Mol. Cells *29*, 229-232, March 31, 2010
DOI/10.1007/s10059-010-0051-x

Minireview

Molecules and Cells

MARCH-I: a New Regulator of Dendritic Cell Function

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We and other groups have demonstrated that the expression level of MHC class II (MHC II) is regulated through ubiquitination of the MHC II β chain. We also reported that MARCH-I, an E3 ubiquitin ligase, is critical for this process. At present, however, the importance of MARCH-I-mediated MHC II regulation *in vivo* is still unknown. In this review, we will summarize recent advances in our understanding of MARCH-I-mediated MHC II ubiquitination, and discuss how we can overcome the difficulties inherent in these studies.

INTRODUCTION

Many physiologically important receptors are expressed as cell surface transmembrane proteins that must be downmodulated after interaction with their ligands in order to prevent overstimulation of the cell. In many cases, this is accomplished by receptor degradation, a process that has been intensively investigated in the field of cancer biology. The EGF receptor is one of the best characterized molecules, and many studies have demonstrated that ubiquitination is an important protein modification required for degradation of this receptor. The E3 ubiquitin ligase c-Cbl was found to be a critical enzyme for ubiquitination of the phosphorylated EGF receptor (Ryan et al., 2006; Thien and Langdon, 2005). Recently, we and other groups have demonstrated that ubiquitination contributes to the homeostasis of membrane proteins involved in immune responses (Coscoy and Ganem, 2003; Ishido et al., 2009; Lehner et al., 2005; Nathan and Lehner, 2009). Thus, many membrane proteins involved in diverse biological events are regulated by ubiquitination.

From an immunological perspective, it still remains unclear how the stability of MHC molecules, which are critical membrane proteins for antigen presentation, is regulated *in vivo*. In this vein, we have recently discovered a physiological E3 ubiquitin ligase for MHC class II, which was named MARCH-I (Ishido et al., 2009; Matsuki et al., 2007). Since MHC class II is a key molecule for the initiation of an immune response, this finding has lead to the exiting idea that MARCH-I-mediated MHC class II ubiquitination contributes to the regulation of immunological events (Ishido et al., 2009). At present, however, no one has succeeded in showing directly that MARCH-I regulates antigen presentation through MHC II ubiquitination. Instead, we have fund a surprising and unexpected role of MARCH-I in the immune system (Ohmura-Hoshino et al., 2009). In this short review, we will summarize the present status of the study of MARCH-I and suggest possible future directions for the study of MARCH-I-mediated MHC II ubiquitination.

History of MARCH

Around 2000, we and other groups identified two viral proteins involved in immune evasion. These proteins, named MIR1 and MIR2, are encoded by the Kaposi's sarcoma-associated herpes virus (KSHV) (Coscoy and Ganem, 2000; Ishido et al., 2000). Interestingly, these proteins have an activity as E3 ubiquitin ligases for MHC class I (Coscoy and Ganem, 2003). Therefore, we assumed that MIR-mediated MHC class I downregulation contributes to immune evasion by KSHV by inhibiting MHCI-restricted cytotoxic T cell activity. It is well know that large DNA viruses encode several homologs of mammalian proteins, such as IL-6, that can modulate host immunity (Choi et al., 2001). Therefore, we used a bioinformatic approach to search for candidate mammalian MIR homologs and identified one, which we named c-MIR (Goto et al., 2003). At the same time, the Fruh group reported that c-MIR is a member of a membrane-bound E3 ubiquitin ligase family known as MARCH (Bartee et al., 2004). At present, c-MIR is called MARCH-VIII. The MARCH family has eleven members and is reported to have potentially interesting substrates in the immune system (Jabbour et al., 2009).

What is MARCH-I

Among MARCH family members, several were shown to downregulate surface molecules involved in antigen presentation. However, these data were obtained in overexpression experiments (Bartee et al., 2004; Goto et al., 2003). Therefore, to test whether these candidates are indeed physiological E3s, we have generated genetically modified mice in which the molecules of interest are deleted. Using this strategy, we found that MARCH-I is a physiological E3 ubiquitin ligase for MHC class II (Matsuki et al., 2007). Importantly, MARCH-I is expressed primarily in secondary lymphoid tissues, lymph node and spleen and is highly expressed in antigen-presenting cells (APCs), B cell and dendritic cells (Bartee et al., 2004; Matsuki et al., 2007). Moreover, the Mellman and Stoorvogel groups reported that MHC II expression is regulated by ubiquitination of its β chain in

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Received January 18, 2010; accepted January 20, 2010; published online March 4, 2010

Keywords: antigen presentation, E3 ubiquitin ligase, MHC class II

dendritic cells, an important type of APC (Shin et al., 2006; van Niel et al., 2006). Thus, MARCH-I has been highlighted as a candidate modulator of antigen presentation.

