Hrd1-mediated BLIMP-1 ubiquitination promotes dendritic cell MHCII expression for CD4 T cell priming during inflammation

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The ubiquitin pathway plays critical roles in antigen presentation. However, the ubiquitin ligases that regulate *MHC* gene transcription remain unidentified. We showed that the ubiquitin ligase Hrd1, expression of which is induced by Toll-like receptor (TLR) stimulation, is required for *MHC-II* but not *MHC-I* transcription in dendritic cells (DCs). Targeted *Hrd1* gene deletion in DCs diminished MHC-II expression. As a consequence, *Hrd1*-null DCs failed to prime CD4⁺ T cells without affecting the activation of CD8⁺ T cells. Hrd1 catalyzed ubiquitination and degradation of the transcriptional suppressor B lymphocyte–induced maturation protein 1 (BLIMP1) to promote MHC-II expression. Genetic suppression of Hrd1 function in DCs protected mice from myelin oligodendrocyte glycoprotein (MOG)–induced experimental autoimmune encephalomyelitis (EAE). We identified Hrd1-mediated BLIMP1 ubiquitination as a previously unknown mechanism in programming DC for CD4⁺ T cell activation during inflammation.

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Abbreviations used: BLIMP1: B lymphocyte–induced maturation protein 1; BMDC, BM-derived DC; CIITA, MHC class II transactivator; EAE, experimental autoimmune encephalomyelitis; ER, endoplasmic reticulum; MOG, myelin oligodendrocyte glycoprotein; UPR, unfolded protein response.

MHC class I (MHC-I) and class II (MHC-II) are cell surface glycoproteins on APCs involved in the binding and presentation of peptide antigens to the TCRs of T lymphocytes. MHC-I presents antigen from endogenous sources to CD8⁺ T cells, whereas MHC-II presents peptide from exogenous sources to CD4⁺ T cells (Rudolph et al., 2006; Adams and Luoma, 2013; Merad et al., 2013). These MHC–peptide–TCR complexes are required to generate antigen-specific immune responses. MHC-II is constitutively expressed in APCs at a relatively low level under naive conditions but can be induced by IFN-γ (Collins et al., 1984; Koeffler et al., 1984). In addition, pathogen-associated molecule patterns, including LPS and bacterial DNA, are capable of inducing MHC-II gene expression through binding to TLRs (Reis e Sousa, 2004). TLR-induced MHC-II expression plays critical roles in host immunity against microbial infections and is associated with inflammatory autoimmune diseases (Friese et al., 2005).

The MHC class II transactivator (CIITA) is critical for both constitutive and inducible MHC-II gene transcription (Steimle et al., 1993). IFN-γ and TLR stimulation induce CIITA to activate MHC-II gene transcription during inflammation (Drozina et al., 2005).

CIITA-deficient mice do not express conventional MHC-II molecules on the surface of splenic B cells, macrophages, and DCs (Chang et al., 1994). It has been reported that B lymphocyte–induced maturation protein 1 (BLIMP1), a transcriptional repressor that is capable of triggering plasma cell differentiation, is a repressor of CIITA transcription. BLIMP1-mediated CIITA suppression turns off MHC-II–mediated antigen presentation to induce B cell differentiation into plasma cells (Piskurich et al., 2000). *BLIMP1*-deficient DCs exhibit elevated expression of MHC-II and facilitate CD4⁺ T cell immunity (Piskurich et al., 2000; Tooze et al., 2006; Chen et al., 2007; Zhao et al., 2007; Kim et al., 2013).

The ubiquitin pathway has been shown to play a critical role in regulating MHC-II antigen presentation at multiple levels (Moffat et al., 2013). Ubiquitination of CIITA is required for its transcriptional activation of *MHC-II* gene expression (Greer et al., 2003). Ubiquitination also directly regulates MHC-II degradation,

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catalyzed by the membrane-associated RING (really interesting new gene)-CH (MARCH) family of E3 ubiquitin ligases (Ishido et al., 2009). Although several MARCH family members have been suggested as regulators of both innate and the adoptive immune responses, MARCH 1, which targets CD86 and MHC-II for ubiquitination-mediated degradation, is the most well characterized member (Matsuki et al., 2007; De Gassart et al., 2008; Young et al., 2008; Walseng et al., 2010; Tze et al., 2011). Given the critical roles of MHC-II in antigen presentation and the activation of the adaptive immune system, it is not surprising that a tight regulatory mechanism is necessary to ensure appropriate MHC-II antigen presentation. However, how the ubiquitin pathway controls MHC-II antigen presentation, in particular the specific E3 ubiquitin ligases that are required in this process, remains largely unidentified.

Hrd1, also known as Synoviolin, is a membrane-spanning protein on the endoplasmic reticulum (ER). It has a RING finger domain followed by a long proline-rich C terminus in its cytoplasmic portion, which is likely involved in recruiting cytoplasmic proteins for ubiquitination. Hrd1 was initially identified as a ubiquitin ligase involved in degrading misfolded proteins (Carvalho et al., 2006; Denic et al., 2006). Because Hrd1 expression is often up-regulated in synovial fibroblasts in patients with rheumatoid arthritis, it was renamed Synoviolin (Amano et al., 2003). We recently reported that proinflammatory cytokines, including TNF and IL-1β, are responsible for inducing Hrd1 expression in synovial fibroblasts (Gao et al., 2006). We further observed that Hrd1 ubiquitinates IRE1α (inositol-requiring enzyme 1α), a critical kinase in regulating the ER stress response (Gao et al., 2008). It has been shown that Hrd1 targets the misfolded MHC-I for degradation in the in vitro cultured cell lines (Burr et al., 2011; Huang et al., 2011). Although the ER stress functions of Hrd1 in misfolded protein degradation have been well studied, its physiological roles in immune regulation are not known.

RESULTS

Hrd1 promotes MHC–II expression by DCs

To study the physiological functions of Hrd1 in DCs, we generated *Hrd1* floxed mice. The *Hrd1* gene contains 16 exons (Fig. 1 A), we floxed exons 8–11 that encode a large region of the Hrd1 protein from its fifth transmembrane domain (TM) to the proline-rich sequence leading to deletion of the functional RING finger (Fig. 1, B and C). To exclude the potential effects of the neomycin selection cassette on *Hrd1* expression, this cassette was flanked by two flippase recognition target (*FRT*) sites, which can be deleted by FLP recombinase. This targeting vector was transfected into an embryonic stem cell line generated from C57/BL6 mice. Neomycin selects were screened by PCR. Seven clones were obtained and confirmed to be correct targeting by Southern blotting. Blastocyst injections resulted in several chimeric mice with the capacity for germline transmission. Breeding of heterozygous mice yielded *Hrd1^wt/wt*, *Hrd1^wt/f*, and *Hrd1^f/f* offspring without phenotypic abnormalities in expected Mendelian ratios (Fig. 1 D and not depicted). DC-specific *Hrd1* knockout (*Hrd1^f/f CD11c-Cre+*)
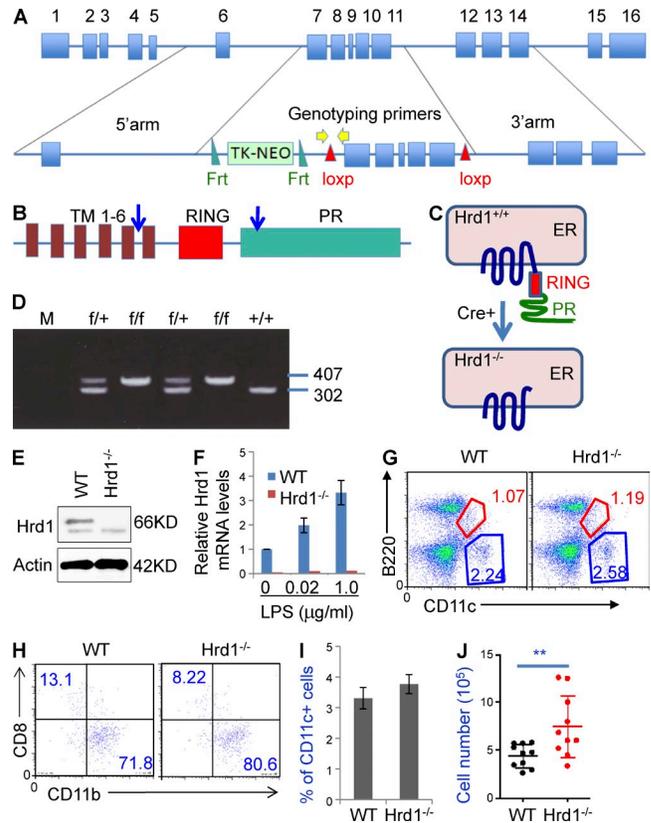
Figure 1. Targeted disruption of the *Hrd1* gene in DCs. (A) Structures of the *Hrd1* WT and targeted alleles. Exons and the neomycin phosphotransferase gene (Neo) driven by the thymidine kinase (TK) promoter are shown. The TK-NEO cassette is flanked by 2 FRT sites and exons 7–11 are flanked by 2 LoxP sites. (B and C) Domain structure of Hrd1 protein. The ER membrane-anchoring protein Hrd1 carries 6 transmembrane (TM) domains, one RING finger domain, and a C terminus proline-rich domain. The deletion of floxed *Hrd1* gene by Cre recombinase destroys Hrd1 protein expression. (D) Genotyping of Hrd1-floxed mice. Tail snips from a litter of Hrd1^flox/wt X Hrd1^flox/wt offspring were collected for DNA extraction and PCR analysis. The 302-bp PCR product is the WT allele and the 407-bp product is the mutant allele. (E and F) BM cells were isolated from WT and *Hrd1* conditional KO (*Hrd1^−/−*) mice and cultured under DC differentiation conditions. (E) Hrd1 protein expression was analyzed by immunoblotting (top) using β-actin as a loading control (bottom). (F) *Hrd1* mRNA levels were determined by real-time quantitative RT-PCR. Hrd1 levels in WT DCs increased with LPS treatment. (G) Cell surface expression of B220 and CD11c in total splenocytes from WT and *Hrd1^−/−* mice was analyzed by flow cytometry. (H) CD11c+B220− cells were gated and the expression of CD8 and CD11b was analyzed. (I and J) The mean percentages (I) and absolute numbers (J) of CD11c+ cells in the spleens of 10 pairs of WT and *Hrd1^−/−* mice are shown (n = 10).

