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Physiological substrates and ontogeny-specific expression of the ubiquitin ligases MARCH1 and MARCH8

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ABSTRACT

MARCH1 and MARCH8 are ubiquitin ligases that control the expression and trafficking of critical immunoreceptors. Understanding of their function is hampered by three major knowledge gaps: (i) it is unclear which cell types utilize these ligases; (ii) their level of redundancy is unknown; and (iii) most of their putative substrates have been described in cell lines, often overexpressing MARCH1 or MARCH8, and it is unclear which substrates are regulated by either ligase *in vivo*. Here we address these questions by systematically analyzing the immune cell repertoire of MARCH1- or MARCH8-deficient mice, and applying unbiased proteomic profiling of the plasma membrane of primary cells to identify MARCH1 and MARCH8 substrates. Only CD86 and MHC II were unequivocally identified as immunoreceptors regulated by MARCH1 and MARCH8, but each ligase carried out its function in different tissues. MARCH1 regulated MHC II and CD86 in professional and "atypical" antigen presenting cells of hematopoietic origin, including neutrophils, eosinophils and monocytes. MARCH8 only operated in non-hematopoietic cells, such as thymic and alveolar epithelial cells. Our results establish the tissue-specific functions of MARCH1 and MARCH8 in regulation of immune receptor expression and reveal that the range of cells constitutively endowed with antigen-presentation capacity is wider than generally appreciated.

1. Introduction

Ubiquitination is a major mechanism for the regulation of membrane proteostasis. In brief, covalent attachment of ubiquitin (Ub) chains to the cytosolic tail of transmembrane proteins promotes endosomal trafficking to multivesicular bodies for subsequent degradation in lysosomes (Komander and Rape, 2012). This post-translational modification enables the fine-tuning of surface protein expression levels. Ub is attached to substrates by E3 Ub ligases. Membrane Associated RING-CH Finger (MARCH, gene symbol *Marchf*) is a family of eleven E3 ligases, all of which possess two or more transmembrane domains, with the exception of MARCH7 and MARCH10 (Liu et al., 2019). They were initially identified as the mammalian homologues of herpesvirus immunoevasins that ubiquitinate host molecules involved in anti-viral immunity to subvert immune responses (Coscoy et al., 2001; Ishido et al., 2000). MARCH E3 Ub ligases are thought to be specialized at ubiquitinating immunoregulatory receptors, but their physiological substrates remain largely unknown (Liu et al., 2019; Samji et al., 2014). It is also unclear if their expression and function is restricted to cells of the immune system and, if so, which.

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MARCH1 and MARCH8 are the most studied members of the MARCH family. As they share approximately 60% overall sequence homology (Liu et al., 2019), they are thought to also share substrate specificity. Indeed, both ubiquitinate major histocompatibility complex class II (MHC II) molecules, the receptor employed by antigen presenting cells (APCs) to display peptide antigens to $CD4^+$ T cells. MARCH1 and MARCH8 regulate the surface expression of MHC II (Young et al., 2008; Cho et al., 2015; Bannard et al., 2016) and thereby play key roles in the activation of naïve $CD4^+$ T cells in the periphery (Ishikawa et al., 2014), as well as their development in the thymus (Liu et al., 2016; von Rohrscheidt et al., 2016; Oh et al., 2013). Furthermore, they have been involved in complex immune reactions such as inflammation (Galbas et al., 2017), infection (Wu et al., 2020; Zhang et al., 2019), cancer (Xie et al., 2019), allergy and autoimmunity (Kishta et al., 2018; Toyomoto et al., 2011). This raises the question whether both ligases regulate the expression of other immune receptors, some of which reportedly include CD44 (Eyster et al., 2011), CD71 (Fujita et al., 2013), CD86 (Corcoran et al., 2011), CD95 (Bartee et al., 2004) and CD98 (Eyster et al., 2011; Ablack et al., 2015) among others (Samji et al., 2014). However, to date CD86 is the only membrane protein apart from MHC II that has been shown to be regulated by MARCH1 *in vivo* (Baravalle et al., 2011), and it is not known if it can also be regulated by MARCH8. All other putative MARCH1 or MARCH8 substrates have been described in cell lines and/or overexpression studies. MARCH proteins are expressed at very low levels in primary cells (Liu et al., 2019; Jabbour et al., 2009; Kaul et al., 2018; Thibodeau et al., 2008), and since E3 ligase overexpression can cause off-target effects, it remains unclear which, if any of the MARCH1 and MARCH8 substrates described in transfected cell lines are ubiquitinated by these ligases in physiological settings. To summarize, the repertoire of MARCH1 and MARCH8 substrates *in vivo* remains largely unknown. This is an important shortcoming because ubiquitination is amenable to pharmacological manipulation (Zhao et al., 2020; Edelmann et al., 2011), and development of drugs targeting MARCH1 or MARCH8 might have therapeutic potential provided their substrates are identified.

Another important knowledge gap in MARCH1 and MARCH8 biology pertains to their expression pattern. Quantitating MARCH1 or MARCH8 protein expression is unfeasible due to their low abundance (Liu et al., 2019) and fast turn-over (Bourgeois-Daigneault and Thibodeau, 2012; Lei et al., 2018), and even their transcription levels are poor predictors of function (Jabbour et al., 2009; Kaul et al., 2018; Thibodeau et al., 2008). Identification of MARCH1- or MARCH8-expressing cells thus relies on analysis of surface expression of membrane protein substrates as a surrogate of activity. MARCH1 ubiquitinates MHC II and CD86 in B cells and conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively) (Young et al., 2008; Cho et al., 2015; Bannard et al., 2016), but it is not functional in thymic epithelial cells (TECs) (Liu et al., 2016; von Rohrscheidt et al., 2016). Whether it is active in other hematopoietic or non-hematopoietic cells remains unknown. In contrast, MARCH8 ubiquitinates MHC II in TECs, not in B cells or DCs (Liu et al., 2016; von Rohrscheidt et al., 2016), but it is not known if it ubiquitinates other receptors in these cells, and whether it is also expressed in other cells. Incomplete understanding of the pattern of MARCH expression again limits the development and potential application of ubiquitination-modulating agents as immunomodulatory drugs.

Here, we present a systematic analysis of the pattern of activity of MARCH1 and MARCH8 in multiple hematopoietic and non-hematopoietic cells isolated from *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. We have also carried out quantitative proteomic comparisons of wild type (WT) vs *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ plasma membrane purified from cDCs and B cells. Our results define physiological substrates regulated by these two ligases and demonstrate functional specializations of MARCH1 and MARCH8 in two ontogenically distinct compartments.

2. Materials and methods

2.1. Mice

Wild type (WT, C57BL/6), *Marchf1*$^{-/-}$ (Matsuki et al., 2007), *Marchf8*$^{-/-}$ (Liu et al., 2016) and *I-Aα*$^{-/-}$ (Köntgen et al., 1993) mice were bred and maintained in specific pathogen-free conditions within the Melbourne Bioresources Platform at the Bio21 Molecular Science and Biotechnology Institute. Analyses were undertaken with male or female mice aged between 6 and 14 weeks and performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Melbourne. All procedures were approved by the Animal Ethics Committee at the University of Melbourne (#1714238 and #1513472).

2.2. Isolation of mouse primary cells and analytical flow cytometry

Single cell suspensions from blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung were generated for analysis of B cells, T cells, DCs, macrophages, monocytes, neutrophils, eosinophils and thymic or alveolar epithelial cells. Blood was collected from submandibular veins and red blood cells were lysed. Whole single cell suspensions from spleen and subcutaneous LN (axillary and inguinal) were generated by digestion with 0.1% DNase I (Roche) and 1 mg/ml collagenase type III (Worthington) and red blood cell lysis. DCs from spleen and LN were further enriched by selection of low-density cells by density gradient centrifugation in 1.077 g/cm^3 Nycodenz® (Axis shield). Thymi were digested in 0.1% DNase I (Roche) and 0.5 U/ml liberase (Roche) and thymic cDCs were further enriched by 1.077 g/cm^3 Nycodenz® density gradient centrifugation. Cells from the peritoneal cavity were harvested by injection and aspiration of PBS. Lungs were perfused with PBS and digested with 50 μg/ml DNase I (Roche) and 0.25 mg/ml liberase (Roche) and red blood cells were lysed.