Biochemical analysis revealed that MARCH-I induces the ubiquitination of lysine residue 225, located in the cytoplasmic tail of the MHC II β chain (Matsuki et al., 2007). By analyzing MARCH-I-deficient B cells, it was concluded that MHC II ubiquitination is not required for internalization of MHC II, but is required for subsequent lysosomal degradation (Matsuki et al., 2007). However, the contribution of ubiquitination to the internalization of MHC II remains controversial, because an MHC II mutant lacking the ubiquitination site was not internalized efficiently (Shin et al., 2006; van Niel et al., 2006). At present, all data support a model in which MARCH-I contributes to the stability of peptide-loaded MHC II (pMHC II) *in vivo*.

A working hypothesis for MARCH-I function

To understand the physiological function of MARCH-I, several groups have examined the regulation of MARCH-I expression at the transcriptional and post-transcriptional levels. The Pierre and Gatti group demonstrated that TLR signals inhibit the expression of MARCH-I mRNA, and inhibit MHC II ubiquitination in dendritic cells (De Gassart et al., 2008). Also, inhibition of DC maturation by IL-10 was shown to rescue the down-regulation of MARCH-I expression and MHC II ubiquitination in DCs (Thibodeau et al., 2008). Given that MARCH-I is a physiological E3 for MHC II, down-regulation of MARCH-I mRNA might be one of the initiation signals for immune responses (Ishido et al., 2009). However, it seems that loss of MHC II ubiquitination is not simply caused by down-regulation of the MARCH-I message, because MHC II ubiquitination disappears before that (van Niel et al., 2008). This finding suggests that post-transcriptional regulation of MARCH-I and regulation of MARCH-I-related signaling pathways are also involved in loss of MHC II ubiquitination. In this line, the Lybarger group generated several MARCH-I deletion mutants and demonstrated that MARCH-I stability is regulated by its trafficking within the endolysosomal pathway (Jabbour et al., 2009). It is important to examine how DC maturation signals intersects with trafficking of MARCH-I. At present, however, it is very difficult to monitor the expression level of MARCH-I protein as its expression level is too low to be detected with available MARCH-I antibodies. Therefore, we need to generate an *in vivo* system to overcome this problem.

Even though there is accumulating evidence supporting the idea that MARCH-I is a modulator of antigen presentation, we have not yet succeeded in proving it. The most convincing data in support of this hypothesis are derived from experiments in which peptide fragments were employed as model antigens (Matsuki et al., 2007). MARCH-I-deficient B cells could present fragmented I-E alpha peptide to T cells much better than wild type APCs (Matsuki et al., 2007). These data demonstrate a strong correlation between down-regulation of MARCH-I and an increase in the expression of peptide-loaded MHC II (pMHC II), however this hypothesis must be validated *in vivo*.

A "surprise" in the MARCH-I KO

As described above, we hypothesize that down-regulation of MARCH-I is an important initiation signal for immune responses. However, the question remains of why constitutive MHC II ubiquitination by MARCH-I is necessary at the steady state *in vivo*? To address this question, we examined T-dependent immune responses in conventional MARCH-I KO mice. So far, we have not observed any evidence of enhanced MHC II function in these mice. Instead, to our surprise, we observed DC abnormalities *in vivo* (Ohmura-Hoshino et al., 2009). MARCH-I-deficient splenic DCs showed high expression of MHC II as expected. Interestingly, the co-stimulatory molecule B7-2/CD86 was also highly expressed in the KO DCs (Ohmura-Hoshino et al., 2009). Since over-expression of MARCH-I inhibited B7-2 expression (Bartee et al., 2004; Jabbour et al., 2009), B7-2 seems to be a physiological MARCH-I substrate. However, it will be necessary to examine whether endogenous B7-2 is indeed ubiquitinated in immature APCs. Surprisingly, even though the molecules required for antigen presentation were highly expressed, the ability of splenic DCs to present the antigens was impaired in the MARCH-I KO (Ohmura-Hoshino et al., 2009). Moreover, LPS-induced cytokine production was impaired in MARCH-I-deficient DCs (Ohmura-Hoshino et al., 2009). Consistent with these observations, T-dependent immune responses were impaired in the MARCH-I KO.