mice were generated by breeding *Hrd1^f/f* mice with *CD11c-Cre+* transgenic mice. Both Hrd1 protein (Fig. 1 E) and mRNA (Fig. 1 F) were eliminated in purified *CD11c+* cells from *Hrd1^f/f CD11c-Cre+* (*Hrd1^−/−*) mice.

Because Hrd1 has been identified as an anti-apoptotic molecule that protects cells from ER stress–induced apoptosis (Amano et al., 2003), we asked whether *Hrd1* gene deletion affects CD11c+ DC survival. Surprisingly, loss of Hrd1 function

in DCs did not reduce survival; rather, it led to a slight increase in the percentage and a statistically significant increase in the total numbers of CD11c+ DCs in the spleen. In addition, the percentages of CD11c+B220− conventional DCs and CD11c+B220low plasmacytoid DCs were not altered in the spleens of $Hrd1^{−/−}$ mice compared with WT mice (Fig. 1 G). Moreover, analysis of the gated CD11c+B220− DCs by their expression of CD11b or CD8 did not detect any changes in the percentages of CD11c+CD11b+CD8−B220− myeloid DCs and CD11c+CD11b−CD8+B220− lymphoid DCs with $Hrd1$ gene deletion (Fig. 1, G and H). In addition, a slight increase in the percentage (Fig. 1 I) and a statistical significant increase in the total numbers (Fig. 1 J) of CD11c+ cells were detected in the spleen of DC-specific Hrd1 knockout mice.

Notably, we detected a significant reduction in MHC-II expression on the surface of immature BM-derived DCs (BMDCs). Stimulation with LPS for 24 h led to a dramatic increase in MHC-II expression in WT DCs but failed to upregulate MHC-II expression in $Hrd1$-null DCs. In contrast, the expression levels of MHC-I, CD80, and CD86 were not altered by $Hrd1$ gene deletion (Fig. 2, A and B). A similar reduction in MHC-II expression in gated CD11c+ cells from the spleens of $Hrd1^{−/−}$ mice was further confirmed (Fig. 2, C and D). These results indicate that $Hrd1$ gene deletion in mice selectively impairs MHC-II expression by DCs.

Because MHC-II expression is critical for CD4+ T cell development and homeostatic proliferation, we asked whether the decrease in MHC-II expression in $Hrd1$-null DCs alters T cell development in mice. Neither the percentages nor the absolute numbers of CD4 and CD8 double-negative, double-positive, and single-positive T cell populations were altered in the thymus of $Hrd1^{−/−}$ mice (Fig. 3, A and B). In contrast, a 20–30% increase in total splenocytes in $Hrd1^{−/−}$ mice was detected (Fig. 3 D). This enlarged spleen size of $Hrd1^{−/−}$ mice appeared to be due to a mean 30% increase in the percentage and total numbers of B220+ B cells (Fig. 3 E) and CD4+ cells (Fig. 3, F–H). Although there is a reduction in the percentage of CD8+ T cells, presumably due to the increase in CD4+ T cell populations in spleen, their absolute numbers were not affected (Fig. 3, F and H). Analysis of CD44 and CD62L expression on the surface of CD4+ T cells showed no changes in CD4+ T cell activation in DC-specific $Hrd1$-null mice (Fig. 3 I), excluding the possibility that chronic activation caused the observed increase in CD4+ T cells. In addition, there was an ∼30% reduction in the percentage of DC25+FoxP3+ T reg cells in the spleens of $Hrd1^{−/−}$ mice (Fig. 3, J and K), but absolute T reg cell numbers were not altered (Fig. 3 L), suggesting that the reduction in T reg cell percentage was likely a consequence of the CD4+ T cell increase. To support this, FoxP3+ T reg cells were not changed in the thymus of DC-specific $Hrd1$-null mice (Fig. 3 C). Further characterization of the splenic B cells did not did not detect any changes in the percentages of follicular and marginal zone B cells by their expression of CD21 and CD23, and no changes in the cell surface IgM and IgD expression (Fig. 3 M). Similar to CD4+ T cells, no increase in chronic B cell activation was detected because their
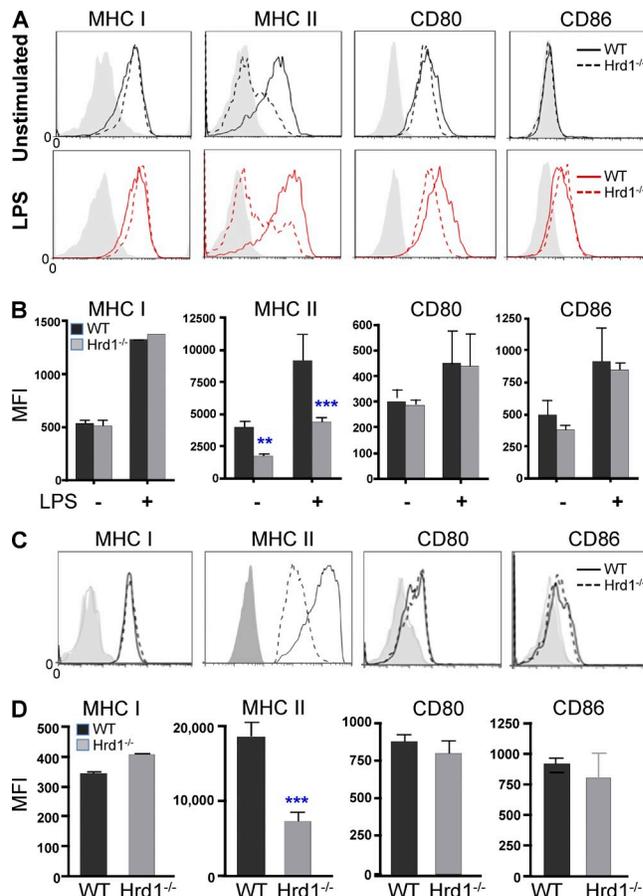

Figure 2. Hrd1 promotes MHC–II expression in DCs. (A and B) WT and $Hrd1^{−/−}$ BMDCs were cultured overnight with or without LPS (200 ng/ml). Expression levels of MHC-I, MHC-II, CD80, and CD86 were analyzed by flow cytometry. (A and B) Representative images (A) and the mean fluorescence identity (MFI; B) from 7 independent experiments are shown (n = 7). (C and D) The expression levels of MHC-I, MHC-II, CD80, and CD86 on gated CD11c+ DCs in WT and $Hrd1^{−/−}$ splenocytes were analyzed. (C and D) Representative images (C) and the average MFI (D) from nine pairs of mice are shown (n = 9). (B and D) Data are reported as mean ± SD and were analyzed by Student's t test. **, P < 0.01; ***, P < 0.005.

expression levels of MHC-I and II, CD80, and CD86 were indistinguishable between WT and DC-specific $Hrd1$ KO mice (Fig. 3 N).

