# CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10–driven MARCH1-mediated ubiquitination and degradation

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**Effective vaccine adjuvants must induce expression of major histocompatability (MHC) class II proteins and the costimulatory molecule CD86 on dendritic cells (DCs). However, some adjuvants elicit production of cytokines resulting in adverse inflammatory consequences. Development of agents that selectively increase MHC class II and CD86 expression without triggering unwanted cytokine production requires a better understanding of the molecular mechanisms influencing the production and degradation of MHC class II and CD86 in DCs. Here, we investigate how CD83, an immunoglobulin protein expressed on the surface of mature DCs, promotes MHC class II and CD86 expression. Using mice with an *N*-ethyl-*N*-nitrosourea–induced mutation eliminating the transmembrane (TM) region of CD83, we found that the TM domain of CD83 enhances MHC class II and CD86 expression by blocking MHC class II association with the ubiquitin ligase MARCH1. The TM region of CD83 blocks interleukin 10–driven, MARCH1-dependent ubiquitination and degradation of MHC class II and CD86 in DCs. Exploiting this posttranslational pathway for boosting MHC class II and CD86 expression on DCs may provide an opportunity to enhance the immunogenicity of vaccines.**

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Abbreviations used: BMDCs, BM-derived DCs; DP, double positive; DsRed, *Discosoma sp.* red fluorescent protein; ENU, *N*-ethyl-*N*-nitrosourea; hCD4, human CD4; MARCH, membrane-associated RING-CH; MCMV, murine cytomegalovirus; TLR, Toll-like receptor; TM, transmembrane.

Immunogenicity of vaccines hinges upon the presence of adjuvants, but few adjuvants are currently licensed for human use because of the need for more knowledge about how to elicit their immunogenic effects without toxic side-effects (Mata-Haro et al., 2007; Reed et al., 2009). T cell activation is promoted by adjuvants through Toll-like receptors (TLRs) and other pattern receptors, which among other effects trigger DCs to mature and display high levels of MHC II molecules and CD86 T cell costimulatory proteins (Ishii and Akira, 2007; McKee et al., 2007; Steinman and Banchereau, 2007; Longhi et al., 2009; Reed et al., 2009). Increased surface display of MHC II on mature DCs results from decreased rates of endocytosis and intracellular degradation caused by decreased ubiquitination of a critical lysine residue in the MHC II-β chain cytoplasmic tail by a ubiquitin ligase, membrane-associated RING-CH 1 (MARCH1) protein (Cella et al., 1997; Villadangos et al., 2001; Shin et al., 2006; van Niel et al., 2006; De Gassart et al., 2008;

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Young et al., 2008). Nevertheless, many potent TLR ligands cannot be used as adjuvants in humans because they also signal the production of cytokines causing fever and adverse inflammatory syndromes. The use of suboptimal adjuvants, on the other hand, risks antigen presentation by inadequately matured DCs with low MHC II and CD86 that induce T cell tolerance (Steinman et al., 2003; Lan et al., 2006). Understanding the endogenous pathways that promote mature DC expression of MHC II and CD86 may enable more specific strategies for enhancing vaccine potency.

The problem of inefficient DC maturation in vaccination is compounded by the effects of IL-10, which is produced by a variety of cells including DCs, macrophages, and Foxp3$^+$ regulatory T cells, because this cytokine decreases MHC II and CD86 expression on DCs and macrophages (de Waal Malefyt et al., 1991; Willems et al., 1994; Redpath et al., 1999). IL-10 is an important physiological silencer of excessive immune and inflammatory responses, and it also interferes with effective immunity in some infections (Couper et al., 2008). This immunosuppressive pathway is exploited by microbes that establish lifelong or recurrent infections, exemplified by Epstein-Barr virus (Hsu et al., 1990), human cytomegalovirus (Spencer et al., 2002; Chang et al., 2004; Jenkins et al., 2008), equine herpesvirus type 2 (Rode et al., 1993), ovine herpesvirus 2 (Jayawardane et al., 2008), and the parapoxvirus ORF (Chan et al., 2006), which have independently captured a host IL-10 gene or cDNA during their evolution. The resulting viral IL-10s retain potent suppressive effects on monocyte and DC MHC II and CD86 expression (de Waal Malefyt et al., 1991; Chang et al., 2004; Spencer et al., 2002; Chan et al., 2006; Jenkins et al., 2008). Other viruses like murine cytomegalovirus (MCMV) do not carry a viral IL-10, but trigger infected macrophages or DCs to make endogenous IL-10, and thereby achieve suppression of MHC II and CD86 (Redpath et al., 1999). How IL-10 suppresses surface MHC II and CD86 on DCs remains unclear.

DC maturation in response to TLR ligands is also marked by induction of CD83, but unlike MHC II and CD86, the function of CD83 in T cell activation remains enigmatic (Prazma and Tedder, 2008). Like CD86, the CD83 protein comprises a single extracellular Ig-like domain, a membrane spanning segment, and a cytoplasmic tail. It is most highly expressed on mature DCs (Zhou et al., 1992), and is induced on activated B and T cells (Cramer et al., 2000; Prazma et al., 2007) by the NF-κB transcription factor (McKinsey et al., 2000; Lenz et al., 2008). CD83 has been hypothesized to function as a T cell costimulatory molecule based upon the observation that soluble CD83 extracellular domain fusion proteins inhibited T cell activation in vitro and in vivo (Lechmann et al., 2001; Zinser et al., 2004; Xu et al., 2007), although these results have not been replicated in some studies (Pashine et al., 2008) and no ligand is currently known. Diminished CD83 expression by DCs caused by herpes simplex virus type 1 infection or siRNA transfection was accompanied by poor stimulation of T cells (Kruse et al., 2000; Aerts-Toegaert et al., 2007; Prechtel et al., 2007). In contrast, no marked difference in T cell activation has been found in assays using DCs from knockout mice lacking CD83 or from an N-ethyl-N-nitrosourea (ENU) mutant mouse strain with low CD83 cell surface expression caused by an extended C-terminal cytoplasmic tail (Fujimoto et al., 2002; García-Martinez et al., 2004; Kretschmer et al., 2008; Kuwano et al., 2007). These *Cd83*-deficient mice nevertheless exhibit a 75% decrease in positive selection of CD4 T cells in the thymus, and a 30–50% decrease in the cell surface expression of MHC II and CD86 on DCs, B cells, and thymic epithelium (Fujimoto et al., 2002; García-Martinez et al., 2004; Kuwano et al., 2007; Kretschmer et al., 2008). The molecular basis for these effects of CD83 remains unknown (Prazma and Tedder, 2008).

In this study, we identify a posttranslational pathway mediated by CD83 that promotes MHC II and CD86 expression on DCs and opposes the effects of IL-10. In this pathway, the transmembrane (TM) domain of CD83 inhibits the actions of MARCH1, a member of a recently discovered family of mammalian and viral TM proteins that ubiquitinate and down-regulate cell surface MHC, CD86, and other proteins (Goto et al., 2003; Bartee et al., 2004; Ohmura-Hoshino et al., 2006a,b; Matsuki et al., 2007). IL-10 induces MARCH1 mRNA and causes low MHC II and CD86 expression on LPS activated DCs, and the latter effects were dependent on MARCH1 and opposed by enforced expression of CD83 TM. These findings reveal a pathway by which TLR signaling promotes MHC II and CD86 display on DCs that provides opportunities for selectively augmenting vaccine or DC immunogenicity.

## RESULTS

### Mutant mouse strain lacking the CD83 TM region

In a screen of ENU mutagenized mouse pedigrees, we identified a strain, anubis, transmitting a Mendelian recessive trait characterized by 25% of normal peripheral CD4 cells and a high fraction with CD44$^{high}$ activated/memory phenotype (Fig. 1 A and Fig S1, A and B). Only 25% of normal CD4$^+$ single-positive T cells were present in the thymus of anubis (*anu*, allele name abbreviation) homozygotes, and CD4$^+$CD8$^+$ double-positive (DP) thymocytes displayed increased TCRβ and CD4 on their surface (Fig. 1 B). The latter resembles the selective increase in CD4 on DP cells in MHC II–null or MHC II mutant thymi, indicating a defect in MHC II signaling to CD4 on DP thymocytes (Cosgrove et al., 1991; Riberdy et al., 1998). These defects were not caused by an effect of the mutation within anubis thymocytes because they developed normally in irradiated WT recipients reconstituted with an equal mixture of WT (CD229.1$^-$) and anu/anu (CD229.1$^+$) BM (Fig. S1 C).

The anubis CD4 T cell deficiency mapped to a 15-Mb interval on chromosome 13, excluding the rest of the genome (Fig. S1 D). *Cd83* lies in the middle of this interval and was sequenced because of a similar decrease in CD4 T cells in *Cd83*-deficient mice (Fujimoto et al., 2002; García-Martinez et al., 2004). Genomic DNA sequencing revealed a G-to-T substitution in the donor splice site at the boundary between

