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, whereas MARCH8 only operated in non-hematopoietic cells. Our results reveal that the range of cells constitutively endowed with antigen-presentation capacity is wider than generally appreciated. They also establish MARCH1 and MARCH8 as specialized regulators of CD4+ T cell immunity in two ontogenically distinct cellular compartments.

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 [1]. 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 [2]. They were initially identified as the mammalian homologues of herpesvirus immunoevasins that ubiquitinate host molecules involved in anti-viral immunity to subvert immune responses [3][4]. MARCH E3 Ub ligases are thought to be specialized at ubiquitinating immunoregulatory receptors, but their physiological substrates remain largely unknown [2][5]. It is also unclear if their expression and function is restricted to cells of the immune system and, if so, which.

MARCH1 and MARCH8 are the most studied members of the MARCH family. As they share approximately 60% overall sequence homology [2], 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 (APC) to display peptide antigens to $CD4^+$ T cells. By regulating MHC II expression [6][7][8], MARCH1 and MARCH8 play key roles in $CD4^+$ T cell development in the thymus [9][10][11] and activation in the periphery [12], respectively. Furthermore, they have been involved in complex immune reactions such as inflammation [13], immunity to infection [14][15], cancer [16], allergy and autoimmunity [17][18]. This poses the question whether both ligases regulate the expression of other immune receptors, some of which reportedly include CD44 [19], CD71 [20], CD86 [21], CD95 [22] and CD98 [19] among others [5][23]. However, to date CD86 is the only membrane protein apart from MHC II that has been shown to be regulated by MARCH1 *in vivo* [21], 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 [2][24][25][26], 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 [27][28], 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 [2] and fast turn-over [29][30], and even their transcription levels are poor predictors of function [24][25][26]. 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 (cDC and pDC, respectively) [6][7][8], but it is not functional in thymic epithelial cells (TEC) [9][10]. Whether it is active in other hematopoietic or non-hematopoietic cells remains unknown. In contrast, MARCH8 ubiquitinates MHC II in TEC, not in B cells or DC [9][10], 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 WT vs *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ plasma membrane purified from cDC 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.

MATERIALS AND METHODS

Mice

Wild type (WT, C57BL/6), *Marchf1*$^{-/-}$ [31], *Marchf8*$^{-/-}$ [9] and *I-Aα*$^{-/-}$ [32] 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 6-14 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.

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, DC, granulocytes, 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 spleen 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 cDC were further enriched by 1.077 g/cm^3 Nycodenz® density gradient centrifugation (Axis shield). 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 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). 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 Figures 1 and 2** summarize gating strategies for cells from blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung.

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 cDC were purified from mice subcutaneously injected with Flt3L-secreting melanoma cells [33], 9 days before purification. cDC were purified from spleens of Flt3L-expanded mice following spleen digestion with DNase I (Roche) and collagenase type III and Nycodenz® density gradient centrifugation (Axis shield) 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 [34]. Preparations were approximately 90-95% pure for $CD11c^+$ MHC II^+ cDC.

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

Subcellular fractionation was performed as previously described [35]. In brief, purified B cells (4-5 x 10^7 cells, 95-98% purity) and cDC (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 (cDC). 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.

Proteomic profiling of differentially expressed PM proteins

Subcellular fractions (PM and IC) were prepared for mass spectrometry analysis from three independent cell preparations using FASP protein digestion (Protein Discovery) as previously described [36], 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 [37]. 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 2 missed cleavages. The minimum required peptide length was 7 amino acids. The "match between runs" option in MaxQuant was used [38]. 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 2. 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 [39] 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 [40] followed by *eBayes* using the default settings [41] 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 [42]. Enrichment was calculated separately for the proteins overrepresented in each fraction, relative to all proteins identified in collected fractions across all the LCMS runs, and GO term association was filtered to include only experimental and high

200 throughput evidence. Enrichment P values were corrected for multiple testing using the
201 function's *'fdr'* method. The mass spectrometry proteomics data have been deposited to the
202 ProteomeXchange Consortium via the PRIDE [43]. The PRIDE database and related tools and
203 resources in 2019: improving support for quantification data. Nucleic Acids Res 47(D1):D442-
204 D450 partner repository with the dataset identifier *PXD023115*.