Hrd1 regulates MHC–II expression at the mRNA level in DCs

To delineate the mechanism by which Hrd1 regulates MHC-II expression in DCs, we analyzed MHC-II mRNA expression in WT and $Hrd1$-null BMDCs. Consistent with the observed reduction in MHC-II protein expression in $Hrd1$-null BMDCs by flow cytometry (Fig. 2), MHC-II mRNA expression was largely diminished in these cells (Fig. 4 A). Importantly, TLR stimulation for 2 h failed to enhance MHC-II transcription in $Hrd1$-null BMDCs. In contrast, the transcription of MHC-I was not affected by $Hrd1$ deficiency (Fig. 4 B). Diminished $Hrd1$ mRNA expression was confirmed in BMDCs from $Hrd1^{−/−}$ mice (Fig. 4 C). In addition, a significant increase
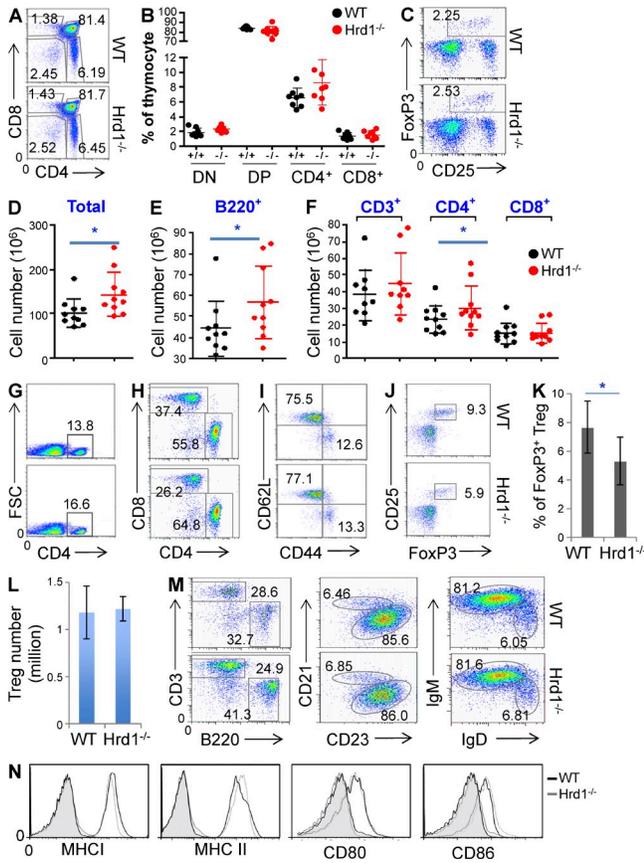
Figure 3. Characterization of T and B lymphocytes in *Hrd1^f/f^ CD11c–Cre^+^ (Hrd1^−/−^) mice. (A–C) Analysis of T cell development in thymus of *Hrd1^−/−^* mice. CD4 and CD8 expression in total thymocytes was analyzed by flow cytometry. (A) Representative images are shown from experiments run using seven pairs of WT and *Hrd1^−/−^* mice. (B) The percentages of CD4/CD8 double-negative (DN), double-positive (DP), and single-positive (SP) cells from seven pairs of mice are shown. (C) The FoxP3^+^CD25^+^ T reg cells in the gated CD4 SP T cells were analyzed; representative images from seven pairs of mice are shown. (D–N) Cellularity analysis in the spleens of *Hrd1^−/−^* mice. The absolute numbers of total splenocytes (D), B220^+^ B cells (E), and T cells (F) from nine pairs of mice are shown. (G) CD4^+^ T cells in the spleens of WT and *Hrd1^−/−^* mice were analyzed by flow cytometry. (H) The ration of CD4 and CD8 T cells in the gated CD3^+^ population was analyzed. (I–L) Gated CD4^+^ T cells were used for the analysis of CD44 and CD62L expression (I) and FoxP3^+^CD25^+^ T reg cells (J). (K) Mean percentages (K) and total numbers of T reg cells (L) in nine pairs of mice are shown. Data are reported as means ± SEM and Student's *t* test was used for the statistical analysis. *, P < 0.05. (M and N) Analysis of B cells in the spleens of *Hrd1^−/−^* mice. (M) The percentages of CD3^+^ T cells and B220^+^ B cells in the spleen of WT and Hrd1 conditional KO mice are analyzed (left). The gated B220^+^ B cells were analyzed for their expression of CD21 and CD23 (middle), and IgM and IgD (right). (N) The expression levels of MHC-I, MHC-II, CD80, and CD86 on the surface of gated B220^+^ B cells were analyzed. Images are representative for at least nine pairs of mice (n = 9).

in *Hrd1* mRNA and protein expression was detected in WT BMDCs upon TLR stimulation (Fig. 4, C and D), indicating that TLR signaling normally induces Hrd1 expression in DCs.

It has been reported that TLR stimulation suppresses *MHC-II* mRNA transcription in DCs 18–24 h after stimulation (Landmann et al., 2001; Pai et al., 2002; Yao et al., 2006), but with a transient increase during the early stage (0.5–3 h) of stimulation (Landmann et al., 2001; Casals et al., 2007). We dynamically analyzed the effects of *Hrd1* deletion on TLR-mediated *MHC-II* transcription. Indeed, consistent with these previous studies, after a transient increase at 2 h after stimulation, a 40–50% reduction in *MHC-II* transcription was detected in WT DCs 24 h after LPS stimulation. In contrast, this dynamic transcription of *MHC-II* in Hrd1-null DCs was dismissed (Fig. 4 E). The transcription factor CIITA has been identified as a critical factor for TLR-induced *MHC-II* transcription in DCs (Steimle et al., 1993). Interestingly, we detected that *CIITA* mRNA expression was diminished in BMDCs and in the gated CD11c^+^ DCs in the spleens of *Hrd1^−/−^* mice (Fig. 4 F), indicating that Hrd1 may regulate MHC-II expression by promoting *CIITA* gene transcription.

DC Hrd1 is a positive regulator for CD4^+^ T cell priming

The finding of reduced MHC-II expression on the surface of *Hrd1*-null DCs led us to test whether Hrd1 positively regulates antigen presentation by DCs. Alexia Fluor 647–conjugated chicken ovalbumin (Ax647-OVA) was developed as a convenient approach to measure antigen uptake and presentation as the processed peptides fluoresce (Mansour et al., 2006). We incubated WT BMDCs with Ax647-OVA and found that mean fluorescence intensity increased in a dose-dependent manner, indicating normal antigen presentation and processing. In contrast, mean fluorescence intensity was significantly reduced in *Hrd1*-null BMDCs incubated with Ax647-OVA (Fig. 5 A). A similar reduction in fluorescence intensity was observed in freshly sorted CD11c^+^ splenic DCs (Fig. 5 B), indicating that Hrd1 functions are required for antigen presentation by DCs.

MHC-II expression by DCs is essential for antigen-specific CD4^+^ T cell priming by directly presenting peptide antigens to their TCRs. Because *Hrd1* deficiency in DCs selectively impaired MHC-II expression, we tested whether *Hrd1*-null DCs were still able to prime CD4^+^ T cells. WT and *Hrd1*-null BMDCs were stimulated with LPS in the presence of OVA overnight, washed, and then incubated with naive CD4^+^ T cells from OT-II TCR transgenic mice. Naive CD8^+^ T cells from the OT-I mice were used as controls. Antigen-specific T cell proliferation was determined by ³H-thymidine incorporation and CFSE dilution as previously described (Zhang et al., 2009). OT-II CD4^+^ T cells proliferated vigorously when co-cultured with WT BMDCs. Conversely, OT-II CD4^+^ T cell proliferation was diminished when co-cultured with *Hrd1*-null BMDCs and OVA protein as measured by ³H-thymidine incorporation (Fig. 5 C) or CFSE dilution (Fig. 5 E). As expected, the proliferation of CD8^+^ OT-I T cells was comparable when co-cultured with either WT or *Hrd1*-null DCs (Fig. 5, D and E). As an E3 ubiquitin ligase that promotes protein degradation, the DC Hrd1 may regulate CD4 T cell activation by controlling the protein antigen processing onto MHC-II complex. To test this
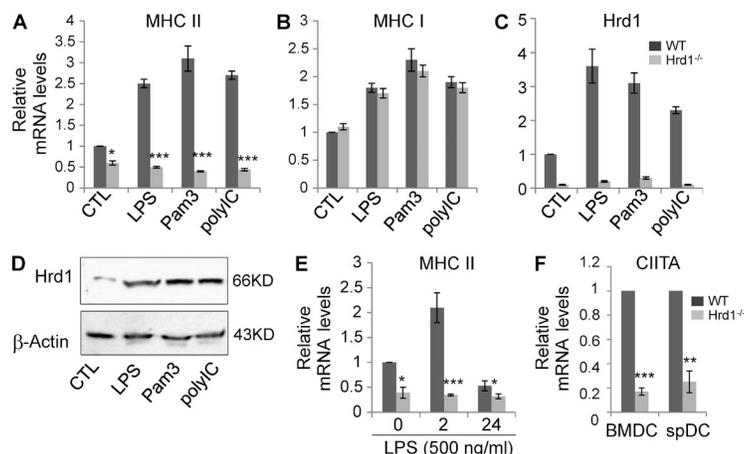
Figure 4. Hrd1 regulates MHC–II expression at the mRNA level. WT and $Hrd1^{-/-}$ BMDCs were stimulated with the indicated TLR agonists for 2 h. Total mRNA was extracted and levels of *MHC-II* (A), *MHC-I* (B), and *Hrd1* (C). (D) WT and $Hrd1^{-/-}$ BMDCs were stimulated with each indicated TLR agonist for 24 h. Hrd1 protein expression levels were determined by Western blotting with β-actin as a loading control. (E) WT and $Hrd1^{-/-}$ BMDCs were stimulated with 500 ng/ml LPS for 2 or 24 h. The expression levels of MHC-II were determined by real-time PCR. (F) *CIITA* expression in BMDCs and sorted CD11c^{+} splenic DCs without TLR stimulation was quantified by real-time RT-PCR using *β-actin* as a control. Data are reported as mean ± SD from five independent experiments ($n = 5$). Student's t test was used for statistical analysis. *, P < 0.05; **, P < 0.01; **, P < 0.01; ***, P < 0.005.

notion, we analyzed the T cell proliferation by co-cultivating with OVA peptides. Similar to the results from OVA protein co-culture experiments, loss of Hrd1 in DCs dramatically impaired their ability to prime CD4^{+} T cells in the presence of the MHC-II–presented $OVA_{323-339}$ peptide as measured by both ^{3}H-thymidine incorporation assay (Fig. 5 F) and CFSE analysis (Fig. 5 H). In contrast, *Hrd1* gene deletion in DCs did not affect CD8+T cell proliferation when co-cultured with the MHC-I–presented $OVA_{257-264}$ peptide (Fig. 5, G and H). Therefore, genetic deletion of *Hrd1* in DCs impairs CD4 T cell priming is largely due to the reduced MHC-II expression.

