)]. Although CD9 and MHC-II have been shown to associate in certain cell types (64), we found the surface display and trafficking of CD9 to be independent of MARCH8 expression (Figure 4A, lower panels). This finding further validates the use of CD9 as a control in our experiments.

We collected the culture medium from cells expressing EYFP-MARCH8 or EYFP as a control. Latex beads were coated with exosomes obtained by ultracentrifugation and analyzed by flow cytometry for the presence of CD9 and DR β (Figure 4B). In our hands, this technique is highly reproducible and more sensitive than immunoblots. Our results show that the levels of DR and CD9 are similar in exosomes from MARCH8- or EYFP-transfected cells. Thus, the strong downmodulation of DR expression induced by MARCH8 is not accompanied by substantial MHC-II shedding into exosomes. We cannot rule out that there is increased delivery of MHC-II in ILVs, but in that case, the targeted MVBs would seem to preferentially fuse with lysosomes rather than with the plasma membrane. We repeated the experiment in HEK293T CIITA cells. As a control, we used a truncated version of MARCH8 devoid ofi ts RING domain (EYFP-MARCH8 Δring) and which does not downregulate MHC-II molecules (Figure 4C). Cell surface expression of DR was again very efficiently downregulated by EYFP-MARCH8, but interestingly, and in contrast to HeLa cells, total DR expression levels were not reduced in EYFP-MARCH8 expressing HEK293T CIITA cells (Figure 4C, upper right panel). Exosomes from both types of transfectants were collected, coated onto latex beads and analyzed by flow cytometry. Despite the fact that similar amounts of DR molecules are present in both cell types and that they are very efficiently retained intracellularly in

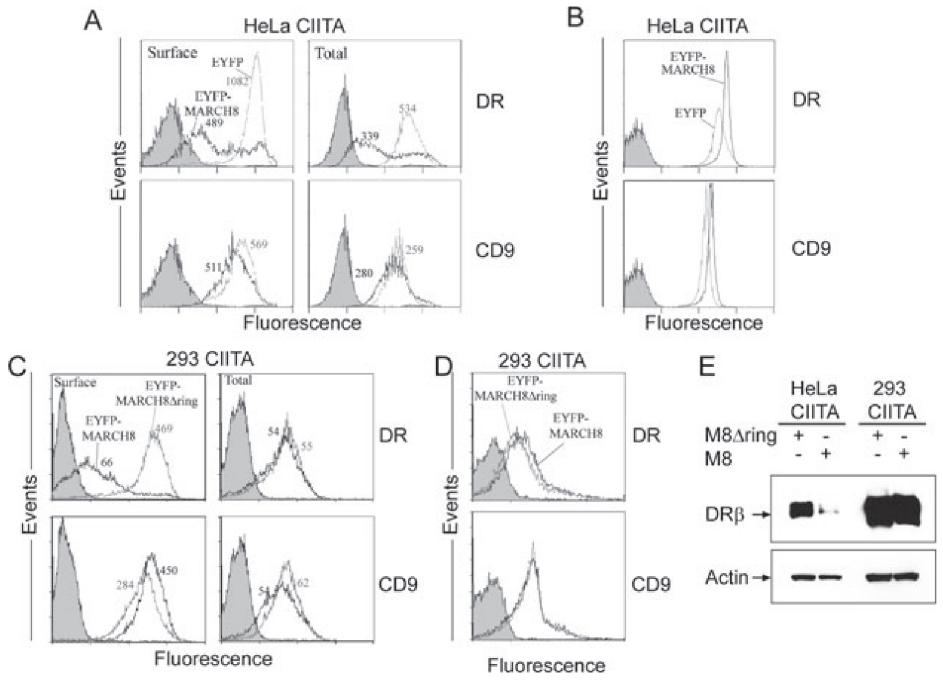

Figure 4: Surface MHC-II molecules downre gulated by MARCH8 are not shed onto exosomes. A) HeLa CIITA cells were transiently transfected with EYFP-MARCH8 or EYFP as a control and analyzed after 48 h. Cells were stained before (surface) or after permeabilization (total) for the expression of DR (L243) or CD9. The negative controls (shaded curve) represent cells stained before (surface) or after permeabilization (total) with the secondary antibody alone. Numbers repr esent the mean fluorescence intensities for each curve. B) Exosomes were purified by di fferential centrifugation, coated onto lat ex beads, stained for DR (XD5.117) or CD9 and analyzed by flow cytometry. The negative con trols (shaded curve) represent exosomes stained with the secondary antibody alone. C) HEK293T CIITA cells were treated as in panel (A) except that an EYFP-MARCH8 Δring was used as control. Numbers represent the mean fluorescence intensities for each curve. D) Exosomes were pur ified from transfected HEK293T CIITA cells and analyzed as in panel (B). E) Cell lysate proteins from HeLA CIITA or HEK293T C IITA cells transfected transi ently with MARCH8 (M8) or MARCH8 Δring (M8 Δring) were separated by SDS–PAGE and analyzed by immunoblotting for DR β (XD5.117) (upper panel) and actin expression as a control (lower panel).

MARCH8-expressing cells, no increased incorporation into exosomes was observed (Figure 4D).

Intracellular retention of MHC-II molecules without degradation or increased shedding was highly reproducible in HEK293 cells expressing MARCH8. Similar results were obtained with MARCH1 or HEK293 cells transfected with HLA-DR instead of CIITA (data not shown). Immunofluorescence microscopy revealed that MHC-II molecules co-localized with the late endosomal markers HLA-DM and CD63, irrespective of MARCH expression (data not shown). The difference between HeLa and HEK293T regarding the loss of MHC-II molecules was confirmed by western blotting (Figure 4E) and suggests the existence of cell-type specific differences in the handling of ubiquitinated cargo. These could be caused, among other things, by variations in the type of ubiquitin chains that are generated or by indirect effects of MARCH activity on the function ofl ysosomes. In addition, deubiquitinating enzymes may vary in composition and localization between the two cell types, affecting the fine sorting of MHC-II (65). Taken together, our results demonstrate that ubiquitination of MHC-II molecules does not necessarily lead to increased release into exosomes nor is ubiquitination and efficient downregulation from the cell surface inevitably linked to increased degradation.

A DR molecule devoid ofl ysine residues in its cytoplasmic domains can still enter exosomes

Although there is no evidence that MHC-II molecules are ubiquitinated in HeLa cells in the absence of co-transfected MARCH proteins (54), we cannot exclude that an unidentified endogenous ubiquitin ligase/deubiquitinase couple is responsible for the entry of some MHC-II molecules into exosomes. To eliminate the possibility that HLA-DR becomes ubiquitinated, the cytoplasmic α- and β-chain lysine residues were mutated (Figure 1). Surface and total expression in HeLa cells of this DR αKR/βKA heterodimer was compared to DR αTM/β as a control (Figure 5). Both molecules were expressed at similar levels and fluorescence microscopy showed that they both co-localize in intracellular vesicles with the late endosomal marker Lamp-1 (Figure 5A,B).