For flow cytometry, cells were incubated with FcR blocking reagent (Miltenyi Biotec), prior to staining with mAb detecting B220/CD45R (RA3-6B2), CD19 (6D5), CD64 (X54-5/7.1), F4/80 (F4/80, Walter Eliza Hall Institute (WEHI) Antibody Facility), CD3 (KT3-1.1, WEHI Antibody Facility), TCRβ (H57-597, WEHI Antibody Facility), CD4 (GK1.5), CD8 (YTS169.4 WEHI Antibody Facility), CD8 (53–6.7), BST-2 (927), Siglec-H (551), MHC II (M5/114), CD11c (N418), CD11b (M1/70), Ly6G (1A8), Ly6C (HK1.4), NK1.1 (PK136, BD Biosciences), Sirpα (P84), XCR1 (ZET), CD45 (30-F11), EpCAM (G8.8), Ly51 (6C3), UEA-1 (Vector Laboratories), MerTK (2B10C42), Siglec-F (E50-2440 BD Biosciences), CD31 (390), CD24 (M1/69, WEHI Antibody Facility), Sca-1 (D7), CD86 (GL-1), CD40 (FGK45.5, Miltenyi Biotec), CD80 (16-10A1, BD Biosciences), CD44 (IM7.81), CD71 (R17217, eBiosciences), CD95 (15A7, eBiosciences), CD98 (RL388), PD-L1 (10F.9G2), PD-L2 (TY25), ICOS-L (HK5.3), B7–H3 (MIH35) or B7–H4 (HMH4-5G1), conjugated to fluorochromes BUV395, BUV805, FITC, PE, PE-Cy7, PerCP/Cy5.5, APC, APC-Cy7, AF700, BV785, BV650, BV510 or BV421 (all from BioLegend, if not stated differently). Cell viability was determined with Fixable Viability Dye eFluor™ 780 (eBiosciences), propidium iodide (PI) or diamidino phenylindole (DAPI) staining. Analysis was performed using a LSRFortessa (BD Biosciences) or CytoFLEX LX (Beckman Coulter) in the Melbourne Cytometry Platform (University of Melbourne). Data was analyzed with FlowJo (Tree Star) and GraphPad Prism. Supplementary Figs. 1 and 2 summarize gating strategies for cells from blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung.

2.3. Isolation of primary immune cells for proteomic analysis

B cells were purified from spleens using Ficoll® Paque Plus (GE Healthcare) gradient centrifugation and negative depletion with FITC-conjugated mAb specific for CD4 (GK1.5), Ly-76 (TER119) and CD43 (S7) and magnetic anti-FITC MicroBeads (Miltenyi Biotec). Preparations were approximately 95–98% pure for $CD19^+$ $B220^+$ B cells. Splenic

cDCs were purified from mice subcutaneously injected with Flt3L-secreting melanoma cells (Mach et al., 2000), nine days before purification. cDCs were purified from spleens of Flt3L-expanded mice following spleen digestion with DNase I (Roche) and collagenase type III and Nycodenz® density gradient centrifugation with subsequent negative depletion using rat mAb specific for CD3 (KT3-1.1), Thy1 (T24/31.7), Ly-76 (Ter119), B220 (RA3-6B2) and Ly-6C/G (RB6-8C5) and anti-rat IgG-coupled magnetic beads (Qiagen) as previously described (Vremec, 2010). Preparations were approximately 90–95% pure for CD11c^{+} MHC II^{+} cDCs.

2.4. Preparation of subcellular fractions enriched in plasma membrane and intracellular compartments for proteomics

Subcellular fractionation was performed as previously described (Segura et al., 2009). In brief, purified B cells (4–5 x 10^7 cells, 95–98% purity) and cDCs (4–5 x 10^7 cells, 90–95% purity) from spleens of WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice were incubated with FITC-conjugated anti-CD19 and anti-B220 mAb (B cells) or anti-CD11c, anti-CD45.2, anti-CD49d and anti-MHC I mAb (cDCs). mAb-labelled cells were homogenized in the presence of cOmplete™ protease inhibitors (Roche) by mechanical disruption using a cell-cracker (HGM Laboratory equipment). Homogenized preparations were centrifuged at low speed to obtain post-nuclear supernatant (PNS). Surface-labelled plasma membrane (PM) microsomes were isolated by magnetic immunoaffinity using anti-FITC mAb-coated magnetic beads (Miltenyi Biotec) and concentrated by ultracentrifugation in thickwall polycarbonate tubes (Beckman Coulter). PNS, with the PM fraction removed, was likewise ultracentrifuged to sediment the "intracellular compartments" (IC) fraction.

2.5. Proteomic profiling of differentially expressed PM proteins

Subcellular fractions (PM and IC) from equal cell numbers of *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ cDCs and B cells were prepared for mass spectrometry analysis from three independent cell preparations using FASP protein digestion (Protein Discovery) as previously described (Wiśniewski et al., 2009), with the following modifications. Proteins were reduced and digested with sequence-grade modified Trypsin Gold (Promega). Peptides were eluted with ammonium bicarbonate and acidified peptide mixtures from each biological replicate were analyzed in technical triplicates by nanoflow reverse-phase liquid chromatography tandem mass spectrometry (LC-MS/MS) on a nanoAcquity system (Waters) coupled to a Q-Exactive mass spectrometer equipped with a nanoelectrospray ion source for automated MS/MS (Thermo Fisher Scientific). High-resolution MS/MS spectra were processed with MaxQuant (version 1.6.7.0) for feature detection and protein identification using the Andromeda search engine (Cox et al., 2011). Extracted peak lists were searched against the UniProtKB/Swiss-Prot Mus musculus database (Oct-2019) and a separate reverse decoy database to empirically assess the false discovery rate (FDR) using a strict trypsin specificity allowing up to two missed cleavages. The minimum required peptide length was seven amino acids. The "match between runs" option in MaxQuant was used (Cox and Mann, 2008). PSM and protein identifications were filtered using a target-decoy approach at a FDR of 1%. LFQ quantification was performed, with a minimum ratio of two. Protein relative quantitative analysis was performed in R using MaxQuant's proteinGroups.txt and LFQ intensities. Missing values were imputed using a random normal distribution of values derived from the measured distribution of intensities (Cox et al., 2014) using a mean with a negative shift of 1.8 standard deviations and a standard deviation equal to 0.3 of the standard deviation of the measured intensities. The probability of differential expression was calculated using the function *lmFit* from the Bioconductor package limma (Ritchie et al., 2015) followed by *eBayes* using the default settings (Phipson et al., 2016) and false-discovery rate correction using the Benjamini–Hochberg method. The output included P value, confidence interval and ratio estimate. GO-term enrichment analysis was performed using the *enrichr* function in the Bioconductor clusterProfiler package (Yu et al., 2012). Enrichment was calculated separately for the proteins overrepresented in each fraction, relative to all proteins identified in collected fractions across all the LC-MS/MS runs, and GO term association was filtered to include only experimental and high throughput evidence. Enrichment P values were corrected for multiple testing using the function's *'fdr'* method. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD023115.

3. Results

3.1. MARCH1, but not MARCH8, is functional in professional APCs

The first objective of this study was to establish which mouse cells express MARCH1 or MARCH8. Their low level of transcription combined with fast turn-over contribute to maintain the two proteins at non-detectable levels in primary cells, hampering definition of their expression pattern. We reasoned that MHC II and/or CD86 could be used as reporters of MARCH1 and MARCH8 activity because in all primary or transformed cells analyzed so far, the surface level of these two receptors decreases by expression of either ligase (Liu et al., 2019). Cells that express MHC II or CD86 and either MARCH1 or MARCH8 should therefore display higher levels of the receptor(s) in *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ mice.