Next, we studied the role of DC Hrd1 in regulating CD4^{+} T cell priming in vivo using an adoptive transfer approach. Naive CD45.2^{+}OT-II CD4^{+} T cells and control CD45.1^{+}CD4^{+} T cells were mixed at a 1:1 ratio, stained with CFSE, and adoptively transferred into lethally irradiated WT and DC-specific *Hrd1* KO mice. 1 d after the adoptive transfer, recipients were immunized with $OVA_{323-339}$/CFA. 5 d after immunization, vigorous proliferation of the CD45.2^{+}CD4^{+} OT-II T cells in the WT recipients was detected. In contrast, proliferation of CD45.2^{+}CD4^{+} OT-II T cells in the DC-specific $Hrd1^{-/-}$ recipients was dramatically impaired (Fig. 5, I and J).
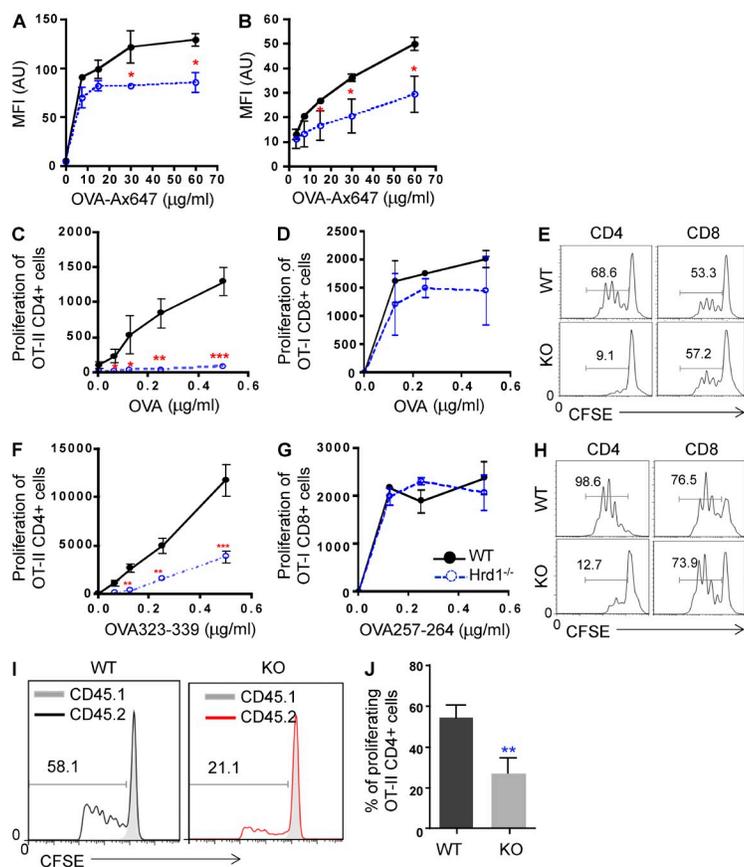
Figure 5. *Hrd1* deficiency impairs DC–mediated CD4^{+} T cell priming. (A and B) BMDCs (A) and sorted CD11c^{+} cells (B) from the spleen were cultured with Ax647-OVA at the indicated concentrations for 1 h and washed. Fluorescence intensity was analyzed by flow cytometry and data are reported as mean ± SD from five independent experiments. (C–E) BMDCs were stimulated with LPS in the presence of OVA protein overnight, washed, and co-cultured with either CD4^{+} OT-II T cells or CD8^{+} OT-II T cells for 3 d. T cell proliferation was analyzed by ^{3}H-thymidine incorporation (C and D) or CFSE dilution (E). (F–H) BMDCs were stimulated with LPS in the presence of either $OVA_{323-339}$ (F and H [left]) or $OVA_{257-264}$ (G and H [right]) peptides overnight, washed, and co-cultured with either CD4^{+} OT-II T cells or CD8^{+} OT-II T cells for 3 d. T cell proliferation was analyzed by ^{3}H-thymidine incorporation (F and G) or CFSE dilution (H). (I and J) In vivo OVA-specific CD4^{+} T cell priming was analyzed as described in Materials and methods. (I and J) Representative images (I) and data reported as mean ± SD (J) from five pairs of WT and $Hrd1^{-/-}$ recipients are shown ($n = 5$). Student's t test was used for statistical analysis. *, P < 0.05; **, P < 0.01; ***, P < 0.0001.
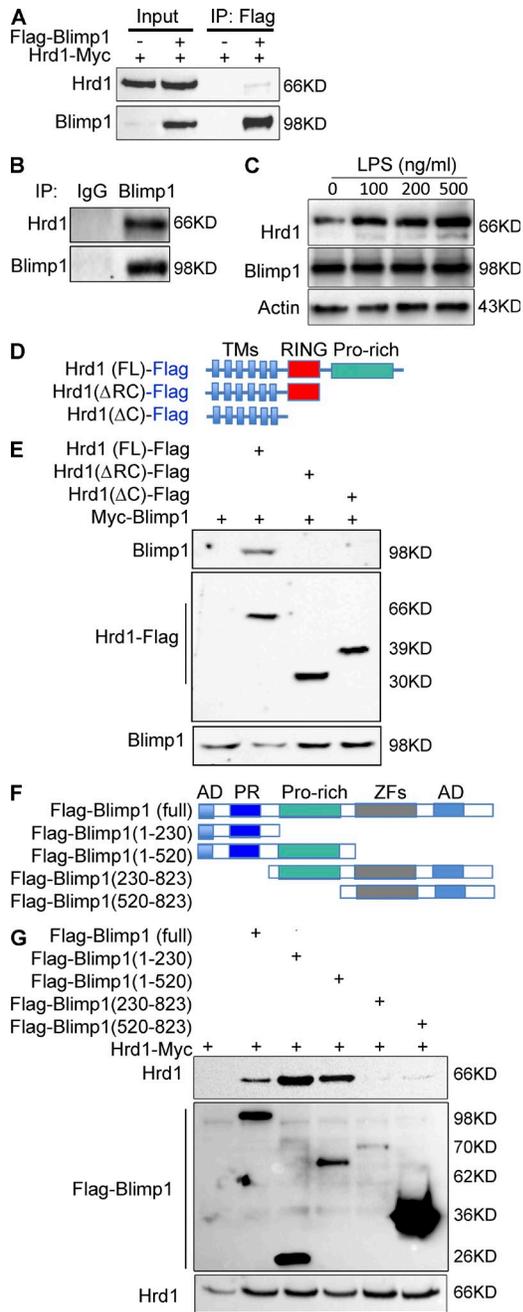
Figure 6. Hrd1 interacts with BLIMP1. (A) A *Myc-Hrd1* expression plasmid was transfected with or without *Flag-BLIMP1* expression plasmid into HEK293 cells. Expression of Hrd1 and BLIMP1 in whole cell lysates was confirmed by immunoblotting (lanes 1 and 2). Hrd1 interaction with BLIMP1 was determined by immunoprecipitation (IP) with anti-Flag antibody followed by immunoblotting with anti-Myc antibody (top, lanes 3 and 4). The same membrane was reprobed with anti-Flag antibody to confirm BLIMP1 expression (bottom). (B) Interaction between endogenous Hrd1 and BLIMP1 in mouse BMDCs was analyzed by IP with anti-BLIMP1 using normal rabbit IgG as a control, followed by immunoblotting with anti-Hrd1 (top). The same membrane was reprobed with anti-BLIMP1 (bottom). (C) BMDCs were stimulated with different doses of LPS as indicated for 2 h. The interaction between BLIMP1 and Hrd1 was determined as in B. (D and E) Truncation mutants of Hrd1 were generated (D) and

These results indicate that DC Hrd1 plays a critical role in antigen-specific priming of CD4^{+} T cells in mice.