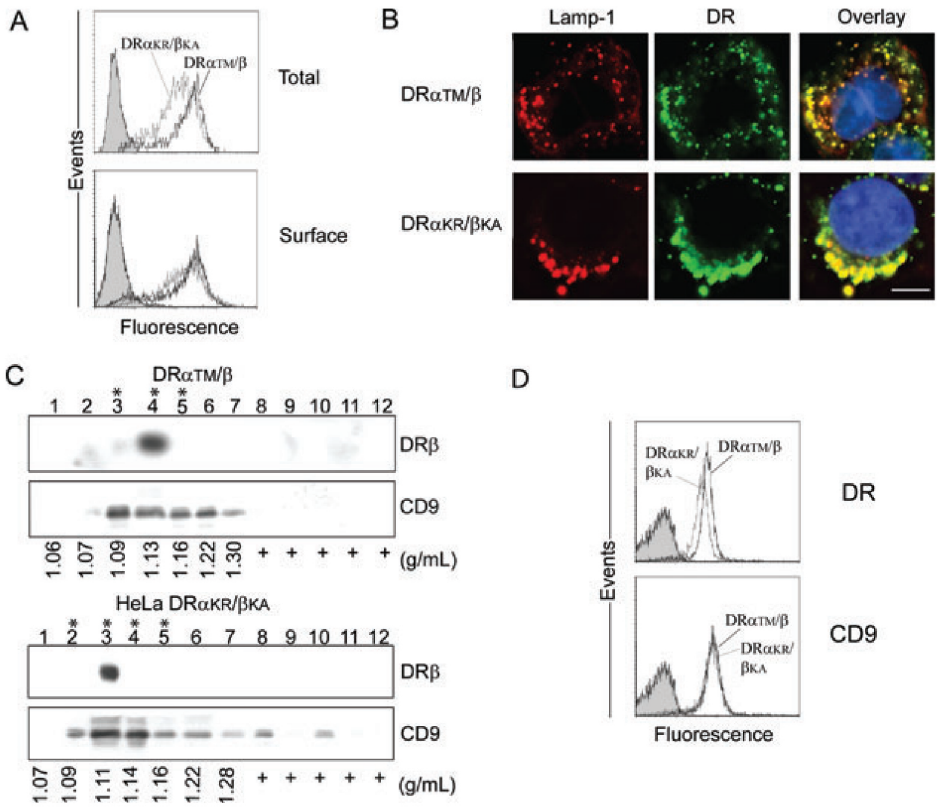

Figure 5: Lysine-less DR molecules are present in exosomes. A) HeLa cells stably transfected with the indicated constructs were stained with the DR-specific L243 antibody before (surface) or after permeabilization (total). B) Cells were analyzed by immunofluorescence microscopy with antibodies specific for DR (L243) and Lamp-1. The nucleus is stained in blue. Scale bar is 10 μm. C) Exosomes were purified on sucrose gradients and fractions were analyzed on immunoblots for the presence of DR (XD5.117) and CD9. Gradient fractions with densities between 1.08 and 1.22 g/mL, where exosomes normally float, are indicated by asterisks (*). D) Latex beads coated with exosomes were analyzed by flow cytometry for the presence of DR (XD5.117, upper panel) and CD9 (lower panel). The negative controls (shaded curve) represent cells or exosomes stained with the secondary antibody alone.

Exosomes were analyzed on immunoblots and by flow cytometry (Figure 5C,D). Both techniques show that comparable levels of MHC-II and CD9 are found between exosomes from DR αTM/β and DR αKR/βKA cells. Much weaker signals were obtained by flow cytometry for these MHC-II-transfected cells as compared to CIITA-expressing HeLa cells (Figure 4B). Whether this only reflects different levels of MHC-II expression or, for example, a role for CIITA expression in the efficiency of exosomal incorporation is under investigation. CIITA induces the expression of many genes that may shape the endocytic pathway and affect trafficking (66,67). An obvious candidate would be the invariant chain but co-expression of this chaperone did not impact on the incorporation of HLA-DR in the exosomes of HeLa cells (data not shown). Altogether, these results demonstrate that ubiquitination of MHC-II lysine residues is not a prerequisite for gaining access to exosomes.

Recent immunoelectron microscopy studies using mouse DCs have demonstrated that an I-A molecule devoid of cytoplasmic lysine residues entered less efficiently into MVBs (46,47). If such a mutant is also excluded from exosomes has not been addressed. Still, the presence of significant levels of lysine-less MHC-II in ILVs suggested to Mellman and collaborators that '*MHC-II may be one of the first examples of a ubiquitin-independent cargo during MVB biogenesis*' (47). Our results are in agreement with the existence of such a ubiquitin-independent pathway to ILVs. Roche and collaborators recently showed that exosome-embedded MHC-II molecules have previously transited via the plasma membrane (68). Thus, the ubiquitin-independent pathway may be based on lipid rafts and/or tetraspanin protein webs, two types of MHC-II$^+$ plasma membrane microdomains that have been suggested to deliver cargo to exosomes and shape their composition (19,40,69). Our results suggest that the MHC-II pool destined to exosomes is independent of the one that ultimately ends up in lysosomes for degradation. Accordingly, we speculate that molecules included in rafts or tetraspanin microdomains are probably protected from the action of the ubiquitin ligases, at least those of the

MARCH family. Future studies will address the distribution of our MHC-II constructs in the context of membrane microdomains.

Neither the expression of MARCH8 nor mutation of the lysines was sufficient to modulate the amount of DR in exosomes. Still, the fact that the ratio of DR in exosomes over total cellular DR is increased in MARCH8 $^{+}$ HeLa cells may suggest that there is preferential shedding in these conditions. However, this was not observed in HEK293T cells. The dramatic reduction in protein amounts of DR in MARCH8-positive HeLa cells demonstrates that a massive transfer of these molecules toward the ILVs ofl ate MVBs had to occur at some point in time. As this subcellular redistribution from the plasma membrane to the endocytic pathway did not impact on the amount of MHC-II in exosomes, we postulate that exosomes do not originate from classical MVBs in some cell types. Interestingly, a similar conclusion was drawn from the fact that lysobisphosphatidic acid (LBPA), a lipid sorted in the ILVs of MVBs and co-localizing with MHC-II molecules, is not found in exosomes released from DCs and B cells (69,70). One possibility is that exosomes are shed from sorting or recycling endosomes (69). Indeed Rab11, a protein involved in TfR recycling, was found to increase exosome release in the human erythroleukemia K562 cell line (71). Further analysis of the impact of various Rab proteins, which regulate different levels of membrane traffic, may give clues as to the origin of exosomes in HeLa and HEK293 cells (16,71,72).

In conclusion, our results demonstrate that the ubiquitination of the MHC-II cytoplasmic tails is not an absolute requirement for inclusion into exosomes. However, it seems likely that such a ubiquitin-independent pathway is indirectly dependent on ubiquitination reactions such as those needed to generate MVBs (16,73–75). Future experiments will investigate if, as described for Pmel17, the luminal portion of MHC-II molecules is important for the targeting to ILVs (43).

Materials and Methods

Plasmids and cDNA mutagenesis

The cytoplasmic tail of HLA-DR β *0101 was replaced by the ubiquitin polypeptide via the polymerase chain reaction (PCR) overlap extension method (76). A first fragment was amplified from pBSDR β008 using the universal primer and a mutagenic primer overlapping the end of the DRβ transmembrane and the N-terminal region of the ubiquitin protein (oligonucleotide DR β3′b: 5′-GAAGATTCTGCATTCGAAAGTAGATGAAC-3′). A cDNA fragment encoding wild-type or K48R-mutated ubiquitin was amplified from pCW7 or pCW8 plasmids (77) (obtained from Dr E. Cohen, University of Montreal) using a complementary 5′ primer (Ubi-DRb5′c: 5′-GTTCATCTACTTTCGAATGCAGATCTTC-3′) and a 3′ primer (Ubi3′*BamH* I-*Cla*I.d: 5′-GGCCTTATCGATGGATCCTCAACCACCT CTTAGTCT-3′). Following the overlap reaction, the PCR product was subcloned into the *Sty* I and *Cla*I sites of pBSDR β and then subcloned into the *BamH* I site of SR αpuro to generate SR αpuroDR β-ubi and SR αpuroDR β-ubiKR. The DR βKA chain devoid ofi ts cytoplasmic lysine was described previously (78).

To create a truncated DR αKR lysine-less mutant, a first fragment was amplified from pBS DR αOri2 using the reverse primer and a mutagenic primer (DR αKG:RGstop.c 5′-TTC ATC ATC AGG GGT TAA CGC AAA AGC-3′). A second fragment was amplified from pBS DR αOri2 using a complementary mutagenic primer (DR αKG:RGstop.b: 5′-GCT TTT GCG TTA ACC CCT GAT GAT GAA-3′) and the universal primer. The two overlapping PCR products were mixed and a final PCR reaction was completed using the flanking primers. This PCR product was subsequently digested with *Pst* I, cloned into RSV.5neo DR α. We also used a DR αTM molecule devoid of most ofi ts cytoplasmic tail but retaining one lysine at the junction with the transmembrane domain (79).

The pcDNA3.1 EYFP plasmid was from Invitrogen. The MARCH8 cDNA was obtained from Dr K. Frueh (Oregon University) (50). EYFP was fused to the MARCH8 N-terminal amino acid 1 (EYFP-MARCH8) or 126 (EYFP-MARCH8 Δring).

All DNA constructs were verified by sequencing.

Antibodies

L243 mAb (IgG 2_a) binds a DR α conformational determinant (80). XD5.117 (IgG1) is a DR β-specific mAb (81). The anti-CD63 (H5C6; IgG1) and the anti-Lamp-1 (H4A3; IgG1) mAbs are from the Developmental Studies Hybridoma Bank, NICHD, University of Iowa, IA. The 50H19 mAb reacts with the human CD9 (82). For fluorescence microscopy analyses, H5C6 and H4A3 were detected using an IgG1-specific goat anti-mouse antibody coupled to Alexa-488. L243 was biotinylated and detected using avidin coupled to Texas Red.

