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MARCH ligases in immunity

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Membrane associated RING-CH (MARCH) ubiquitin ligases control the stability, trafficking and function of important immunoreceptors, including MHC molecules and costimulatory molecule CD86. Regulation of the critical antigen presenting molecule MHC II by MARCH1 and the control of MARCH1 expression by inflammatory stimuli is a key step in the function of antigen presenting cells. MHC II ubiquitination by MARCH8 and CD83 plays a critical role in T cell thymic selection. Recent studies reveal new immune functions of MARCH ligases in innate immunity, regulation of FcγR expression and T_{reg} development. In addition, we review the importance of MARCH in immunomodulation at the host–pathogen interface. Both bacterial and viral pathogens manipulate MARCH function, while MARCH ligases act as an important host anti-viral defence mechanism. Here, we review the role of membrane-bound MARCH ligases in immune function and provide an update on new substrates and concepts. Understanding the increasingly complex roles of MARCH E3 ligases will be vital to develop therapeutic strategies for their regulation.

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Introduction

Ubiquitination is a highly conserved and versatile post-translational modification which can control the localisation and degradation of cytosolic and membrane proteins [1]. It requires the concerted action of ubiquitin E1, E2 and E3 ligases (Box 1). As determinants of substrate specificity, the E3s have attracted preferential research interest (reviewed in Ref. [2]). The membrane-associated RING-CH (MARCH) family are E3 ligases. They were first discovered in viruses as immunoevasins that were able to downregulate MHC class I molecule (MHC I) expression. These include K3 and K5 (also called modulator of immune recognition, or MIR1 and MIR2) encoded by Kaposi's sarcoma associated herpesvirus (KSHV) [3,4], murine mK3 from gammaherpesvirus-68 [5] and pK3 from rodent herpesvirus Peru [6]. Although structurally similar, the viral MARCH ligases employ different immune evasion strategies by targeting different subsets of substrates in addition to MHC I (reviewed in Ref. [7]). Additionally, K3 and K5 are differentially regulated during lytic replication and may have distinct roles in KSHV immune evasion [8].

Bioinformatic studies identified eleven mammalian homologues of K3 and K5 which contained the RING-CH domain and were called MARCH1–11 ([9], reviewed in Refs. [10,11•]). Apart from MARCH7/10, which do not possess transmembrane domains, all MARCH enzymes are similarly organised with one N-terminal RING-CH domain followed by two or more transmembrane domains (Figure 1). These domains also play a major role in substrate recognition via interaction with the transmembrane regions of MARCH targets [12–14]. Phylogenetic analysis defines four MARCH groups comprising MARCH1 and 8, MARCH2 and 3, MARCH7 and 10, and MARCH4, 9 and 11 [9,11•]. Here, we review the role of the MARCH family members involved in immunity, namely MARCH1, 2, 3, 4, 8 and 9 (Figure 2). The mitochondrial MARCH5, endoplasmic reticulum-associated MARCH6, cytosolic MARCH7 and 10, as well as MARCH11 have not yet been described to play immunological roles and will not be discussed here, but have been reviewed elsewhere [10].

Regulation of MHC II expression, trafficking and turnover by MARCHs

The paradigm of activity and function of the MARCHs is the regulation of MHC II surface expression and turnover by MARCH1. The cytoplasmic tail of the MHC II β chain contains a conserved Lys residue which is constitutively polyubiquitinated in resting mouse and human B cells [15], plasmacytoid dendritic cells (pDC), conventional dendritic cells (cDC) [16,17] and monocytes [18]. The cellular location where MHC II is ubiquitinated remains unknown, though it is most likely the plasma membrane or early endosomes accessed by constitutively pinocytosed molecules [16]. Ubiquitinated MHC II is recognised by the endosomal sorting complexes required for transport (ESCRT) and delivered to late endosomes and lysosomes for destruction [19]. Not all endocytosed

Box 1 Mechanism of ubiquitination.

