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Inhibition of MHC Class II Expression and Immune Responses by c-MIR

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We previously reported a novel E3 ubiquitin ligase (E3), designated as c-MIR, which targets B7-2 to lysosomal degradation and down-regulates the B7-2 surface expression through ubiquitination of its cytoplasmic tail. B7-2 is well known as a costimulatory molecule for Ag presentation, suggesting that the manipulation of c-MIR expression modulates immune responses in vivo. To examine this hypothesis, we generated genetically modified mice in which c-MIR was expressed under an invariant chain (Ii) promoter. Dendritic cells derived from genetically engineered mice showed low ability to present Ags. In addition, these mice showed resistance to the onset of experimental autoimmune encephalomyelitis and an impaired development of CD4 T cells in the thymus and the periphery. These findings led us to conclude that MHC class II (MHC II) is an additional target for c-MIR. Indeed, forced expression of c-MIR in several B cell lines down-regulated the surface expression of MHC II, and down-regulation was found to depend on the presence of a single lysine residue in the cytoplasmic tail of the I-A β-chain. In a reconstitution system using 293T cells, we found that the lysine residue at position 225 in the I-A β-chain was ubiquitinated by c-MIR. To our knowledge, c-MIR is the first example of an E3 that is capable of inhibiting MHC II expression. Our findings suggest that c-MIR might potently regulate immune responses in vivo. The Journal of Immunology, 2006, 177: 341–354.

Previously we reported a novel E3 ubiquitin ligase (E3), designated c-MIR, which targets B7-2 to lysosomal degradation by ubiquitination of its cytoplasmic tail (1). c-MIR has been proposed to belong to a novel family of E3, designated as the modulator of immune recognition (MIR) family, of which catalytic domain is a variant RING domain (RING-CH domain) (2–4). MIR family proteins share a secondary structure and a catalytic domain as a variant RING domain, which is located at the N terminus (3). MIR family proteins bind to the membrane through their hydrophobic domains located at the center and possess two intracellular regions. The initially discovered MIR family proteins are viral E3s: MIR1 and MIR2 of Kaposi’s sarcoma-associated herpesvirus (KSHV), mK3 of murine γ-herpesvirus 68, and m153R of myxomavirus (3, 5–9). Each molecule targets a different set of Ag presentation-related molecules: CD1d and MHC class I (MHC I) for MIR1; B7-2, ICAM-1, CD1d, and MHC I for MIR2; MHC I for mK3; MHC I, CD4, and Fas for m153R (3, 5, 9–12).

It has been proposed that down-regulation of MHC I by viral E3s contributes to viral strategies for the evasion from host immunity. This idea emerged from an experiment, which was performed using genetically modified murine γ-herpesvirus 68 (13). First, an mK3-deficient virus, which is not able to down-regulate MHC I expression efficiently, was generated. The propagation of this mK3-deficient virus was not impaired in vitro, but fibroblasts infected with the mK3-deficient virus were more sensitive to Ag-specific CTL clones than those infected with wild-type virus. In addition, in mice infected with the mK3-deficient virus, the efficiency of the latent infection in the spleen was reduced, and the frequency of virus-specific CTL was increased. Thus, at least, mK3 was clearly shown to function as an immune modulator that helps the virus to evade host immunity in vivo.

c-MIR was found as a functional and structural homolog of KSHV MIR1 and MIR2 by searching the public databases (1). At present, its physiological function remains unknown. However, forced expression of c-MIR in B cell lines induces strong down-regulation of B7-2 surface expression, suggesting its involvement in immune regulation in vivo. Furthermore, c-MIR has been shown to down-regulate the expression of transferrin receptor and Fas, an important molecule for the induction of apoptosis (14). Again, the biological relevance of these findings remains unknown. Up to date, several mammalian E3s related to c-MIR have been characterized, and these mammalian E3s including c-MIR were termed MARCH (membrane-associated RING-CH) proteins, and c-MIR is now also known as MARCH VIII (14).

The molecular machinery for MIR family protein-mediated down-regulation of target molecules is under intensive examination by several groups. As shown in the case of other families of E3, MIR family proteins also induce the ubiquitination through binding to target molecules (1). The lysine residues in the cytoplasmic tail of target molecules have been identified as the sites for ubiquitination by MIR family proteins. In the most cases, the
ubiquitination of cytoplasmic tail is thought to induce a rapid endocytosis and lysosomal degradation of the target molecules (15). In contrast, mK3 encoded by murine γ-herpesvirus 68, a member of MIR family, has been shown to induce the retrograde transport of MHC I at endoplasmic reticulum and finally degrade the targets at the proteasome (16). There is no clear picture that explains why mK3 uses a different way for down-regulation of MHC I. As to the molecular mechanism for specific targeting by MIR family proteins, an experiment was performed (17). Ganem and colleagues (11, 12, 17) showed that a chimeric MIR1 whose transmembrane regions were substituted by those of MIR2 was able to down-regulate B7-2 and ICAM-2, which are targets for MIR2 but not MIR1. This result suggests the involvement of transmembrane regions of MIR1 and MIR2 and the targets for substrate specificity.

B7-2 is well known as a costimulatory molecule in Ag presentation. B7-2-deficient mice have been reported to be resistant to the onset of autoimmune diseases (18), suggesting that B7-2 might be a candidate target for immune therapy. Therefore, our previous observation led us to examine the attractive hypothesis that artifical manipulation of c-MIR expression might modulate immune responses in vivo.

In this report, we show that forced expression of c-MIR in APCs in vivo inhibits Ag presentation and prevents the onset of experimental autoimmune encephalomyelitis (EAE). Furthermore, we show that MHC class II (MHC II) is an additional target of c-MIR, and down-regulation of its surface expression depends on the presence of a single lysine residue in the cytoplasmic tail of β-chain of MHC II. These findings reveal an additional function of c-MIR and suggest c-MIR might potentely regulate immune responses in vivo.