RESULTS

MARCH1, but not MARCH8, is functional in professional APC

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 [44]. 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 APC (defined as cells that express detectable levels of MHC II in the steady-state [45][46]) and T cells across various tissues. B cells, cDC1, cDC2, pDC and macrophages from blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung of *Marchf1*$^{-/-}$ mice displayed elevated surface MHC II and CD86 relative to WT cells, while no changes were observed in their *Marchf8*$^{-/-}$ counterparts (**Figure 1A-F**). CD4^{+} and CD8^{+} T cells in spleen and LN showed no detectable surface MHC II and their CD86 expression [47] was not altered by MARCH1- nor MARCH8-deficiency (**Figure 1B-C**). MHC II and CD86 expression in peritoneal cDC deficient in both MARCH1 and MARCH8 (*Marchf1*$^{-/-}$ x *Marchf8*$^{-/-}$) was not elevated above that of *Marchf1*$^{-/-}$ cells (**Supplementary Figure 3)**. These results indicate that MARCH1 is expressed and active in all professional APC 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 cDC, the archetypical professional APC. Toll-like receptor (TLR) ligands trigger an activation program in DC, known as DC maturation, that includes up-regulation of MHC II and CD86 expression on the plasma membrane, among other receptors [48]. 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 [6][49][50][51]. 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 cDC, it would be expected that the expression of these two

molecules would not vary during activation of *Marchf1*$^{-/-}$ cDC. However, activation of *Marchf1*$^{-/-}$ cDC further increased surface expression of MHC II by ~1.5 times, and increased CD86 by ~4 times, when compared than their resting counterparts (**Figure 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*$^{-/-}$ cDC at both resting and activated states, so up-regulation of MHC II and CD86 in *Marchf1*$^{-/-}$ cDC could not be attributed to overall dysregulation of surface receptor expression (**Figure 2**). These results indicate that the main contributor to MHC II and, especially, CD86 up-regulation during DC activation is not reduced ubiquitination and degradation, but sustained deposition of newly synthesized molecules on the cell surface [52][53]. DC lacking MARCH8 were indistinguishable from WT cDC in these experiments, again indicating it has no role in resting or activated cDC (**Figure 2**).

Granulocytes 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 APC", this is, immune cells that are not considered professional APC but have been suggested to play antigen-presenting roles under certain conditions [46]. 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 [54], they are not thought to perform antigen presenting functions in their undifferentiated state [55]. We examined these atypical APC 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 (**Figure 3**). Strikingly, all four cell types deficient in MARCH1 expressed MHC II at levels comparable to WT B cells or cDC (compare **Figures 1B** and **F** to **Figures 3A** and **B**, respectively), though expression was higher in spleen than it was in their lung counterparts (**Figure 3A** and **B)**. CD86 was also highly expressed on all four MARCH1-deficient cell types, in this case both in spleen and lungs (**Figure 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 (**Figure 3**). Of note, MARCH1-deficient T cells lacked surface MHC II and did not exhibit enriched CD86 expression when deficient in MARCH1 (**Figure 1B),** so neither mutation caused ectopic or increased expression of either molecule. We conclude that neutrophils, eosinophils, monocytes and possibly other atypical APC types [46] 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 APC might be capable of $CD4^+$ T cell priming under certain conditions.

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 [5][56]). 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*$^{-/-}$ cDC and B cells compared to WT cells (**Figure 4A**). Furthermore, *Marchf1*$^{-/-}$ cDC and B cells also expressed normal levels of the three receptors (**Figure 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. Expression of all these receptors on cDC1, cDC2, pDC and B cells was unaltered in the absence of MARCH1 (**Figure 4B**).

Proteomic profiling of the plasma membrane of MARCH1- and MARCH8-deficient cDC 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*$^{-/-}$ cDC 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, cDC1 and cDC2 [57]. To obtain sufficient numbers of primary cDC for this purpose, these cells were expanded in WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice bearing a melanoma cell line that secretes the DC growth factor, Flt3L [33]. The cDC expanded using this approach are phenotypically and functionally equivalent to their counterparts in untreated mice [57]. Splenic B cells were 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 IDed proteins regardless of any restrictions**)**. Of note, the subcellular 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 (**Supplementary Table 2**, total number of IDed 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 (**Figure 5A** and **Supplementary Figure 4)**.

Comparison of the PM proteomes of WT and *Marchf1*$^{-/-}$ cDC 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)] (**Figure 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*$^{-/-}$ cDC was complement component 3 (C3) (**Figure 5B, Supplementary Table 5**). 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 [58]. As our main goal was to identify immunoregulatory MARCH1 substrates, we did not investigate further whether these were true or artifactual "hits" of the proteomic analysis. Comparison of the PM fractions of WT and *Marchf8*$^{-/-}$ cDC did not reveal any differentially

expressed proteins (**Figure 5B,** 922 proteins in total, **Supplementary Table 3**), supporting the previous results indicating that MARCH8 is not expressed/active in cDC.

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)] (**Figure 5B, Supplementary Table 6** and **Supplementary Table 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 (**Figure 5B** and **Supplementary Table 6**), but neither of the four were enriched in *Marchf8*$^{-/-}$ B cells (**Figure 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 Table 6** and **Supplementary Table 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 Figure 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 DC.