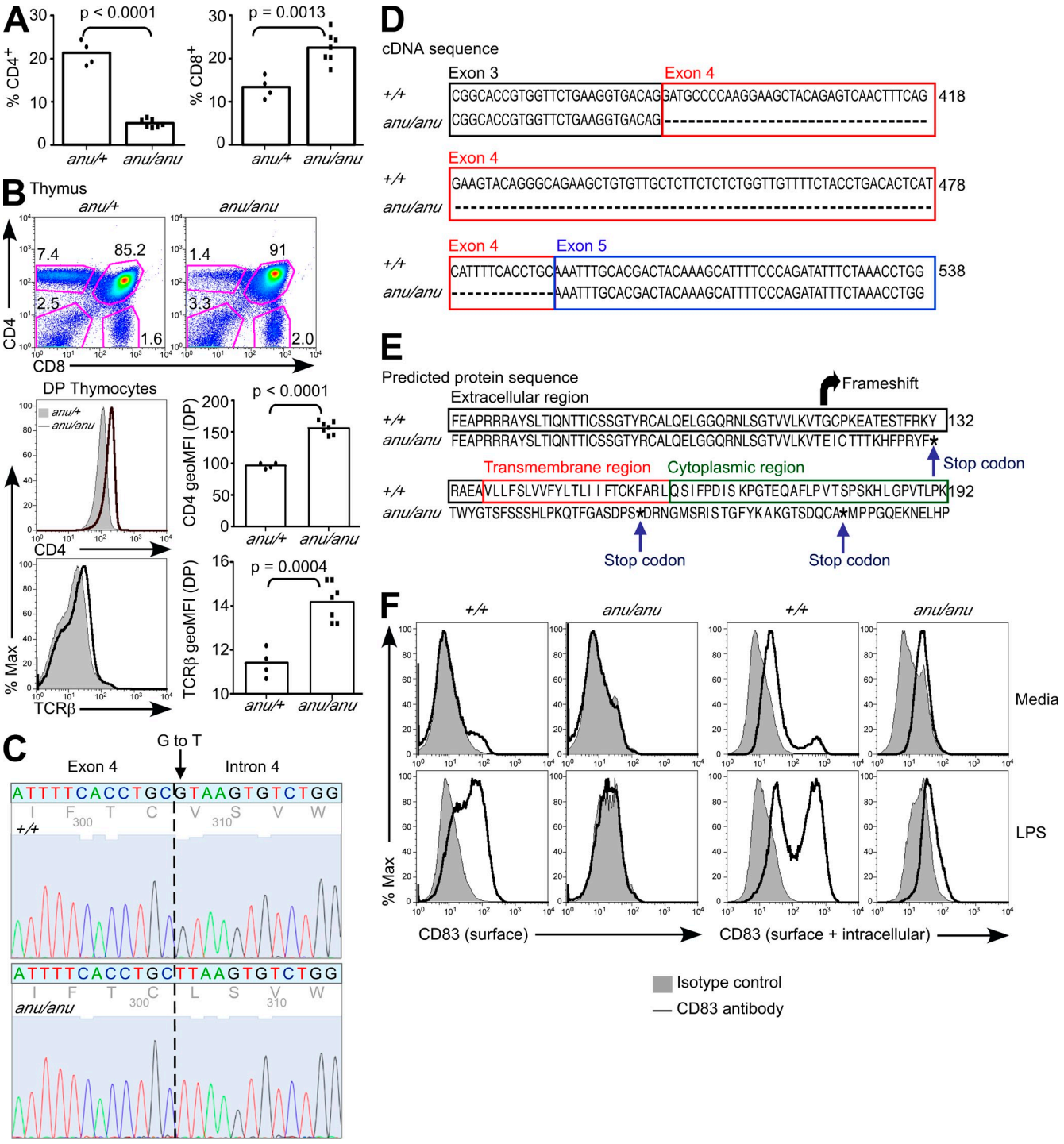


**Figure 1. ENU mouse mutation, *anubis*, eliminating the CD83 TM segment.** (A) Percentage of CD4⁺ and CD8⁺ lymphocytes in spleen of individual anu/anu or anu/+ mice (dots), with means (columns) and statistical comparison by Student's *t* test. Data are representative of three independent experiments. (B) Thymocytes from anu/+ (shaded) or anu/anu mice were stained with antibodies to CD4, CD8, and TCRβ. Histograms show CD4 and TCRβ on CD4⁺CD8⁺ (DP) thymocytes. Scatter plots show CD4 and TCRβ geometric mean fluorescence intensity (MFI) of DP thymocytes from individual *anu/anu* or *anu*/+ mice, with means (columns) and statistical comparison by Student's *t* test. Data are representative of two independent experiments. (C) Genomic DNA sequence traces across the *Cd83* exon 4-intron 4 boundary (dashed line). Arrow shows mutated nucleotide. Data are representative from three independent samples. (D) cDNA sequences showing the skipped exon 4 in the $Cd83^{anu/anu}$ mutant transcript. Data are representative of three independent samples. (E) Amino acid sequence encoded by $Cd83^{anu/anu}$ mRNAs, showing the frameshift and premature stop codons in the mutant protein. (F) Cell surface or total CD83 expression on 6-d GM-CSF cultures of BM-derived CD11c⁺ DCs from $Cd83^{+/+}$ and $Cd83^{anu/anu}$ mice, either unstimulated or stimulated with 1 µg/ml LPS during the last 16–20 h of culture. Data are representative of two independent experiments, each done using two individual animals.

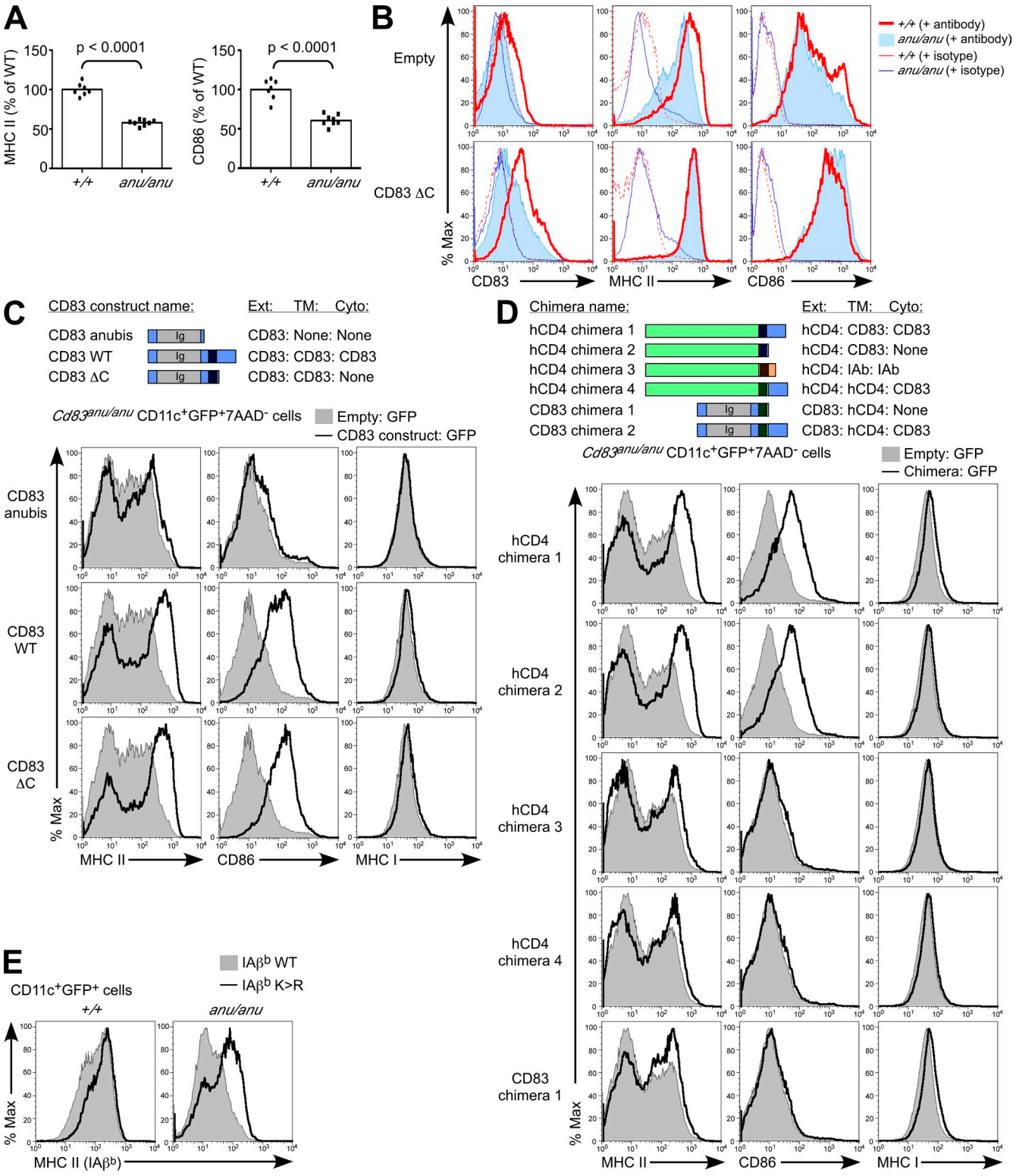


**Figure 2. CD83 TM segment is necessary and sufficient to enhance cell surface expression of MHC II and CD86 in DCs.** (A) Relative expression levels of MHC II or CD86 on ex vivo splenic CD11c+ DCs from individual *Cd83*$^{+/+}$ ($n = 7$) or *Cd83*$^{anu/anu}$ ($n = 8$) mice (dots), with means (columns) and statistical comparison by Student's *t* test. Shown are data combined from three independent experiments by using the geometric (geo) MFI of MHC II and CD86 of each sample normalized to the mean geo MFI of the *Cd83*$^{+/+}$ group within the same experiment (set to 100%). (B) Flow cytometric staining with antibodies to MHC II, CD86, and CD83, or with isotype-matched control antibodies on 5-d GM-CSF cultures of BMDCs from *Cd83*$^{+/+}$ and *Cd83*$^{anu/anu}$ mice. On day 2 of culture, BMDCs were transduced with retrovirus encoding IRES-GFP (empty) or CD83 ΔC-IRES-GFP. Histograms show CD11c+GFP+ BMDCs.

*Cd83* exon 4 and intron 4 (Fig. 1 C). Sequencing of *Cd83* cDNA from spleen showed that all detectable mRNA in $Cd83^{anu/anu}$ cells was aberrantly spliced from exon 3 to exon 5, excluding exon 4 (Fig. 1 D). A frameshift in the translation product introduced premature stop codons within exon 5, truncating the CD83 protein just upstream of the TM region (Fig. 1 E). The loss of cell surface CD83 was confirmed by flow cytometric staining of LPS-activated GM-CSF–cultured BM-derived DCs (BMDCs; Fig. 1 F) or anti-IgM–treated splenic B cells (Fig. S2 B), with no appreciable staining above that of an isotype-specific control antibody. Flow cytometric staining of surface and intracellular CD83 protein revealed very low levels of intracellular CD83 in $Cd83^{anu/anu}$ BMDCs (Fig. 1 F).

### The CD83 TM region stabilizes surface MHC II and CD86 display

Analysis of $Cd83^{anu/anu}$ splenic DCs or BMDCs (Fig. 2, A and B) and B cells (Fig S2 A) revealed decreased cell surface MHC II and CD86, which is also observed in CD83 knockout mice and mice with decreased CD83 expression (Fujimoto et al., 2002; García-Martinez et al., 2004; Kretschmer et al., 2008; Kuwano et al., 2007). This was also seen in $Cd83^{anu/anu}$ B cells cultured overnight with or without anti-IgM stimulation (Fig. S2 B). In mixed BM chimeras, MHC II and CD86 were also decreased on anti-IgM or LPS-activated $Cd83^{anu/anu}$ B cells that developed in a $Cd83^{+/+}$ environment despite normal B cell activation based on CD69 or CD25 (Fig. S2 C), indicating that CD83 acts cell autonomously to promote MHC II and CD86. $Cd83^{anu/anu}$ B cells consistently showed accelerated clearance of antibody-labeled MHC II and CD86 from the cell surface (Fig S3). This finding contrasts with the absence of any accelerated turnover observed in the LCD4.1 mutant cells with low CD83 expression (Kretschmer et al., 2008), but is consistent with the accelerated MHC II turnover observed in *Cd83* knockout B cells (Kuwano et al., 2007) and extends this result to CD86. Collectively, these data establish that the TM segment of CD83 is essential to stabilize surface display of MHC II and CD86 by regulating their rate of cell surface turnover.