Cell lines and transfections

Cells were cultured in DMEM supplemented with 10% FBS (Wisent) and appropriate selective agents (see below). HeLa and HEK293T cells were transfected using Fugene6 (Roche Diagnosis) and calcium phosphate co-precipitation, respectively. To generate stable lines of HeLa cells, G-418 (Gibco-BRL) was added to a final concentration of 500 μg/mL and DR-positive cells were sorted using magnetic beads (Dynal Inc.).

Fluorescence microscopy and flow cytometry

Cells were plated on cover slips in 24 well plates and cultured for 3 days before immunostaining, as described (83,84). Nuclear staining was achieved using Hoechst 33342, as recommended by the manufacturer (Invitrogen). Epifluorescence micrographs were obtained on a Zeiss Cell Observer system equipped with an Axiovert 200M microscope. Images were digitally deconvoluted with the AxioVision 3.1 software using the nearest neighbor deconvolution method that uses Agard's formula (85).

For flow cytometry analysis of MHC-II su rface expression, live cells were incubated on ice with L243. After washing, cells were incubated with a secondary goat anti-mouse antibody coupled to Alexa-Fluor 647 (Invitrogen) and analyzed on a FACSCalibur (Becton Dickinson). To determine the total expression of MHC-II or CD9 molecules, cells were fixed with paraformaldehyde (PFA) and permeabilized as described (83,84). Stainings were performed as described above for live cells.

Exosome purification and characterization

Cells were grown in 10 175-cm ^{2} flasks in medium supplemented with FBS that had been spun at 100 000 × *g* for 60 min to remove bovine exosomes. When cells reached near confluence (3×10^{7} cells per flask), fresh exosome-free cell culture media was added to flasks and cells were incubated for 24 h. Cell culture medium was harvested and centrifuged for 10 min at 1200 × *g* to remove cells and debris. The supernatant was filtered on a 0.22 μm Qiagen stericup. After a second centrifugation at 1200 × *g*, the medium was centrifuged for 30 min at 10 000 × *g* and exosomes were collected from the supernatant by ultracentrifugation at 100 000 × *g* for 60 min. The pellets (approximately 60 μg of proteins) were either used as such and processed for flow cytometry or resuspended

in 5 mL of 2.6 M sucrose, 20 m M Tris–HCl, pH 7.2 and overlaid with a linear density gradient (2.0–0.25 M sucrose in 20 m M Tris–HCl, pH 7.2). Exosomes were centrifuged in a SW41Ti rotor for 18 h at 270 000 × *g*. Fractions of 1 mL were collected and precipitated in 20% trichloroacetic acid (TCA) for 30 min on ice. Density off ractions was determined by refractometry. Exosomes were spun, washed with cold acetone and spun again. Supernatant was removed and pellets were air dried.

For flow cytometry analyses, we used exosomes obtained by ultracentrifugation. The protein concentration was determined by Bradford (Bio-Rad). Exosomes (1 μg) were incubated for 2 h at room temperature with 25 000 latex beads (Interfacial Dynamics Corp) as described (40,86). Beads were washed and saturated overnight with 10% powdered milk in PBS. Beads were stained as described above for live cells except that XD5.117 was used for MHC-II molecules. Beads were resuspended in PBS and analyzed by flow cytometry.

Whole-mount immunoelectron microscopy of exosomes

After flotation gradients, exosome fractions (approximately 10^7 particles per mL) were loaded into micro-ultracentrifuge tubes with formvar-coated electron microscopy grids placed into the bottom of the tubes. Exosomes were spun at 120 000 × *g* (20 psig) for 5 min in an Airfuge ultracentrifuge (Beckman). Exosomes were fixed in PBS-PFA 4% and preincubated with PBS/ovalbumin 1% to block non-specific binding. Grids were then incubated with primary antibodies in PBS for 1 h at room temperature and washed 3× for 5 min in PBS. Immunogold labeling was done using 15-nm beads coupled to a goat anti- mouse secondary antibody (Jackson Laboratories) for 1 h in PBS. After three washes, samples were stained with a drop of 3% phosphotungstic acid (pH 6.0) and dried out (87). The grids were examined on a transmission electron microscope (Hitachi 7100).

Endoglycosidase H treatment and western blotting

The exosome fractions were solubilized in reducing SDS sample buffer and treated or not with Endo H, as described (88). Samples were boiled and loaded on 12% SDS–PAGE. Proteins were transferred to Hybond ECL membranes (Amersham) and analyzed with specific mAbs. Secondary peroxydase-coupled goat anti-rabbit or anti-mouse antibodies were used (Bio/Can Scientific) and detected by chemiluminescence (Boehringer-Mannheim).

Acknowledgments

We thank Serge S énéchal for helping with flow cytometry. We thank the Developmental Studies Hybridoma Bank at the University of Iowa for hybridomas. This work was supported by grants to JT from the Canadian Institutes for Health Research (MOP 36355) and the Canadian Cancer Society (#17230).

References

1. Unanue ER. Cellular studies on antigen presentation by class II MHC molecules. Curr Opin Immunol 1992;4:63–69.
2. Cresswell P. Assembly, transport, and function of MHC class-II molecules. Annu Rev Immunol 1994;12:259–293.
3. Roche PA, Marks MS, Cresswell P. Identification of the HLA class II-invariant chain complex as a nine subunit transmembrane glycoprotein. Nature 1991;354:392–394.
4. Sekaly RP, Tonnelle C, Strubin M, Mach B, Long EO. Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. J Exp Med 1986;164:1490–1504.
5. Miller J, Germain RN. Efficient cell surface expression of class II MHC molecules in the absence of associ ated invariant chain. J Exp Med 1986;164:1478–1489.
6. Nijman HW, Kleijmeer MJ, Ossevoort MA, Oorschot VMJ, Vierboom MPM, Van de Keur M, Kenemans P, Kast WM, Geuze HJ, Melief CJM. Antigen capture and major histocompatibility class II compartments off reshly isolated and cultured human blood dendritic cells. J Exp Med 1995;182:163–174.
7. Murk JL, Humbel BM, Ziese U, Griffith JM, Posthuma G, Slot JW, Koster AJ, Verkleij AJ, Geuze HJ, Kleijmeer MJ. Endosomal compartmentalization in three dimensions: implications for membrane fusion. Proc Natl Acad Sci U S A 2003;100:13332–13337.
8. Murk JL, Stoorvogel W, Kleijmeer MJ, Geuze HJ. The plasticity of multivesicular bodies and the regulation of antigen presentation. Semin Cell Dev Biol 2002;13:303–311.
9. Zwart W, Griekspoor A, Kuijl C, Marsman M, van RJ, Janssen H, Calafat J, van HM, Janssen L, van Lith M, Jalink K, Neefjes J. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escap e. Immunity 2005;22:221–233.
10. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol 2002;3:893–905.
11. Pan BT, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalizatio n of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol 1985;101:942–948.
12. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte mat uration. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 1987;262:9412–9420.
13. Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G. The biogenesis and functions of exosomes. Traffic 2002;3:321–330.
14. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol 2002;2:569–579.
15. Raposo G, Nijman HW, Stoorvogel W, Leijendekker R, Harding CV, Melief CJM, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996;183:1161–1172.
16. Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellu lar compartment distinct from apoptotic vesicles. J Immunol 2001;166:7309–7318.
17. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem 1998;273:20121–20127.
18. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. J Biochem 2006;140:13–21.
19. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem 2003;278:10963–10972.
20. Caby MP, Lankar D, Vincendeau-Sc herrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol 2005;17:879–887.
21. Vincent-Schneider H, Stumptner-Cuvelette P, Lankar D, Pain S, Raposo G, Benaroch P, Bonnerot C. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. Int Immunol 2002;14:713–722.
22. Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic 2008;9:871–881.
23. Chaput N, Flament C, Viaud S, Taieb J, Roux S, Spatz A, Andre F, LePecq JB, Boussac M, Garin J, Amigorena S, Thery C, Zitvogel L. Dendritic cell derived-exosomes: biology and clinical implementations. J Leukoc Biol 2006;80:471–478.
24. Viaud S, Ullrich E, Zitvogel L, Chaput N. Exosomes for the treatment of human malignancies. Horm Metab Res 2008;40:82–88.