Hrd1 interacts with BLIMP1 in DCs

The significant reduction in both *MHC-II* and *CIITA* mRNA levels in *Hrd1*-null DCs led us to ask whether Hrd1 regulates MHC-II expression by targeting upstream regulatory factors. The transcription factor BLIMP1 has been shown to suppress the expression of both *CIITA* and *MHC-II* (Piskurich et al., 2000). We speculated that Hrd1 controls *MHC-II* and *CIITA* expression through targeting BLIMP1 in DCs. To test this hypothesis, we first determined whether Hrd1 interacts with BLIMP1. Indeed, Hrd1 protein was detected in anti–BLIMP1 immunoprecipitates from HEK293 cells cotransfected with Hrd1 and BLIMP1, but not in cells transfected with Hrd1 alone (Fig. 6 A). Interaction between endogenous Hrd1 and BLIMP1 in mouse primary BMDCs was confirmed by coimmunoprecipitation and immunoblotting because Hrd1 was detected in immunoprecipitates from BMDC lysates using anti–BLIMP1 but not normal rabbit IgG (Fig. 6 B). In addition, the interaction between Hrd1 and BLIMP1 appears to be regulated by TLR signaling as LPS stimulation of BMDCs for 2 h enhanced their interaction in a dose–dependent manner (Fig. 6 C).

Hrd1 protein contains six transmembrane (TM) domains and its cytoplasmic tail carries an E3 ligase catalytic RING finger and a long proline–rich C terminus (Fig. 6 D). Analysis of BLIMP1 interaction with Hrd1 truncation mutants identified that the Hrd1 proline-rich region mediates its interaction with BLIMP1, as deletion of this region completely abolished the Hrd1–BLIMP1 interaction (Fig. 6 E). The BLIMP1 protein carries 2 acidic domains (AD), one positive regulatory (PR) domain, a proline-rich region, and 5 DNA-binding zinc finger (ZF) regions (Fig. 6 F). We generated a series of truncated BLIMP1 mutants and mapped the domains that interact with Hrd1. As indicated in Fig. 6 G, the BLIMP1 PR domain appears to be required for its interaction with Hrd1, as the N-terminal portion containing the PR domain interacted with Hrd1 and deletion of the PR domain-containing C terminus completely abolished the Hrd1 interaction.

Hrd1 promotes BLIMP1 protein degradation through ubiquitination in DCs

E3 ligases often catalyze ubiquitin conjugation onto interacting proteins to modulate their function. We asked whether Hrd1 catalyzes BLIMP1 ubiquitination. Indeed, transient Hrd1 expression in HEK293 dramatically enhanced BLIMP1 ubiquitination. In contrast, only a low level of BLIMP1 ubiquitination was detected in cells without Hrd1 co-transfection,

their interactions with BLIMP1 in transiently transfected HEK293 cells were determined by IP and immunoblotting (E) as described in A. (F and G) Truncation mutants of BLIMP1 were generated (F) and their interactions with full-length Hrd1 in the transiently transfected HEK293 cells were determined by IP and immunoblotting (G) as described in A. TM, transmembrane; Pro, proline; AD, acidic domain; PR, positive regulatory; ZF, zinc finger.
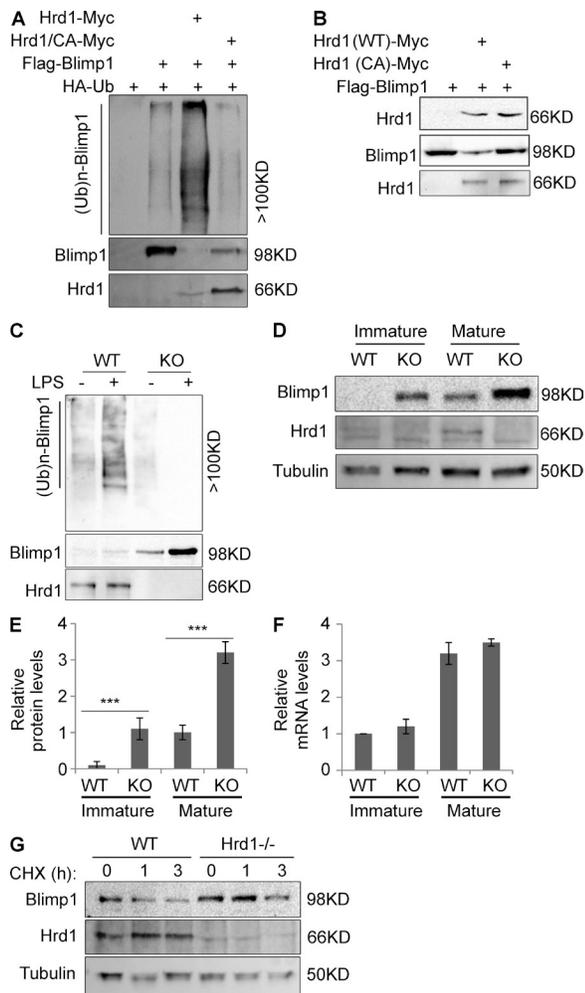
Figure 7. Hrd1 is an E3 ubiquitin ligase of BLIMP1. (A) *Flag-BLIMP1* and *HA-ubiquitin* (*Ub*) plasmids were cotransfected with WT Hrd1 or its CA mutant. BLIMP1 protein in the lysates of transfected cells was immunoprecipitated (IP) with anti-Flag antibody and polyubiquitination ((Ub)n) of BLIMP1 was analyzed by immunoblotting with anti-HA antibody (top). The same membrane was reblotted with anti-BLIMP1 (middle) and again with anti-Hrd1 to confirm expression in cell lysates (bottom). (B) Interaction of BLIMP1 with WT Hrd1 or its CA mutant in transiently transfected HEK293 cells was determined by IP and immunoblotting as described in A. (C) WT and *Hrd1*$^{-/-}$ BMDCs were treated with or without LPS (200 ng/ml) for 24 h and lysed. BLIMP1 protein in the lysates was immunoprecipitated with anti-BLIMP1, followed by immunoblotting with anti-Ub antibody (top). The same membrane was reprobed with anti-BLIMP1 (middle) and again with anti-Hrd1 to confirm expression in cell lysates (bottom). (D–F) WT and *Hrd1*$^{-/-}$ BMDCs were stimulated with (mature) or without (immature) LPS (200 ng/ml) for 24 h. (D) Protein expression levels of BLIMP1 (top), Hrd1 (middle), and Tubulin (bottom) in the whole cell lysates were determined by immunoblotting. (E) Relative levels of BLIMP1 protein expression reported as mean ± SD from three independent experiments. Student's *t* test was used for statistical analysis. ***, P < 0.005. (F) Total RNA was extracted from immature and mature DCs and the levels of *BLIMP1* mRNA were determined by real-time quantitative PCR. Data represent means ± SD. (G) Mature WT and *Hrd1*$^{-/-}$ BMDCs were treated with cycloheximide (CHX) for the indicated times. Expression levels of BLIMP1 (top), Hrd1 (middle), and Tubulin (bottom) in the whole cell lysates were

presumably catalyzed by endogenous Hrd1 (Fig. 7 A). Mutation of a critical cysteine to alanine in the RING finger of Hrd1 (Hrd1/CA) that inactivates its E3 ligase catalytic activity (Gao et al., 2008) completely abolished its ability to catalyze BLIMP1 ubiquitination (Fig. 7 A), although interaction between Hrd1 and BLIMP1 was not affected (Fig. 7 B). These results indicate that Hrd1 is an E3 ubiquitin ligase of BLIMP1 and its functional RING finger is required for Hrd1-mediated BLIMP1 ubiquitination. We then speculated that loss of Hrd1 function would impair or abolish BLIMP1 ubiquitination in DCs. Compared with *Hrd1*-null DCs, an anti-ubiquitin antibody detected bands with higher molecular weights in anti-BLIMP1 immunoprecipitates from WT BMDCs, suggesting that BLIMP1 protein ubiquitination in WT DCs is lost in *Hrd1*-null DCs (Fig. 7 C, lanes 1 and 3). Similar to our results that TLR signaling positively regulates Hrd1–BLIMP1 interaction (Fig. 6 C), LPS stimulation further enhanced BLIMP1 ubiquitination in WT DCs (Fig. 7 C, lanes 1 and 2) but failed to enhance BLIMP1 ubiquitination in *Hrd1*-null BMDCs (Fig. 7 C, lanes 3 and 4). These results indicate that Hrd1 is required for BLIMP1 ubiquitination in mouse DCs and that TLR signaling enhances BLIMP1 ubiquitination.

We noticed a significant reduction in BLIMP1 protein expression in cells when WT Hrd1, but not the Hrd1/CA mutant, was coexpressed (Fig. 7, A and B). This prompted us to ask whether Hrd1 is involved in regulating BLIMP1 protein degradation. We sorted CD11c$^+$ splenic DCs from WT and *Hrd1*$^{-/-}$ mice and analyzed the expression levels of both BLIMP1 protein and its mRNA. BLIMP1 protein expression was relatively low in the immature mouse splenic DCs, and stimulation with LPS for 24 h significantly induced BLIMP1 protein expression (Fig. 7 D, lane 1 vs. 3). Notably, compared with immature WT DCs, a dramatically higher level of BLIMP1 protein was detected in immature *Hrd1*-null DCs, which again was further enhanced by LPS stimulation (Fig. 7, D and E). Conversely, *BLIMP1* mRNA levels were indistinguishable between WT and *Hrd1*-null DCs, before or after LPS stimulation (Fig. 7 F), indicating that Hrd1 suppresses BLIMP1 expression posttranscriptionally. To confirm this conclusion, we demonstrated that BLIMP1 protein expression level and half-life are significantly increased in *Hrd1*-null BMDCs (Fig. 7 G). Therefore, our results collectively indicate that Hrd1 is an E3 ubiquitin ligase of BLIMP1 that controls BLIMP1 protein stability in DCs.