The role of the CD83 TM segment in MHC II and CD86 surface display was further delineated by expressing CD83 truncated or chimeric molecules from bi-cistronic retroviral vectors together with GFP in GM-CSF cultures of BMDCs (Fig. 2). Flow cytometric analysis of $GFP^+$ populations allowed the effects of a given vector to be measured independently in thousands of single cells with different integration sites and expression patterns, with the distribution among the cell population visualized as histograms. $Cd83^{anu/anu}$ splenic DCs or BMDCs had lower surface MHC II expression compared with those from $Cd83^{+/+}$ mice (Fig. 2, A and B, top). Expression of CD83 was low for the majority of WT BMDCs, which is consistent with the immature phenotype of DCs generated in GM-CSF cultures. Transduction of $Cd83^{anu/anu}$ BMDCs with GFP vector encoding the mis-spliced $Cd83^{anubis}$ lacking the TM segment had no effect on MHC II, CD86, or MHC I levels (CD83 anubis, Fig. 2 C). In contrast, vector encoding full-length CD83 (CD83 WT) increased MHC II and CD86 on the majority of $GFP^+$ cells, as did a vector (CD83 ΔC) encoding truncated CD83 containing the TM, but lacking the cytoplasmic tail (Fig. 2 B, bottom, and C). Confocal microscopy of the transduced BMDCs was unable to detect intracellular CD83 anubis protein in the $GFP^+$ cells (Fig S4 A), whereas the CD83 ΔC protein was distributed intracellularly and on the plasma membrane in a pattern that colocalized with MHC II and CD86 (Fig. S4, B and C). The latter is consistent with published evidence for colocalization of CD83 with MHC II and CD86 (Klein et al., 2005; Kretschmer et al., 2008). None of the CD83 constructs significantly affected surface MHC I expression, indicating the relative specificity of CD83. Likewise, there was no change in CD9 surface expression on $Cd83^{anu/anu}$ BMDCs or $Cd83^{+/+}$ or $Cd83^{anu/anu}$ BMDCs expressing CD83 ΔC (Fig S5), despite the association between CD9 and MHC II in tetraspanin microdomains that promote MHC II–dependent signaling, colocalization of MHC II and CD86, and antigen presentation to CD4 T cells (Kropshofer et al., 2002; Zilber et al., 2005; Unternaehrer et al., 2007). These data indicate that the CD83 TM, but not cytoplasmic region, is specifically required for normal MHC II and CD86 surface expression.

Chimeric constructs were transduced into $Cd83^{anu/anu}$ BMDCs to define the minimal CD83 region for normal MHC II and CD86 display (Fig. 2 D). MHC II and CD86 expression were increased on many $Cd83^{anu/anu}$ BMDCs expressing chimeric proteins with the human CD4 (hCD4) extracellular domain and the CD83 TM domain, either with or without the CD83 cytoplasmic domain (hCD4 chimera 1 and 2, respectively). In contrast, expression of the hCD4 extracellular domain with other TM segments from MHC II I-$A\beta^b$ (hCD4 chimera 3) or hCD4 (hCD4 chimera 4) had no effect. Similarly, the capacity of CD83 to enhance MHC II and CD86 was lost if the CD83 TM region was selectively replaced by the hCD4 TM segment (Fig. 2 D, CD83 chimera 1). The CD83 TM segment therefore represents a minimal functional domain both necessary and sufficient for normal MHC II and CD86 display on DCs.

Data are representative of two independent experiments. (C) Schematic of full-length CD83 or variants lacking the TM or cytoplasmic (Cyto) segments encoded in bicistronic GFP vectors. Histograms show MHC II, CD86, and MHC I staining of $CD11c^+GFP^+7AAD^-$ $Cd83^{anu/anu}$ BMDCs transduced with the indicated vectors, overlaid on cells transduced with empty GFP vector. Data are representative of four independent experiments. (D) Schematic of chimeric proteins comprising the indicated segments from CD83, human CD4 (hCD4), and mouse MHC class II I-$A\beta^b$ (IAb). Histograms show $CD11c^+GFP^+7AAD^-$ $Cd83^{anu/anu}$ BMDCs cells analyzed as in C. Data are representative of two independent experiments. (E) $CD11c^+GFP^+$ $Cd83^{+/+}$ or $Cd83^{anu/anu}$ BMDCs transduced with bi-cistronic GFP retroviral vectors encoding $IA\beta^b$ WT or $IA\beta^b$ K>R were stained with $IA\beta^b$–specific antibody. Data are representative of two independent experiments.

### CD83 promotes surface display of MHC II and CD86 in opposition to MARCH1

We next asked if the lower surface MHC II expression on DCs in the absence of the CD83 TM segment requires ubiquitination of lysine 225 on the MHC II β chain cytoplasmic tail (Ohmura-Hoshino et al., 2006b; Shin et al., 2006). MHC II IAβ$^b$ chains with either WT lysine 225 (IAβ$^b$ WT) or a K225R mis-sense mutation (IAβ$^b$ K>R) were expressed in *H2$^k$ Cd83$^{+/+}$* or *Cd83$^{anu/anu}$* BMDCs from bi-cistronic GFP retroviral vectors (Fig. 2 E). The vector-encoded "b" allotype IAβ$^b$ chains were distinguished from endogenous "k" allotype IAβ$^k$ chains by staining with IA$^b$ allotype-specific antibody, and GFP$^+$ cells were gated to analyze cells with comparable retroviral bi-cistronic mRNA expression. Surface expression of IAβ$^b$ WT was significantly lower on *Cd83$^{anu/anu}$* BMDCs (Fig. 2 E, gray histogram, right) compared with WT BMDCs (Fig. 2 E, gray histogram, left), confirming the requirement for the CD83 TM segment in the surface display of WT MHC II. In *Cd83$^{+/+}$* BMDCs, expression of IAβ$^b$ K>R (Fig. 2 E, bold line, left) was slightly higher than IAβ$^b$ WT (Fig. 2 E, gray histogram, left panel), in agreement with previously published studies (Ohmura-Hoshino et al., 2006b; Shin et al., 2006). Importantly, in *Cd83$^{anu/anu}$* BMDCs, the expression of I-Aβ$^b$K>R (bold line, right) restored expression almost to the levels of cells with WT CD83. We conclude that the loss of the CD83 TM region has little effect on surface MHC II display in the absence of the K225 ubiquitination residue.

In light of the aforementioned results and the recent observation that MARCH1 and MARCH8 ubiquitin ligases diminish surface MHC II and CD86 expression (Goto et al., 2003; Bartee et al., 2004; Ohmura-Hoshino et al., 2006b; Matsuki et al., 2007), we asked if the CD83 TM domain promoted MHC II and CD86 surface display by opposing the action of MARCH1 or MARCH8. MARCH1 or MARCH8 were expressed from bi-cistronic GFP retroviral vectors in *Cd83$^{+/+}$* or *Cd83$^{anu/anu}$* B cells (Fig. 3, A and B). Because MARCH and GFP proteins were encoded in a single, bicistronic proviral mRNA, GFP levels could be used to infer relative expression of MARCH in individual transduced splenic B cells. This revealed a dramatic GFP (MARCH) dose-dependent decrease in MHC II and CD86 expression per cell (Fig. 3 A). Down-regulation of MHC II and CD86 occurred at a lower threshold of GFP for the MARCH8 vector, which may reflect either increased activity of MARCH8 or increased translation from the bicistronic vector. By gating on cells expressing low, intermediate or high relative levels of MARCH:GFP (Fig. 3 B, top), surface expression of MHC II or CD86 was decreased at a lower threshold of GFP(MARCH) in *Cd83$^{anu/anu}$* B cells (bold lines) compared with *Cd83$^{+/+}$* cells (gray histograms). These results establish that the endogenous CD83 TM domain opposes the effects of MARCH1 and MARCH8 to promote surface MHC II and CD86 display.

To cross-titrate CD83 against MARCH1, the latter was cloned into a bi-cistronic retroviral expression vector containing a variant *Discosoma sp.* red fluorescent protein (DsRed) so that it could be cotransduced with bi-cistronic GFP vectors expressing MHC II or CD83 (Fig. 4). In B cells doubly transduced with MARCH1:DsRed and I-Aβ$^b$WT or I-Aβ$^b$K>R:GFP, MHC II down-regulation by MARCH1 required the MHC II cytoplasmic K225 residue (Fig. 4 A). Likewise, endogenous MHC II and CD86 were expressed on the surface of B cells doubly transduced with empty GFP and empty DsRed vectors (Fig. 4 B, top, thin line), but both were down-regulated on cells doubly transduced with empty GFP vector and MARCH1:DsRed (Fig. 4 B, top, gray histograms labeled "Empty"). Surface expression of MHC II and CD86 was partially restored on cells cotransduced with GFP vector encoding full length CD83 WT, and almost fully restored on most cells transduced with CD83 ΔC lacking the cytoplasmic tail (Fig. 4 B, left middle histogram, bold line). In contrast, there was no restoration of MHC II or CD86 on MARCH1:DsRed-expressing cells doubly transduced with CD83 anubis lacking the TM segment or CD83 molecules with hCD4 TM segments (CD83 chimera 1 and 2, bottom left, bold line). CD83 chimeras 1 and 2 were nevertheless expressed on the cell surface at higher levels than CD83 WT or CD83 ΔC (unpublished data). Thus the CD83 TM region is the essential element antagonizing MARCH1-mediated MHC II and CD86 down-regulation, and removal of the cytoplasmic segment in CD83 ΔC yields a more potent form of CD83.

These conclusions were reinforced in reciprocal experiments in B cells doubly transduced with MARCH1:DsRed and GFP vectors encoding hCD4 chimeras. hCD4 chimera 2 containing extracellular hCD4 and the TM region of CD83 partially restored MHC II and CD86 surface display in MARCH1-overexpressing cells (Fig. 4 B, right). In contrast, hCD4 chimeras 3 and 4 with other TM regions were unable to restore MHC II or CD86 (Fig. 4 B, bottom right) despite high expression on the cell surface (unpublished data).