First, we examined professional APCs (defined as cells that express detectable levels of MHC II in the steady-state (Lassila et al., 1988; Kambayashi and Laufer, 2014) and T cells across various tissues. B cells, cDC1s, cDC2s, pDCs and macrophages from spleen, blood, thymus, peritoneal cavity, lung, and subcutaneous lymph nodes (LN) of *Marchf1*$^{-/-}$ mice displayed elevated surface MHC II and CD86 relative to WT cells, while no changes were observed in their *Marchf8*$^{-/-}$ counterparts (Fig. 1A–E and Supplementary Fig. 3A–E). CD4^{+} and CD8^{+} T cells in spleen and LN showed no detectable surface MHC II and their CD86 expression was not altered by MARCH1- nor MARCH8-deficiency (Fig. 1A and Supplementary Fig. 3B). MHC II and CD86 expression in peritoneal cDCs deficient in both MARCH1 and MARCH8 (*Marchf1*$^{-/-}$ x *Marchf8*$^{-/-}$) was not elevated above that of *Marchf1*$^{-/-}$ cells (Supplementary Fig. 3F). These results indicate that MARCH1 is expressed and active in all professional APCs across various organs/tissues whereas MARCH8 is not or, if it is, does not display enough activity to compensate for the loss of MARCH1.

Next, we assessed the contribution of MARCH1 to activation-dependent regulation of MHC II and CD86 expression in cDCs, the archetypical professional APCs. Toll-like receptor (TLR) ligands trigger an activation program in DCs, known as DC maturation, that includes up-regulation of MHC II and CD86 expression on the plasma membrane, among other receptors (Wilson et al., 2004). Activation also leads to down-regulation of *Marchf1* transcription which, combined with fast turn-over of MARCH1, results in negligible expression of the protein in activated DCs (Young et al., 2008; Vega-Ramos et al., 2014; De Gassart et al., 2008; Galbas and Thibodeau, 2012). It has been assumed that this change is responsible for the accumulation of MHC II and CD86 on the plasma membrane during cDC activation, but this has not been directly examined. If ubiquitination were the dominant mechanism controlling how much MHC II and CD86 is displayed on cDCs, it would be expected that the expression of these two molecules would not vary during activation of *Marchf1*$^{-/-}$ cDCs. However, activation of *Marchf1*$^{-/-}$ cDCs further increased surface expression of MHC II by ~1.5 times, and increased CD86 by ~4 times, when compared with their resting counterparts (Fig. 2). CD40, which also increases in expression during activation, though it is not a MARCH1 substrate, was expressed at equivalent levels in WT and *Marchf1*$^{-/-}$ cDCs at both resting and activated states, so up-regulation of MHC II and CD86 in *Marchf1*$^{-/-}$ cDCs

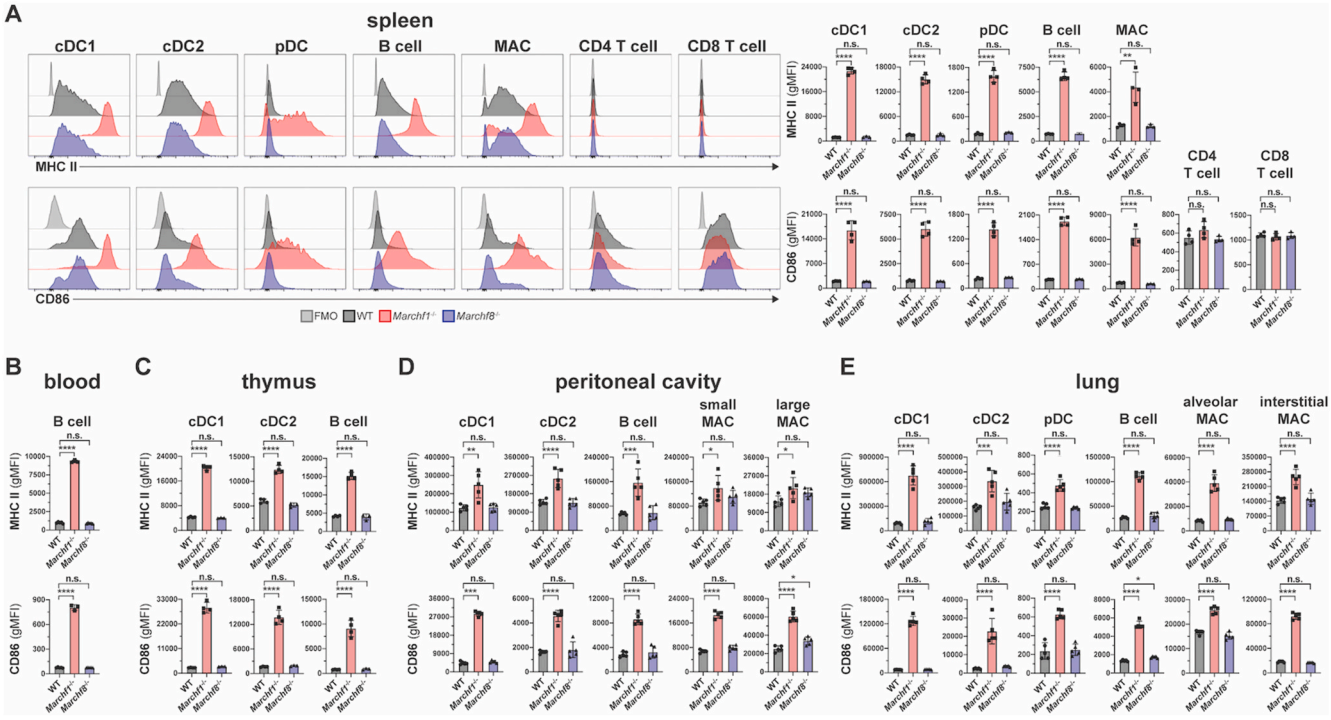


Fig. 1. Ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in haemopoietic professional APCs. Surface expression levels of MHC II and CD86 in (A) splenic cDC1s, cDC2s, pDCs, B cells, macrophages (MAC), and $CD4^+/CD8^+$ T cells, (B) blood B cells, (C) thymic cDC1s, cDC2s, and B cells, (D) peritoneal cDC1s, cDC2s, B cells, and small/large MAC, and (E) lung cDC1s, cDC2s, pDC, B cells, and alveolar/interstitial MAC, all purified from WT mice or mice deficient in either MARCH1 or MARCH8. Bars represent mean ± SD with each symbol representing an individual mouse (n = 4–5). Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. ****p < 0.0001, ***p < 0.0002, **p < 0.002, *p < 0.03, n.s. not significant.