Why are splenic DCs abnormal in MARCH-I KO mice? First, we examined whether DCs that are generated from MARCH-I-deficient bone marrow cells (BM) *in vitro* (BMDCs) show abnormalities similar to the *ex vivo* splenic DCs. However, MARCH-I-deficient BMDCs were functionally normal in terms of cytokine production, suggesting that some aspect of the *in vivo* environment causes the DC abnormalities (Ohmura-Hoshino et al., 2009). To test this hypothesis, we examined whether the function of MARCH-I-deficient BMDCs is modified after their transfer into normal mice. Recently, the Shortman group demonstrated that BMDCs generated with Flt-3L can differentiate into $CD8^+$ DCs after being transferred into mice (Naik et al., 2005). Therefore, we also examined the level of CD8 expression on the transferred BMDCs. We found that MARCH-I-deficient BMDCs showed abnormalities similar to that of endogenous MARCH-I-deficient splenic DCs (Ohmura-Hoshino et al., 2009). Thus, the DC abnormality appears to be due to an *in vivo* environmental effect.

DC maintenance by MHC II ubiquitination

Our experiments with the transplanted DCs indicated that an interaction between DCs and the *in vivo* environment might cause the DC abnormality. Since MHC II and B7-2 are both highly expressed on DCs and potentially interact with the microenvironment (e.g. T cells), the contribution of these molecules to the DC abnormality was examined. Each molecule was deleted from MARCH-I KO mice by crossing them with MHC II or B7-2 KOs. Interestingly, deletion of MHC II completely rescued the abnormality of MARCH-I-deficient DCs, even though B7-2 was still highly expressed (Ohmura-Hoshino et al., 2009). Consistent with the lack of a role for B7-2, its deletion did not rescue the abnormality (Ohmura-Hoshino et al., 2009). These results showed the requirement of MHC II for the DC abnormality induced by MARCH-I deletion.

This result strongly suggested that the abnormal stabilization of MHC II in the MARCH-I KO causes the DC abnormality. To test this hypothesis, MHC II ubiquitination was inhibited introducing a mutant MHC II that cannot be ubiquitinated *in vivo*. Since we and others have already shown that K225 of the MHC II β chain is the site ubiquitinated by MARCH-I, K225R mutation was introduced to MHC II β chain gene (MHC II KI). As expected, the surface expression level of MHC II was enhanced in the MHC II KI, and again, splenic DCs of MHC II KI were impaired in terms of both cytokine production and antigen presentation (Ohmura-Hoshino et al., 2009). Thus, MHC II ubiquitination is required for DC homeostasis *in vivo*.