Materials and Methods

Transgene construction and generation of transgenic (Tg) mice

Murine c-MIR cDNA was obtained from total RNA of the spleen by RT-PCR. The sequence of the hemagglutinin (HA) epitope tag was inserted at the 3′ terminus of cDNA by PCR. The resultant DNA fragment was inserted into a unique ClaI site of pDOI-6 vector, which was provided by C. Benoist and D. Mathis (Joslin Diabetes Center, Boston, MA) (19). The linearized DNA was microinjected into the pronuclei of fertilized eggs from BDF1 mice, and Tg founders were backcrossed onto the C57BL/6 background at least six times. In all experiments, nontransgenic littermates were used as control mice. All mice were maintained under specific pathogen-free conditions according to the instructions of Kobe University School of Medicine and RIKEN (The Institute of Physical and Chemical Research) guidelines and used for analysis at 8–12 wk of age.

Histological analysis

Various organs were removed and fixed in 10% formalin/PBS. Frozen sections were prepared and stained with H&E. Serial frozen sections were stained with anti-HA Ab, and the signal was enhanced using the TSA system (tyramide signal amplification; Invitrogen Life Technologies). For double staining analysis, the frozen sections were fixed with cold acetone, stained with anti-HA Ab, and the signal was enhanced using the TSA system, and then each section was stained with indicated Abs.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde/PBS for 15 min and cold acetone for 15 min. Stained tissue sections or fixed cells were mounted in mounting media (Vector Laboratories) and examined by a DP-70 Immunofluorescence digital camera (Olympus).

Quantification by real-time RT-PCR

For the quantification of c-MIR mRNA level, TaqMan Gene Expression Assays (Applied Biosystems) was used. Total RNA was extracted using an RNaseasy kit (Qiagen) from the indicated cells prepared from each mouse, and reverse-transcribed using the SuperScript RT kit (Invitrogen Life Technologies) according to the manufacturer’s protocol. Synthesized cDNAs were transferred into TaqMan Universal PCR Master Mix containing a FAM-labeled TaqMan probe and specific primers for murine c-MIR, and subsequent PCR amplification was performed. The cycle threshold number, at which amplification entered exponential phase, was determined in each sample by using ABI PRISM 7000 sequence detection system (Applied Biosystems). Relative quantitation was performed using comparative Ct method. All results were normalized to an internal control: the 18S mRNA. For the quantitative analysis of the expression of I-Å β-chain and invariant chain (Ii), we used the QuantiTect SYBR Green PCR kit (Qiagen). First-strand cDNA was generated from the B cells of control or c-MIR Tg mice, and short (∼150-bp) fragment from MHC II or Ii cDNA was amplified by PCR with each specific set of primers. The intensity of incorporated SYBR Green was monitored by using an ABI PRISM 7000 sequence detection system (Applied Biosystems). The expression level of β-actin was determined for normalization of the data.

Immunoblotting, metabolic labeling, and immunoprecipitation

Cells were lysed with RIPA buffer (0.15 M NaCl, 1% Nonident P-40, 0.1% SDS, and 50 mM HEPES buffer (pH 8.0)) containing protease inhibitors, and the supernatants were cleared by centrifugation. Whole cell lysates were subjected to immunoprecipitarting and/or serial immunoblotting as indicated for each experiment. Immunoprecipitation was performed with the indicated Ab together with 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology). For PNGase-F digestion, washed immunoprecipitates were resuspended in 50 μl of 1× denaturing buffer (0.5% SDS, 1% 2-ME), heated for 10 min at 100°C and incubated for 1 h at 37°C with 1 μl of PNGase-F (New England Biolabs). For metabolic labeling, cells were incubated in medium containing 50 μCi of [35S]methionine and [35S]cysteine (PerkinElmer) for 6 h. Labeled protein samples were extracted and precipitated with 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology). For pulse-chase analysis, cells were labeled for 30 min, chased for the indicated time, and labeled proteins were extracted with Nonidet P-40 buffer (0.5% Nonidet P-40, 300 mM NaCl, and 50 mM Tris buffer (pH 7.4)) containing protease inhibitors.

Cell surface biotinylation and immunoprecipitation

Pulse-labeled A20 cells at each chase time were incubated with NHS-S-SS-biotin (sucinimidyl 2-(biotinamido)-ethyl-1,3’-dithiopropionate, 2 mg/ml; Pierce) in PBS for 2 min at 4°C, and biotinylated surface proteins were extracted with Nonidet P-40 buffer and precipitated with MKD6 Ab. Precipitated samples were eluted from the protein A/G-Sepharose beads by boiling for 2 min in 2% SDS in PBS. Eluted biotinylated proteins were precipitated with streptavidin-agarose (Pierce) and analyzed by SDS-PAGE.

Flow cytometry analysis and Abs

Cells were washed with PBS containing 2% FCS and incubated with the following fluorescein-conjugated mAbs for 30 min at 4°C. After being washed, each sample was fixed with 2% paraformaldehyde solution, and flow cytometry analysis was performed with a FACScalibur (BD Biosciences). H57-597 Ab for TCR-β; M1/69 Ab for CD24, 30-F11 Ab for CD45, G8-8 Ab for EpCAM, M5/114.15.2 Ab for MHC II, GL-1 Ab for B7-2, 53-6.7 Ab for CD8, HL3 Ab for CD11c, 116-10A1 Ab for B7-1, 28-14-8 or 34-5-8S Ab for MHC I, 145-2C11 Ab for CD3, RA3-6B2 Ab for B220, L3T3 Ab for CD4, and 1C10 Ab for CD40 used for FACS analysis were obtained from BD Biosciences or eBioscience. Y-H anti-HA rabbit polyclonal Ab (Santa Cruz Biotechnology) was used for immunohistochemistry and immunoprecipitation. MKD6 anti-I-Å β-chain Ab (American Type Culture Collection (ATCC)) and M5/114.15.2 anti-MHC II Ab were used for pulse-chase analysis of I-Å β-chain. M2 anti-FLAG mAb (Sigma-Aldrich) was used for immunoprecipitation and immunoblot analysis. FK2 anti-ubiquitin Ab (AFFINITI Research Products), KL295 anti-I-Å-chain Ab (ATCC), In-1 anti-Ii Ab (BD Biosciences), and clone B-5-1-2 anti-α-tubulin Ab (Sigma-Aldrich) were used for immunoblot analysis.