25. Saksena S, Sun J, Chu T, Emr SD. ESCRTing proteins in the endocytic pathway. Trends Biochem Sci 2007;32:561–573.
26. Hurley JH. ESCRT complexes and the biogenesis of multivesicular bodies. Curr Opin Cell Biol 2008;20:4–11.
27. Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol 2003;19:141–172.
28. Staub O, Rotin D. Role of ubiquitylation in cellular membrane transport. Physiol Rev 2006;86:669–707.
29. Ciechanover A, Ben-Saadon R. N-te rminal ubiquitination: more protein substrates join in. Trends Cell Biol 2004;14:103–106.
30. Pickart CM, Fushman D. Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol 2004;8:610–616.
31. Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. EMBO J 2006;25:1635–1645.
32. Piper RC, Luzio JP. Ubiquitin-dependent sorting ofi ntegral membrane proteins for degradation in lysosomes. Curr Opin Cell Biol 2007;19:459–465.
33. Buschow SI, Liefhebber JM, Wubbolts R, Stoorvogel W. Exosomes contain ubiquitinated proteins. Blood Cells Mol Dis 2005;35:398–403.
34. Amerik A, Sindhi N, Hochstrasse r M. A conserved late endosome-targeting signal required for Doa4 deubiquitylating enzyme function. J Cell Biol 2006;175:825–835.
35. Ma YM, Boucrot E, Villen J, Affar eB, Gygi SP, Gottlinger HG, Kirchhausen T. Targeting of AMSH to endosomes is required for epidermal growth factor receptor degradation. J Biol Chem 2007;282:9805–9812.
36. Row PE, Liu H, Hayes S, Welchman R, Charalabous P, Hofmann K, Clague MJ, Sanderson CM, Urbe S. The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation. J Biol Chem 2007;282:30929–30937.
37. Richter C, West M, Odorizzi G. Dual mechanisms specify Doa4-mediated deubiquitination at multivesicular bodies. EMBO J 2007;26:2454–2464.
38. Nikko E, Andre B. Evidence for a direct role of the Doa4 deubiquitinating enzyme in protein sorting into the MVB pathway. Traffic 2007;8:566–581.
39. Reggiori F, Pelham HR. Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. EMBO J 2001;20:5176–5186.
40. De Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. Bl ood 2003;102:4336–4344.
41. Geminard C, De GA, Blanc L, Vidal M. Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. Traffic 2004;5:181–193.
42. Delcayre A, Estelles A, Sperinde J, Roulon T, Paz P, Aguilar B, Villanueva J, Khine S, Le Pecq JB. Exosome display technology: applications to the development of new diagnostics and therapeutics. Blood Cells Mol Dis 2005;35:158–168.
43. Theos AC, Truschel ST, Tenza D, Hurbain I, Harper DC, Berson JF, Thomas PC, Raposo G, Marks MS. A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. Dev Cell 2006;10:343–354.
44. Huang J, Reggiori F, Klionsky DJ. The transmembrane domain of acid trehalase mediates ubiquitin-independent multivesicular body pathway sorting. Mol Biol Cell 2007;18:2511–2524.
45. Zeelenberg IS, Ostrowski M, Krumeich S, Bobrie A, Jancic C, Boissonnas A, Delcayre A, Le Pecq JB, Combadiere B, Amigorena S, Thery C. Targeting tumor antigens to secreted membrane vesicles in vivo induces efficient antitumor immune responses. Cancer Res 2008;68:1228–1235.
46. van Niel G, Wubbolts R, Ten BT, Buschow SI, Ossendorp FA, Melief CJ, Raposo G, van Balkom BW, Stoorvogel W. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. Immunity 2006;25:885–894.
47. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature 2006;444:115–118.
48. van Niel G, Wubbolts R, Stoorvogel W. Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. Curr Opin Cell Biol 2008;20:437–444.
49. Ohmura-Hoshino M, Goto E, Matsuki Y, Aoki M, Mito M, Uematsu M, Hotta H, Ishido S. A novel family of membrane-bound E3 ubiquitin ligases. J Biochem 2006;140:147–154.
50. Bartee E, Mansouri M, Hovey Nerenberg BT, Gouveia K, Fruh K. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J Virol 2004;78:1109–1120.
51. Matsuki Y, Ohmura-Hoshino M, Goto E, Aoki M, Mito-Yoshida M, Uematsu M, Hasegawa T, Koseki H, Ohara O, Nakayama M, Toyooka K, Matsuoka K, Hotta H, Yamamoto A, Ishido S. Novel regulation of MHC class II function in B cells. EMBO J 2007;26:846–854.
52. Ohmura-Hoshino M, Matsuki Y, Aoki M, Goto E, Mito M, Uematsu M, Kakiuchi T, Hotta H, Ishido S. Inhibition of MHC class II expression and immune responses by c-MIR. J Immunol 2006;177:341–354.
53. De Gassart A, Camosseto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, Gatti E. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc Natl Acad Sci U S A 2008;105:3491–3496.
54. Thibodeau J, Bourgeois-Daigneault MC, Huppe G, Tremblay J, Aumont A, Houde M, Bartee E, Brunet A, Gauvreau ME, De GA, Gatti E, Baril M, Cloutier M, Bontron S, Fruh K, Lamarre D, Steimle V. Interleukin-10-induced MARCH1 med iates intracellular sequestration of MHC class II in monocytes. Eur J Immunol 2008;38:1225–1230.
55. Bania J, Gatti E, Lelouard H, David A, Cappello F, Weber E, Camosseto V, Pierre P. Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. Proc Natl Acad Sci U S A 2003;100:6664–6669.
56. McCormick PJ, Martina JA, Bonifacino JS. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. Proc Natl Acad Sci U S A 2005;102:7910–7915.
57. Kirk E, Chin LS, Li L. GRIF1 binds Hrs and is a new regulator of endosomal trafficking. J Cell Sci 2006;119:4689–4701.
58. Sloma I, Zilber MT, Vasselon T, Setterblad N, Cavallari M, Mori L, De LG, Charron D, Mooney N, Gelin C. Regulation of CD1a surface expression and antigen presentation by invariant chain and lipid rafts. J Immunol 2008;180:980–987.
59. van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M. Intestinal epi thelial cells secrete exosome-like vesicles. Gastroenterology 2001;121:337–349.
60. Mallegol J, van NG, Heyman M. Phenotypic and functional characterization ofi ntestinal epithelial exosomes. Blood Cells Mol Dis 2005;35:11–16.
61. Simpson RJ, Jensen SS, Lim JW. Proteomic profiling of exosomes: current perspectives. Proteomics 2008;8:4083–4099.
62. Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 2007;315:201–205.
63. Lapaque N, Jahnke M, Trowsdale J, Kelly AP. The HLA-DRalpha chain is modified by poly-ubiquitination. J Biol Chem 2008;284:7007–7016.
64. Unternaehrer JJ, Chow A, Pypaert M, Inaba K, Mellman I. The tetraspanin CD9 mediates lat eral association of MHC class II molecules on the dendritic cell surface. Proc Natl Acad Sci U S A 2007;104:234–239.
65. Sun SC. Deubiquitylation and regulation of the immune response. Nat Rev Immunol 2008;8:501–511.