### CD83 TM inhibits MARCH1 association with MHC II and ubiquitination

The findings above showed that CD83 TM opposes the effects of MARCH1 and promotes MHC II surface display, raising the possibility that it antagonized MARCH1-MHC II association and ubiquitination. To test this, immunoprecipitation and Western blotting experiments were performed in HEK293T cells transfected with Flag-tagged hCD4 chimera 3 (Fig. 2 D), containing the MHC II I-Aβ$^b$ TM and cytoplasmic regions and the hCD4 extracellular domain (Flag-hCD4-IAb), together with V5-tagged MARCH1. Comparing cells transfected with Flag-hCD4-IAb alone (Fig. 5 A, lane 2) or together with MARCH1-V5 (Fig. 5 A, lane 3), anti-ubiquitin Western blotting of Flag immunoprecipitates showed that ubiquitination of the MHC II chimeric protein (top panels, solid arrowheads) was consistently increased in MARCH1 cotransfected cells and abolished by the K225R (KR) mis-sense mutation in the Flag-hCD4-IAb cytoplasmic tail (Fig. 5 A, lane 4). MARCH1-induced ubiquitination of the FLAG-hCD4-IAb chimeric protein was also abolished by cotransfecting WT CD83 (Fig. 5, A [lane 5] and B [lane 4]), but was unaffected by cotransfecting CD83 chimera 2 where the TM

segment was substituted with that from hCD4 (Fig. 5, A [lane 6] and B [lane 5]). Flow cytometric analysis of the cells used to prepare these immunoprecipitates established that CD83 chimera 2 was nevertheless expressed at higher levels on the cell surface than WT CD83 (Fig. 5, A and B, far right, orange versus green histograms). Flow cytometric measurement of Flag-hCD4-IAb on the surface of the same cells showed that WT CD83, but not CD83 chimera 2, inhibited

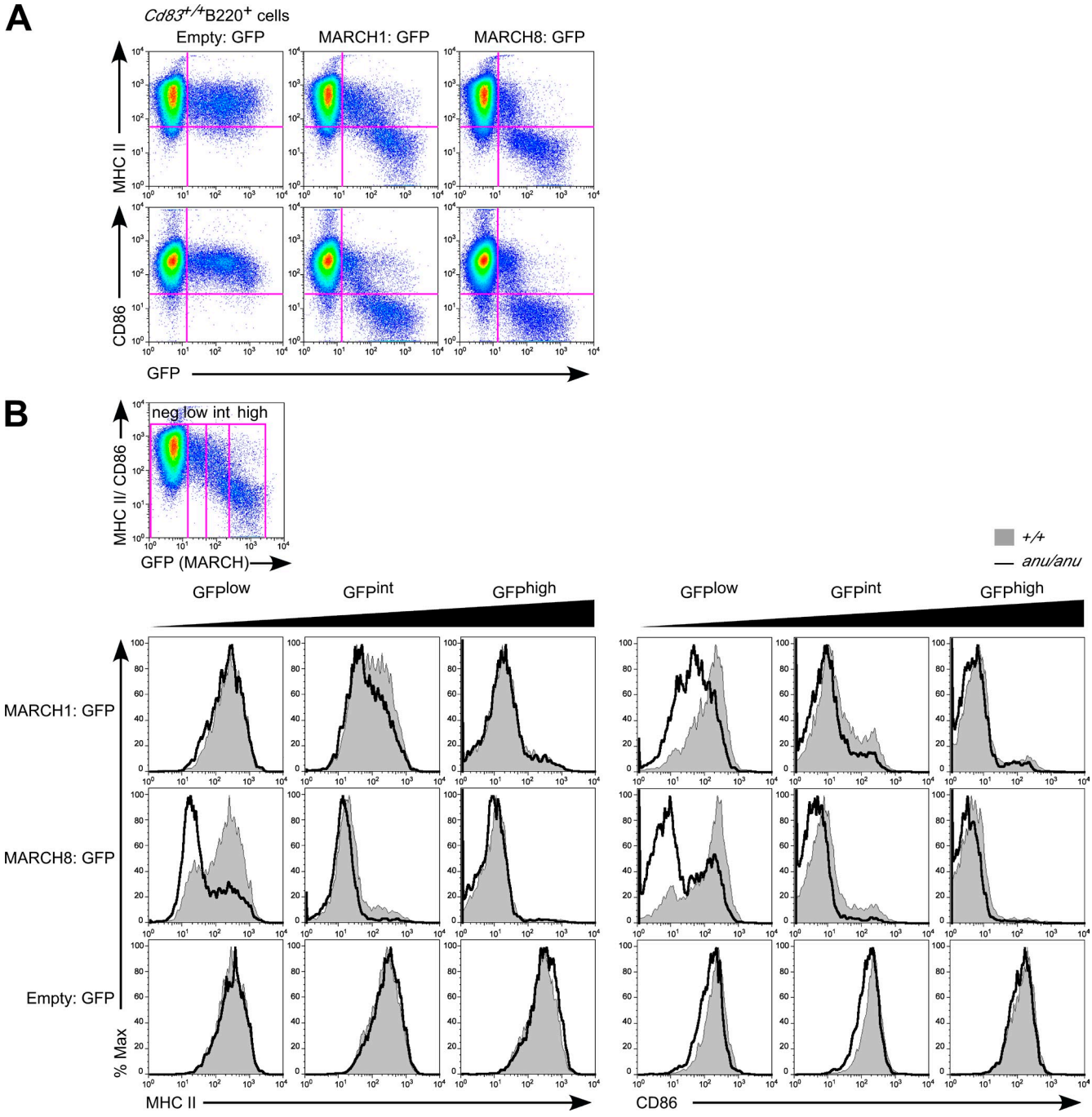


**Figure 3. CD83 TM antagonizes MHC II and CD86 down-regulation by MARCH1 and MARCH8 ubiquitin ligases.** (A) *Cd83*$^{+/+}$ splenic B cells activated by LPS were transduced with the empty bi-cistronic GFP vector or GFP vector encoding MARCH1 or MARCH8, and were subjected to flow cytometric analysis the next day. Cell surface staining for MHC II or CD86 versus GFP fluorescence is shown on gated B220$^{+}$ cells. Data are representative of two independent experiments. (B) Relationship between relative GFP (MARCH1 or MARCH8) expression and surface MHC II or CD86 in splenic B cells from *Cd83*$^{+/+}$ or *Cd83*$^{anu/anu}$ mice transduced as in A. Transduced cells were gated into subsets with different levels of GFP (low, intermediate, or high) and surface MHC II or CD86 expression on each subset displayed as overlaid histograms of *Cd83*$^{+/+}$ or *Cd83*$^{anu/anu}$ cells. Data are representative of two independent experiments.

down-regulation of the MHC II chimeric protein by MARCH1 cotransfection (Fig. 5, A and B, left), indicating that CD83 does not require DC- or B cell-specific cofactors to block the effects of MARCH1.

To test if CD83 TM inhibited MHC II ubiquitination by blocking association between MARCH1 and MHC II, the Flag-hCD4-IAb immunoprecipitates were reblotted for MARCH1-V5 (Fig. 5, A and B). Although MARCH1-V5 was

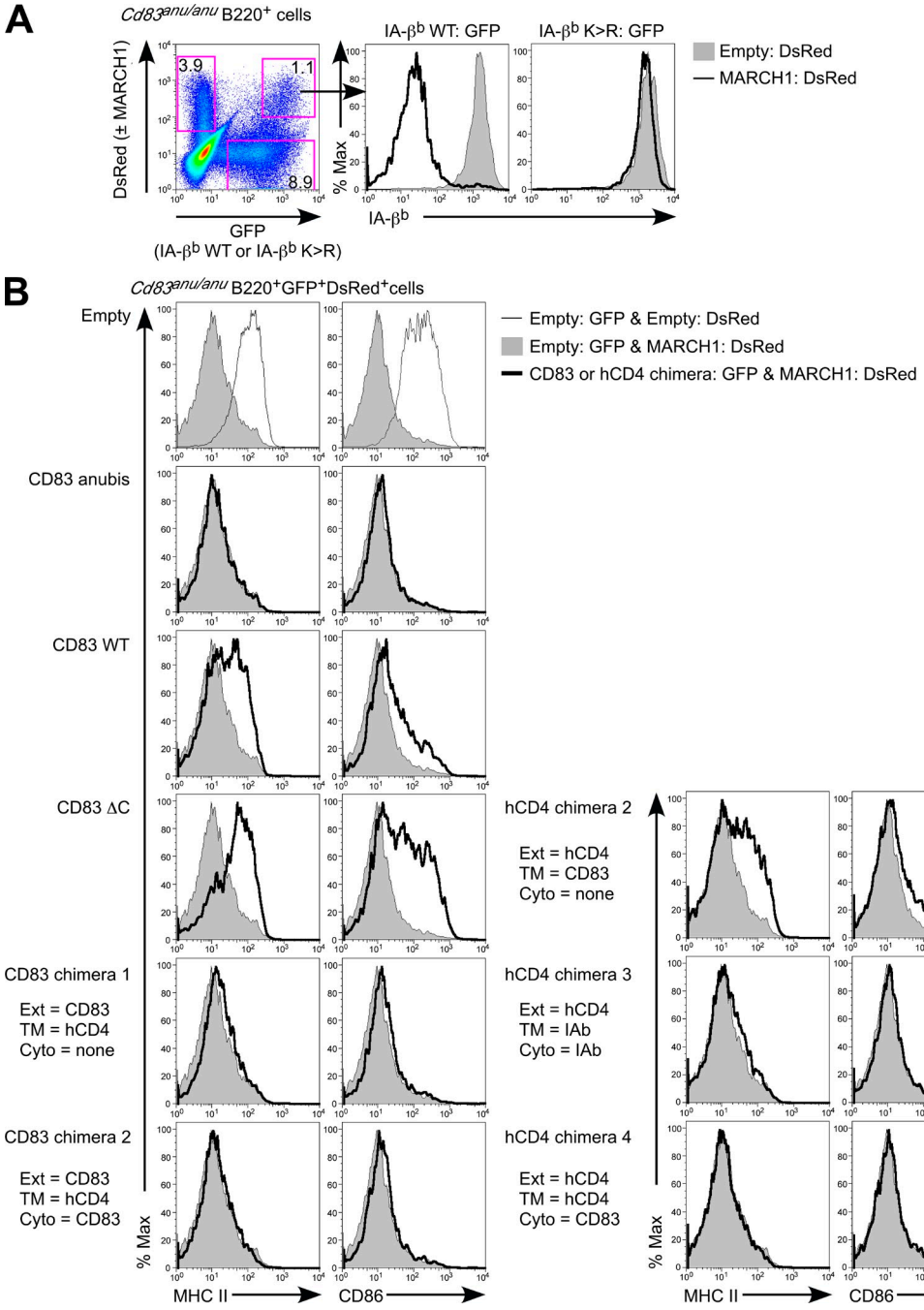


**Figure 4. CD83 TM antagonizes MARCH1-mediated MHC II and CD86 down-regulation.** (A) LPS-activated *Cd83*$^{anu/anu}$ splenic B cells were transduced with bi-cistronic GFP vectors encoding IAβ$^{b}$ WT or IAβ$^{b}$ K>R in combination with bicistronic DsRed vectors that were either empty or encoding MARCH1. Histograms show cell surface IAβ$^{b}$ gated on B220$^{+}$GFP$^{+}$DsRed$^{+}$ doubly transduced cells (top right gate in plot) expressing empty DsRed vector or MARCH1:DsRed vector. Data are representative of two independent experiments. (B) Endogenous MHC II or CD86 expression on B220$^{+}$GFP$^{+}$DsRed$^{+}$ *Cd83*$^{anu/anu}$ B cells coexpressing the indicated GFP and DsRed vectors. Histograms show doubly transduced cells expressing a particular GFP:CD83 or GFP:hCD4 chimera (as described in Fig. 2, C and D) and MARCH1:DsRed overlaid with double-transduced cells expressing empty GFP and MARCH1:DsRed vectors. Data are representative of at least two independent experiments.