Cell preparation

Immature dendritic cells (DCs) were prepared by culturing bone marrow (BM) cells obtained from each mouse with GM-CSF (20 ng/ml) (PeproTech) for 8 days. Mature DCs were generated from immature DCs by stimulation with LPS (1 μg/ml) (Sigma-Aldrich) for 24 h. CD4 T cells, T cells, B cells, splenic DCs, and splenic macrophages were purified from the spleens of mice using the MACS system (Miltenyi Biotec). CLA3-1 biotin-conjugated anti-F4/80 Ab for macrophages, 30-H12 biotin-conjugated anti-CD90.2 Ab for T cells, RA3-1B2 biotin-conjugated anti-CD45R B Ab for B cells were obtained from eBioscience to prepare each cells. CD11c microbeads for preparation of DCs were obtained from Miltenyi Biotec. The cell purity of all type of cells isolated by MACS system was
Secreted IL-2 was measured by ELISA (BD Biosciences).

Student’s t test. Values of p < 0.05 were considered significant.

Results

Generation of c-MIR Tg mice

To examine the immune regulatory functions of c-MIR, we generated genetically modified mice in which HA-tagged c-MIR was expressed under an l35 promoter using pDO6f, which allows examined molecules to be expressed in APCs. We thereby finally generated two lines of Tg mice: no. 64 and no. 65, in which the expression of exogenous c-MIR protein could be visualized in situ by immunohistochemistry. Fig. 1, A and C, shows the expression of HA-tagged c-MIR in the spleen and the thymus of mouse no. 64. This specific expression profile was confirmed by immunohistochemical and immunoprecipitation analysis (Fig. 1, B and D).

As shown in Fig. 1B, the expression of HA-tagged c-MIR was observed in most of the B cells and in part of DCs. To confirm that c-MIR is overexpressed in APCs in the spleen of c-MIR Tg mice, quantitative real-time PCR analysis was performed. In this experiment, we generated a pair of primers to amplify one part of the open reading frame of endogenous c-MIR so that this assay can detect endogenous c-MIR mRNA as well as exogenous mRNA. As we reported in previous work (1), in control littermates, the expression level of endogenous c-MIR mRNA was low in B and T cells, whereas macrophages and DCs moderately expressed c-MIR mRNA (Fig. 1E). In c-MIR Tg mice, the expression level of c-MIR mRNA was remarkably increased in macrophages, DCs, and B cells and was slightly increased in T cells, compared with littermate controls (Fig. 1E). Thus, in c-MIR Tg mice, the expression of c-MIR was increased predominantly in APCs. The growth of c-MIR Tg mice was not impaired, and there were no significant differences in the size and shape of the organs including the lymphoid tissues (Fig. 1, A and C). In contrast, there were small, but statistically significant differences in the proportions of splenic B and T cells (Fig. 1F); the percentage of CD3+ cells in c-MIR Tg mice was decreased (34.9 ± 2.5% in c-MIR Tg vs 41.9 ± 1.9% in control littermates; p < 0.05; n = 6), and the percentage of B220+ cells in c-MIR Tg mice was contrastingly increased (50.1 ± 1.6% in c-MIR Tg vs 46.2 ± 3.2% in control littermates; p < 0.05; n = 6). These findings were exactly the same in two lines of c-MIR Tg mice (data not shown).

Expression of B7-2 is significantly inhibited in the BMDCs generated from c-MIR Tg mice

To confirm that exogenously expressed c-MIR is functionally active in Tg mice, we examined the expression level of B7-2 in BMDCs generated from c-MIR Tg mice and control littermates. Both types of BM cells differentiated into CD11c+ cells, showing that normal development of BM cells occurred in c-MIR Tg mice (Fig. 2A). However, we found that up-regulation of B7-2 upon LPS stimulation was significantly inhibited in BMDCs generated from c-MIR Tg mice by measuring the mean fluorescence intensity (MFI) of B7-2; MFI of B7-2 in LPS-treated BMDCs (mature DC) generated from c-MIR Tg was lower than that in mature DC generated from control littermates (96 ± 11.5 vs 532 ± 17.4, respectively; p < 0.01; n = 3) (Fig. 2B). Also, this inhibition was confirmed by measuring the proportion of the cells whose B7-2 expression level is high (above 10^2 as its fluorescence intensity in Fig. 2B) compared with total LPS-treated BMDCs. The percentage of B7-2high cell in total LPS-treated BMDCs from c-MIR Tg mice was significantly lower than that in total LPS-treated BMDCs from control littermates (42.6 ± 2.8% vs 88.7 ± 3.6%, respectively; p < 0.01; n = 3). This inhibition was specific to B7-2 because up-regulation of B7-1 and MHC I expression were not significantly inhibited in BMDCs generated from c-MIR Tg mice by immunoprecipitation analysis (Fig. 2C).