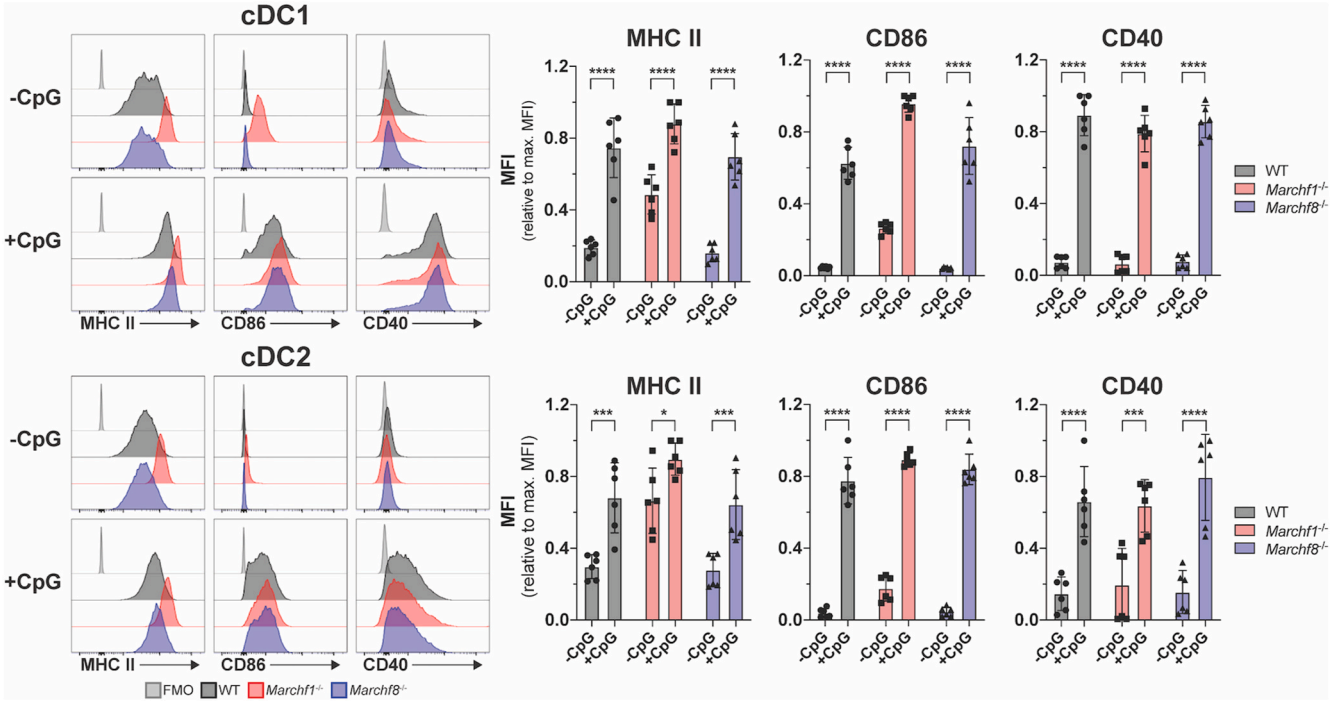


Fig. 2. The role of ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in DC maturation. Surface expression of MHC II and CD86 in CpG-activated cDCs purified from the spleen of WT mice or mice deficient in either MARCH1 or MARCH8. Purified splenic cDCs ($2x10^5$ cells) were kept on ice (-CpG) or incubated for 16 hours *ex vivo* with 50 nm CpG in 96-well plates, then washed and analyzed by flow cytometry for MHC II and CD86 surface expression. A fluorescence-minus-one (FMO) control was included, for which cells were incubated with the corresponding multi-colour staining panel, excluding the fluorescently labelled antibody species of interest (i.e. anti-CD86 or anti-MHC II mAb). Bars represent mean ± SD with each symbol representing an individual mouse (n = 6). Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. ****p < 0.0001, ***p < 0.0002, *p < 0.03.

could not be attributed to overall dysregulation of surface receptor expression (Fig. 2). These results indicate that deposition of newly synthesized molecules on the cell surface (Villadangos et al., 2001; Wilson et al., 2004) is the main contributor to MHC II and, especially, CD86 up-regulation during DC activation. cDCs lacking MARCH8 were indistinguishable from WT cDCs in these experiments, again indicating it has no role in resting or activated cDCs (Fig. 2).

3.2. *Neutrophils, eosinophils and monocytes express MHC II and CD86, but MARCH1 ubiquitination maintains their surface expression at negligible levels*

Next we assessed MARCH1 and MARCH8 activity in "atypical" APCs, that is, immune cells that are not considered professional APCs but have been suggested to play antigen-presenting roles under certain conditions (Kambayashi and Laufer, 2014). These include neutrophils, eosinophils and "inflammatory" (Ly6C^{+}) and "patrolling" (Ly6C^{-}) monocytes. While monocytes have the potential to develop into macrophages or DCs in inflamed sites (Auffray et al., 2009), they are not thought to perform antigen presenting functions in their undifferentiated state (Jakubzick et al., 2017). We examined these "atypical" APCs in spleen and lung. MHC II expression in WT neutrophils, eosinophils and monocytes was barely detectable by flow cytometry, staining at just above the background level observed in cells of mice that do not express any surface MHC II at all (Fig. 3). Strikingly, all four cell types deficient in MARCH1 expressed MHC II at levels comparable to WT B cells or cDCs (compare Figs. 1A to 3A), though expression was higher in spleen than it was in their lung counterparts (Fig. 3A and B). CD86 was also highly expressed at the surface of all four MARCH1-deficient cell types, in this case both in spleen and lungs (Fig. 3). MARCH8-deficient cells did not display altered MHC II or CD86 expression, confirming this member of the MARCH family is not expressed and/or active in hematopoietic cells (Fig. 3). Of note, MARCH1-deficient T cells lacked surface MHC II and did not exhibit enriched CD86 expression when deficient in MARCH1 (Fig. 1A), so neither mutation caused ectopic or increased expression of either molecule. We conclude that neutrophils, eosinophils, monocytes and possibly other atypical APC types (Kambayashi and Laufer, 2014) produce receptors for antigen presentation and T cell stimulation constitutively. While MARCH1 ubiquitination maintains the surface expression of these proteins at barely detectable levels, these "atypical"

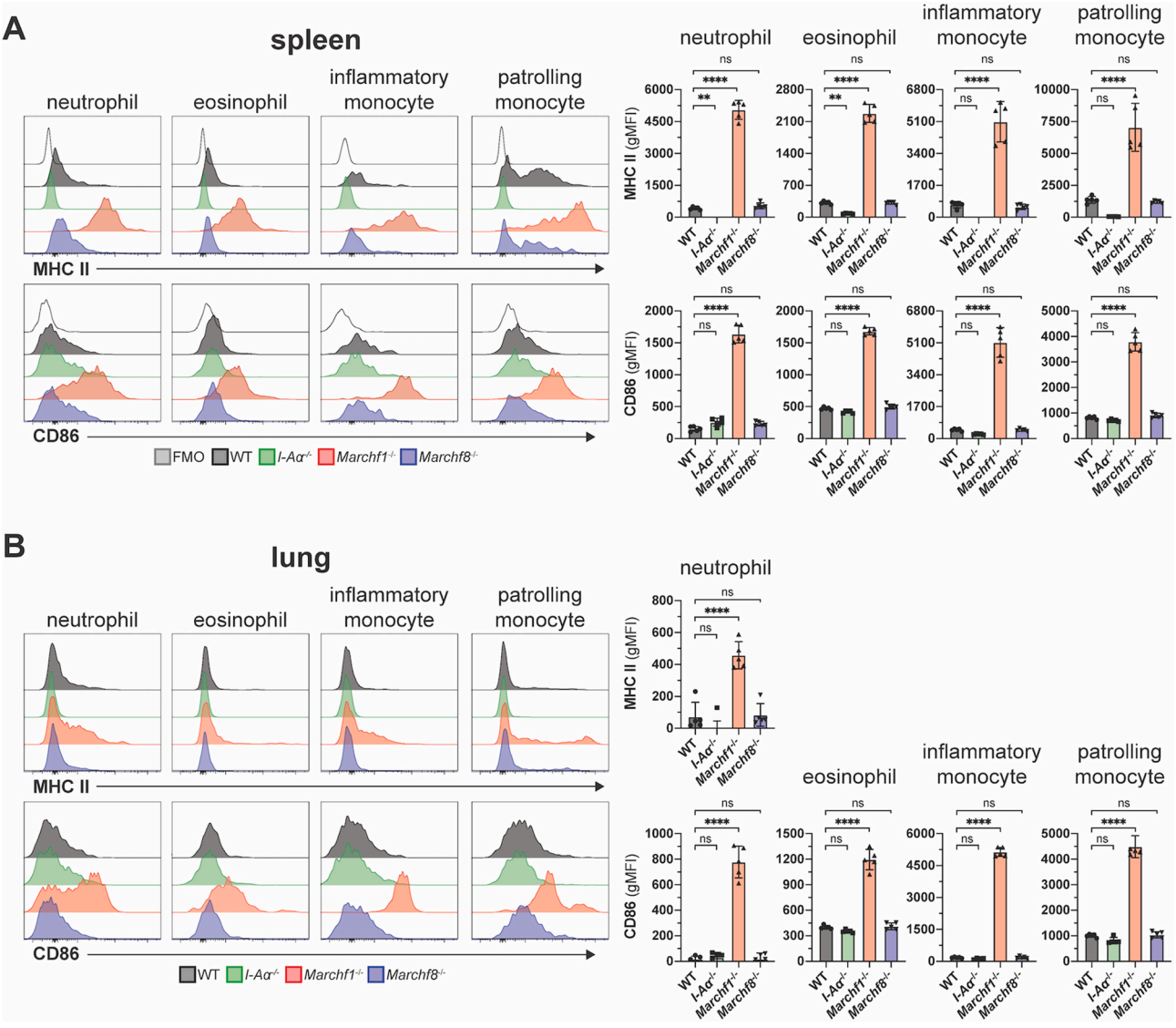


Fig. 3. Ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in granulocytes and monocytes. Surface expression of MHC II and CD86 in neutrophils, eosinophils and inflammatory and patrolling monocytes purified from (A) spleen and (B) lung of WT mice or mice deficient in I-Aα, MARCH1, or MARCH8. Bars represent mean ± SD with each symbol representing an individual mouse (n = 5). Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. ****p < 0.0001, **p < 0.002, n.s. not significant.