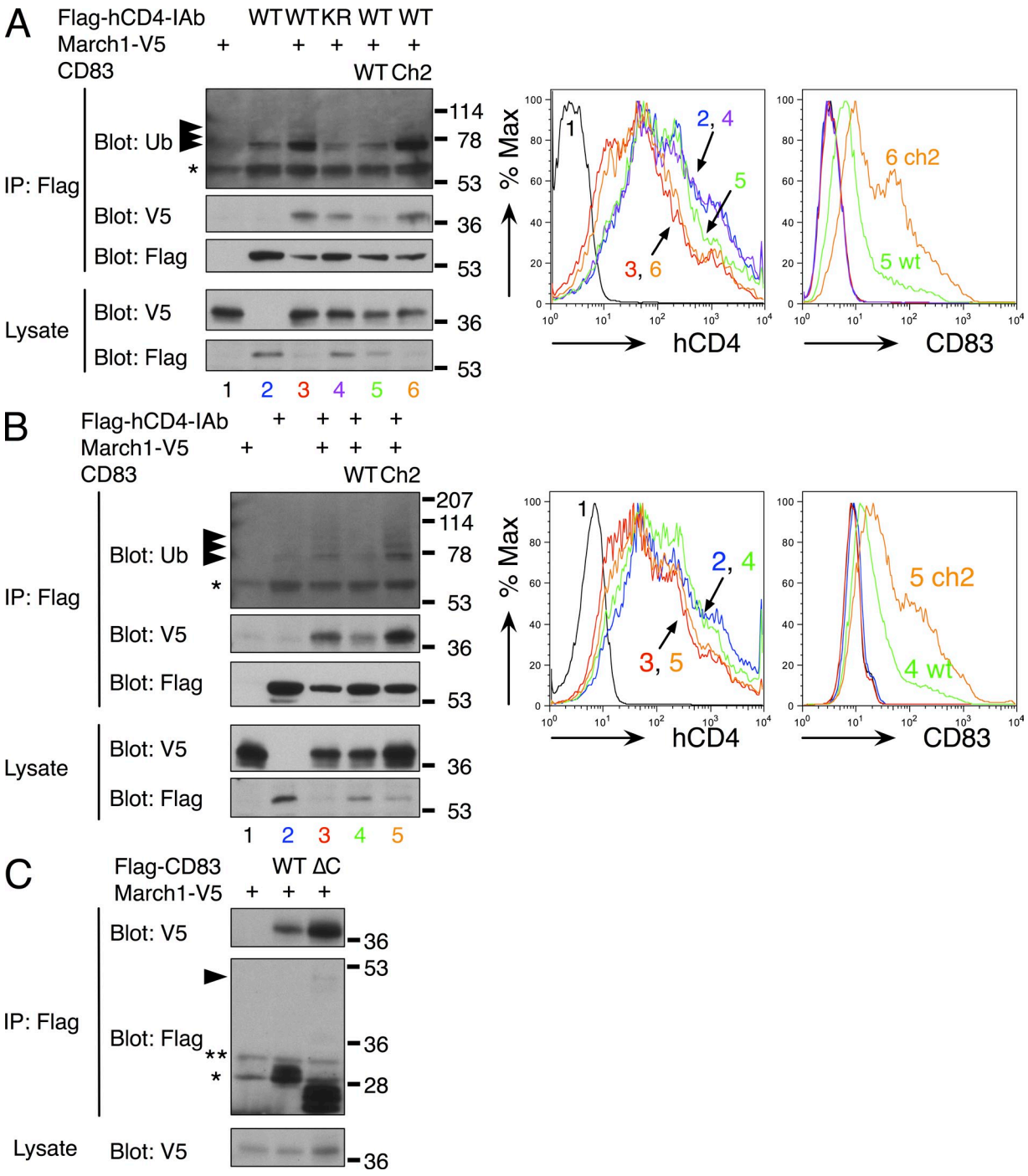


**Figure 5. CD83 TM inhibits MARCH1 association with MHC II and ubiquitination of MHC II.** (A) HEK293T cells were transfected with the indicated vectors encoding Flag-hCD4 IAb WT or KR, March1-V5, CD83 WT, or CD83 chimera 2, where the TM segment is derived from hCD4. Cells were lysed and anti-Flag immunoprecipitates were resolved on 12% SDS-PAGE gel, and then analyzed by immunoblotting with anti-ubiquitin, -V5, or -Flag antibodies. Unfractionated NP-40–soluble cell lysates were analyzed in parallel to compare the levels of Flag-hCD4-IAb and MARCH1-V5. The arrows indicate ubiquitinated Flag-hCD4 IAb. The asterisk indicates the heavy chain band of the antibody used for immunoprecipitation. Histograms show flow cytometric analysis of cell surface expression of Flag-hCD4-IAb and CD83 on the same samples of transfected cells. (B) Independent replicate experiment of A. (C) HEK293T cells were transfected with the indicated vectors encoding MARCH1-V5 and Flag-CD83 WT or CD83 lacking the cytoplasmic tail (ΔC). Anti-Flag immunoprecipitates were resolved on SDS-PAGE gel and analyzed by immunoblotting with anti-V5 to detect MARCH1 associated with Flag-CD83, or with anti-FLAG to detect the Flag-CD83 protein itself. Unfractionated cell lysates were analyzed in parallel to compare the levels of MARCH1-V5. The arrows indicate the glycosylated form of CD83, the asterisk and double asterisk depict light chain and protein G eluent, respectively. Data shown are representative of two independent experiments.

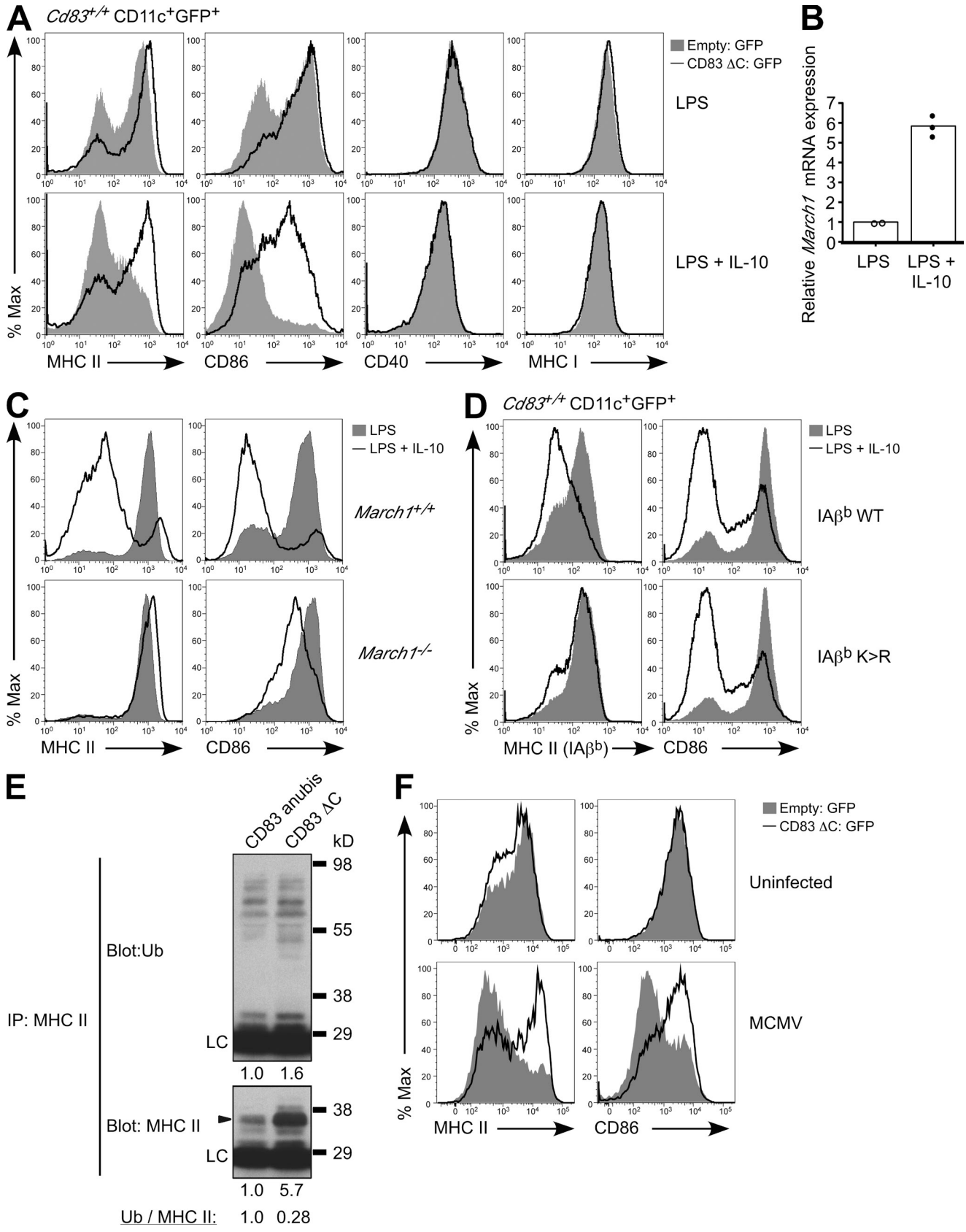


**Figure 6. Expression of CD83 ΔC in DCs blocks MHC II and CD86 down-regulation by IL-10.** (A) *Cd83*$^{+/+}$ BMDC were transduced with empty bicistronic GFP vector or vector encoding CD83 ΔC, and then cultured with or without 20 ng/ml IL-10 for 2 d with addition of 1 µg/ml LPS in the last 16–20 h

not detected in Flag immunoprecipitates from control cells transfected with MARCH1-V5 alone (Fig. 5, A and B, lane 1) or in cells transfected with Flag-hCD4-IAb but not MARCH1-V5 (Fig. 5, A and B, lane 2), MARCH1-V5 was readily detected in Flag immunoprecipitates from cells transfected with both (Fig. 5, A and B, lane 3). MARCH1-V5 also precipitated with Flag-hCD4-IAb bearing the MHC II K225R (KR) mis-sense mutation (lane 4, Fig. 5 A), establishing that ubiquitination of the MHC II tail is not required for MARCH1 association. Much less MARCH1-V5 immunoprecipitated with Flag-hCD4-IAb in cells cotransfected with WT CD83 (Fig. 5, A [lane 5] and B [lane 4]), but was unaffected by CD83 chimera 2 bearing the hCD4 TM region, despite it being expressed at higher level on these cells (Fig. 5, A [lane 6] and B [lane 5]). These results establish that CD83 TM inhibits association between MHC II and MARCH1.