Hrd1 regulates MHC–II expression through suppression of BLIMP1

Because BLIMP1 has been shown to suppress CIITA and MHC–II expression (Kim et al., 2013), and we demonstrated that protein expression of BLIMP1 is elevated in *Hrd1*-null DCs (Fig. 7 D), we hypothesized that Hrd1 promotes MHC–II expression by catalyzing BLIMP1 protein degradation. To test this hypothesis, we used an shRNA–mediated knockdown

determined by immunoblotting. Representative results from 3 independent experiments are shown (*n* = 3).

approach and examined whether BLIMP1 suppression rescues MHC-II expression in *Hrd1*-null DCs. shRNA–mediated knockdown inhibited >95% of BLIMP1 protein expression in both WT and *Hrd1*-null DCs (Fig. 8 A). Suppression of BLIMP1 expression rescued MHC-II expression in *Hrd1*-null BMDCs to levels comparable to those of WT BMDCs (Fig. 8, B and C). We also noticed that the MHC-II expression level in *Hrd1*-null BMDCs with BLIMP1 knockdown was slightly but statistically significantly lower than that in WT BMDCs with BLIMP1 knockdown; this may be due to incomplete BLIMP1 knockdown in *Hrd1*-null DCs. We also found that BLIMP1 knockdown rescued *CIITA* mRNA expression in *Hrd1*-null DCs (Fig. 8 D). Collectively, these data suggest that Hrd1 diminishes BLIMP1-mediated CIITA suppression to promote MHC-II expression by ubiquitination and degradation of BLIMP1 protein.

Next, we determined whether BLIMP1 knockdown could rescue the ability of *Hrd1*-null BMDCs to prime CD4$^+$ T cells. As shown in Fig. 8 E, CD4$^+$ T cells from OT-II mice failed to proliferate when co-cultured with *Hrd1*-null DCs and OVA$_{323-339}$ peptides, confirming that Hrd1 is required for DC function in priming CD4$^+$ T cells. BLIMP1 knockdown in *Hrd1*-null BMDCs rescued CD4$^+$ OT-II T cell proliferation (Fig. 8, E and F). BLIMP1 knockdown in WT BMDCs also resulted in a slight but statistically significant increase in CD4$^+$ T cell proliferation (Fig. 8, E and F). Therefore, BLIMP1 protein accumulation in *Hrd1*-null BMDCs appears to interfere with the function of these cells in CD4$^+$ T cell priming, supporting the model that DC Hrd1 regulates CD4$^+$ T cell activation through BLIMP1 degradation.

Hrd1 has been initially identified as an E3 ubiquitin ligase critical for misfolded protein degradation, raising the possibility that loss of Hrd1 expression attenuates *MHC-II* expression through unfolded protein response (UPR). However, analysis of the downstream UPR genes, including *ERdj3*, *ERdj4*, and *Edem1* (ER–associated degradation), *Pdi4* and *Fkbp11* (protein folding), *Ire1α* (ER stress transducer), and *Sec22L1* (ER–Golgi transport; Zhang et al., 2011), did not detect any significant increases in their transcription levels in *Hrd1*-null BMDCs, even after stimulation with the pharmacological UPR inducer tunicamycin (Fig. 8 G). Together with our observation that *Hrd1* deficiency did not facilitate the ER stress–induced DC death (Fig. 2), our results largely exclude the possibility of misfolded protein responses caused by *Hrd1* deficiency in DCs. In addition, we have previously reported that Hrd1 promotes the degradation of IRE1α (Gao et al., 2008). Indeed, we did detect that the IRE1α protein expression levels are increased in Hrd1-null DCs (unpublished data), raising a possibility that loss of Hrd1 expression may inhibits MHC-II expression due to the elevated IRE1α functions. However, the MHC-II expression levels, both its protein and mRNA, are not affected in IRE1α-null DCs (Fig. 8 H), excluding the possible role of IRE1α in MHC-II expression in DCs. Collectively, our results indicate that Hrd1 regulates MHC-II expression through targeting BLIMP1 degradation.

Genetic Hrd1 deletion attenuates autoimmune response in mice

When primed by specific antigens, CD4$^+$ T cells undergo clonal expansion and then differentiate into effector T helper
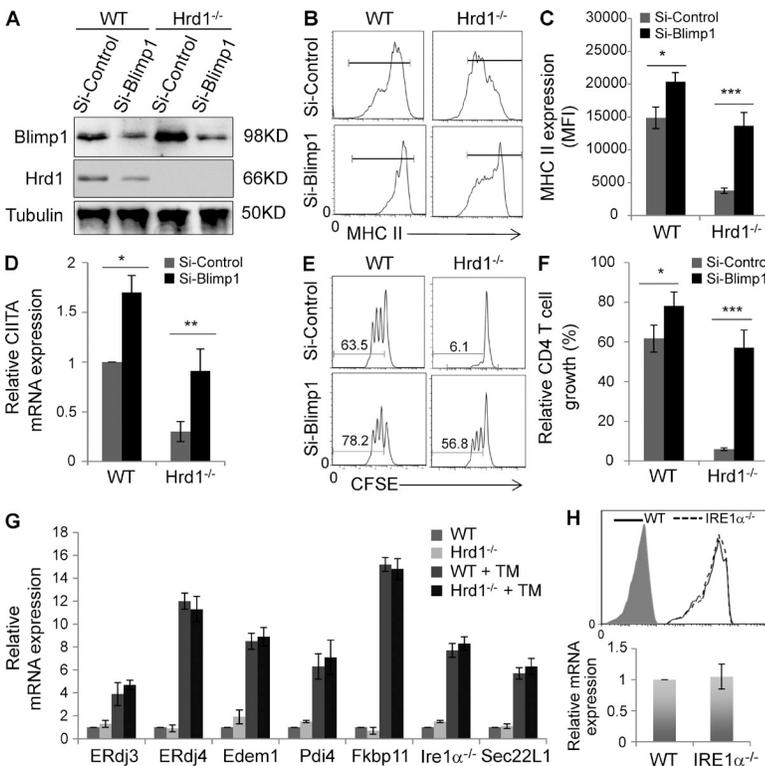
Figure 8. Hrd1 promotes MHC–II expression through degradation of BLIMP1. WT and *Hrd1*$^{-/-}$ BMDCs were transfected with control or *BLIMP1*-specific shRNA. (A) 3 d after transfection, protein expression levels of BLIMP1 (top) and Hrd1 (middle) were determined by immunoblotting, using Tubulin as a loading control (bottom). (B and C) MHC-II expression levels were analyzed by flow cytometry. Representative images (B) and mean fluorescence intensities ± SD (C) from 3 independent experiments are shown. (D) *CIITA* mRNA expression levels were determined by real-time RT-PCR. (E and F) BLIMP1 knockdown and control BMDCs were stimulated with LPS (200 ng/ml) and OVA$_{323-339}$ peptide overnight, washed, and co-cultured with CD4$^+$ T cells from OT-II mice. Representative images of CD4$^+$ T cell proliferation (E) and mean percentages of dividing CD4$^+$ cells ± SD (F) from three independent experiments are shown. Student's *t* test was used for the statistical analysis of the data in (C, D, and F). *, P < 0.05; **, P < 0.01; ***, P < 0.005. (G) WT and *Hrd1*$^{-/-}$ splenic DCs were stimulated with or without tunicamycin for 2 h and the mRNA expression levels of each indicated UPR genes were analyzed by real-time PRC. (H) The expression levels of MHC-II on the surface of WT and *IRE1α*$^{-/-}$ splenic DCs (top) and MHC-II mRNA were analyzed. Error bars represent data from five pairs of mice (*n* = 5).