66. Nagarajan UM, Bushey A, Boss JM. Modulation of gene expression by the MHC class II transactivator. J Immunol 2002;169:5078–5088.
67. Krawczyk M, Leimgruber E, Seguin-Estevez Q, Dunand-Sauthier I, Barras E, Reith W. Expression of RAB4B, a protein governing endocytic recycling, is co-regulated with MHC class II genes. Nucleic Acids Res 2007;35:595–605.
68. Muntasell A, Berger AC, Roche PA. T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. EMBO J 2007;26:4263–4272.
69. De Gassart A, Geminard C, Hoekstra D, Vidal M. Exosome secretion: the art of reutilizing nonrecycled proteins? Traffic 2004;5:896–903.
70. Stern LJ, Potolicchio I, Sant ambrogio L. MHC class II compartment subtypes: structure and function. Curr Opin Immunol 2006;18:64–69.
71. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. J Cell Sci 2002;115:2505–2515.
72. Vidal MJ, Stahl PD. The small GTP-binding proteins Rab4 and ARF are associated with released exosomes during reticulocyte maturation. Eur J Cell Biol 1993;60:261–267.
73. Komada M, Soriano P. Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. Genes Dev 1999;13:1475–1485.
74. Hislop JN, Marley A, Von ZM. Role of mammalian vacuolar protein-sorting proteins in endocytic trafficking of a non-ubiquitinated G protein-coupled receptor to lysosomes. J Biol Chem 2004;279:22522–22531.
75. Babst M. A protein's final ESCRT. Traffic 2005;6:2–9.
76. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51–59.
77. Ward CL, Omura S, Kopito RR. Degradation of CFTR by the ubiquitin-proteasome pathway. Cell 1995;83:121–127.
78. Khalil H, Brunet A, Thibodeau J. A three-amino-acid-long HLA-DRbeta cytoplasmic tail is sufficient to overcome ER retention ofi nvariant-chain p35. J Cell Sci 2005;118:4679–4687.
79. Robadey C, Ammerlaan W, Muller C, Cloutier I, S ékaly RP, Haefliger JA, Demotz S. The processing routes determined by negatively charged residues in DR1-restricted T cell determinants. J Immunol 1997;159:3238–3246.
80. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:293–299.
81. Radka SF, Machamer CE, Cresswell P. Analysis of monoclonal antibodies reactive with human class II beta chains by two-dimensional electrophoresis and western blotting. Hum Immunol 1984;10:177–188.
82. Masellis-Smith A, Shaw AR. CD9-regulated adhesion. Anti-CD9 monoclonal antibody induce pre-B cell adhesion to bone marrow fibroblasts through de novo recognition of fibronectin. J Immunol 1994;152:2768–2777.
83. Brunet A, Samaan A, Deshaies F, Kindt TJ, Thibodeau J. Functional characterization of a lysosomal sor ting motifi n the cytoplasmic tail of HLA-DObeta. J Biol Chem 2000;275:37062–37071.
84. Faubert A, Samaan A, Thibodeau J. Functional analysis of tryptophans alpha 62 and beta 120 on HLA-DM. J Biol Chem 2002;277:2750–2755.
85. Agard DA, Hiraoka Y, Shaw P, Seda t JW. Fluorescence microscopy in three dimensions. Methods Cell Biol 1989;30:353–377.
86. Lamparski HG, Metha-Damani A, Yao JY, Patel S, Hsu DH, Ruegg C, Le Pecq JB. Production and characterization of clinical grade exosomes derived from dendritic cells. J Immunol Methods 2002;270:211–226.
87. Alain R, Nadon F, Seguin C, Payment P, Trudel M. Rapid virus subunit visualization by direct sedimentation of samples on electron microscope grids. J Virol Methods 1987;16:209–216.
88. Khalil H, Brunet A, Saba I, Terra R, Sekaly RP, Thibodeau J. The MHC class II beta chain cytoplasmic tail overcomes the invariant chain p35-encoded endoplasmic reticulum retention signal. Int Immunol 2003;15:1249–1263.

Annexe III

Cutting Edge

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Cutting Edge: HLA-DO Impairs the Incorporation of HLA-DM into Exosomes

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In multivesicular bodies, HLA-DM (DM) assists the loading of antigenic peptides on classical MHC class II molecules such as HLA-DR. In cells expressing HLA-DO (DO), DM is redistributed from the internal vesicles to the limiting membrane of these organelles. This suggests that DO might reduce DM incorporation into exosomes, which are shed upon fusion of multivesicular bodies with the plasma membrane. To test this hypothesis, we used the 721.45 B lymphoblastoid cell line and different HeLa cell transfectants. We demonstrate that the poor recovery of DM in exosomes as compared with HLA-DR is not the mere reflection of differences in protein expression. Indeed, we found that DO contributes to the inefficient transfer of DM to exosomes. This negative regulation requires an intact di-leucine endosomal sorting motif in the cytoplasmic tail of HLA-DOβ. These results demonstrate that canonical sorting signals and protein–protein interactions modulate the selection of MHC protein cargos. ***The Journal of Immunology*****, 2011, 187: 1547–1551.**

Multivesicular bodies (MVBs) are characterized morphologically by the presence of intralumenal vesicles (ILVs) that form subsequently to the inward budding of the outer membrane (1). The intralumenal membranes enclose cytoplasm and carry transmembrane proteins, such as endocytosed activated receptors, that will be degraded by acid hydrolases upon fusion of MVBs with lysosomes (2). Alternatively, fusion may occur with the plasma membrane, causing the release of microvesicles (3). By analogy to the transferrin receptor-loaded vesicles produced by reticulocytes, the internal membranes of MVBs were called exosomes upon release (4). These 50–100-nm exocytic membrane vesicles have been described in hematopoietic cells but now appear to be part of a general physiological cellular process (5). Some of the roles attributed to exosomes overlap cell-specific effector functions (5).

One persistent feature of exosomes secreted by human and mouse APCs, including immortalized and tumor cells, is the display of classical MHC class II (MHC II) molecules at their surface (6–8). The mechanisms that regulate cargo inclusion into such suborganelle structures are beginning to emerge. The evidence that ubiquitination events regulate entry into ILVs is compelling. Katzmann's group (2) has shown that this posttranslational modification serves as a signal for cargo transport into MVBs and demonstrated the importance of the ESCRT machinery in this pathway. Accordingly, exosomes accumulate a vast array of ubiquitinated proteins (9).

Other mechanisms involved in MVB sorting are less well characterized. In yeast, both monoubiquitination-dependent and -independent mechanisms have been invoked (10). It has also been proposed that lipid raft domains might constitute the platform for entry into ILVs. Proteins with low recycling kinetics can be packaged into these microdomains for degradation or for exosomal secretion (11, 12). More recently, a ceramide-dependent mechanism of membrane invagination allowing formation of internal vesicles and cargo entry has been described (13). Although the coexistence of different types of MVBs has been reported, it is still unclear if exosome-secreting MVBs are specific entities and if, for example, ubiquitin-dependent and -independent mechanisms take place simultaneously on a given organelle (11, 13, 14).

Although ubiquitination of the cytoplasmic region of HLA-DR (DR) regulates its subcellular trafficking, we and others have shown that it is dispensable for exosome entry (15–18). Interestingly, the nonclassical MHC II molecule HLA-DM (DM) appears to be excluded from exosomes (19, 20). DM assists the loading of short peptides to classical MHC II molecules and mainly resides in the endocytic pathway. In certain dendritic cell subtypes and B cells, the activity of DM is regulated by another nonclassical MHC II molecule called HLA-DO (DO). Those two chaperones form a complex in the endoplasmic reticulum before gaining access to the endosomes. The presence of DO causes a redistribution of DM from the internal vesicles to the limiting membrane of MVBs, a phenomenon that could have repercussions on the

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Received for publication January 24, 2011. Accepted for publication June 21, 2011.

This work was supported by Canadian Cancer Society Grant 017230.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DM, HLA-DM; DO, HLA-DO; DR, HLA-DR; HeLa CIITA DOLL, HeLa CIITA devoid of its HLA-DOβ di-leucine motif; ILV, intralumenal vesicle; MFV, mean fluorescence value; MHC II, MHC class II; MVB, multivesicular body.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100199

protein composition of exosomes (21). In the current study, we have addressed the mechanisms of action of DO, the regulation of DM trafficking, and the structural basis behind the selective incorporation of cargo proteins into exosomes.

Materials and Methods

Abs and cell lines

L243 (HLA-DR), H4A3 (Lamp-1), 50H19 (CD9), XD5.117 (HLA-DRβ), MaP.DM1 (HLA-DM), Mags.DO5 (HLA-DO) mAbs, and the rabbit serum against the cytoplasmic tail of DMβ have been previously described (22–24). The EBV-transformed MHC-hemizygous B cell line 721.45 was obtained from Dr. Robert DeMars (25). HeLa DR1, HeLa DM.5, HeLa DM.5/DO5, HeLa CIITA, HeLa CIITA/DO, and HeLa DR/DOcyto stable transfectants were described previously (22, 24, 26).

Exosomes characterization

Exosomes were prepared and analyzed on immunoblots or by flow cytometry as described (18). Briefly, exosomes from the culture media of cells grown in exosome-free serum were obtained by ultracentrifugation. The exosome pellets were resuspended and coupled to aldehyde beads for flow cytometry analyses. Alternatively, exosomes were purified on a linear sucrose density gradient. Fractions were collected, acid-precipitated, and analyzed by SDS-PAGE.

Flow cytometry

Cells were harvested, fixed, permeabilized using saponin, and stained with primary mouse mAbs (22). After 45 min at 4°C, cells were washed twice in PBS and incubated for another 45 min with Alexa Fluor 488-conjugated goat anti-mouse secondary Abs (Invitrogen) in PBS. Cells were analyzed on an FACScalibur (BD Biosciences).

Western blotting

The exosomes were solubilized in reducing sample buffer, boiled, and analyzed by immunoblotting following separation of proteins on 12% acrylamide gels, as described (18).