Forcibly expressed c-MIR suppresses the function of APCs

The results shown in Fig. 2B suggest that BMDCs with forcibly expressed c-MIR are not able to present the Ags to T cells efficiently. To this end, we performed MLR assay. BMDCs from c-MIR Tg mice or control littermates were stimulated with LPS for 24 h, irradiated and mixed with allogenic CD4 T cells. The ability of allogenic T cells to proliferate was analyzed. As shown in Fig.
2D, the ability of c-MIR Tg mice-derived DCs to stimulate allogenic T cells was lower than the ability of control littermate-derived DCs. To confirm this suppression of the Ag-presenting activity, we examined the ability of Ag-specific presentation using OT-II Tg mice-derived T cells, which recognize OVA peptide loaded by the I-A<sup>B</sup> molecule. Again, the ability of c-MIR Tg mice-derived BMDCs to stimulate OT-II T cells was lower than ability found in control littermates-derived BMDCs. Next, to examine whether c-MIR functions as an immune suppressor in other cells, we generated a B cell line in which c-MIR is forcibly expressed under the control of the elongation factor <i>EF1α</i> promoter. A20.2J cells were chosen for this purpose because these cells express B7-2
at moderate levels (Fig. 2E) (24). A20.2J cells with forced expression of c-MIR were mixed with a Th1 clone, 42-6A, which recognizes OVA323–339 peptide presented by the I-A d molecule. As shown in Fig. 2F, the c-MIR-expressing A20.2J cells were not able to stimulate 42-6A cells efficiently in the presence of either OVA polypeptide or OVA323–339 peptide.

Forcibly expressed c-MIR prevents the onset of EAE

As shown in Fig. 2, D and F, c-MIR is able to inhibit the function of APCs efficiently, suggesting that forced expression of c-MIR might inhibit T cell-mediated immunity in vivo. To test this hypothesis, we chose EAE, an autoimmune disease caused by the occurrence of autoreactive CD4 T cells against myelin Ags and which can be prevented by inhibition of APCs (25). The clinical score of control littermates was elevated to 3 at 15–18 days. In contrast, c-MIR Tg mice did not show any clinical symptoms during the entire observation period (Fig. 3A). To examine the frequency of autoreactive T cells among CD4 T cells, we collected CD4 T cells from the spleens of control littermates or c-MIR Tg mice 10 days after EAE induction and measured the frequency of
autoreactive CD4 T cells by ELISPOT. As we expected, the frequency of autoreactive CD4 T cells was remarkably reduced in the spleen of the c-MIR Tg mice (Fig. 3A). During analysis of autoreactive CD4 T cells, we found that the total number of splenic CD4 T cells in c-MIR Tg mice was only about half of that in control littermates (data not shown). These results suggest that the...
suppression of the development of EAE by c-MIR is due to the low frequency of autoreactive T cells and the low number of CD4 T cells. Therefore, we assessed the development of T cells in the thymus and the spleen of c-MIR Tg mice. In the spleens of c-MIR Tg mice, the percentage of CD4 T cells was slightly lower than that of control littermates, but the percentage of CD8 T cells was not remarkably different between the two types of mice (Fig. 3B). The percentage of CD4 T cells was more significantly decreased in the thymus (Fig. 3B). Furthermore, the frequency of matured CD4 single-positive thymocytes, but not that of matured CD8 single-positive thymocytes, was significantly reduced in the thymus of c-MIR Tg mice (Fig. 3B). These results demonstrate that the development of CD4 T cells is selectively impaired in c-MIR Tg mice.

We then assessed whether impaired CD4 T cell development in c-MIR Tg mice is due to intrinsic defects of blood-derived hemoipoietic cells, including T cells, or caused by a nonhemopoietic microenvironment, including the thymic environment, through adoptive transfer experiments. BM cells from control littermates were transplanted into lethally irradiated c-MIR Tg mice (Cont > c-MIR Tg) or control littermates (Cont > Cont). Moreover, BM cells from c-MIR Tg mice were transplanted into lethally irradiated control littermates (c-MIR Tg > Cont). As shown in Fig. 3C, the development of CD4 single-positive thymocytes was impaired in control littermates transplanted into lethally irradiated c-MIR Tg mice, but development was normal in thymocytes in c-MIR Tg mice transplanted into lethally irradiated control littermates, compared with cells in control littermates transplanted into lethally irradiated control littermates, suggesting that an impaired CD4 T cell development in c-MIR Tg mice was probably due to the defects in the nonhemopoietic environment, such as the thymic microenvironment.

Next, we investigated the phenotypes of splenic DCs as physiologic APCs in c-MIR Tg mice. As shown in Fig. 3D, the surface expression of B7-2, but not that of B7-1 and MHC I, was significantly down-regulated on splenic DCs obtained from LPS-treated c-MIR Tg mice (Cont > c-MIR Tg) or control littermates (Cont > Cont). Moreover, BM cells from c-MIR Tg mice were transplanted into lethally irradiated control littermates (c-MIR Tg > Cont). As shown in Fig. 3C, the development of CD4 single-positive thymocytes was impaired in control littermates transplanted into lethally irradiated c-MIR Tg mice, but development was normal in thymocytes in c-MIR Tg mice transplanted into lethally irradiated control littermates, compared with cells in control littermates transplanted into lethally irradiated control littermates, suggesting that an impaired CD4 T cell development in c-MIR Tg mice was probably due to the defects in the nonhemopoietic environment, such as the thymic microenvironment.