To test the possibility that CD83 blocked association between MHC II and MARCH1 by direct binding to the latter, Flag-CD83 immunoprecipitates were tested for presence of MARCH1 in HEK293T cells cotransfected with Flag-CD83 and MARCH1-V5 (Fig. 5 C). Although no MARCH1-V5 was detected in Flag immunoprecipitates from control cells transfected with MARCH1-V5, but not Flag-CD83 (Fig. 5 C, lane 1), it was readily detected in Flag-immunoprecipitates from cells cotransfected with WT Flag-CD83 (Fig. 5 C, lane 2) or Flag-CD83 ΔC lacking the cytoplasmic tail (Fig. 5 C, lane 3).

### CD83 inhibits the effect of IL-10 on DC surface MHC II and CD86

Because IL-10 has a well established but poorly understood effect on DCs by decreasing cell surface MHC II and CD86 (de Waal Malefyt et al., 1991; Willems et al., 1994; Redpath et al., 1999), we asked if this action of IL-10 might be inhibited by expression of the potent MARCH1-inhibitor, CD83 ΔC. *Cd83*$^{+/+}$ BMDCs were transduced with empty bi-cistronic GFP retroviral vector or GFP vector encoding CD83 ΔC, and cultured in the presence or absence of IL-10 for 2 d with the addition of LPS for the last 16–20 h to induce DC maturation. Flow cytometric staining showed that IL-10 dramatically reduced cell surface MHC II and CD86 on GFP$^+$ DCs expressing the control vector (Fig. 6 A, gray histograms). The effect of IL-10 was almost completely negated in GFP$^+$ DCs expressing CD83 ΔC (Fig. 6 A, bold lines). A modest reduction of cell surface CD40 and MHC I was also observed in IL-10–treated cells; however, the expression of CD83 ΔC had no effect on these cell surface proteins (Fig. 6 A), lending further support for the specificity of CD83 action on MHC II and CD86.

Measurement of *March1* mRNA by quantitative PCR in these DC cultures demonstrated that IL-10 increased *March1* mRNA by approximately sixfold (Fig. 6 B). To directly address if the suppressive effect of IL-10 upon surface MHC II and CD86 was a result of MARCH1 action, BMDCs from *March1*$^{+/+}$ or *March1*$^{-/-}$ mice were cultured in the presence or absence of IL-10 and LPS as in Fig. 6 A. The addition of IL-10 to *March1*$^{-/-}$ BMDCs failed to reduce surface MHC II and CD86 expression on these cells (Fig. 6 C, bottom, bold lines) as compared with *March1*$^{+/+}$ BMDCs (Fig. 6 C, top, bold lines). Down-regulation of surface MHC II expression by IL-10 was also abolished by mutation of the critical lysine 225 residue on the MHC II β chain cytoplasmic tail (Fig. 6 D, left). The effect of the K225R mutation was specific to MHC II in these cells because surface expression of CD86 was down-regulated by IL-10 in a manner identical to cells expressing WT MHC II (Fig. 6 D, right). These results address a longstanding question about how IL-10 down-regulates MHC II, by showing that it requires MHC II ubiquitination by MARCH1.

Given the requirement for ubiquitination in IL-10 action, and the inhibition of ubiquitination by CD83 in MARCH1-transfected HEK293T cells (Fig. 5), we examined the effect of CD83 ΔC expression on endogenous MHC II ubiquitination in DCs exposed to IL-10. BMDCs were transduced with bi-cistronic GFP retroviral vectors encoding either CD83 anubis or CD83 ΔC and cultured in the presence of IL-10. After 3.5 d in IL-10, GFP$^+$ cells were sorted, endogenous MHC II was immunoprecipitated, and the immunoprecipitates were analyzed by Western blot to detect ubiquitin and MHC II (Fig. 6 E). Immunoprecipitates from CD83 ΔC–expressing cells contained 5.7 times more MHC II than CD83 anubis–expressing cells (Fig. 6 E, bottom), but only 1.6 times more ubiquitinated

of culture. Histograms show cell surface MHC II, CD86, CD40, and MHC I expression on CD11c$^+$GFP$^+$ cells and are representative of three independent experiments. (B) RNA and cDNA were prepared from nontransduced *Cd83*$^{+/+}$ BMDCs cultured as in A, and quantitative real-time PCR reactions performed using March1 and β-actin specific primers. March1 expression normalized to β-actin expression is shown by dots for independent cultures, with the mean value in LPS-treated samples set to equal 1. Data are representative of two independent experiments. (C) *March1*$^{+/+}$ or *March1*$^{-/-}$ BMDCs cultured with or without IL-10 with the addition of LPS in the last 16–20 h of culture as in A were stained with antibodies to MHC II and CD86. Histograms shown are on CD11c$^+$ cells and are representative of two independent experiments. (D) *Cd83*$^{+/+}$ BMDC were transduced with GFP retroviral vectors encoding IAβ$^b$ WT or IAβ$^b$ K>R, and then cultured with or without IL-10 and LPS, as in A. Histograms show cell surface MHC II (IAβ$^b$) and endogenous CD86 on transduced CD11c$^+$GFP$^+$ cells and are representative of two independent experiments. (E) *Cd83*$^{+/+}$ BMDCs were transduced with the bi-cistronic GFP vector encoding CD83 anubis or CD83 ΔC, and then cultured in the presence of IL-10. GFP$^+$ cells were sorted and MHC II proteins were immunoprecipitated and resolved by SDS-PAGE and analyzed by immunoblotting with antibody to ubiquitin or MHC II I-Aβ. Arrowhead, position of unmodified MHC II β chain; LC, light chain of the antibody used to immunoprecipitate MHC II, providing an internal loading control. Relative intensities of the ubiquitin and MHC II bands, and the calculated ratio of ubiquitin to MHC II, are indicated. (F) J774 macrophage cells transduced with either the empty bi-cistronic GFP vector or vector encoding CD83 ΔC, were then left uninfected or infected with MOI of 10 of MCMV 2 d later. Flow cytometric analysis of these cells was done 4 d after infection. Histograms show MHC II and CD86 on transduced GFP$^+$ cells.

MHC II (Fig. 6 E, top), so that the relative ubiquitination of MHC II was decreased to 28% by CD83 ΔC.

The immunosuppressive effect of IL-10 is exploited by many microbial pathogens, notably *Herpesviridae*, to subvert host immune responses (Redpath et al., 2001; Slobedman et al., 2009). For example, infection by the herpesvirus MCMV has been shown to induce IL-10 production by host macrophages, which in turn decreased surface MHC II levels on infected cells (Redpath et al., 1999). To test whether or not vector-delivered CD83 ΔC could protect infected macrophages from down-regulation of surface MHC II, J774 macrophages were transduced with CD83 ΔC vector or empty vector control and infected with MCMV. CD83 ΔC vector (Fig. 6 F, bottom, bold lines), but not empty vector (Fig. 6 F, bottom, gray histograms), protected infected cells from losing surface MHC II and CD86. This result provides a proof-of-principle for future in vivo studies incorporating CD83 ΔC within other viral vaccine antigen delivery vectors.

## DISCUSSION

These experiments identify a posttranslational pathway by which MHC II and CD86 expression on DCs can be induced both to emulate or augment a key action of TLR-ligands and to oppose one of the main immunosuppressive effects of IL-10 (summarized in Fig. 7). CD83 is induced by TLR signals in DCs, and through its TM domain CD83 inhibits MHC II association with MARCH1, preventing MHC II ubiquitination and down-regulation by MARCH1. A similar mechanism presumably explains how CD83 TM blocks CD86 down-regulation by MARCH1. We show that *March1* mRNA is induced by the antiinflammatory cytokine IL-10 in DCs, which has also been found in human monocytes (Thibodeau et al., 2008), and show that IL-10 is unable to down-regulate surface MHC II or CD86 in March1-deficient DCs when the lysine 225 residue in MHC II β chain cytoplasmic tail is mutated or when endogenous MHC II ubiquitination is inhibited by CD83 TM proteins. Enforced expression of CD83 TM proteins negates the suppressive effect of IL-10 on DC MHC II and CD86 surface display and blocks the down-regulation of these proteins on MCMV-infected macrophages, which are known to be IL-10 dependent, leading us to conclude that this key effect of IL-10 is mediated via its ability to induce *March1* mRNA, in opposition to the decreased *March1* caused by LPS. The CD83 pathway for promoting MHC II and CD86 display on DCs and opposing the actions of virus-induced IL-10 provides opportunities to enhance the potency of T cell–directed vaccine strategies.

The discovery that CD83 promotes surface display of MHC II by opposing its association with MARCH1 and ubiquitination provides a mechanistic explanation for the accelerated turnover of cell surface MHC II and CD86 molecules in cells lacking the CD83 TM segment (Fig. S3; Kuwano et al., 2007). This result connects the function of CD83 with a body of work demonstrating that surface MHC II is negatively regulated by MARCH1-dependent ubiquitination of MHC II-β chain tail on lysine 225, resulting in the retention of MHC II intracellularly and its subsequent degradation (Ohmura-Hoshino et al., 2006b; Shin et al., 2006; Matsuki et al., 2007). Increased mean MHC II ubiquitination was suggested in CD83$^{null}$ B cells by Western blotting (Kuwano et al., 2007), although because the difference did not achieve statistical significance the authors concluded that accelerated MHC II turnover was not caused by alterations in ubiquitination.