APCs might be capable of $CD4^+$ T cell priming under certain conditions.

3.3. Previously predicted MARCH1 substrates display normal expression in *Marchf1*$^{-/-}$ mice

The second objective of this study was to identify which of the receptors found to be ubiquitinated by MARCH1 or MARCH8 in (transfected) cell lines are also substrates *in vivo* under physiological conditions. Such receptors include CD44, CD71, CD95 and CD98 (reviewed in (Samji et al., 2014)). Carrying out this analysis also allowed us to address the possibility that, contrary to our conclusions above, MARCH8 might be expressed and active in these cells but dedicated to ubiquitinate these receptors rather than MHC II and CD86. This was not the case; expression of CD44, CD71, and CD98 was unaltered in *Marchf8*$^{-/-}$ cDCs and B cells compared to WT cells (Fig. 4A). Furthermore, *Marchf1*$^{-/-}$ cDCs and B cells also expressed normal levels of the three receptors (Fig. 4A). We extended our analysis to other regulatory receptors of T cell activation, including CD40 and members of the B7 family to which CD86 (B7.2) belongs: CD80 (B7.1), CD274 (PD-L1), CD273 (PD-L2), CD275 (ICOS-L), CD276 (B7–H3) and B7–H4. No surface expression for PD-L2, B7–H3, and B7–H4 was detected on cDC1s, cDC2s, pDCs, and B cells, and PD-L1 and ICOS-L expression was unaltered in the absence of MARCH1 (Fig. 4B).

3.4. Proteomic profiling of the plasma membrane of MARCH1- and MARCH8-deficient cDCs and B cells

To more comprehensively address the role of MARCH1 and MARCH8 in APC membrane proteostasis, we performed an unbiased proteomic screen where we compared the proteomes of subcellular microsomal fractions enriched in plasma membrane (PM) of WT versus *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDCs and B cells. We have previously shown this is a robust approach to identify differentially expressed PM proteins between closely related cell populations such as the two major cDC subtypes, cDC1s and cDC2s (Segura et al., 2010). To obtain sufficient numbers of primary cDCs for this purpose, they were expanded in WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice bearing a melanoma cell line that secretes the DC growth factor, Flt3L (Mach et al., 2000). The cDCs expanded using this approach are phenotypically and functionally equivalent to their counterparts in untreated mice (Segura et al., 2010). Splenic B cells were

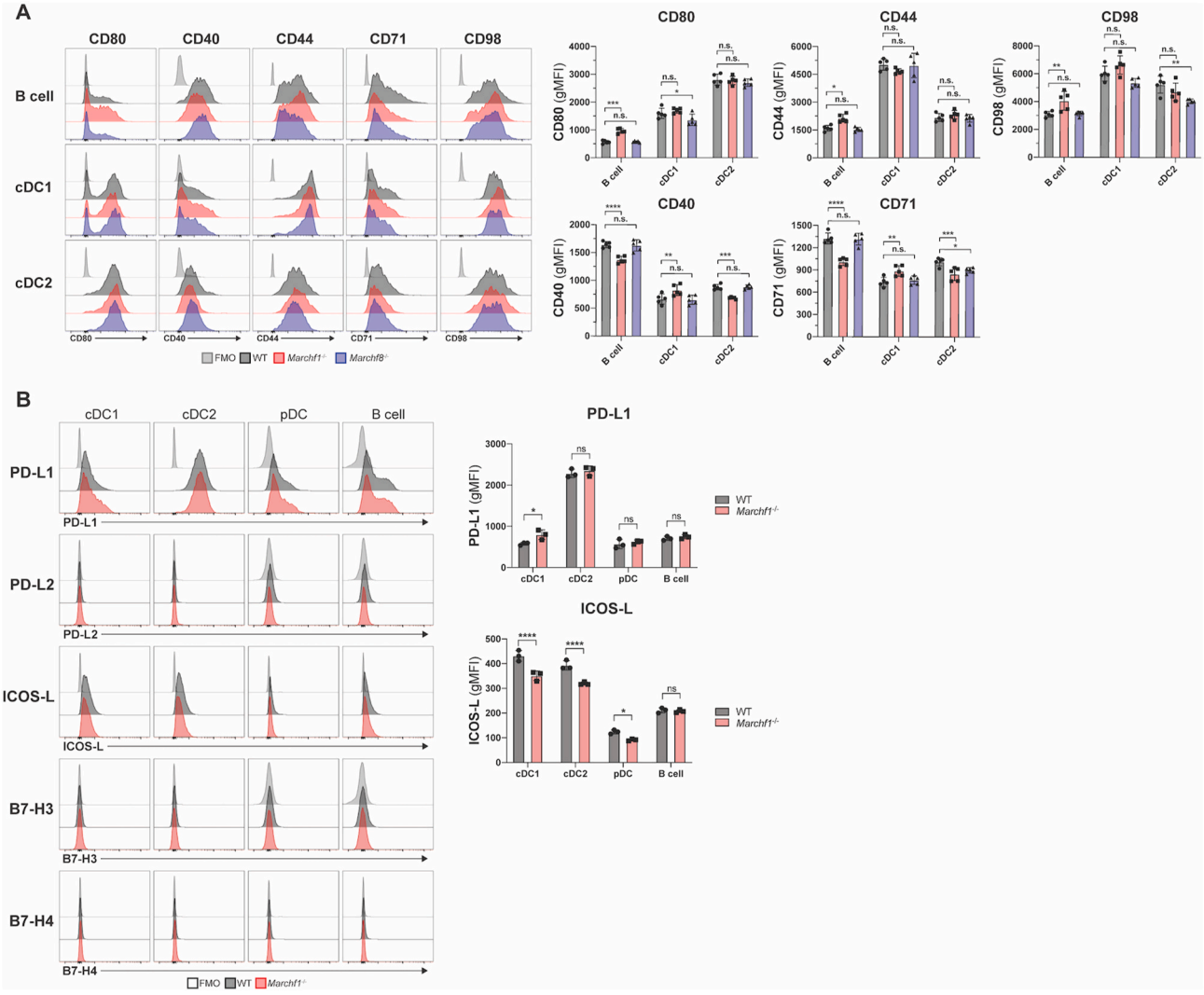


Fig. 4. Analysis of putative MARCH1 and MARCH8 substrates in haemopoietic APCs. (A) Surface expression of CD80, CD40, CD44, CD71, and CD98 in splenic B cells, cDC1s, and cDC2s from WT, *Marchf1*$^{-/-}$, and *Marchf8*$^{-/-}$ mice. (B) Surface expression of B7 costimulatory molecules, PD-L1, PD-L2, ICOS-L, B7–H3 and B7–H4, in splenic cDC1s, cDC2s, pDCs, and B cells purified from WT or *Marchf1*$^{-/-}$ mice. In all cases a fluorescence-minus-one (FMO) control was included, for which cells were incubated with the corresponding multi-colour staining panel, excluding the fluorescently labelled antibody species of interest. Bars represent mean ± SD with each symbol representing an individual mouse (n = 3–6). Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. ****$p < 0.0001$, ***$p < 0.0002$, **$p < 0.002$, *$p < 0.03$, n.s. not significant.

purified from untreated mice. The protein profiles of each fraction were identified by semi-quantitative mass spectrometry from three biological replicates, each measured in technical triplicates.