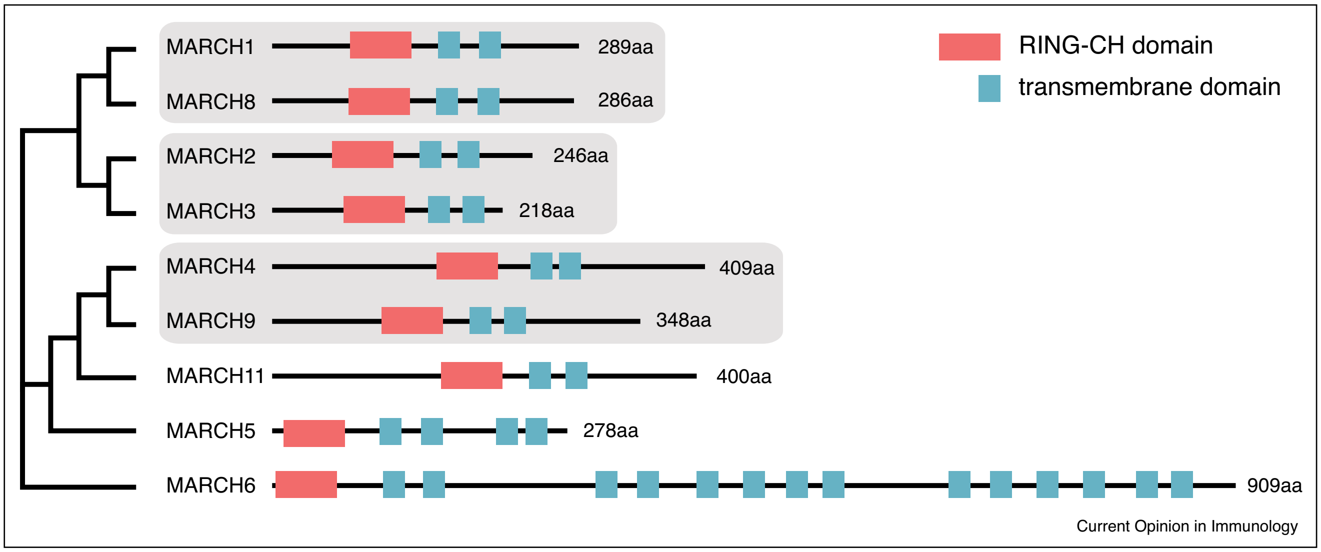
Ubiquitination is a highly conserved and versatile ‘molecular switch’ which can control the localisation and degradation of target proteins in a specific and reversible manner. The most well characterised consequences of ubiquitination are the recognition and degradation by the proteasome of cytosolic polypeptides [57], and the delivery of membrane proteins to late endosomes, intra-luminal vesicles and lysosomes by ESCRT [58]. Target protein ubiquitination involves a three-step reaction in which free ubiquitin is first covalently bound to a ubiquitin-activating enzyme (E1), subsequently transferred to a ubiquitin-conjugating enzyme (E2), and finally attached to a substrate residue by a ubiquitin ligase (E3) [1]. Among E3 ligases, the family of RING (Really Interesting New Gene) type ligases represent the largest group with over 600 predicted enzymes [2]. They contain a catalytic zinc finger domain, which is further classified by the order of conserved cysteine (C) and histidine (H) residues into RING-HC (C3HC4), RING-H2 (C3H2C3) and RING-CH (C4HC3) subfamilies [2].

MHC II molecules are ubiquitinated, and those that are not return to the plasma membrane via recycling [20]. In the absence of MARCH1, all (non-ubiquitinated) MHC II recycles, causing a net 5–10-fold increase in surface expression. MHC II ubiquitination decreases or ceases when B cells, pDC, cDC or monocytes become activated by TLR ligands such as LPS or CpG due to partial or complete downregulation of MARCH1 transcription [16,17,21•]. This leads to the accumulation of long-lived MHC II molecules, produced during the period post-activation, on the surface of the stimulated cells.