To confirm the down-regulation of MHC II expression by c-MIR in vivo, we next examined the expression of MHC II in the spleen. As observed in the thymus, down-regulation of MHC II expression was observed in the B cell follicles (data not shown). In addition, double staining analysis with anti-MHC II and anti-β2Mc Abs showed that the regions brightly stained with anti-β2Mc showed low expression of MHC II, but the regions that were not stained with anti-β2Mc showed high expression of MHC II in the same B cell follicle, suggesting that exogenous expression of c-MIR suppressed the expression of MHC II in vivo (Fig. 3C). This finding was confirmed by FACs analysis of the splenocytes (Fig. 3D). Given our previous report that showed c-MIR induces rapid degradation of B7-2, down-regulation of MHC II is also speculated to occur at the posttranscriptional level. Therefore, to rule out the possibility of transcriptional alteration of the MHC II gene and its related genes, we examined the mRNA level of I-A β-chain gene and li gene in the splenic B cells by quantitative real-time PCR. As we expected, there was no significant difference in the amount of I-A β mRNA and li mRNA between c-MIR Tg mice and control littermates (Fig. 3E). Finally, we examined whether c-MIR directly influenced the surface expression of MHC II using A20 cells, a murine B cell line. c-MIR was expressed by electroporation of a vector DNA that expresses both enhanced GFP and c-MIR from the BMDCs (Fig. 2, B and D), the function of physiologic APCs was impaired in c-MIR Tg mice, giving rise to the question as to whether inhibition of Ag presentation contributes to the prevention of EAE induction by c-MIR. To address this question, we examined the susceptibility of cells in c-MIR Tg mice transplanted into lethally irradiated control littermates used in Fig. 3C to the induction of EAE because in these mice, the CD4 T cell development is normal and physiologic APCs are thought to be derived from c-MIR Tg mice. As shown in Fig. 4, A and B, c-MIR Tg mice transplanted into lethally irradiated control littermates were resistant to the induction of EAE to some extent, and the frequency of autoreactive CD4 T cells was significantly reduced in the spleen of these mice, compared with frequency of cells in control littermates transplanted into lethally irradiated control littermates. In addition in c-MIR Tg mice transplanted into lethally irradiated control littermates, the surface expression of B7-2 was significantly down-regulated on splenic DCs; the MFI of B7-2 in DCs obtained from LPS-treated c-MIR Tg mice transplanted into lethally irradiated control littermates were not able to stimulate OT-II CD4 T cells efficiently (Fig. 4D), indicating that physiologic APCs were derived from c-MIR Tg mice. Thus, these results suggest that inhibition of Ag presentation partially contributes to the prevention of EAE induction in c-MIR Tg mice.

MHC II molecule is an additional target of c-MIR

In c-MIR Tg mice, the development of CD4 T cells was significantly impaired presumably due to the defects in the nonhemopoietic environment, such as the thymic microenvironment. Also, as shown in Fig. 2F, the Ag recognition by 42-6A Th1 clone was more strongly inhibited by c-MIR, compared with that by primary/naive T cells, OT-II T cells. These findings cannot be explained by the inhibition of B7-2 because B7-2-deficient mice do not show any abnormalities in the development of CD4 T cells (26), and T cell clones are thought to be less dependent on costimulation for optimal responses than primary T cells. In contrast, MHC II-deficient mice have been reported to show impaired development of CD4 T cells and strongly reduced T cell immunity (27). Therefore, we examined the level of MHC II expression in the thymus in c-MIR Tg mice. Histochemical analysis of the thymus revealed that the expression of MHC II was significantly down-regulated in c-MIR Tg mice (Fig. 5A). Specific down-regulation of MHC II expression was confirmed by FACs analysis of thymic EpCAM+ epithelial cells (Fig. 5B). Interestingly, FACs analysis of thymic epithelial cells showed two populations of EpCAM+ epithelial cells; one population whose MHC II expression was remarkably down-regulated, and another population whose MHC II expression was not significantly inhibited.

A single lysine residue in the cytoplasmic tail of MHC II β-chain is responsible for its down-regulation

The mature MHC II molecule is a heterodimer that consists of α-chain and β-chain. In addition, li molecule is a third component of the immature MHC II molecule (28). Therefore, we examined...
which components are targeted by c-MIR. First, the amount of Ii molecule was examined by immunoblot analysis in parental and c-MIR-overexpressing A20.2J cells. This analysis revealed that the amount of Ii molecule was not reduced by c-MIR (Fig. 6). We and other groups previously showed that lysine residues located in the cytoplasmic tail of targets were responsible for down-regulation of targets by viral and mammalian E3s (1, 2, 5). Examination of the peptide sequences of the \( \alpha \)- and \( \beta \)-chains of MHC II molecule revealed that the \( \beta \)-chain but not the \( \alpha \)-chain of I-A has one lysine residue in its cytoplasmic tail, leading us to focus on the

FIGURE 4. Analysis of BM chimeras. A, EAE was induced in indicated BM chimeras, and the mice were scored as in Fig. 3A. Data present mean clinical score of each group plotted against time (\( n = 10 \) for each group). Data are mean ± SEM. \(*, p < 0.01\), compared with control littermates transplanted into lethally irradiated control littermates (Cont > Cont mice). Data shown are representative of two independent experiments. B, IFN-\( \gamma \)- or IL-2-producing CD4 T cells were detected by ELISPOT 10 days after EAE induction as in Fig. 3A. Data are expressed as the mean ± SD of triplicate samples, and the values shown are representative of two independent experiments. \(*, p < 0.01\), compared with control littermates transplanted into lethally irradiated control littermates (Cont > Cont). C, DCs obtained from the spleens of nontreated mice (open histograms) or mice that were i.v. injected with 30 \( \mu \)g LPS (shaded histograms), were analyzed as in Fig. 3D. Results are from one representative experiment of three performed. As to the expression level of each surface molecule in the different groups, the average values of MFI obtained from three independent experiments are presented at the far right of each panel. LPS- and LPS+ indicate nontreated BMDC and LPS-treated BMDC, respectively. Data are mean ± SD. \(*, p < 0.05\) (\( n = 3 \)). D, DCs obtained from the spleens of LPS-treated BM chimeras were mixed with OT-II Tg-derived CD4 T cells at different concentration of OVA peptide as indicated. Proliferation of each CD4 T cells was measured by \([3H] \)thymidine incorporation. Data are expressed as the mean ± SD of triplicate samples, and the values shown are representative of three independent experiments. \(*, p < 0.05\), compared with control littermates transplanted into lethally irradiated control littermates (Cont > Cont).
\(\beta\)-chain of MHC II as a target. First, we examined the amount of \(\beta\)-chain of the I-A\(^d\) molecule in c-MIR-overexpressing A20.2J cells. The same number of c-MIR-expressing A20.2J cells and parental cells were subjected to immunoblot analysis using a specific Ab against the \(\beta\)-chain of the I-A molecule. This analysis revealed a drastic reduction of the amount of I-A\(^d\) \(\beta\)-chain (Fig. 6A). Next, we examined whether the lysine at position 225 (K225) in the I-Ad \(\beta\)-chain is responsible for the down-regulation by c-MIR. For this purpose, we used M12 C3 cells in which the I-A\(^d\) \(\beta\)-chain but not the I-A\(^d\) \(\alpha\)-chain is expressed (23). We reconstituted the surface expression of MHC II by transfection of the wild-type I-Ad \(\beta\)-chain or its mutant form whose K225 was mutated to arginine. The two types of reconstituted cells showed equivalent expression of MHC II (data not shown). As we expected, transiently expressed c-MIR down-regulated the expression of MHC II molecules that were reconstituted with I-A\(^d\) \(\alpha\)-chain and FLAG-tagged I-A\(^d\) \(\beta\)-chain whose K225 was mutated to arginine. c-MIR mutant form whose cysteine residues in RING-variant domain were mutated to serine (c-MIR RING-variant mt) did not remarkably down-regulate the surface expression of MHC II molecules reconstituted with I-A\(^d\) \(\alpha\)-chain and FLAG-tagged I-A\(^d\) \(\beta\)-chain. Based on these findings, we concluded that this assay system is able for use in further analysis.