We identified 1868–3108 proteins in the PM fraction of each cell type (Supplementary Table 1, total number of identified proteins regardless of any restrictions). Of note, the subcellular PM fractions are comprised of microsomes generated during mechanical homogenization of cells, so their composition includes PM but also cytosolic and extracellular content 'trapped' inside microsomes or tethered to the cell surface. This method enables analysis of proteins loosely associated with the inner or outer leaflet of the PM. To test the efficiency of the PM-enrichment method, we also sedimented and analyzed in parallel the compartments that remained in the post-nuclear supernatant (PNS) of homogenized cells after retrieval of the PM fraction (mitochondria, endosomes, etc., henceforth termed intracellular compartments, IC). We identified 2073–3537 proteins in the IC fraction of each cell type

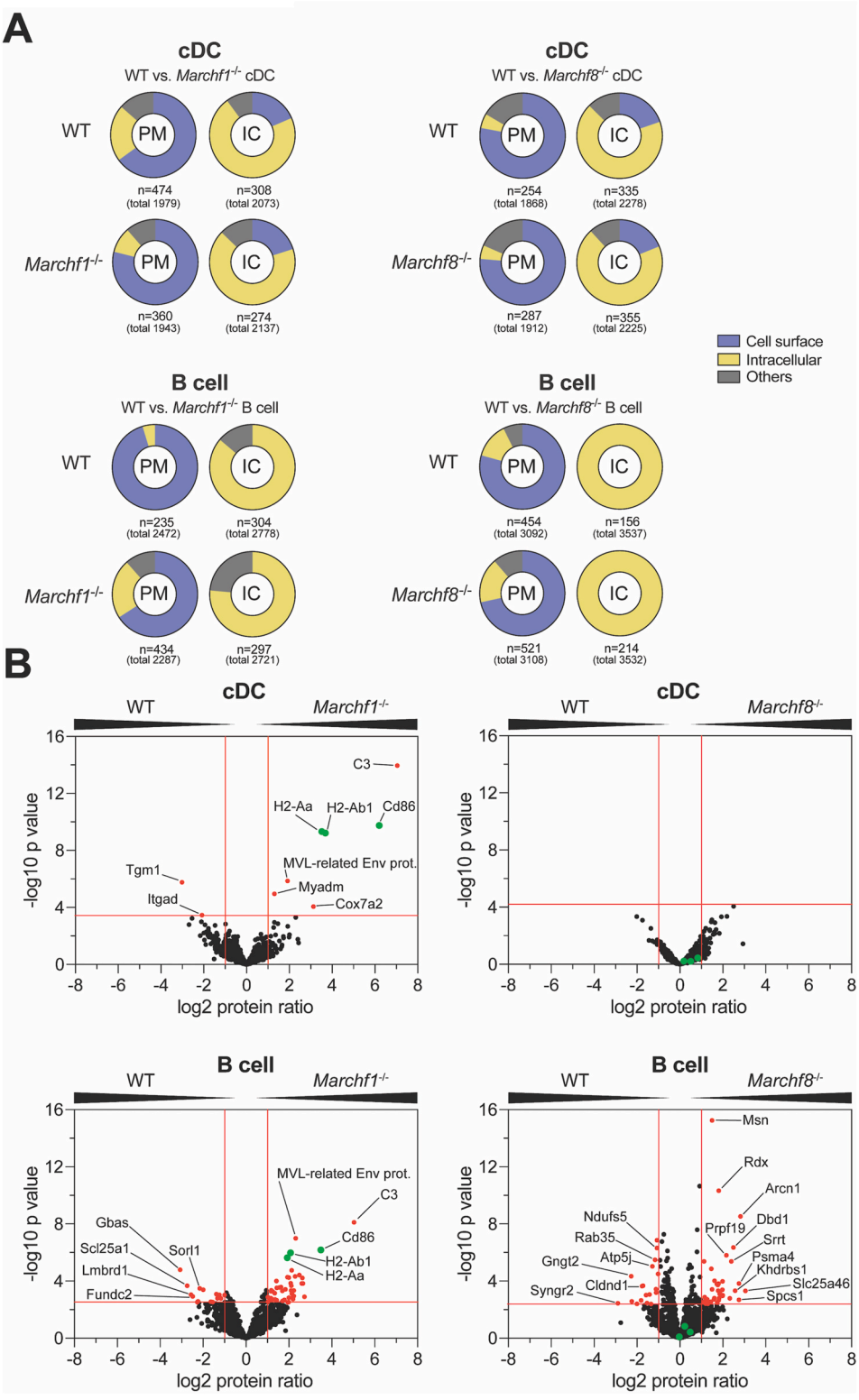


Fig. 5. Proteomic analysis of differentially expressed proteins in the plasma membrane between WT and *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDCs and B cells. PM fractions were purified from PNS of mAb surface stained cDCs and B cells via magnetic immunoaffinity and analyzed by semi-quantitative mass spectrometry from three biological replicates (in total 3x 8 samples; WT vs. *Marchf1*$^{-/-}$ and WT vs. *Marchf8*$^{-/-}$ cDCs as well as WT vs. *Marchf1*$^{-/-}$ and WT vs. *Marchf8*$^{-/-}$ B cells). The remaining compartments (mitochondria, endosomes, etc.) sedimented from the PNS of homogenized cells following PM fraction retrieval was termed intracellular compartment (IC). (A) Enrichment analysis (performed using the function enricher included in the Bioconductor clusterProfiler package (Yu et al., 2012) of detected proteins via MS from PM or IC fractions from cDCs and B cells of WT versus *Marchf1*$^{-/-}$ and WT versus *Marchf8*$^{-/-}$ mice. Only experimentally verified Gene Ontology (GO) annotations were used (n = XY) from all detected proteins (total number in parenthesis), and grouped into categories of 'Cell surface', 'Intracellular Compartment (IC)' and 'others'. 'Cell surface' category included the GO terms 'plasma membrane', 'external side of plasma membrane' and 'cell surface' among others, while the categories 'Intracellular Compartment (IC)' and 'others' included GO terms such as 'mitochondrial membrane' and 'endoplasmic reticulum' as well as 'myelin sheath', respectively. A detailed list of all annotated GO terms of all fractions can be found in Supplementary Fig. 4. (B) Detection of differentially expressed proteins in the PM fraction of cDCs and B cells of WT versus *Marchf1*$^{-/-}$ and WT versus *Marchf8*$^{-/-}$ mice. Equivalent amounts of PM fractions (based on cell count) of three biological replicates were analyzed by mass spectrometry and semi-quantitative proteomics in three technical replicates, for which the foldchange of the label-free quantification (LFQ) peptide peak intensities of each protein was used. Proteins detected in both WT and *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDCs or B cells were displayed in volcano plots (1020 proteins for WT vs. *Marchf1*$^{-/-}$ cDCs, 922 proteins for WT vs. *Marchf8*$^{-/-}$ cDCs, 1275 proteins for WT vs. *Marchf1*$^{-/-}$ B cells and 1819 proteins for WT vs. *Marchf8*$^{-/-}$ B cells) with differentially expressed proteins [red dots] identified based on two-fold ratio (log2 protein ratio >1 or <1) and significance (5% FDR) across three biological replicates, each measured in technical triplicates. The known MARCH1 substrates, MHC II (H2-Aa and H2-Ab1) and CD86 in B cells and cDCs, are highlighted in green in each volcano plot.

(Supplementary Table 2, total number of identified proteins regardless of any restrictions). In order to assess enrichment of the PM by this methodology, we compared Gene Ontology (GO) terms/annotations of the proteins identified in the PM and IC fractions of each cell type. This comparison clearly demonstrated enrichment of proteins known to be expressed at the cell surface in the PM fractions, and enrichment of proteins known to occur in intracellular compartments in the IC fractions, validating the subcellular fractionation protocol (Fig. 5A and Supplementary Fig. 4).