The proteins that drive MARCH1 transcription are unknown, but their activity is finely regulated and maintained at a very low level [22], with TLR signalling reducing transcription even further. IL-10 causes the opposite effect in macrophages and monocytes (though not in DC or B cells), leading to reduced MHC II expression that contributes to the immunosuppressive effects of this cytokine [16,17,21•,23•]. The MARCH1 protein itself has a short half-life because it undergoes self-ubiquitination and degradation [24]. This combines with low transcription/translation to keep MARCH1 at very low levels in resting cells, and to be rapidly lost when transcription decreases in activated cells. Low expression has hampered the determination of MARCH1 residence and activity within cells. MARCH1 ubiquitination is further reduced by CD83 (see below). Maintenance of low expression and/or activity is probably necessary to avoid uncontrolled ubiquitination of membrane proteins. Indeed, many receptors found to be ubiquitinated in transfected cells overexpressing MARCH1 were not so in primary cells, indicating they were off-targets (our unpublished results).

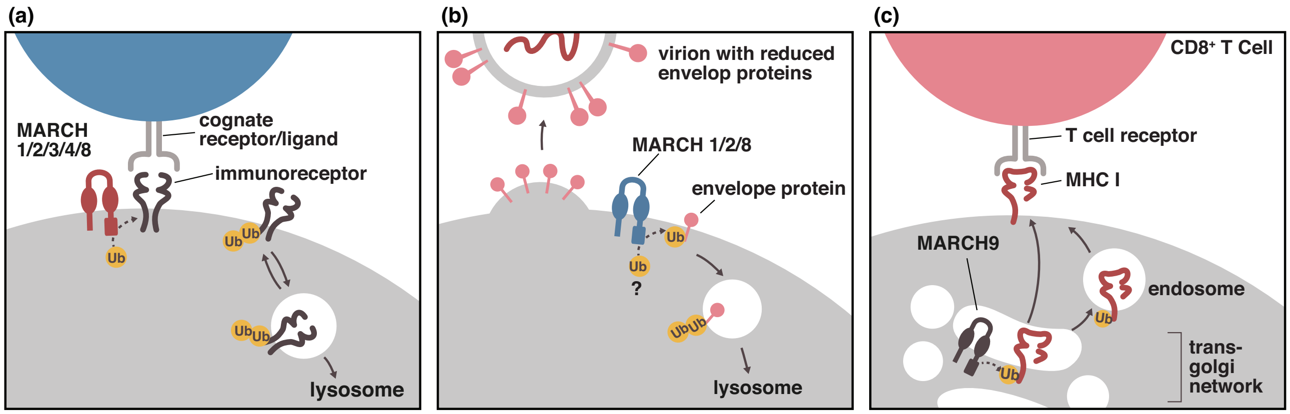
The impact of MARCH1 ubiquitination on MHC II function has not been fully characterised. Studies of *March1*$^{-/-}$ mice can be difficult to interpret due to the confounding effects of MARCH1 ubiquitination of other targets (see below), but can be complemented with analyses of MHC II-K225R knock-in mice where MHC II is not ubiquitinated but other MARCH1 targets are [25]. Thymic cDC of mice lacking MARCH1 or expressing MHC II-K225R are inefficient at driving natural T$_{reg}$ cell selection [26,27]. Their splenic counterparts are likewise defective at activating naïve CD4$^+$ T cells [25,27]. Induction of MARCH1 expression in donor

Figure 1



Schematic illustration of the membrane-bound murine MARCH ligases. The RING-CH domain (red boxes) and transmembrane domains (blue boxes) are shown at their respective positions in the protein, with the total size (in amino acid residues) indicated on the right of each protein. MARCH ligases with high homology are grouped in light grey boxes. The phylogenetic tree was generated with Clustal Omega alignment using the protein sequences in the Uniprot database.

Figure 2



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Summary of the different immunomodulatory roles of MARCH ligases. **(a)** MARCH ligases (including MARCH1, MARCH2, MARCH3, MARCH4, and MARCH8) can reduce immunoreceptor binding to cognate receptors or ligands by increasing their turnover from the cell membrane and/or lysosomal degradation. **(b)** MARCH1, MARCH2 and MARCH8 can impair virus production of infected cells by reducing cell surface abundance of viral envelope proteins. Whether these proteins are ubiquitinated directly or trafficked through an indirect mechanism is unclear. **(c)** MARCH9 regulates antigen cross-presentation by ubiquitinating MHC I molecules and inducing their transport from the trans-Golgi network to the plasma membrane, where they can be recognised by CD8$^+$ T cells.

graft DC with IL-10 can reduce MHC II expression and alloimmunity [23•]. In B cells, MHC II ubiquitination is involved in B cell receptor affinity maturation in the germinal centre [28], although another study found no difference in antibody responses in MHC II-K225R mice [29]. Of note, the effects of impaired MHC II ubiquitination may not be caused by increased MHC II levels *per se*, but by reduced turnover of MHC II-peptide complexes.