(Th) cells including Th1, Th2, Th17, and T reg cells. Both Th1 and Th17 are critical pathogenic factors in multiple sclerosis in humans and experimental autoimmune encephalomyelitis (EAE) in mice (Pierson et al., 2012). Because *Synv*-null DCs are incapable of priming CD4$^+$ T cells, we reasoned that genetic deletion of *Hrd1* in DCs would attenuate the CD4$^+$T cell–mediated autoimmune inflammatory response and disease progression. We used a myelin oligodendrocyte glycoprotein (MOG)–induced EAE mouse model to test this hypothesis. Because both CD4$^+$T cells and B cells are increased in DC-specific *Hrd1$^{-/-}$* mice (Fig. 3), we generated *Hrd1$^{f/f}$CD11c-Cre$^+$/RAG1$^{-/-}$* double KO (*Hrd1$^{-/-}$/RAG1$^{-/-}$*) mice and used an adoptive transfer approach. CD11c$^+$ cell–depleted splenocytes from WT C57/B6 mice were adoptively transferred into *Hrd1$^{-/-}$/RAG1$^{-/-}$* double KO and *Hrd1$^{+/+}$/RAG1$^{-/-}$* mice. EAE induction with MOG was initiated 1 d after adoptive transfer and the clinical symptoms were scored daily. As shown in Fig. 9 A, in contrast to the severe disease that developed in *Hrd1$^{+/+}$ RAG1$^{-/-}$* recipients after MOG immunization, only modest symptoms with a dramatic delay in onset were observed in *Hrd1$^{-/-}$/RAG1$^{-/-}$* mice, indicating that Hrd1 suppression in DCs protects mice from MOG-induced EAE. MOG-specific CD4$^+$ T cell proliferation (Fig. 9 B) and IL-2 production (Fig. 9 C) were largely diminished in the draining lymph nodes of *Hrd1$^{-/-}$/RAG1$^{-/-}$* recipients. As a consequence of impaired CD4$^+$ T cell priming during disease progression in *Hrd1*-null mice, the differentiation of MOG-specific Th1 and Th17 cells was significantly inhibited (Fig. 9 D). In addition, we confirmed a significant reduction in MHC-II, but not MHC-I, expression on CD11c$^+$ DCs in the draining lymph nodes of *Hrd1$^{-/-}$/RAG1$^{-/-}$* mice (Fig. 9, E and F). Therefore, our studies collectively indicate that DC-specific Hrd1 suppression attenuates autoimmune EAE through down-regulation of MHC-II expression, which inhibits MOG-specific CD4$^+$ T cell priming.

DISCUSSION

Our study shows that the ER membrane-spanning E3 ubiquitin ligase Hrd11 is required for DC-controlled CD4$^+$ T cell priming in the autoimmune inflammatory response. This conclusion is supported by the following observations: targeted deletion of the *Hrd1* gene in DCs impaired MHC-II expression at the transcriptional level; *Hrd1*-null DCs failed to prime CD4$^+$ T cells without affecting CD8$^+$ T cell activation; Hrd1 targets BLIMP1, a nuclear transcriptional repressor, for ubiquitin-mediated degradation in DCs; Hrd1 promotes *MHC-II* gene transcription and CD4$^+$ T cell priming through BLIMP1 degradation; and genetic deletion of *Hrd1* gene in DCs partially protects mice from MOG-induced EAE.

Hrd1 appeared to specifically regulate MHC-II expression without affecting the expression of either MHC-I or costimulation molecules, including CD80 and CD86. As a consequence, *Hrd1*-null DCs failed to prime CD4$^+$ T cells, but antigen-specific CD8$^+$ T cell activation was not affected. Recent studies showed that Hrd1 catalyzes the degradation of misfolded MHC-I and is possibly involved in MHC-I–restricted antigen

presentation (Burr et al., 2011; Huang et al., 2011). However, we found that in mouse primary DCs, Hrd1 is not required for MHC-I protein and mRNA expression. Therefore, it is likely that Hrd1 selectively regulates MHC II gene transcription but degrades the misfolded MHC I molecules in DCs.

We identified Hrd1 as a specific E3 ubiquitin ligase that catalyzes BLIMP1 ubiquitination and degradation in mouse DCs. Loss of Hrd1 led to an accumulation of BLIMP1 in DCs, which reduced MHC-II expression; BLIMP1 knockdown rescued CIITA and MHC-II expression as well as CD4$^+$ T cell priming. Therefore, Hrd1-mediated BLIMP1 degradation promotes the transcription of both *CIITA* and *MHC-II* genes. To our knowledge, Hrd1 is the first identified E3 ubiquitin ligase that targets BLIMP1. In addition to its important physiological functions in DCs, BLIMP1 has been shown as a critical regulator in B cell differentiation of plasma cells (Kikuchi et al., 1995; Simard et al., 2011; Maseda et al., 2012), follicular T helper cell differentiation (Crotty, 2011), CD8$^+$ memory T cell development (Martins and Calame, 2008), and myeloid cell functions (Chang et al., 2000). It will be interesting to determine whether Hrd1-mediated BLIMP1 ubiquitination and degradation regulate the functions of these immune cell types. In addition to BLIMP1, this ER membrane-anchoring E3 ubiquitin ligase Hrd1 has been shown to target transcription factors including Nrf1 (Steffen et al., 2010; Tsuchiya et al., 2011) and p53 (Yamasaki et al., 2007). More recently, we have shown that Hrd1 catalyzes Nrf2 degradation to suppress Nrf2-mediated cellular protection during liver cirrhosis (Wu et al., 2014). As expected, similar to BLIMP1, Hrd1 recognizes these target proteins through its cytoplasmic C terminus domain. More importantly, the regulation of these transcription factors is unlikely triggered by misfolded protein response. Therefore, the accumulated evidences suggest that Hrd1 is involved in a variety of pathobiological functions by degrading cellular signaling molecules through its cytoplasmic domain.

MHC-II expression is critical for CD4$^+$ T cell development. Mice with targeted gene deletion of MHC-II, or factors required for MHC-II gene transcription such as CIITA, lack CD4$^+$ T cells (Chang et al., 1994; Madsen et al., 1999). Although the DC-specific *Hrd1* knockout mice showed a significant reduction in MHC-II expression, the development of CD4$^+$ and CD8$^+$ T cells in their thymus was not affected. On the contrary, CD4$^+$ T cells and B cells, but not CD8$^+$ T cells, in the peripheral lymphoid organs were slightly increased in the DC-specific *Hrd1* knockout mice compared with WT mice. The mechanisms underlying the increase in CD4$^+$ T cells and B cells are not clear. It is possible that the relatively lower percentage of FoxP3$^+$ T reg cells, which normally suppress the homeostatic proliferation of both T cells and B cells, permits an increase in the CD4$^+$ T cells and B cell populations in the *Hrd1* KO mice. However, T reg cell reduction cannot completely explain why the increase is specific to CD4$^+$ T cells and B cells without affecting CD8$^+$ T cells. The reduced percentage of T reg cells is probably not related to a developmental defect because their absolute numbers in the
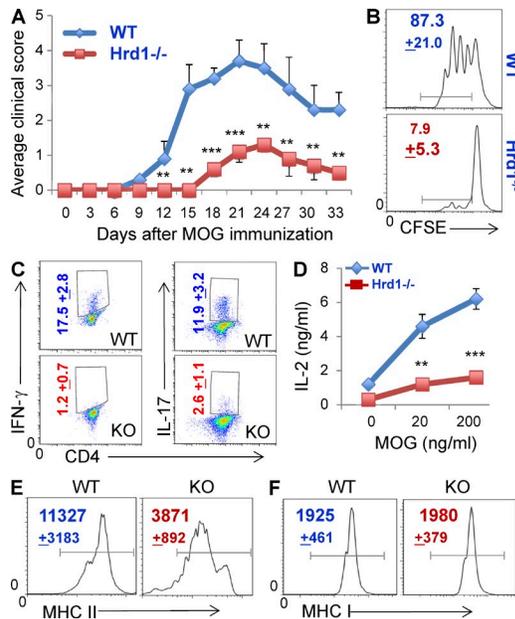
Figure 9. Genetic deletion of *Hrd1* gene in DCs partially protects mice from MOG-induced EAE. (A) CD11c-depleted splenocytes from WT C57/B6 mice were adoptively transferred into 8-wk-old *RAG1*⁻/⁻ and *Hrd1*⁻/⁻/*RAG1*⁻/⁻ double KO mice. 1 d after transfer, recipients were immunized with MOG₃₅₋₅₅ peptide (100 µg per mouse, emulsified with CFA). Mice were also given pertussis toxin (200 ng per mouse) on days 0 and 2 via tail vein injection. All mice were weighed and examined for clinical symptoms. Error bars represent data from six pairs of mice (mean ± SD; $n = 6$). **, P < 0.01. (B–F) Splenocytes from MOG-immunized mice were isolated, stained with CFSE, and co-cultured with MOG peptide for 3 d. (B) Proliferation of CD4⁺ T cells was analyzed by flow cytometry. (C) Percentages of IFN-γ–producing Th1 and IL-17–producing Th17 cells were analyzed by intracellular staining. (D) IL-2 production in the culture supernatant was examined by ELISA. Data represent means ± SD from 6 pairs of mice. **, P < 0.01. (E and F) CD11c⁺ conventional DCs in the draining lymph nodes from mice during disease were analyzed for their expression of MHC-II (E) and MHC-I (F) by flow cytometry. The mean fluorescence identity is indicated (mean + SD).

peripheral lymphoid organs of *Hrd1*-null mice were not different than in WT controls. Rather, it is likely that the reduction in T reg cell percentage is a consequence of the increase in CD4⁺ T cells. However, because MHC-II–restricted expression of self-antigen is critical for FoxP3⁺ T reg cell development (Sakaguchi, 2005), the possibility that Hrd1 regulates DC MHC-II expression to modulate T reg cell development cannot be fully excluded. Further, in addition to DCs, CD11c expression can be detected in other cell types (Miller et al., 2012). Therefore, further studies are needed to delineate how Hrd1 expression in DCs, as well as other types of CD11c⁺ cells, affects CD4⁺ and B cell homeostatic proliferation in mice.