Results and Discussion

DM and DR are differentially sorted into exosomes

We have re-evaluated the efficiency of DM incorporation into exosomes using different techniques, including quantitative flow cytometry. First, we characterized our exosomal preparations obtained from the culture media of the 721.45 B cell line and purified by sucrose density gradients (6). The different gradient fractions were analyzed for the presence of DR, CD9, and DM. The CD9 glycoprotein, used as a control, is a 24-kDa tetraspan family member associated with plasma and exosome membranes (27, 28). Immunoblot analysis showed that the bulk of DR molecules resides mostly in fractions of ≤1.21 g/ml (Fig. 1*A*, identified by asterisks). Exosomes isolated from culture medium of various cell types float in sucrose densities that vary between 1.08 and 1.20 g/ml (6). The CD9 distribution overlaps that of DR, confirming the exosomal nature of these membranes. Some DM was also found in the corresponding fractions (Fig. 1*A*). All three molecules were also detected in some heavier density fractions (fractions 7 and 8), probably due to cross-contamination during collection of the fractions. Alternatively, these membranes may originate from other types of vesicles (e.g., apoptotic bodies float at densities above 1.24 g/ml) (28). The presence of DR, DM, and CD9 on exosomes was confirmed by immunoelectron microscopy using material from fraction 3 of the gradient (Supplemental Fig. 1*A*).

To get a quantitative assessment of the efficiency of DM incorporation into exosomes, we turned to flow cytometry. Comparing the steady-state amounts of DR and DM in cells versus exosomes indicates if one molecule is selectively enriched comparatively to the other. 721.45 B cells were grown and exosomes were purified by ultracentrifugation of the culture media. The pellet was coupled to aldehyde beads and stained for the presence of DR or DM. In parallel, the producing cells were permeabilized and stained using the same Abs. Fig. 1*B* shows the expression profile for DR, DM, and CD9 in permeabilized cells and exosomes. The mean fluorescence values (MFVs) were used to derive ratios (exosomes over cells) for both DR and DM (Fig. 1*C*). This ratio is ~20-fold lower for DM, demonstrating that the poor recovery of DM in exosomes is not the mere reflection of lower cellular protein expression when compared with DR.

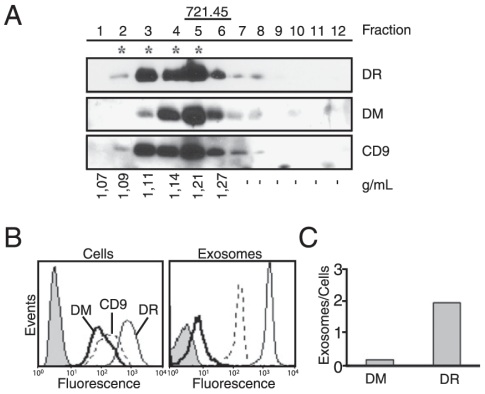

FIGURE 1. Inclusion of DM and DR in exosomes is differentially regulated. *A*, Culture medium from the 721.45 B lymphoblastoid cell line was centrifuged, and the pellet was subjected to ultracentrifugation in a continuous sucrose gradient. Fractions were collected and blotted for the presence of DRβ, CD9, and DMβ. Asterisks (*) indicate exosomal fractions. *B*, The expression of DR, DM, and CD9 was analyzed in fixed and permeabilized cells using specific mAbs (*left panels*). Exosomes shed from these cells were obtained by ultracentrifugation and coupled to beads for flow cytometry analyses (*right panel*). *C*, The MFVs obtained in *B* were plotted as the ratio of DM or DR expression in exosomes over permeabilized cells. These experiments were done twice, and representative results are shown.

The conclusion that DM and DR are differentially incorporated into exosomes was also drawn from the analysis of a second cellular model (Supplemental Fig. 1*B–D*). HeLa cells were stably transfected with CIITA, a transactivating factor regulating the constitutive and inducible expression of class II-related genes (18, 29, 30). As described for B cells, flow cytometry analyses of permeabilized cells and bead-coupled exosomes confirmed the poor recovery of DM in exosomes.

DO negatively regulates the incorporation of DM

The fact that DM is barely found in exosomes suggests that the selection of cargo proteins is tightly controlled. One potential regulator of DM trafficking is DO, which is expressed by most APCs as well as the 721.45 and HeLa CIITA cell lines (25, 26, 31, 32). DO inhibits DM and prevents movement of the complex from the limiting membrane to the internal vesicles of MVBs, where peptide loading occurs (21, 33). To test the hypothesis that DO downregulates incorporation of DM into exosomes, we used DM-expressing HeLa cells (HeLa DM.5) stably transfected with DO (HeLa DM.5/DO5) (26). Exosomes were prepared, coated to beads, and analyzed by flow cytometry for the presence of DM and CD9 (Fig. 2*A*). In parallel, cells were permeabilized and stained for CD9 and

DM. In these experiments, as opposed to the results presented in Fig. 1, the incorporation of a given marker is compared between two cell lines that inevitably express a different quantity of DM and will yield different amounts of exosomes. Thus, the results were expressed as the ratio of DM over CD9 for any given exosome preparation, and, most importantly, this value is compared with the one obtained for the permeabilized producer cells. In Fig. 2*A* (*bottom panel*), the DM/CD9 ratios show that there is relatively more DM in the HeLa DM.5/DO5 cells than in HeLa DM.5. In exosomes, however, the ratio is smaller for DM.5/DO5 as compared with DM.5 cells, demonstrating that the presence of DO reduced the incorporation of DM.

To rule out trivial clonal variations in the ability of the different cell lines to shed DM, we tested another independent set of transfectants. HeLa CIITA cells supertransfected with DO (HeLa CIITA DO) are characterized by an increased display of MHC II molecules complexed with the CLIP fragment of invariant chain (24). Exosomes were prepared from HeLa CIITA and HeLa CIITA DO cells, coupled to beads, and stained for CD9 or DM. Fig. 2*B* shows that the DM over CD9 ratio is about one third smaller for HeLa CIITA DO cells (0.82) as compared with HeLa CIITA (1.28). However, in exosomes, there is a 5-fold difference in the DM/CD9 ratios between HeLa CIITA DO (0.14) and HeLa CIITA (0.7), in line with a negative role of DO in the incorporation of DM. Of note, the effect appears to be direct as Lamp-1, which, like DM, is not preferentially enriched in B cell exosomes (19), was not affected by the presence of DO (Supplemental Fig. 2*A*).

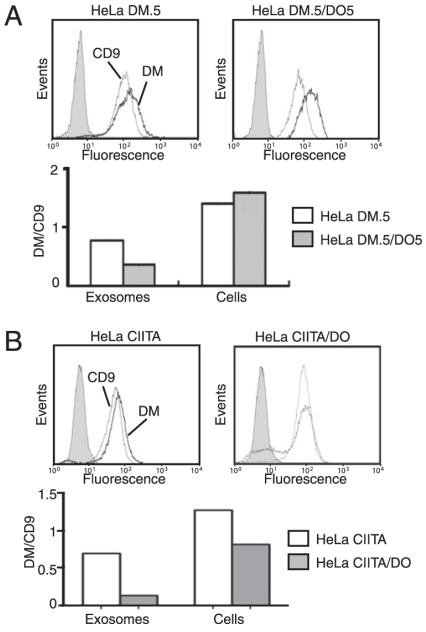

FIGURE 2. DO negatively regulates the incorporation of DM in exosomes. *A*, HeLa DM.5 and HeLa DM.5/DO5 cells were fixed, permeabilized, and stained. CD9 and DM expression profiles are shown (*upper panels*). The MFVs for DO in HeLa DM.5 and HeLa DM.5/DO5 were 5 and 64, respectively. Exosomes from these cells were coupled to beads and analyzed by flow cytometry after staining for CD9 and DM. The ratios of DM over CD9 MFVs were plotted (*bottom panel*). *B*, HeLa CIITA and HeLa CIITA/DO cells were analyzed as in *A*. The MFVs for DO in HeLa CIITA and HeLa CIITA/DO cells were 26 and 51, respectively. These experiments were done twice, and representative results are shown.