A tissue that expresses MHC II but not MARCH1 is the thymic epithelium, which plays a crucial role in CD4$^+$ T cell selection. Instead, thymic epithelial cells express MARCH8 to restrict surface MHC II expression [30,31]. These studies establish the concept that MHC II is regulated in antigen presenting cells of hematopoietic versus non-hematopoietic origin by MARCH1 and MARCH8, respectively, to keep surface MHC II levels within narrow limits. The importance of this regulation is illustrated in mice that lack CD83, which is proposed to bind to the transmembrane domain of MARCH1 or 8 to prevent interaction with, and ubiquitination of their substrate proteins [13]. In CD83-deficient mice, unchecked MARCH8 ubiquitination reduces MHC II expression in thymic epithelium, causing impaired CD4$^+$ T cell development. Deletion of MARCH8 in CD83-deficient mice restores MHC II expression and rescues the CD4$^+$ T cell defect [30,31].

Regulation of other immune receptors by MARCHs

Multiple studies have described immunomodulatory functions of MARCH1 beyond control of MHC II expression. *March1*$^{-/-}$ cDC produce less TNF-α and IL-12 upon TLR stimulation [25,32,33]. Another study found that *March1*$^{-/-}$ mice produce higher inflammatory blood cytokines than wild type mice in response to a sublethal injection of LPS and succumb quicker to LPS-induced endotoxic shock [34]. The mechanism of this anti-inflammatory role of MARCH1 is not clear, but involves altering the recruitment of Ly6C^{hi} monocytes. Another role for MARCH1 was identified in a model of allergic asthma, where *March1*$^{-/-}$ mice exhibited increased neutrophilic inflammation, but reduced lung IL-6 and serum IgE, after antigen challenge [35]. Interestingly, these phenotypic alterations are independent of MHC II, as they were not phenocopied in MHC II-K225R knock-in mice [34,35].

These studies establish MARCH1 as a multi-functional regulator of both innate and adaptive immunity, but it remains unclear which are the putative MARCH1substrates that cause these alterations when they are no longer ubiquitinated. The only molecule apart from MHC II that has been shown to be ubiquitinated by MARCH1 *in vivo* is CD86 [36]. Although MARCH1 can ubiquitinate other proteins when overexpressed [10], it is uncertain how many of these reported substrates, if any, are actual targets *in vivo*. It can also be difficult to distinguish whether a particular phenotype in *March1*$^{-/-}$ mice is due to altered ubiquitination of a MARCH1 substrate, or to indirect effects on, for example, T$_{reg}$ [26] or DC [25] development, or even to non-immunological effects, for example altered expression of insulin receptors [37]. Furthermore, we have recently shown that lack of MHC II ubiquitination can affect the expression and function of non-MARCH1 targets. Splenic cDC from *March1*$^{-/-}$ and MHC II-K225R mice have reduced MHC class I surface expression and antigen presentation to CD8^{+} T cells [38]. This may be due to disruption of lipid rafts and/or tetraspanin membrane microdomains enriched in MHC II, indirectly affecting MHC I trafficking and expression [27]. Such a phenomenon may also explain why cDC expressing the MHC II-K225R mutation produce less cytokines in response to TLR ligands [25,32,33]. In summary, there are still many open questions with regards to the true range of membrane proteins ubiquitinated by MARCH1, and the functional consequences of this activity. Unbiased analysis of the impact of MARCH1 deficiency on membrane protein expression using high-throughput methods, and direct validation of the role of ubiquitination on these proteins, will help fill this knowledge gap.