Hrd1 has been identified as an anti-apoptotic factor that protects cells from ER stress–induced cell death (Carvalho et al., 2006; Denic et al., 2006), raising the possibility that loss of Hrd1 might cause DC apoptosis. However, the percentages of CD11⁺ cells in the spleens of *Hrd1*⁻/⁻ mice were not altered and the absolute numbers of CD11⁺ cells in the spleen

were increased. It has been shown that the ER stress responsive transcription factor XBP-1 (X-box binding protein 1) is required for the development and survival of CD11c⁺ DCs (Iwakoshi et al., 2007). We have recently reported that Hrd1 can promote degradation of IRE1α (Gao et al., 2008), the only known enzyme that activates XBP-1 (Shen et al., 2001), and our unpublished data show that the IRE1α protein, but not its mRNA, is increased in *Hrd1*-null DCs. Together with our observations that *Ire1α* gene deletion did not alter MHC-II expression on DCs, these studies suggest that loss of Hrd1 may promote DC survival due to an elevation in IRE1α–mediated XBP-1 activation, but not for the impaired MHC-II expression. We are currently generating DC-specific *IRE1α*⁻/⁻/*Hrd1*⁻/⁻ double knockout mice to study the role of the Hrd1–IRE1α pathway in DC survival.

In summary, our studies reveal a previously unappreciated molecular mechanism that regulates *MHC-II* gene transcription, involving the ER membrane-spanning E3 ubiquitin ligase Hrd1. In this model, TLR signaling induces Hrd1 expression to promote BLIMP1 ubiquitination and degradation. Because BLIMP1 is a critical transcriptional suppressor of MHC-II expression, TLR-induced Hrd1 expression enhances MHC-II expression to facilitate CD4⁺ T cell response.

MATERIALS AND METHODS

Generation of *Hrd1* floxed mice. The Hrd1-targeting vector was generated as in Fig. 1 A, and then transfected into an embryonic stem cell line generated from C57BL/6 mice. Neomycin selects were screened by PCR. Seven clones were obtained and confirmed by Southern blotting. Blastocyst injections resulted in several chimeric mice with the capacity for germline transmission. Breeding of heterozygous mice yielded *Hrd1*^wt/wt^, *Hrd1*^wt/f^, and *Hrd1*^f/f^ mice without phenotypic abnormalities in expected Mendelian ratios (Fig. 1 C). The DC-specific *Hrd1*-null mice were generated by breeding *Hrd1* floxed mice with *CD11c-Cre* transgenic mice.

CD11c-Cre transgenic mice, OT-I and OT-II *TCR* transgenic mice, and *RAG1* knockout mice, all of which are at the C57BL/6 genetic background, were purchased from The Jackson Laboratory. IRE1α floxed mice were used as previously described (Qiu et al., 2013). All mice used in this study were maintained and used at the Northwestern University mouse facility under pathogen-free conditions according to institutional guidelines. All animal study proposals have been approved by the institutional animal care and use committees (IACUC) at Northwestern University.

Cell lines, antibodies, and plasmids. Human embryonic kidney (HEK) 293 cells were maintained in DMEM (Invitrogen). The media was supplemented with 10% FBS, 100 U/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Polyclonal antibodies against the epitope tags (Flag, HA, and Myc), Ubiquitin, BLIMP1, and β-actin were obtained from Santa Cruz Biotechnology, Inc. Anti-Hrd1 and anti-Tubulin were purchased from Sigma-Aldrich. Fluorescence-labeled antibodies, including CD11c, CD11b, CD4, CD8, CD45.1, CD45.2, MHC-I, MHC-II, CD80, and CD86, were used for flow cytometry analysis (eBioscience). Hrd1 and the ubiquitin expression plasmids were obtained as reported previously (Gao et al., 2008). Flag-BLIPM1 expression plasmids were purchased from Addgene. The truncation mutants of both Hrd1 and BLIMP1 were generated by PCR and subcloned into pCMV-Flag (Sigma-Aldrich) or pCMV-Myc vectors (Invitrogen).

BMDC cultivation and activation. BMDCs were generated as previously described (Yang et al., 2013). BM cells were isolated from leg bones of 8–10-wk-old WT and *Hrd1*^f/f^*CD11c-Cre*⁺ (*Hrd1*⁻/⁻) mice and were cultured in RPMI medium containing 10% FCS and GM-CSF (20 ng/ml; BioLegend). Cell cultures were fed on days 3, 6, and 8 and used on day 9 or 10. To select

pure DCs, cells were purified by CD11c microbeads (Miltenyi Biotec) and stimulated with TLR agonists LPS (Sigma-Aldrich), Pam3 (Sigma-Aldrich), and polyIC (InvivoGen).

Antigen presentation and the antigen–specific T cell proliferation assay. For the analysis of antigen presentation, WT and Hrd1–null DCs were activated with 200 µg/ml LPS overnight and then incubated with different doses of OVA–Alexia Fluor 647 (Invitrogen) for 1 h at 37°C. Fluorescence intensity was analyzed by flow cytometry. For the study of antigen-specific T cell proliferation, WT and *Hrd1*–null DCs were cultivated with LPS in the presence of either OVA protein or with OVA peptides (OT-I: OVA$_{257-264}$; OT-II: OVA$_{323-339}$). The DCs were then washed and co-cultured with naive CD4$^+$ T cells from OT-II TCR transgenic mice or CD8$^+$ T cells from OT-I TCR transgenic mice for 3 d. Proliferation of co-cultured T cells was determined by either CFSE dilution or ^{3}H-thymidine incorporation.

For in vivo antigen-specific CD4$^+$ T cell activation, naive CD4$^+$ T cells were isolated from OT-II TCR transgenic mice with CD4$^+$ T cells from B6/Sjl mice as controls and stained with CFSE. The CFSE-stained cells were adoptively transferred into lethally irradiated recipient mice ($RAG1^{-/-}$ or $RAG1^{-/-}/Hrd1^{-/-}$ double KO mice) by intravenous injection. 1 d after transfer, mice were immunized with OVA/CFA and CD4$^+$ T cell growth was analyzed by flow cytometry.

Cell surface receptor expression by flow cytometry and IL-2 production analysis. Single cell suspensions of thymocytes, splenocytes, or BMDCs were used for staining using specific antibodies against CD11c, CD11b, MHC-I, MHC-II, CD80, CD86, CD4, CD8, CD25, B220, CD44, and CD62L for 30 min on ice. The stained cells were washed with ice-cold PBS, fixed, and analyzed by flow cytometry. The levels of IL-2 in the culture supernatants were determined by ELISA as previously described (Zhang et al., 2009).

Real-time quantitative RT PCR. WT and *Hrd1*–null CD11c$^+$ BMDCs were activated with or without TLR agonists for 4 h. Total RNA was extracted from DCs with TRIzol reagent according to the manufacturer's instructions (Invitrogen). cDNA was synthesized with qScript cDNA Synthesis kit (Quanta Biosciences). iQ5 and SYBRGreen Detection system (Bio-Rad Laboratories) was used for qPCR as previously described (Lee et al., 2008, 2009). Data were normalized to the expression of β-actin in each sample. Primers used to detect the expression of *MHC-I*, *MHC-II*, *CIITA*, *BLIMP1*, and *Hrd1* are shown in Table S1.

Cell transfection, immunoblotting, and coimmunoprecipitation assay. Transient transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, in 60-mm dishes and using 2–3 µg of total DNA per transfection. 2 d after transfection, cells were lysed in 1× Nonidet P-40 lysis buffer and freshly added protease inhibitor cocktail. The cell lysates were mixed with antibodies (1 µg) for 2 h, followed by the addition of 30 µl of fast flow protein G–Sepharose beads (GE Healthcare) for an additional 2 h at 4°C. Immunoprecipitates were washed four times with Nonidet P-40 lysis buffer and boiled in 20 µl of 2× Laemmli's buffer. Samples were subjected to 8–12% SDS-PAGE analysis and electro-transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with the indicated primary antibodies against Hrd1 (Sigma-Aldrich), BLIMP1 (Santa Cruz Biotechnology, Inc.), and Tubulin (Santa Cruz Biotechnology, Inc.), followed by horseradish peroxidase–conjugated secondary antibodies. Membranes were then washed and visualized with an enhanced chemiluminescence detection system (ECL; GE Healthcare). When necessary, membranes were stripped by incubation in stripping buffer (Bio-Rad Laboratories), washed, and then reprobed with other antibodies as indicated.

EAE induction, immunohistochemistry, and isolation of infiltrated lymphocytes from brain and spinal cord. 6–8-wk-old C57/BL6 mice were immunized with MOG$_{35-55}$ peptide (emulsified with CFA [200 µg per mouse]). Mice were also given pertussis toxin (200 ng per mouse) on days 0 and 2 via tail vein injection. All mice were weighed and examined daily for clinical symptoms

and assigned scores on a scale of 0–5 as follows: 0, no overt signs of disease; 1, limp tail; 2, limp tail and partial hindlimb paralysis; 3, complete hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; 5, moribund state or death.

Online supplemental material. Table S1 shows the sequence details of primers used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20140283/DC1.

Author contributions: H. Yang, S. Kong, Q. Qiu, Z. Lin, and B. Gao performed the experiments and analyzed the data. D. Fang analyzed the data and wrote the manuscript.

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