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

The only cell type in which MARCH8 activity has been demonstrated is thymic epithelial cells (TEC), where it regulates MHC II surface expression but not CD86 [9][10]. Analysis of CD40, CD44, CD95 and CD98 expression in WT and *Marchf8*$^{-/-}$ medullar and cortical TEC showed that neither of these receptors, which have been shown to be ubiquitinated in cell lines overexpressing MARCH8, are physiological substrates (**Figure 6A**).

Although TEC constitutively present antigens via MHC II, they are not hematopoietic cells, but of endodermal origin [59]. Therefore, we asked the question whether other cells

ontogenically related to TEC 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 (AEC) [60][61][62]. We found that MARCH8-deficient type II AEC showed enriched MHC II surface expression (**Figure 6B**), but MHC II levels in mutant endothelial cells, type I AEC and bronchial epithelial cells was not altered (**Figure 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 (**Figure 6B**). In conclusion, not all epithelial cells regulate MHC II expression via ubiquitination, but those that do employ MARCH8.

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 TEC and in type II AEC, 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 [63]. 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 and cathepsin L in non-hematopoietic cells [64]. 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 APC confirmed previous observations, we were surprised to observe high MHC II and CD86 expression in non-professional APC 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 APC express and deposit on their plasma membrane 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 [46], 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 [65] and neutrophils [66][67][68] contribute to enhancing antigen-specific CD4^{+} T cell responses. The realization

that these cells regulate MHC II and CD86 via ubiquitination utilizing the same mechanism as professional APC 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 plasma membrane of infected cells and that will be incorporated in the envelop of the virion upon budding [69][70][71]. 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 substrates 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 Ub ligase overexpression. Our unbiased proteomic profiling of B cells and DC 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. Further investigations will be required to determine if enriched levels of surface C3 in these cells is a direct or indirect effect of MACRH1 deficiency, as we have also shown that high MHC II expression in *Marchf1*$^{-/-}$ cells indirectly induces higher or lower expression of other surface receptors that are not direct MARCH1 substrates [72]. However, the magnitude of these changes is below the level of resolution afforded by high-throughput, unbiased proteomic analysis of subcellular fractions. We did not observe changes in expression of any protein on the plasma membrane of *Marchf8*$^{-/-}$ DC. The "hits" detected *Marchf8*$^{-/-}$ B cell membrane could be attributed to contamination with other subcellular compartments because they were not classified as plasma membrane proteins and/or could not be validated as differentially expressed by flow cytometry. The proteomic analysis thus confirmed that neither B cells nor DC 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.

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REFERENCES

[1] D. Komander and M. Rape, "The Ubiquitin Code," *Annu. Rev. Biochem.*, vol. 81, no. 1, pp. 203–229, Jul. 2012, doi: 10.1146/annurev-biochem-060310-170328.

[2] H. Liu, J. D. Mintern, and J. A. Villadangos, "MARCH ligases in immunity," *Curr. Opin. Immunol.*, vol. 58, no. Box 1, pp. 38–43, Jun. 2019, doi: 10.1016/j.coi.2019.03.001.

[3] L. Coscoy, D. J. Sanchez, and D. Ganem, "A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition," *J. Cell Biol.*, vol. 155, no. 7, pp. 1265–1274, Dec. 2001, doi: 10.1083/jcb.200111010.

[4] S. Ishido, C. Wang, B.-S. Lee, G. B. Cohen, and J. U. Jung, "Downregulation of Major Histocompatibility Complex Class I Molecules by Kaposi's Sarcoma-Associated Herpesvirus K3 and K5 Proteins," *J. Virol.*, vol. 74, no. 11, pp. 5300–5309, Jun. 2000, doi: 10.1128/JVI.74.11.5300-5309.2000.

[5] T. Samji, S. Hong, and R. E. Means, "The Membrane Associated RING-CH Proteins: A Family of E3 Ligases with Diverse Roles through the Cell," *Int. Sch. Res. Not.*, vol. 2014, pp. 1–23, 2014, doi: 10.1155/2014/637295.

[6] L. J. Young *et al.*, "Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells," *Nat. Immunol.*, vol. 9, no. 11, pp. 1244–1252, Nov. 2008, doi: 10.1038/ni.1665.

[7] K.-J. Cho, E. Walseng, S. Ishido, and P. A. Roche, "Ubiquitination by March-I prevents MHC class II recycling and promotes MHC class II turnover in antigen-presenting cells," *Proc. Natl. Acad. Sci.*, vol. 112, no. 33, pp. 10449–10454, Aug. 2015, doi: 10.1073/pnas.1507981112.

[8] O. Bannard *et al.*, "Ubiquitin-mediated fluctuations in MHC class II facilitate efficient germinal center B cell responses," *J. Exp. Med.*, vol. 213, no. 6, pp. 993–1009, May 2016, doi: 10.1084/jem.20151682.