Reports describing immunoregulatory roles for other MARCHs are scarce. MARCH3 was reported to ubiquitinate the inhibitory FcγRIIb and may increase antibody-mediated responses and/or target cell killing by monocytes and macrophages [39]. MARCH3 also regulates histamine-induced endothelial permeability, though the direct substrate and mechanisms are not clear [40]. Recently, MARCH3 was reported to attenuate IL-1β mediated inflammation by ubiquitinating IL-1RI. *March3*$^{-/-}$ mice have increased serum TNFα and IL-1β, and are more susceptible to IL-1β or *L. monocytogenes*-induced inflammatory death [41••]. MARCH8 can ubiquitinate CD81 [42], CD86 [43] and IL1RAP in transfected cells [44], but it is unclear if this is also the case *in vivo*. Although a handful of target proteins, including MHC I, have been described for MARCH9 [11•], only recently were such interactions confirmed to occur under physiological conditions. MARCH9 ubiquitinates MHC I and the MHC I-like molecule CD1a, and splenic DCs from *March9*$^{-/-}$ mice were inferior at cross-presentation of ovalbumin-coated beads. Intriguingly, MHC I ubiquitination by MARCH9 appears to induce its exit from the trans-Golgi network, increasing MHC I expression on the plasma membrane [45••]. This is a striking contrast to the consequences of MARCH1 and MARCH8-mediated ubiquitination, which induce lysosomal degradation [20].

Emerging roles for MARCH ligases in host–pathogen interactions

A growing number of pathogens have been discovered to usurp host MARCHs during infection. For example, pathogenic bacteria such as *Francisella tularensis* [46], *Streptococcus suis* [47] and *Salmonella enterica* Typhimurium [48] reduce surface MHC II in infected antigen presenting cells by increasing its ubiquitination, though the mechanisms involved are different. While *Francisella* infection induces IL-10-mediated MARCH1 expression via stimulating prostaglandin E2 production in infected cells [49], *Streptococcus* induces MARCH1 mRNA transcription without affecting IL-10 [47]. In contrast, *Salmonella* does not modulate MARCH ligase expression, but produces and secretes the effector protein SteD, which is proposed to directly bind MARCH8 and its substrates to promote MARCH8-mediated ubiquitination of MHC II and CD86 [50•]. Whether SteD can also hijack MARCH1 is an interesting question that remains to be determined.

MARCH ligases also have important functions in viral immunity. Findings by Tada *et al.* suggest that MARCH8 can limit viral budding from the plasma membrane by ubiquitinating viral envelope proteins [51]. Unlike in murine hematopoietic antigen presenting cells, where MARCH8 is not active [30,52], MARCH8 is highly expressed in human monocyte-derived macrophages and monocyte-derived dendritic cells (MDDCs), and silencing MARCH8 strikingly increases the infectivity of viruses produced by these cells. Similar anti-viral functions have been reported for human MARCH1, which is induced by type I interferons in monocyte-derived macrophages (MDMs) [53], and for MARCH2, which is upregulated during infection with human immunodeficiency virus (HIV) 1 [54]. MARCH2 inhibits HIV-1 production by infected cells by reducing cell surface levels of envelope protein [54]. Of note, neither of these studies provided direct evidence of ubiquitination of the viral envelope proteins that were downregulated.

In an interesting development, KSHV actively induces cellular MARCH8 expression via lytic replication and transcription activator (RTA) to downregulate human leucocyte antigen (HLA) DRα [55], in addition to downregulating MHC class I molecules through expression of its own ligases K3 and K5 [3,4]. Recently, MARCH8 was shown to mediate intracellular budding of hepatitis C virus (HCV) by ubiquitinating non-structural (NS) 2 protein, with MARCH8 activity being critical for the infectivity of HCV, Dengue and Zika viruses [56]. Taken together, these findings suggest that MARCH E3 ligase activity can serve a dual role in immunity: on the one hand, they participate in the normal cellular anti-viral response by interfering with virus production, but the same MARCH ligases can be repurposed by other pathogens to increase their own infectivity, or to downregulate important immunoreceptors to dampen the immune response.

Conclusion

In summary, MARCH E3 ligases have now emerged as potent modulators of the immune system. Newly discovered roles of MARCH1, MARCH2 and MARCH8 in bacterial infection, anti-viral defence, as well as T cell development highlight the need to increase our understanding of MARCH biology to exploit MARCH ligases for improved immunological outcomes.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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