The activity of DO depends on a functional di-leucine motif

Site-directed mutagenesis of the di-leucine cytoplasmic motif of DOβ was shown to minimize the effect of DO on the subvesicular localization of DM (21). Accordingly, we reasoned that the same sorting signal is probably required to exclude DO–DM complexes from exosomes. Thus, we generated HeLa CIITA cells supertransfected with a mutated form of DOβ (HeLa CIITA devoid of its DOβ di-leucine motif [HeLa CIITA DOLL]) in which the two leucines were replaced by alanines (22) (Fig. 3). Exosomes from HeLa CIITA DO and HeLa CIITA DOLL were prepared in parallel and stained for DM and CD9. Although cells express very similar amounts of DM, the exosomes purified from HeLa CIITA DOLL contained more than twice as much DM, demonstrating that the effect of DO requires a functional di-leucine motif (Fig. 3*A*). Although disruption of this motif affected the recovery of DM, we have not been able to detect DO in exosome preparations (Supplemental Fig. 2*B*). This may be due to sensitivity of the Ab or degradation of DO in MVBs. Alternatively, as some DO appears to dissociate from DM in the endocytic pathway (31, 34), one can

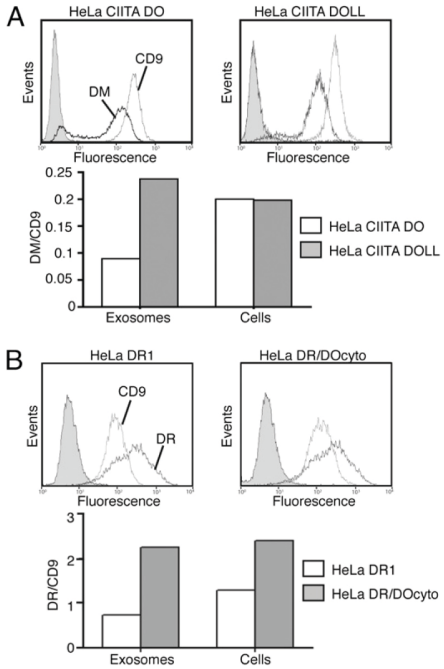

FIGURE 3. The activity of HLA-DOβ depends on its di-leucine motif. *A*, HeLa CIITA cells stably transfected with DO or with a variant devoid of its DOβ di-leucine motif (DOLL) were fixed, permeabilized, and stained. DM and CD9 expression patterns are shown (*top panels*). The MFVs for DO in HeLa CIITA/DO and HeLa CIITA/DOLL were 29 and 17, respectively. Exosomes from these cells were coupled to beads and analyzed by flow cytometry after staining for CD9 and DM. The ratios of DM over CD9 MFVs were plotted (*bottom panel*). *B*, HeLa cells stably transfected with DR1 or with a chimeric molecule in which the DRβ cytoplasmic tail was changed for the one of DOβ were analyzed as in *A*. These experiments were done twice, and representative results are shown.

speculate that the trafficking of free DO is modulated by factors independent of its cytoplasmic tail, causing its exclusion from ILVs. Indeed, MVBs are in constant reorganization, and molecules such as the mannose 6-phosphate receptors can be retrieved from the internal membranes, showing bidirectional exchange with the limiting membrane (35).

The cytoplasmic tail of DO fused to DR does not prevent incorporation into exosomes

A corollary to the above-described results is that a DR molecule with its β-chain cytoplasmic tail replaced by the one of DO would be excluded from exosomes. To test this hypothesis, we used HeLa cells stably transfected with a chimeric DR/DOβcyto molecule. We have shown previously that the di-leucine motif of DO is functional in this context, as the chimeric molecule accumulated more abundantly in lysosomal-like compartments (22). Exosomes from these cells were prepared and stained as above for the presence of DR and CD9. Interestingly, Fig. 3 shows that the chimeric molecule was not excluded from exosomes and was even more incorporated than the wild-type DR. This may be explained by the fact that at steady state, the subcellular localization of this chimeric molecule is slightly shifted from the plasma membrane to the endocytic pathway (22). The fact that the DR backbone overrides the negative effect of DO's cytoplasmic tail confirms that the luminal and/or transmembrane regions have intrinsic properties regulating intracellular trafficking (36, 37). This may translate, for example, in the selective incorporation of DR in large protein networks or into lipid rafts, which are abundant on exosomes (12). Interestingly, DM is excluded from classical rafts and is rather found in distinct tetraspan microdomains (38).

It was recently demonstrated that DR and DM interact in internal vesicles but not at the limiting membrane of MVBs (33). Thus, the capacity of DO to prevent DM entry into MVBs (or exosomes) is in line with the inhibitory activity of DO on CLIP removal. Paradoxically, in transfected cells, disruption of the di-leucine motif increased the inhibitory potential of DO while allowing more DM to access ILVs (21). Thus, we could hypothesize that the ensuing shedding of DM contributes to the inhibitory potential of DO. However, one must exercise caution in interchangeably referring to ILVs and exosomes, as the latter may originate from specific subsets of MVBs (11, 14, 18).

Taken together, our results demonstrate that: 1) DM is incorporated less efficiently than DR into exosomes; 2) the canonical sorting signal of DO regulates the trafficking of DM in *trans*; and 3) the nature of the transmembrane and/or luminal domains have a strong impact on protein incorporation into exosomes. Future experiments should investigate other potential mechanisms regulating cargo selection as, for example, mouse DCs express low levels of DO but do not appear to shed DM in exosomes (28).

Acknowledgments

We thank Eric O. Long and Robert DeMars for providing cDNAs and cell lines. We also thank Lisa Denzin for Abs.

Disclosures

The authors have no financial conflicts of interest.

References

1. Stoorvogel, W., M. J. Kleijmeer, H. J. Geuze, and G. Raposo. 2002. The biogenesis and functions of exosomes. *Traffic* 3: 321–330.
2. Katzmann, D. J., G. Odorizzi, and S. D. Emr. 2002. Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol.* 3: 893–905.
3. Murk, J. L., B. M. Humbel, U. Ziese, J. M. Griffith, G. Posthuma, J. W. Slot, A. J. Koster, A. J. Verkleij, H. J. Geuze, and M. J. Kleijmeer. 2003. Endosomal compartmentalization in three dimensions: implications for membrane fusion. *Proc. Natl. Acad. Sci. USA* 100: 13332–13337.
4. Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262: 9412–9420.
5. Thery, C., M. Ostrowski, and E. Segura. 2009. Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* 9: 581–593.
6. Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. M. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* 183: 1161–1172.
7. van Niel, G., G. Raposo, C. Candalh, M. Boussac, R. Hershberg, N. Cerf-Bensussan, and M. Heyman. 2001. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 121: 337–349.
8. Chaput, N., N. E. Schartz, F. Andre, and L. Zitvogel. 2003. Exosomes for immunotherapy of cancer. *Adv. Exp. Med. Biol.* 532: 215–221.
9. Buschow, S. I., J. M. Liefhebber, R. Wubbolts, and W. Stoorvogel. 2005. Exosomes contain ubiquitinated proteins. *Blood Cells Mol. Dis.* 35: 398–403.
10. Reggiori, F., and H. R. Pelham. 2001. Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *EMBO J.* 20: 5176–5186.
11. de Gassart, A., C. Géminard, D. Hoekstra, and M. Vidal. 2004. Exosome secretion: the art of reutilizing nonrecycled proteins? *Traffic* 5: 896–903.
12. de Gassart, A., C. Geminard, B. Fevrier, G. Raposo, and M. Vidal. 2003. Lipid raft-associated protein sorting in exosomes. *Blood* 102: 4336–4344.
13. Trajkovic, K., C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger, and M. Simons. 2008. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319: 1244–1247.
14. White, I. J., L. M. Bailey, M. R. Aghakhani, S. E. Moss, and C. E. Futter. 2006. EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation. *EMBO J.* 25: 1–12.
15. van Niel, G., R. Wubbolts, T. Ten Broeke, S. I. Buschow, F. A. Ossendorp, C. J. Melief, G. Raposo, B. W. van Balkom, and W. Stoorvogel. 2006. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity* 25: 885–894.
16. Thibodeau, J., M. C. Bourgeois-Daigneault, G. Huppé, J. Tremblay, A. Aumont, M. Houde, E. Bartee, A. Brunet, M. E. Gauvreau, A. de Gassart, et al. 2008. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *Eur. J. Immunol.* 38: 1225–1230.
17. Buschow, S. I., E. N. Nolte-'t Hoen, G. van Niel, M. S. Pols, T. ten Broeke, M. Lauwen, F. Ossendorp, C. J. Melief, G. Raposo, R. Wubbolts, et al. 2009. MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic* 10: 1528–1542.
18. Gauvreau, M. E., M. H. Côté, M. C. Bourgeois-Daigneault, L. D. Rivard, F. Xiu, A. Brunet, A. Shaw, V. Steimle, and J. Thibodeau. 2009. Sorting of MHC class II molecules into exosomes through a ubiquitin-independent pathway. *Traffic* 10: 1518–1527.
19. Escola, J. M., M. J. Kleijmeer, W. Stoorvogel, J. M. Griffith, O. Yoshie, and H. J. Geuze. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273: 20121–20127.
20. Buschow, S. I., B. W. van Balkom, M. Aalberts, A. J. Heck, M. Wauben, and W. Stoorvogel. 2010. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunol. Cell Biol.* 88: 851–856.
21. van Lith, M., M. van Ham, A. Griekspoor, E. Tjin, D. Verwoerd, J. Calafat, H. Janssen, E. Reits, L. Pastoors, and J. Neefjes. 2001. Regulation of MHC class II antigen presentation by sorting of recycling HLA-DM/DO and class II within the multivesicular body. *J. Immunol.* 167: 884–892.
22. Brunet, A., A. Samaan, F. Deshaies, T. J. Kindt, and J. Thibodeau. 2000. Functional characterization of a lysosomal sorting motif in the cytoplasmic tail of HLA-DObeta. *J. Biol. Chem.* 275: 37062–37071.
23. Denzin, L. K., N. F. Robbins, C. Carboy-Newcomb, and P. Cresswell. 1994. Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1: 595–606.
24. Deshaies, F., A. Brunet, D. A. Diallo, L. K. Denzin, A. Samaan, and J. Thibodeau. 2005. A point mutation in the groove of HLA-DO allows egress from the endoplasmic reticulum independent of HLA-DM. *Proc. Natl. Acad. Sci. USA* 102: 6443–6448.
25. Ceman, S., R. Rudersdorf, E. O. Long, and R. Demars. 1992. MHC class II deletion mutant expresses normal levels of transgene encoded class II molecules that have abnormal conformation and impaired antigen presentation ability. *J. Immunol.* 149: 754–761.
26. Khalil, H., F. Deshaies, A. Bellemare-Pelletier, A. Brunet, A. Faubert, G. A. Azar, and J. Thibodeau. 2002. Class II transactivator-induced expression of HLA-DO(β) in HeLa cells. *Tissue Antigens* 60: 372–382.
27. Wright, M. D., and M. G. Tomlinson. 1994. The ins and outs of the transmembrane 4 superfamily. *Immunol. Today* 15: 588–594.
28. Théry, C., M. Boussac, P. Véron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* 166: 7309–7318.
29. Stumptner-Cuvelette, P., S. Morchoisne, M. Dugast, S. Le Gall, G. Raposo, O. Schwartz, and P. Benaroch. 2001. HIV-1 Nef impairs MHC class II antigen