During DC maturation in the absence of IL-10, our findings, together with published data, indicate that two mechanisms cooperate in bringing about decreased MHC II ubiquitination and increased surface display (Cella et al., 1997; Villadangos et al., 2001; Shin et al., 2006; van Niel et al., 2006; De Gassart et al., 2008). First, in the absence of IL-10 there is a progressive decrease in expression of *March1* mRNA (De Gassart et al., 2008; Young et al., 2008). A similar extinction of *March1* mRNA expression occurs in B cells (Fig S6; Hijikata et al., 2007). In parallel, CD83 mRNA and protein is induced and opposes the remaining pool of MARCH1 protein. This paired mechanism for limiting the action of MARCH1 presumably explains the display of MHC II and CD86 on CD83 TM mutant DCs at levels that are approximately half of those found on WT mature DCs, and the retention of immunogenicity by CD83-deficient DCs (Fujimoto et al., 2002). In the presence of IL-10 and LPS, however, *March1* mRNA remains high and under these conditions the CD83 TM provides a

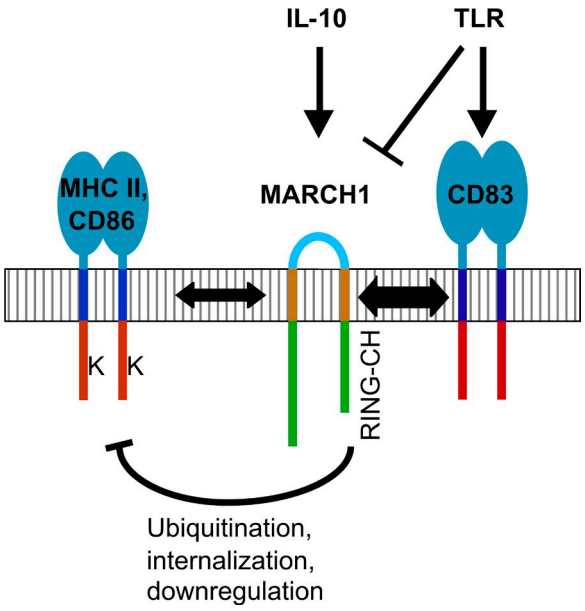


**Figure 7. Summary of CD83 pathway regulating display of MHC II and CD86 on DCs.** CD83 mRNA is induced by TLR signaling, whereas MARCH1 mRNA is induced by IL-10 and diminished by TLR signaling. The CD83 TM binds to MARCH1, decreasing MARCH1 association with the TM segments of MHC II or CD86 and preventing ubiquitination of lysines in their cytoplasmic tails by the MARCH1 RING-CH domain, thereby promoting MHC II or CD86 surface display.

potent posttranslational mechanism to promote MHC II and CD86 display.

An important focus for future research will be the mechanism by which the CD83 TM segment opposes MARCH1 association with and ubiquitination of MHC II. In the case of viral MIR1 and MIR2 homologues of the MARCH family, down-regulation of surface MHC I or CD86 has been shown to require two elements: (1) lysines in the substrate cytoplasmic tail that are the target of the RING-CH catalytic domain in MIR; and (2) TM segments of the substrate and of MIR that promote substrate binding (Coscoy and Ganem, 2000; Coscoy et al., 2001; Hewitt et al., 2002; Ishido et al., 2000a,b; Sanchez et al., 2002; Ohmura-Hoshino et al., 2006a). Specific association between MARCH8 (cMIR) and chimeric proteins containing TM and cytoplasmic segments of its substrate, CD86, has been demonstrated by immunoprecipitation and immunofluorescence (Goto et al., 2003). We show here by immunoprecipitation that MARCH1 associates with MHC II and CD83. Nevertheless, the antagonistic effect of the CD83 TM cannot be explained by simple competition between CD83 and MHC II TM segments for binding to MARCH1, because overexpression of hCD4 chimera 3 containing the MHC II TM and cytoplasmic domains does not oppose the effects of MARCH1 on endogenous MHC II or CD86 (Fig. 4 B). Selective antagonism by CD83 TM and not by MHC II TM could be explained by higher affinity association between MARCH1 and CD83 TM compared with MHC II TM. Alternatively, CD83 TM may engage MARCH1 at a distinct site from MHC II, causing allosteric changes that inhibit MARCH1 E3 ligase activity or causing it to redistribute to membrane subdomains that are less accessible to MHC II. It will also be important to understand how CD83 is itself regulated by MARCH1 or other similar proteins, particularly because CD83 expression by DCs is diminished by herpes simplex virus type 1 infection (Kruse et al., 2000). In unpublished preliminary data on this point, we find that enforced MARCH1 expression also down-regulates cell surface CD83, indicating that MARCH1 may be an inhibitor of its inhibitor, CD83, to create a sharp titration between the actions of MARCH1 and CD83 in controlling MHC II display. Finally, it will be important to determine whether functional interactions between CD83, MARCH1 and MHC II or CD86 occur on the plasma membrane or in the membranes of intracellular sorting compartments. MHCII, CD86, and CD83 colocalize on the cell surface and in recycling endosomes of B cells and DCs (Klein et al., 2005; Kretschmer et al., 2008). Increased endocytosis of surface MHC II has been shown to occur during DC maturation and in cells overexpressing MARCH8 (Villadangos et al., 2001; Ohmura-Hoshino et al., 2006b; van Niel et al., 2006). In contrast, Matsuki et al. (2007) have shown in MARCH1-deficient B cells that the rate of turnover of cell surface MHC II is decreased, but the rate of internalization (presumably into recycling endosomes) is unaffected, indicating that MARCH1 is rate limiting at the level of intracellular retention in sorting endosomes. Consistent with *March1* mRNA induction found here, IL-10 has also been shown to induce retention of MHC II molecules in intracellular compartments (Koppelman et al., 1997; Thibodeau et al., 2008). The biochemical questions raised by our findings will be interesting to explore in the future.

The discovery that IL-10 suppression of MHC II and CD86 can be negated by the CD83 TM-derivative CD83 ΔC suggests a new strategy for augmenting vaccine potency. Delivery of CD83 TM vectors or peptides, either to DCs in culture or encoding CD83 ΔC within viral vaccine antigen delivery vectors such as adeno-associated virus or modified virus ankara, may provide a way to enhance vaccine potency and reverse the effects of viral or host IL-10 in the setting of chronic infection or cancer. The demonstration here that vector-encoded CD83 ΔC can reverse the negative effects of MCMV infection on display of MHC II and CD86 provides a first step proof-of-principle toward this longer-term goal.

## MATERIALS AND METHODS

**Mice.** ENU mutagenesis of male C57BL/6 and breeding was done as previously described (Vinuesa et al., 2005). C57BL/6, CBA, and $Rag^{-/-}$ strains were originally obtained from The Jackson Laboratory and maintained in our specific pathogen facility. *March1*$^{-/-}$ mice were as previously described (Matsuki et al., 2007). All procedures were done according to the approved protocols of the Australian National University Animal Ethics and Experimentation Committee. The anubis strain was maintained on C57BL/6xCBA background. Controls used in experiments were either $CD83^{+/+}$ or $CD83^{anu/+}$ littermates or WT C57BL/6xCBA F2 mice, with matching H-2 genotypes. All mice used were generally between 8 and 14 wk old.

**Mapping and genotyping.** Mapping was done using a set of SNP markers distinguishing C57BL/6 and CBA alleles, based on validated SNP information (www.well.ox.ac.uk/mouse/INBREDS). SNP typing was done using the Amplifluor kit (Millipore) according to the manufacturer's recommendation. Genotyping was done using SNP amplifluor assay using the following primers: WT allele, 5′-GAAGGTCGGAGTCAACGGATTGGTGCTTTGACCAGACACTTAC-3′; anubis allele, forward, 5′-GAAGGTGACCAAGTTCATGCTAGGTGCTTTGACCAGACACTTAA-3′, and reverse, 5′-GCAGAAGCTGTGTTGCTCTT-3′. All mapping and genotyping were done at the Australian Phenomics Facility.

**Sequencing.** Total RNA was extracted using TRIzol reagent and cDNA amplified using Elongase Enzyme Mix (both Invitrogen) according to the manufacturer's recommendations. Sequencing was done using BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's protocol (Applied Biosystems). The following primers were used for sequencing (Geneworks): For gDNA sequencing, CD83e4F, 5′-GGTAGTCAACAACGCTGCAA-3′, and CD83e4R, 5′-TGCCTAACCTCACAGGCTCT-3′; For cDNA sequencing, CD83RNAfor, 5′-GCCTCCAGCTCCTGTTTCTA-3′, CD83RNArev, 5′-GAAAGGTTGCCATCTGAGGA-3′.

**Flow cytometry.** Single-cell suspensions were stained with the following antibodies: CD83 (Michel-17, eBioscience; or Michel-19, BioLegend), rat IgG1 isotype control (eBioscience), human CD4 (S3.4; Invitrogen), or (all following antibodies were obtained from BD) I-$A\beta^k$ (10–3.6), $IA\beta^b$ (25–9-17), $IA\alpha^k$ (11–5.2), CD86 (GL1), B220 (RA3-6B2), CD69 (H1.2F3), CD25 (7D4), CD4 (GK1.5, RM4-5), CD8 (53–6.7), CD44 (IM7), TCRβ (H57-597), MHC I ($H\text{-}2K^k$; 36–7.5), CD11c (HL3), CD229.1 (30C7), CD9 (KMC8), CD40 (3/23), mouse IgG2a isotype control (G155-178), rat IgG2a isotype control (R35-95), and streptavidin-PE and streptavidin-APC. The exclusion of dead cells was done by 7-aminoactinomycin-D staining (Invitrogen). Intracellular staining was done using the Fixation and Permeabilization buffers (eBioscience) according to the manufacturer's instructions. Samples

were collected on a FACSCalibur or LSRII (BD) and data were analyzed using FlowJo software (Tree Star, Inc.).

**In vitro cell stimulation and endocytosis assay.** Splenocytes from mice were prepared and cultured as previously described (Jun et al., 2003). Cells were unstimulated or stimulated with 10 μg/ml goat anti–mouse IgM $F(ab')_2$ (Jackson Immunoresearch Laboratories) or 20 μg/ml LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich) in overnight cultures. For endocytosis assay, splenocytes stimulated with 20 μg/ml LPS in overnight cultures were harvested, stained with biotinylated-CD86 (GL1; BD) or biotinylated-IA$\beta^k$ (10–3.6; BD) at 4°C for 30 min, washed twice in media, and returned to 37°C for the indicated times. The surface MHC II or CD86 expression that remained after incubation time was detected by streptavidin-PE (BD) and analyzed by flow cytometry.