Comparison of the PM proteomes of WT and *Marchf1*$^{-/-}$ cDCs showed that, as expected, most proteins were present at similar levels in the two preparations (1020 proteins in total, Supplementary Table 3). Nine proteins were differentially expressed between WT and *Marchf1*$^{-/-}$ cDC PM [$\log_2$ protein ratio >1 or <1 and -log10 adjusted p value > 3.47 (5% FDR)] (Fig. 5B and Supplementary Table 5). These included MHC IIα and β chains (H2-Aa and H2-Ab1), as well as CD86, confirming the validity of our approach to detect MARCH1 substrates. Surprisingly, the protein that appeared most significantly overexpressed in the PM of *Marchf1*$^{-/-}$ cDCs was complement component 3 (C3) (Fig. 5B, Supplementary Table 5). This was due to covalent binding of constitutively activated C3 to MHC II molecules (Schriek et al., 2021). The remaining three proteins appearing over-expressed in the *Marchf1*$^{-/-}$ cDC PM fraction are not known to be immunoreceptors expressed at the PM: Cox7a2 is a mitochondrial protein, Myadm a component of the cytoskeleton, and MLV-related proviral Env polyprotein, a protein endogenously encoded by a retrovirus integrated in the genome of commonly used mouse strains (Stocking and Kozak, 2008). As our main goal was to identify immunoregulatory MARCH1 substrates, we did not investigate further whether these were true or false "hits" of the proteomic analysis. Comparison of the PM fractions of WT and *Marchf8*$^{-/-}$ cDCs did not reveal any differentially expressed proteins (Fig. 5B, 922 proteins in total, Supplementary Table 3), supporting the previous results indicating that MARCH8 is not expressed or active in cDCs.

Marchf1$^{-/-}$ and *Marchf8*$^{-/-}$ B cells exhibited 45 and 40 enriched and 15 and 17 reduced proteins, respectively, in their PM fractions [$\log_2$ protein ratio >1 or <1. and -log10 adjusted p value > 2.5 and > 2.36 for *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$, respectively (both 5% FDR)] (Fig. 5B, Supplementary Tables 6 and 7, 1275 and 1819 proteins in total, Supplementary Table 3). MHC IIα and β chains (H2-Aa and H2-Ab1), as well as CD86 and C3 were the most significantly enriched proteins in the PM fraction of *Marchf1*$^{-/-}$ B cells (Fig. 5B and Supplementary Table 6), but neither of the four were enriched in *Marchf8*$^{-/-}$ B cells (Fig. 5B and Supplementary Table 7). Only 14 of the 60 proteins differentially expressed in the PM fraction of *Marchf1*$^{-/-}$ B cells, and 10 of the 57 proteins differentially expressed in the PM fraction of *Marchf8*$^{-/-}$ B cells, were immunoreceptors and/or proteins known to be expressed at the plasma membrane (Supplementary Tables 6 and 7). They included aminopeptidase N (CD13, gene *Anpep*), antigen-presenting glycoprotein CD1d, T cell differentiation antigen CD6 and the immunoglobulin epsilon Fc receptor CD23 (gene *Fcer2*). However, analysis by flow cytometry did not confirm differential expression in either *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ B cells (Supplementary Fig. 5). The most likely explanation for detection of these "false positives" is that they were caused by subtle differences in the purity of the B cell preparations or their subcellular fractions. In conclusion, MHC class II and CD86 were the only membrane proteins that we could unequivocally confirm as MARCH1 substrates in B cells, and while we cannot discard the possibility that some of the "hits" found in the proteomic screen of *Marchf8*$^{-/-}$ B cells are indeed MARCH8 substrates, it is more likely that MARCH8 is not active in B cells, just as it is not in DCs.

3.5. MARCH8, not MARCH1, is active in non-hematopoietic cells

The only cell type in which MARCH8 activity has been demonstrated is thymic epithelial cells (TECs), where it regulates MHC II surface expression but not CD86 (Liu et al., 2016; von Rohrscheidt et al., 2016). Analysis of CD40, CD44, CD95 and CD98 expression in WT and *Marchf8*$^{-/-}$ medullary and cortical TECs showed that neither of these receptors, which have been shown to be ubiquitinated in cell lines overexpressing MARCH8, are physiological substrates (Fig. 6A).

Although TECs constitutively present antigens via MHC II, they are not hematopoietic cells, but of endodermal origin (Gordon et al., 2004). Therefore, we asked the question whether other cells ontogenically related to TECs also use MARCH8 to regulate surface MHC II expression. Epithelial cells in the respiratory tract are known to express MHC II, with the highest level found on type II alveolar epithelial cells (AECs) (Wosen et al., 2018; Nakano et al., 2018; Hasegawa et al., 2017). We found that MARCH8-deficient endothelial cells, bronchial epithelial cells and type I and II AECs showed enriched MHC II surface expression (Fig. 6B), though only in type II AECs this difference was clear and statistically significant (Fig. 6B). Neither cell type displayed increased CD86 expression in the absence of MARCH8, and lack of MARCH1 did not affect MHC II nor CD86 expression in any of the cell types analyzed (Fig. 6B). In conclusion, not all epithelial cells regulate MHC II expression via ubiquitination to the same extent, but those that do employ MARCH8.

4. Discussion

Determining which cells utilize MARCH1 and MARCH8 has been hampered by their low level of expression, but analysis of MHC II and CD86 as surrogate markers of activity has allowed us to establish the role of MARCH1 as a master regulator of MHC II and CD86 expression in all hematopoietic cells. MARCH8 plays an equivalent role in the two major types of TECs, in type II AECs and, probably, other epithelial cells, where it ubiquitinates MHC II. We did not observe high CD86 expression in any *Marchf8*$^{-/-}$ cell, but this could be because these cells do not ubiquitinate CD86 or because they do not express it.

There are at least two precedents for ontogeny-specific differences in the use of components of MHC II antigen presentation machinery. Expression of CIITA, which directs transcription of the genes for MHC II and for several accessory molecules involved in antigen presentation, is driven by distinct promoters in hematopoietic and non-hematopoietic cells (Reith and Mach, 2001). Proteolysis of the chaperone invariant chain, a critical step in the MHC II antigen presentation pathway, is carried out by cathepsin S in hematopoietic cells and by cathepsin L in non-hematopoietic cells (Villadangos and Ploegh, 2000). It is unclear why this dichotomy exists, which is probably caused by the establishment of cell lineage-specific gene programs during embryonic development.

While our finding that MARCH1 is operative in professional APCs confirmed previous observations, we were surprised to observe high MHC II and CD86 expression in non-professional APCs lacking MARCH1. This was not caused by ectopic induction or overexpression of either molecule because MARCH1-deficient T cells maintained WT levels of MHC II (negative) and CD86 (low) expression. As MARCH1 ubiquitinates substrates that have already trafficked through the cell surface, this finding implies that "atypical" APCs express and deposit on their PM larger amounts of MHC II and CD86 than is usually appreciated, but their steady-state levels are kept low by virtue of MARCH1 ubiquitination and accelerated turn-over. Eosinophils are associated with inflammatory responses during allergy or parasitic infections, while neutrophils are recruited in abundant numbers to sites of tissue damage or infection. The role of MHC II antigen presentation by either cell type is controversial. While there is evidence for both purified eosinophils and neutrophils that demonstrates their capacity to present antigen via MHC II (Kambayashi and Laufer, 2014), it is difficult to exclude the possibility of DC contamination in these assays. *In vivo* evidence of their antigen presentation capacity is scarce but there are reported examples where both eosinophils (Padigel et al., 2006) and neutrophils (Abi Abdallah et al., 2011; Vono et al., 2017; Lin and Loré, 2017) contribute to enhancing antigen-specific $CD4^+$ T cell responses.