presentation and surface expression. *Proc. Natl. Acad. Sci. USA* 98: 12144–12149.
30. Bania, J., E. Gatti, H. Lelouard, A. David, F. Cappello, E. Weber, V. Camosseto, and P. Pierre. 2003. Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. *Proc. Natl. Acad. Sci. USA* 100: 6664–6669.
31. Fallas, J. L., W. Yi, N. A. Draghi, H. M. O'Rourke, and L. K. Denzin. 2007. Expression patterns of H2-O in mouse B cells and dendritic cells correlate with cell function. *J. Immunol.* 178: 1488–1497.
32. Hornell, T. M., T. Burster, F. L. Jahnsen, A. Pashine, M. T. Ochoa, J. J. Harding, C. Macaubas, A. W. Lee, R. L. Modlin, and E. D. Mellins. 2006. Human dendritic cell expression of HLA-DO is subset specific and regulated by maturation. *J. Immunol.* 176: 3536–3547.
33. Zwart, W., A. Griekspoor, C. Kuijl, M. Marsman, J. van Rheenen, H. Janssen, J. Calafat, M. van Ham, L. Janssen, M. van Lith, et al. 2005. Spatial separation of HLA-DM/HLA-DR interactions within MIIC and phagosome-induced immune escape. *Immunity* 22: 221–233.
34. Gondré-Lewis, T. A., A. E. Moquin, and J. R. Drake. 2001. Prolonged antigen persistence within nonterminal late endocytic compartments of antigen-specific B lymphocytes. *J. Immunol.* 166: 6657–6664.
35. Jiang, L., A. Erickson, and J. Rogers. 2002. Multivesicular bodies: a mechanism to package lytic and storage functions in one organelle? *Trends Cell Biol.* 12: 362–367.
36. Theos, A. C., S. T. Truschel, D. Tenza, I. Hurbain, D. C. Harper, J. F. Berson, P. C. Thomas, G. Raposo, and M. S. Marks. 2006. A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev. Cell* 10: 343–354.
37. Huang, J., F. Reggiori, and D. J. Klionsky. 2007. The transmembrane domain of acid trehalase mediates ubiquitin-independent multivesicular body pathway sorting. *Mol. Biol. Cell* 18: 2511–2524.
38. Kropshofer, H., S. Spindeldreher, T. A. Röhn, N. Platania, C. Grygar, N. Daniel, A. Wölpl, H. Langen, V. Horejsi, and A. B. Vogt. 2002. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nat. Immunol.* 3: 61–68.

<u>Supplemental figure 1</u>: DR, CD9 and DM are differentially sorted in exosomes

(A) IEM was performed on whole-mounted exosomes from fraction 3 (see figure 1A). Exosomes were deposited on grids and stained with rabbit anti-DR serum, 50H.19 (CD9) or Map.DM1 (HLA-DM) mouse mAbs After three washes, samples were stained with a drop of 3% phosphotungstic acid (pH 6.0) and dried out. Secondary Abs were 12nm bead-coupled goat anti-mouse and 18nm bead-coupled goat anti-rabbit antibodies. Bar, 100nm. (**B**) Culture media from the HeLa CIITA cells line was centrifuged and the pellet was subjected to ultracentrifugation in a continuous sucrose gradient. Fractions were collected and blotted for the presence of DRβ, CD9 and DMβ. Asterisks (*) indicate exosomal fractions. **(C)** The expression of DR, DM and CD9 was analyzed in fixed and permeabilized cells using specific mAbs (left panel). Exosomes shed from these cells were obtained by ultracentrifugation and coupled to beads for flow cytometry analyses (right panel). **(D)** The mean fluorescence values (MFVs) obtained in panel B were plotted as the ratio of DM or DR expression in exosomes over permeabilized cells. These experiments were done twice and representative results are shown.

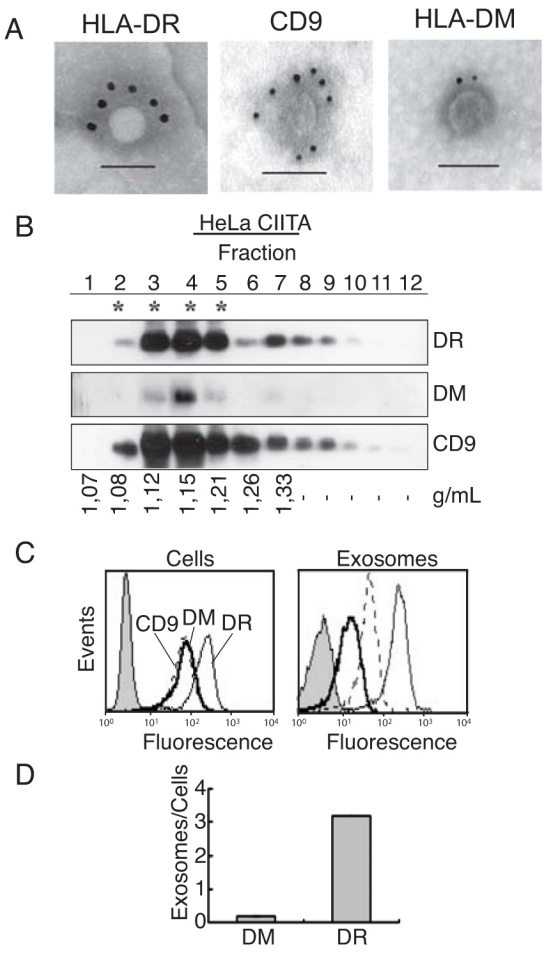

Figure S1

Supplemental figure 2: DO exerts its effect directly on DM

(A) Exosomes were prepared by ultracentrifugation from HeLa CIITA and HeLa CIITA DO cell culture media. Exosomes were coupled to beads, stained with DM-, CD9- or Lamp-1-specific mAbs and analyzed by flow cytometry. Permeabilized cells were stained with the same mAbs. The results were expressed as the ratio of MFVs obtained for DM or Lamp-1 over CD9.

The DM over CD9 ratio is about one third smaller for HeLa CIITA DO cells (3.07) as compared to HeLa CIITA (8.37). However, in exosomes, there is more than a 5 fold difference in the DM/CD9 ratios between HeLa CIITA DO (0.14) and HeLa CIITA (1.03), in line with the negative role of DO in the incorporation of DM observed in Figure 2. However, for Lamp-1, the situation was different. The Lamp-1 over CD9 ratio is about the same for HeLa CIITA DO cells (12.1) and HeLa CIITA (14) while in exosomes, we found about twice as much Lamp-1 in HeLa CIITA DO (ratio of 1,98) as compared to HeLa CIITA (0,94). This clearly demonstrates that Lamp-1 does not behave like DM in the presence of DO. **(B)** Exosomes were coupled to beads and analyzed by flow cytometry as above. Beads were also stained with the Mags.DO5 mAb.