Ubiquitination and degradation of MHC II by c-MIR

Next, we examined whether c-MIR induces ubiquitination and degradation of MHC II. To address this question, we constructed an assay system using a FLAG-tagged I-A \(\beta\)-chain in which a FLAG epitope was fused to the N terminus of the extracellular region of I-A \(\beta\)-chain. I-A \(\alpha\)-chain and FLAG-tagged I-A \(\beta\)-chain were coexpressed in 293T cells, and the surface expression of MHC II was analyzed by flow cytometry. As shown in Fig. 7A, M5/114.15.2 Ab recognized MHC II molecules that were reconstituted by I-A \(\alpha\)-chain and FLAG-tagged I-A \(\beta\)-chain on the cell surface, suggesting that the reconstituted MHC II molecules were correctly folded and transported to the cell surface. Moreover, c-MIR was able to down-regulate the surface expression of MHC II reconstituted with I-A \(\alpha\)-chain and FLAG-tagged I-A \(\beta\)-chain, but not with I-A \(\alpha\)-chain and FLAG-tagged I-A \(\beta\)-chain whose K225 was mutated to arginine. c-MIR mutant form whose cysteine residues in RING-variant domain were mutated to serine (c-MIR RING-variant mt) did not remarkably down-regulate the surface expression of MHC II reconstituted with I-A \(\alpha\)-chain and FLAG-tagged I-A \(\beta\)-chain. Based on these findings, we concluded that this assay system is able for use in further analysis.

FIGURE 5. Down-regulation of MHC II by c-MIR. A, Serial frozen sections of the thymus were stained with anti-HA Ab, anti-MHC II Ab or H&E staining (HE) (magnification, \(\times 40\)). B, Thymic stromal cells were stained with anti-CD45, anti-EpCAM, and anti-MHC I or MHC II Ab. After gating on CD45\(^+\) stromal cells, expression of EpCAM and MHC I or MHC II were examined by three-color flow cytometry. Data shown are representative of two independent experiments. C, A frozen section of the spleen was stained with anti-HA and anti-MHC II Abs simultaneously (magnification, \(\times 200\)). D, Splenocytes obtained from each type of mice were stained with anti-MHC II and anti-B220 Ab and analyzed by two-color flow cytometry. Data shown are representative of four independent experiments. E, Relative expression level of Ii or I-A \(\beta\)-chain was determined by real-time PCR using SYBR Green in the splenic B cells of control littermates (Cont) or c-MIR Tg mice. Data shown are representative of two independent experiments. F, A20 cells were transfected with the GFP-c-MIR vector by electroporation. The surface expression of each molecule and the expression of GFP were analyzed 12 h posttransfection by two-color flow cytometry. Data shown are representative of three independent experiments.
FIGURE 6. A single lysine residue in the cytoplasmic tail of the I-A β-chain is critical for MHC II down-regulation by c-MIR. A, Protein samples obtained from A20.21 (Cont) or c-MIR-overexpressing A20.21 cells (c-MIR) were analyzed by Western blotting with anti-I-A β-chain Ab, anti-II Ab and anti-tubulin Ab. B, Expression of MHC II on M12 C3 cells was reconstituted with wild-type I-A β-chain (I-A β) or mutant type of I-A β-chain (I-A β (K>R)). Cells reconstituted with either were transfected with the GFP-c-MIR vector by electroporation. The surface expression of MHC II molecule and the expression of GFP were analyzed 12 h post-transfection as in Fig. 5F. Data shown are each representative of three independent experiments.

Next, we examined whether c-MIR enhances degradation of MHC II in this system. I-A β-chain or I-A β-chain whose K225 was mutated to arginine was coexpressed with I-A α-chain and c-MIR in the 293T cells. The transfected cells were pulse-labeled with [35S]methionine and [35S]cysteine and chased for the indicated periods of time. As shown in Fig. 7E, I-A β-chain was faster degraded by c-MIR than I-A β-chain whose K225 was mutated to arginine. Taken together, these results suggest that c-MIR enhances degradation of MHC II through ubiquitination at the K225 of I-A β-chain.