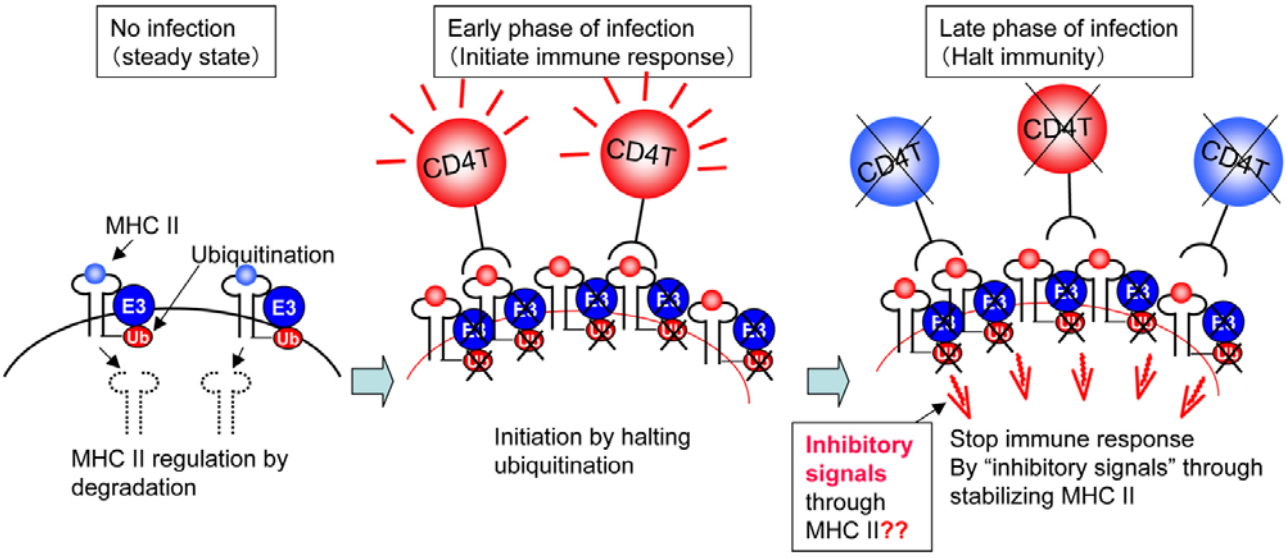

Fig. 1. Possible contribution of MARCH-I to immune responses. In the steady state, MARCH-I (shown as "E3") is constitutively expressed in APCs and regulates the expression of MHC II through ubiquitination-mediated lysosomal degradation (left panel). Once APCs are activated and maturated by infection, MARCH-I expression is down-regulated and pathogen-derived peptides (shown in red) are presented by MHC II stabilized due to loss of ubiquitination (middle panel). However, this stabilization results in sustained MHC II expression that eventually delivers yet to be identified "inhibitory signals" to APCs, directly or indirectly, halting the immune response.

How does stabilized MHC II interfere with DC homeostasis?

As described above, the DC abnormality appears to be due to the interaction between MHC II and the *in vivo* microenvironment. If so, what is the molecular basis of the abnormal DC homeostasis *in vivo*? Previously, several groups have demonstrated that cross linked-MHC II can induce a variety of signals into APCs (Al-Daccak et al., 2004; Cambier et al., 1987). Therefore, we thought that the MARCH-I-deficient DCs might receive a very strong signal after cross linking with an MHC II antibody. Indeed, cross linking of MHC II resulted in the inhibition of LPS-mediated B7-1 upregulation. However, this inhibitory signal was not enhanced in MARCH-I-deficient BMDCs (Ohmura-Hoshino et al., 2009). Since previous reports indicated that, as expected, the cytoplasmic region of MHC II contributes to MHC II-mediated signaling (Al-Daccak et al., 2004), we examined the contribution of β chain cytoplasmic tail by generating MHC II knock-in mice whose I-A β cytoplasmic region was completely deleted. As expected, the mutant MHC II was highly expressed on DCs, but the DC abnormality was still observed in this KI mouse (unpublished data). Thus, so far, we have no evidence indicating that MHC II-mediated signaling contributes to the DC abnormality. We are now using different approaches to try and identify the cells that contribute to this abnormality through MHC II.

What do these "surprise findings" imply?

It has been demonstrated that stimuli that induce DC maturation stabilize surface MHC II molecules by inhibiting MHC II ubiquitination. Furthermore, loss of MHC II ubiquitination is accompanied by the downregulation of MARCH-I expression. As these events are similar to those that occur in MARCH-I KO and MHC II KI, we now hypothesize that matured/activated DCs receive signals that cause DC abnormalities indirectly, via stabilized MHC II. If this is the case, such an inhibitory signal may prevent excessive immune reactions, which may prevent detrimental immune responses, such as occur in autoimmune diseases. Consistent with this idea, several groups have reported on LPS-induced DC malfunction *in vivo* (Young et al., 2008). Taken together, our results and those of others lead us to propose a new model for the physiological role of MARCH-I-mediated MHC II ubiquitination in the immune system (Fig. 1).

PERSPECTIVES

Since the discovery of ubiquitin-mediated MHC II regulation, several groups have begun to examine how MHC II ubiquitination contributes to antigen presentation and immune responses *in vivo*. However, it has been difficult to address this issue, because the functions of APCs have somehow been impaired in MARCH-I KO and MHC II KI. To overcome this problem, we have to improve the experimental systems that we are using. For example, one approach would be to make genetically modified mice in which MHC II ubiquitination is conditionally inhibited. Hopefully, we can validate our hypothesis for MARCH-1 function in the near future with such improved experimental systems. In the same vein, MHC II-medicated modulation of DCs may play an important role in immunological homeostasis. Again, however, this will be difficult to address experimentally since DC abnormalities can only be examined *in vivo*. As an initial step, we have to identify the molecules involved in inducing the DC abnormalities. One important recent finding in the MARCH-I field is that B7-2 appears to be regulated by ubiquitination in the steady state. If so, this raises the question of whether such regulation contributes to antigen presentation. Although at this stage in the study of MARCH-I it is too early to highlight MARCH-I as an essential immune regulator, in future studies we plan to examine the physiological relevance of MARCH-I-mediated MHC II ubiquitination *in vivo*.

ACKNOWLEDGMENTS

We thank all members of our laboratory for their great contribu-

tions to our recent progress. Also, we thank P. Burrows for critical reading of the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by the Japan Society for the Promotion of Science (JSPS).

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