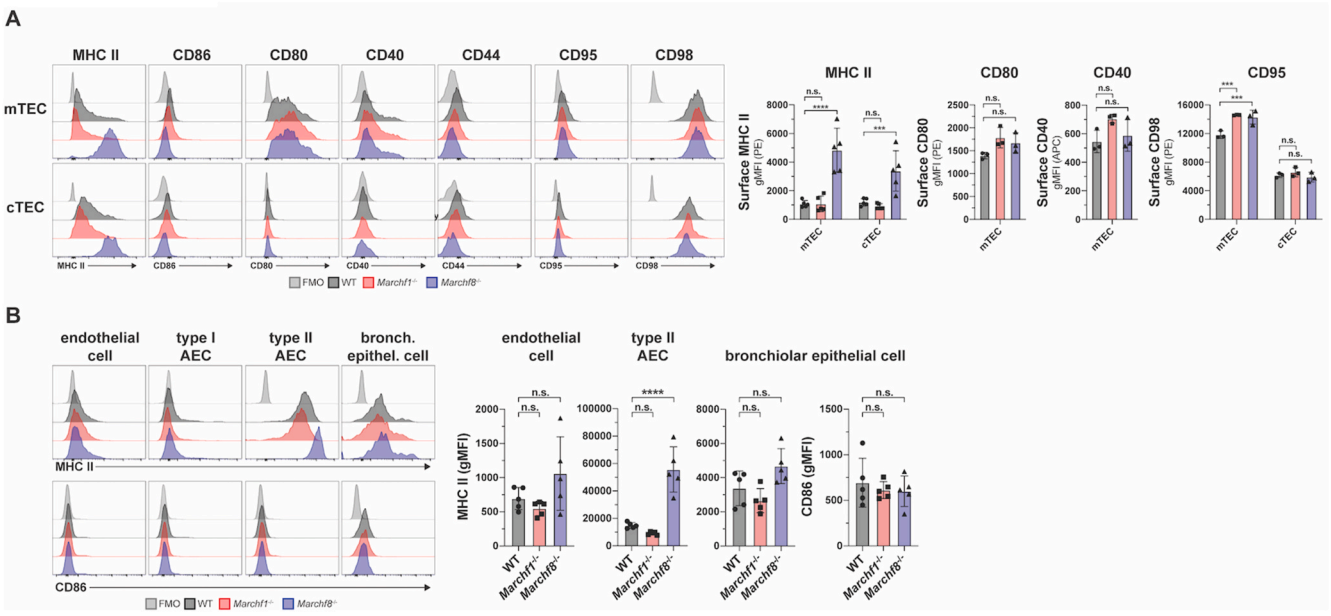


Fig. 6. Ubiquitination of MHC II, CD86 and putative substrates by MARCH1 and MARCH8 in non-haemopoietic APCs. (A) Surface expression of MHC II, CD86, CD80, CD40, CD44, CD95, and CD98 in medullary and cortical thymic epithelial cells (mTECs and cTECs) purified from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. (B) Surface expression of MHC II and CD86 in endothelial cells, type I and type II alveolar epithelial cells (AECs) as well as bronchiolar epithelial cells, purified from the lung of WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. In all cases a fluorescence-minus-one (FMO) control was included, for which cells were incubated with the corresponding multi-colour staining panel, excluding the fluorescently labelled antibody species of interest. Bars represent mean ± SD with each symbol representing an individual mouse (n = 5). Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. ****p < 0.0001, ***p < 0.0002, n.s. not significant.

The realization that these cells regulate MHC II and CD86 via ubiquitination utilizing the same mechanism as professional APCs lends weight to the notion that they perform antigen presentation *in vivo*.

One of the functions attributed to MARCH8 in humans is to ubiquitinate viral proteins deposited on the PM of infected cells and that will be incorporated in the envelop of the virion upon budding (Tada et al., 2015; Zhang et al., 2020; Kumar et al., 2019). The reduction of viral protein expression that ensues inhibits spread of the infection, protecting the host. This activity has not been described in mice, but our results suggest that if it occurs in this species, it is unlikely to be operative in hematopoietic cells, where perhaps other members of the MARCH family replace the function of MARCH8.

While several membrane proteins have been identified for MARCH1 and MARCH8 based on studies using overexpression and/or cell lines, our flow cytometry analysis rules out CD44, CD71, CD95 and CD98 as bona fide MARCH1 or MARCH8 substrates in all primary cells examined. This highlights that caution needs to be taken when interpreting studies that rely on E3 ligase overexpression. Our unbiased proteomic profiling of B cells and DCs unequivocally confirmed the role of MARCH1 in MHC II and CD86 ubiquitination in both cell types, but did not reveal any other MARCH1 substrate that we could validate by flow cytometry with the exception of complement C3. We have recently shown that C3 is not a MARCH1 substrate but constitutively forms covalent complexes with MHC II on the surface of APCs (Schriek et al., 2021). Its enhanced level on MARCH1-deficient DCs and B cells is therefore an indirect consequence of increased MHC II expression. We have also shown that lack of MHC II ubiquitination induces higher or lower expression of other surface receptors that are not direct MARCH1 substrates (Wilson et al., 2018). However, the magnitude of these changes (~less than 2-fold) is below the level of resolution afforded by high-throughput, unbiased proteomic analysis of subcellular fractions. Analyses of whole spleen cell lysates by western blot revealed increased expression of the cytosolic proteins MAVS, STING, TRAF3 and TRAF6 in MARCH1-deficient mice (Wu et al., 2020). We could not replicate these findings by mass spectrometry because our analysis focused on proteins associated with microsomal subcellular fractions, excluding the cytosol. MARCH ligases recognize their substrates via transmembrane region interactions (Liu et al., 2019; Trenker et al., 2021) so cytosolic proteins appear unlikely targets of MARCH1 ubiquitination. It is possible their altered abundance in whole spleens of mutant mice is due to changes in cellular composition or to indirect effects of MARCH1 deficiency on transcription programs in APCs (Kim et al., 2021). Confirmation that MAVS, STING, TRAF3 and TRAF6 are MARCH1 substrates will require direct assessment of their ubiquitination status in cells that do or do not express MARCH1.

We did not observe changes in expression of any protein on the PM of *Marchf8*$^{-/-}$ DCs. The "hits" detected in *Marchf8*$^{-/-}$ B cell PM by proteomics could be attributed to contamination with other subcellular compartments because they were not classified as PM proteins and/or could not be validated by flow cytometry as differentially expressed. The proteomic analysis thus confirmed that neither B cells nor DCs express functional MARCH8.

In summary, MHC II is the only membrane protein unequivocally regulated by MARCH1 and MARCH8 in primary mouse cells, with each ligase playing its role in haemopoietic and non-haemopoietic cells, respectively. CD86 is also a MARCH1 substrate in hematopoietic cells. These results help to predict the potential effects of genetic or pharmacological manipulation of MARCH1 or MARCH8 activities as a treatment for immunological disorders.

CRediT authorship contribution statement

Patrick Schriek: Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Haiyin Liu:** Methodology, Investigation. **Alan C. Ching:** Methodology, Investigation. **Pauline Huang:** Methodology, Investigation. **Nishma Gupta:** Methodology, Investigation. **Kayla R. Wilson:** Methodology, Investigation. **Min-Hsuang Tsai:** Methodology, Investigation. **Yuting Yan:** Methodology, Investigation. **Christophe F. Macri:** Methodology, Investigation. **Laura F. Dagley:** Methodology, Investigation. **Giuseppe Infusini:**

Methodology, Investigation. **Andrew I. Webb:** Methodology, Investigation. **Hamish E.G. McWilliam:** Methodology, Investigation, Funding acquisition. **Justine D. Mintern:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Jose A. Villadangos:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jose A. Villadangos Justine D. Mintern and Hamish E.G. McWilliam report financial support was provided by National Health and Medical Research Council. Jose A. Villadangos and Justine D. Mintern report financial support was provided by Australian Research Council. Jose A. Villadangos and Satoshi Ishido reports financial support was provided by Human Frontier Science Program. Patrick Schriek reports financial support was provided by Australian Government Department of Education Skills and Employment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2021.10.004.

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