Rapid endocytosis of MHC II by c-MIR

Next, we examined how c-MIR influences the assembly, export and traffic of MHC II molecules in APCs. To this end, we used A20 cells because this cell line expresses a large amount of MHC II as shown in Fig. 5F, and the mechanism of MHC II transport in this cell line is well characterized (29). A20 cells were engineered to have stable and excess expression of c-MIR by electroporation (c-MIR cells) and compared with empty vector-transfected A20 cells (control cells). c-MIR cells and control cells were pulse-labeled with [35S]methionine and [35S]cysteine and chased for the indicated periods of time. At the end of the chase periods, pulse-labeled proteins were immunoprecipitated with MKD6 anti-I-A 4 β-chain mAb, which preferentially recognizes mature αβ dimers, and precipitated samples were analyzed by SDS-PAGE without boiling the samples before electrophoresis. As shown in Fig. 8A, in both control cells and c-MIR cells, completely assembled MHC II molecules, which consisted of α-chain and β-chain, were equally detected at 60 min of chase, and the amount of these was not remarkably different. Also, bands corresponding to SDS-stable compact dimers, which reflect peptide-loaded αβ dimers, were equally detected at 60 or 180 min of chase in both control and c-MIR cells. Consistent with the results obtained from a reconstitution system in 293T cells, in c-MIR cells, the amount of mature αβ dimers was decreased at 360 min of chase, compared with control cells. These results clearly demonstrate that c-MIR enhances the degradation of MHC II molecules without alteration of its translation, assembly and peptide-loading process in APCs. Next, we examined whether c-MIR interferes with the transport of mature αβ dimers to the cell surface. c-MIR cells and control cells were pulse-labeled as in Fig. 8A, and at the end of the chase periods, the labeled cells were biotinylated with a membrane-impermeable reagent. The pulse-labeled and biotinylated MHC II molecules were immunoprecipitated with MKD6, followed by purification with a streptavidin-agarose. Purified samples were boiled in sample buffer and analyzed by SDS-PAGE. As shown in Fig. 8B, the same amount of mature αβ dimers was transported to the cell surface in both control cells and c-MIR cells at 60 or 120 min of chase. These results suggest that, like KSHV MIR1 or MIR2, c-MIR might induce rapid endocytosis of MHC II. To test this hypothesis, surface MHC II molecules on c-MIR cells and control cells were labeled with FITC-conjugated MHC II Ab, and chased for 30 min at 37°C. As shown in Fig. 8C, c-MIR cells rapidly endocytosed MHC II molecules, compared with control cells. Taken together, these results strongly suggest that c-MIR downregulates the surface expression of MHC II through induction of rapid endocytosis.
Discussion

In this report, we demonstrated that force-expressed c-MIR functions as a potent immune modulator in vivo and in vitro, and that MHC II is an additional target of c-MIR. Genetically modified mice in which c-MIR is overexpressed in APCs showed marked immune defective phenotypes, such as resistance to the onset of autoimmune disease or impaired CD4 T cell development. Consistent with these phenotypes, surface expression of MHC II was down-regulated on thymic epithelial cells and peripheral APCs in c-MIR Tg mice. Also, forced expression of c-MIR down-regulated the expression of MHC II, and its down-regulation depended upon ubiquitination of a single lysine residue in the cytoplasmic tail of I-A β-chain. To our knowledge, c-MIR is the first example of an E3 that is capable of targeting MHC II. Hence,
with $^{35}$S-methionine and $^{35}$S-cysteine for 30 min and chased for 0–6 h. (Cont) or c-MIR-overexpressing A20 cells (c-MIR) were pulse-labeled containing Tris buffer and immunoprecipitated with MKD6 anti-I-A $\beta$-chain Ab. Each precipitated protein sample was analyzed by SDS-PAGE without boiling the samples before electrophoresis. The SDS-stable compact dimer ($c(\alpha/\beta)$) is indicated. Data shown are representative of two independent experiments.

At present, the physiological role of c-MIR is as yet unknown. Further analysis of c-MIR might provide new insight into the molecular mechanism of Ag presentation.

At present, the physiological role of c-MIR is as yet unknown. However, there is indirect evidence that c-MIR might be a physiological immune modulator in vivo. We previously showed that forced expression of c-MIR down-regulates the expression of B7-2 and that c-MIR is expressed in human monocyte-derived DCs (1). In this study, we showed that the expression of MHC II was significantly inhibited by c-MIR in APCs, and that c-MIR was moderately expressed in splenic macrophages and splenic DCs, which are physiological APCs in vivo. Furthermore, we found that during LPS-induced maturation of DCs, the expression of c-MIR was down-regulated, whereas the surface expression of B7-2 and MHC II were increased remarkably (our unpublished data). In addition, Shortman and colleagues (30) reported that immature DCs degrade MHC II-peptide complexes much faster than the matured DCs. Based on these findings, we propose the hypothesis that c-MIR might function as a modulator of MHC II expression in immature DCs. To examine this hypothesis, we are presently generating c-MIR-deficient mice.

Our results showed that forced expression of c-MIR strongly prevented the onset of EAE. As shown in Fig. 3, the development of CD4 T cells and the ability of splenic DCs to present Ags were remarkably impaired in c-MIR Tg mice, suggesting that both factors contribute to complete prevention of EAE by c-MIR. We speculated that the latter factor partially contributes to the prevention of EAE, based on results obtained from experiments with a BM chimera (in c-MIR Tg mice transplanted into lethally irradiated control littermates). In c-MIR Tg mice transplanted into lethally irradiated control littermates, CD4 T cells developed normally, presumably due to normal expression of MHC II in the thymus (see Fig. 3C), whereas peripheral APCs were derived from c-MIR Tg and failed to present Ags efficiently (Fig. 4D). As shown in Fig. 4A, we found that the induction of EAE was partially prevented in c-MIR Tg mice transplanted into lethally irradiated control littermates. If impaired development of CD4 T cells was the main reason for the prevention of EAE, prevention would not be observed in this mouse model because CD4 T cell development is normal in this BM chimera judged by the expression profile of surface markers. However, this hypothesis should be considered more cautiously because the expression of exogenous c-MIR was faintly detected in samples from splenic T cells in c-MIR Tg mice (see Fig. 1, D and E). In this connection, we found that an induced proliferation of splenic CD4 T cell by anti-CD3 Ab was significantly reduced in c-MIR Tg mice (our unpublished observation). This finding may reflect not only the impaired development of CD4 T cells, but also some other functional defects of CD4 T cells caused by a faint expression of exogenous c-MIR in these mice. However, anti-CD3 Ab-induced proliferation of CD4 T cells from c-MIR Tg mice transplanted into lethally irradiated control littermates was equivalent to that of control littermates transplanted into lethally irradiated control littermates (our unpublished observation). Because mature CD4 T cells of c-MIR Tg mice transplanted into lethally irradiated control littermates are thought to be derived from c-MIR Tg mice, a faint expression of exogenous c-MIR is probably present in the mature CD4 T cells of this BM chimera. Therefore, this amount of exogenous c-MIR protein is unlikely to influence the function of mature CD4 T cells. Thus, so far, we do not have any clear evidences that reject our hypothesis.