**Vector construction.** The full-length sequences for CD83, MHC II (I-A$\beta^b$), March1, and March8 were amplified from C57BL/6 spleen cDNA by *Pfx* DNA polymerase (Invitrogen) and subcloned into pBluescript II SK+ (Stratagene). The human CD4 cytoplasmic deletion sequence was amplified from pR-IRES-CD4 vector. All site-directed mutants and chimeric receptors were generated by PCR-based mutagenesis. The full-length sequences were cut and ligated into pcDNA3.1+ (Invitrogen), pMXs-IRES-GFP vector (Kitamura et al., 2003; provided by T. Kitamura, University of Tokyo, Tokyo, Japan) or pMXs-IRES-DsRed, in which GFP sequence was replaced with DsRed sequence from DsRed-Express-C1 (Clontech). The following primers (Geneworks; all sequences are listed in 5′-3′ orientation) were used for cloning (restriction enzyme sites are depicted as underline): Cd83 CS1, 5′-TTGGATCCGCCACCATGTCGCAAGGCCTCCAGCTCCTG-3′; Cd83 CA1, 5′-AAAACTCGAGTCATACCGTTTCTGTCTTAGGAAG-3′; H2-Ab1 CS1, 5′-TTGGATCCGCCACCATGGCTCTGCAGATCCCC-AGCCTC-3′; H2-Ab1 CA1, 5′-AAAAGTCGACTCACTGCAGGAG-CCCTGCTGGAGG-3′; March1 CS4, 5′-TTGGATCCGCCACCAT-GACCAGCAGCCACATTTGCTG-3′; March1 CA1, 5′-AAAACTC-GAGTCAGACTGGTATAACCTCAGGTGG-3′; March8 CS1, 5′-TTGG-ATCCGCCACCATGAGCATGCCATTGCACCAGATC-3′; March8 CA1, 5′-AAAACTCGAGTCAGACGTTAATAATTTCTGCTCC-3′; Cd4 CS1, 5′-TTGGATCCGCCACCATGAACCGGGGAGTCCCTTTTAG-3′; Cd4 CA1, 5′-AAAACTCGAGTCAGTGCCGGCACCTGACACAG-3′.

The following primers were used for CD83 mutant ΔC and MHC II I-A$\beta^b$ (H2-Ab1) K253R (restriction enzyme sites or inserted mutations are depicted as underline or italic, respectively): Cd83 MA3, 5′-AAAA-CTCGAGTCATTGTAGTCGTGCAAATTTGC-3′; H2-Ab1 CA2, 5′-AAAAGTCGACTCACTGCAGGAGCCCTGCTGGAGGAGGGCC-TCGAGGTCCTCTCTGACTCCTGTGACGGATG-3′.

The following primers were used for generating chimeric receptors: Cd4 MS2, 5′-CAGGTCCTGCTGGAATCCAACATCAAGGTGACAG-GATGCCCCAAGGAAGC-3′; Cd4 MA2, 5′-TAGCTTCCTTGGGG-CATCCTGTCACCTTGATGTTGGATTCCAGCAGGAC-3′; Cd4 MS3, 5′-GGTCGACCCCGGTGCAGCCAATGGCAGAAGCTGTGTTGCT-CTTC-3′; Cd4 MA3, 5′-GAGAGAAGAGCAACACAGCTTCTGC-CATTGGCTGCACCGGGGTC-3′; Cd4 MS4, 5′-AGTCAACTTTCAG-GAAGTACAGGGCCCTGATTGTGCTGGGGG-3′; Cd4 MA4, 5′-ACG-CCCCCCAGCACAATCAGGGCCCTGTACTTCCTGAAAGTTG-3′; Cd4 MS5, 5′-GGCTAGGCATCTTCTTCTGTGTCCGACTACAAA-GCATTTTCC-3′; Cd4 MA5, 5′-TATCTGGGAAAATGCTTTGTAG-TCGGACACAGAAGAAGATGCC-3′; Cd4 MS6, 5′-GTCGACCCC-GGTGCAGCCAATGTTGAGCGGCATCGGGGGC-3′; Cd4 MA6, 5′-CACGCAGCCCCCGATGCCGCTCAACATTGGCTGCACCGG-GGTC-3′.

The following primers were used for inserting Flag epitope after the signal peptide sequence of CD83 and human CD4. V5 epitope was introduced into c-terminus of MARCH1 by ligating with home made pBluescript II SK+ V5-tag vector. Cd83 MS1, 5′-GGCGATGGACTACAAGGACGAC-GATGACAAGGGTGGCGGTCGGGAGGTGACGGTGGCTTG-3′; Cd83 MA1, 5′-ACCTCCCGACCGCCACCCTTGTCATCGTCGTCCTTG-TAGTCCATCGCCATCGCGGGTGCC-3′; Cd4 MS1, 5′-AGGGAA-AGGACTACAAGGACGACGATGACAAGGGTGGCGGTAAAGTGG-TGCTGGGCAAAAAAGG-3′; Cd4 MA1, 5′-CACCACTTTACCGC-CACCCTTGTCATCGTCGTCCTTGTAGTCCTTTCCCTGAGTG-GCTGCTGGG-3′; March1 CA2, 5′-AAAAGTCGACGACTGGTATA-ACCTCAGG-3′.

**Retroviral transduction.** Supernatant containing retroviruses were harvested from transfected Phoenix packaging cell line (a gift from G. Nolan, Stanford University, Palo Alto, CA) and used to transduce B cells as described (Horikawa and Takatsu, 2006). In brief, murine splenocytes were activated overnight with 10 μg/ml LPS (from *E. coli* 055:B5; Sigma-Aldrich). Cells were spinoculated at 2,800 rpm at 29°C for 90 min with retroviral supernatant and DOTAP liposomal transfection reagent (Roche), washed, and returned to cultures with 10 μg/ml LPS for another 1–2 d. BMDCs were generated and transduced as previously described with some modifications (Shin et al., 2006). B and T cells, granulocytes, and erythrocytes were removed from BM cell preparations using Ficoll-Paque Plus density gradient (GE Healthcare) and standard negative selection method by MACS separation (Miltenyi Biotec). The remaining cells were cultured in the presence of 20 ng/ml recombinant mouse GM-CSF (R&D Systems) for 5 d. On day 2 of culture, cells were spinoculated as described. In some cultures, 20 ng/ml recombinant mouse IL-10 (R&D Systems) was added from day 3 onwards, with the addition of 1 μg/ml LPS for the last 16–20 h of culture. In some experiments, J774 macrophages transduced with retroviral constructs as described above were either left uninfected or infected with 10 MOI of MCMV-K181-Perth strain 2 d later and analyzed 4 d after infection as previously described (Andoniou et al., 2005).

**Immunoprecipitation and Western blotting.** HEK293T cells were transfected using PolyFect transfection reagent (QIAGEN) and analyzed by flow cytometric and biochemical assays at 24 h after transfection. To detect ubiquitination, MG-132 (Calbiochem) was added at final concentration of 20 μM 2–3 h before cells were harvested. Cells were lysed with TNE buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM sodium orthovanadate, and complete protease inhibitor [Roche]). Flag-tagged proteins were immunoprecipitated with anti-Flag M2 antibody (Sigma-Aldrich) and protein G–Sepharose (GE Healthcare), and then fractionated by 12% SDS-PAGE. The membranes were blocked with TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Triton X-100) and 5% skim milk (Difco), and then stained with anti-Flag (M2), anti-V5 (SV5-Pk1;, Serotec), or anti-ubiquitin (P4D1; Santa Cruz Biotechnology, Inc.) antibodies, followed by detection using mouse IgG TrueBlot (eBioscience). The bands were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

In some experiments, FACS-sorted $GFP^+$ cells from IL-10–treated, BM-derived DCs generated from *Cd83*$^{+/+}$ (on H-2$^b$ background) mice were lysed with lysis buffer (1% Nonidet P-40, 20 mM TrisHCl, pH 8.0, 150 mM NaCl, and 20 mM *N*-ethylmaleimide) with complete protease inhibitor (Roche). MHC class II protein was immunoprecipitated with anti-MHC class II antibody (clone: Y-3P; American Type Culture Collection [ATCC]) and separated in 10% SDS-PAGE gel. Transferred membrane was stained with anti-ubiquitin (clone: P4D1; Santa Cruz Biotechnology, Inc.) or anti–MHC class II I-A β chain (clone: KL295; ATCC) antibody and detected using horseradish peroxidase–conjugated anti–mouse IgG light chain–specific antibody (Jackson ImmunoResearch Laboratories). Band intensities were measured using ImageJ software (Abramoff et al., 2004).

**Quantitative real-time RT-PCR.** Total RNA was extracted from BMDCs using TRIzol (Invitrogen), and cDNA was synthesized using Superscript III reverse transcription (Invitrogen). Relative levels of March1 cDNA, as compared with the housekeeping gene β-actin, were ascertained using real-time RT-PCR and SybrGreen on the ABI 7900 Real-Time PCR system (Applied Biosystems). 7 technical replicates per culture replicate/gene were averaged (mean SD = 0.05), and the fold change of the LPS + IL-10 samples compared with the LPS only group using the formula $2^{\wedge}(\Delta Ct^{LPS\ only}-\Delta Ct^{LPS+IL10})$. The following

primers (Geneworks) were used: March1 forward, 5′-AAGAGAGCCCACTCATCACACC-3′; March1 reverse, 5′-ATCTGGAGCTTTTCCCACTTCC-3′ (Young et al., 2008); β-actin forward, 5′-TGTTACCAACTGGGACGACA-3′; β-actin reverse, 5′-AAGGAAGGCTGGAAAAGAGC-3′.

**Statistical analysis.** P values were calculated using Prism 5 (GraphPad Software) with unpaired, two-tailed Student's *t* test.

**Online supplemental material.** Fig. S1 describes identification of the anubis mouse strain. Fig. S2 shows the reduction of surface MHC II and CD86 on anubis B cells. Fig. S3 shows a decrease in surface MHC II and CD86 persistence on anubis B cells. Fig. S4 shows the distribution of CD83, MHC II, and CD86 on BMDCs. Fig. S5 shows normal CD9 expression on anubis BMDCs. Fig. S6 shows a decrease in *March1* transcript in activated B cells and BMDCs. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092203/DC1.

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