Besides the two factors we have described, uncharacterized effects of forcibly expressed c-MIR on the immune system might contribute to the complete prevention of EAE by c-MIR. Indeed, we found an increased population of splenic B cells in c-MIR Tg mice; this population consisted of IgM$^{low}$IgD$^{low}$B220$^+$ cells (our unpublished observation). In I-A $\beta$-chain-deficient or B7-2-deficient mice, B cell development was reported to be normal (26, 27). In contrast, an increased percentage of IgM$^{low}$IgD$^{low}$B220$^+$ cells was reported in the spleen of I-A $\alpha$-chain or II-deficient mice (31). Thus, this finding cannot be explained by findings in MHC II or II-deficient mice, indicating that forced expressed c-MIR has other functions than down-regulation of MHC class II and B7-2. Because recent evidence suggests that B cells contribute to EAE induction (32), a detailed analysis of B cell function in c-MIR Tg mice is necessary to understand the detailed mechanisms for c-MIR-mediated prevention of EAE.

**FIGURE 8.** Rapid endocytosis of MHC II by c-MIR. A, A20 cells (Cont) or c-MIR-overexpressing A20 cells (c-MIR) were pulse-labeled with $^{35}$S-methionine and $^{35}$S-cysteine for 30 min and chased for 0–6 h. At the end of the chase periods, cells were lysed in 0.5% Nonidet P-40-containing Tris buffer and immunoprecipitated with MKD6 anti-I-A $\beta$-chain Ab. Each precipitated protein sample was analyzed by SDS-PAGE without boiling the samples before electrophoresis. The SDS-stable compact dimer ($c(\alpha/\beta)$) is indicated. Data shown are representative of two independent experiments. B, A20 cells (Cont) or c-MIR-overexpressing A20 cells (c-MIR) were pulse-labeled and chased as in A. At the end of the chase periods indicated, each type of cell was biotinylated with a NHS-SS-biotin in PBS. The samples were precipitated with MKD6 anti-I-A $\beta$-chain Ab, followed by precipitation with Streptavidin-agarose. Data shown are representative of two independent experiments. C, A20 cells (Cont) or c-MIR-overexpressing A20 cells (c-MIR) were stained with M5/114,15,2 FITC-labeled anti-MHC II Ab at 4°C and washed twice with PBS, and incubated for various periods of time at 37°C. At the indicated time points, the localization of MHC II was examined by an immunofluorescence microscope. Data shown are representative of two independent experiments.
As shown in Fig. 5B in control littermates, there are two populations of EpCAM+ epithelial cells; one population with high expression of MHC II and the other in which MHC II is expressed moderately. These results are consistent with the previous reports by Boyd and colleagues (20), but at this moment, the cellular origins of these two populations remain unknown. Moreover, remarkable down-regulation of MHC II expression was observed only in the population whose MHC II expression level is moderate. This heterogeneous down-regulation was also observed in splenic B cells, as is shown in Fig. 5D. These findings might reflect the activity of the promoter used to generate c-MIR Tg mice. Indeed, Mathis and colleagues (19) reported that the expression level of Ii-moth cytochrome c chimeric molecule driven by this promoter was heterogeneous in IgM-positive cells, compared with that of endogenous Ii molecules. Thus, the activity of this promoter might be different from that of an authentic promoter for Ii molecules in some APCs, and this might be the cause of the unexpected heterogeneous down-regulation of MHC II in vivo.

Recently, several mammalian E3s related to c-MIR have been characterized, and these mammalian E3s including c-MIR were termed MARCH proteins, and c-MIR is now also known as MARCH VIII (14). Among MARCH proteins, there is a closely related molecule to c-MIR, MARCH I. c-MIR and MARCH I have 63% amino acid identity and share the same secondary structure and the same positioning of the catalytic domain that is a variant RING domain. Especially, within their two helical transmembrane regions, MARCH I exhibits 88% amino acid identity as compared with c-MIR. Because the transmembrane regions of MIR family proteins are thought to determine the specificity for targeting (17), one would expect MARCH I to target MHC II and B7-2 molecules as well, and there is a possible functional redundancy of c-MIR and MARCH I. Indeed, preliminary data obtained in our laboratory showed that the forced expression of MARCH I down-regulates the expression of MHC II, and also that MARCH I is expressed in APCs. In addition, Fruh and colleagues (14) and Cadwell and Cos coy (4) demonstrated the down-regulation of B7-2 by MARCH I. Given the apparent functional similarity of c-MIR and MARCH I, mouse models deficient in both molecules might constitute a powerful strategy to explore the physiological function of c-MIR.

Finally, given the striking effect of c-MIR overexpression on the immune system, manipulation of the function and/or expression of c-MIR might be used as a strategy for artificial immune modulation in vivo. At present, there are several projects that are attempting to identify and produce the novel subsets of DCs that induce immunological tolerance (33). These DCs, which have been designated as tolerogenic DCs, or regulatory DCs show a very limited capability of Ag presentation (33, 34). As shown in Fig. 2, c-MIR Tg-derived BMDCs did not present several Ags to T cells efficiently, suggesting that APCs forcibly expressing c-MIR might function as tolerogenic DCs. Preliminary experiments in our group showed that allogenic T cells stimulated with c-MIR Tg-derived mature BMDCs were not able to proliferate efficiently upon stimulation when compared with control littermate mice-derived BM- DCs, whereas T cell responsiveness was improved by adding an excess amount of IL-2. Therefore, we are now investigating possible applications of c-MIR for immune tolerance induction.

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Disclosures

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