[9] H. Liu *et al.*, "Ubiquitin ligase MARCH 8 cooperates with CD83 to control surface MHC II expression in thymic epithelium and CD4 T cell selection," *J. Exp. Med.*, vol. 213, no. 9, pp. 1695–1703, Aug. 2016, doi: 10.1084/jem.20160312.

[10] J. von Rohrscheidt *et al.*, "Thymic CD4 T cell selection requires attenuation of March8-mediated MHCII turnover in cortical epithelial cells through CD83," *J. Exp. Med.*, vol. 213, no. 9, pp. 1685–1694, Aug. 2016, doi: 10.1084/jem.20160316.

[11] J. Oh *et al.*, "MARCH1-mediated MHCII ubiquitination promotes dendritic cell selection of natural regulatory T cells," *J. Exp. Med.*, vol. 210, no. 6, pp. 1069–1077, Jun. 2013, doi: 10.1084/jem.20122695.

[12] R. Ishikawa, M. Kajikawa, and S. Ishido, "Loss of MHC II ubiquitination inhibits the activation and differentiation of CD4 T cells," *Int. Immunol.*, vol. 26, no. 5, pp. 283–289, May 2014, doi: 10.1093/intimm/dxt066.

[13] T. Galbas *et al.*, "MARCH1 E3 Ubiquitin Ligase Dampens the Innate Inflammatory Response by Modulating Monocyte Functions in Mice," *J. Immunol.*, vol. 198, no. 2, pp. 852–861, Jan. 2017, doi: 10.4049/jimmunol.1601168.

[14] J. Wu *et al.*, "The E3 ubiquitin ligase MARCH1 regulates antimalaria immunity through interferon signaling and T cell activation," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 117, no. 28, pp. 16567–16578, 2020, doi: 10.1073/pnas.2004332117.

[15] Y. Zhang *et al.*, "Membrane-associated RING-CH (MARCH) 1 and 2 are MARCH family members that inhibit HIV-1 infection," *J. Biol. Chem.*, vol. 294, no. 10, pp. 3397–3405, Mar. 2019, doi: 10.1074/jbc.AC118.005907.

[16] L. Xie *et al.*, "MARCH1 encourages tumour progression of hepatocellular carcinoma via regulation of PI3K-AKT-β-catenin pathways," *J. Cell. Mol. Med.*, vol. 23, no. 5,

pp. 3386–3401, 2019, doi: 10.1111/jcmm.14235.

[17] O. A. Kishta *et al.*, "March1 E3 Ubiquitin Ligase Modulates Features of Allergic Asthma in an Ovalbumin-Induced Mouse Model of Lung Inflammation," *J. Immunol. Res.*, vol. 2018, pp. 1–17, 2018, doi: 10.1155/2018/3823910.

[18] M. Toyomoto, S. Ishido, N. Miyasaka, H. Sugimoto, and H. Kohsaka, "Anti-arthritic effect of E3 ubiquitin ligase, c-MIR, expression in the joints," *Int. Immunol.*, vol. 23, no. 3, pp. 177–183, Mar. 2011, doi: 10.1093/intimm/dxq470.

[19] C. A. Eyster, N. B. Cole, S. Petersen, K. Viswanathan, K. Früh, and J. G. Donaldson, "MARCH ubiquitin ligases alter the itinerary of clathrin-independent cargo from recycling to degradation," *Mol. Biol. Cell*, vol. 22, no. 17, pp. 3218–3230, Sep. 2011, doi: 10.1091/mbc.e10-11-0874.

[20] H. Fujita, Y. Iwabu, K. Tokunaga, and Y. Tanaka, "Membrane-associated RING-CH (MARCH) 8 mediates the ubiquitination and lysosomal degradation of the transferrin receptor," *J. Cell Sci.*, vol. 126, no. 13, pp. 2798–2809, 2013, doi: 10.1242/jcs.119909.

[21] K. Corcoran, M. Jabbour, C. Bhagwandin, M. J. Deymier, D. L. Theisen, and L. Lybarger, "Ubiquitin-mediated Regulation of CD86 Protein Expression by the Ubiquitin Ligase Membrane-associated RING-CH-1 (MARCH1)," *J. Biol. Chem.*, vol. 286, no. 43, pp. 37168–37180, Oct. 2011, doi: 10.1074/jbc.M110.204040.

[22] E. Bartee *et al.*, "Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins," *J. Virol.*, vol. 78, no. 3, pp. 1109–1120, 2004, doi: 10.1128/JVI.78.3.1109.

[23] E. Bartee, A. McCormack, and K. Früh, "Quantitative membrane proteomics reveals new cellular targets of viral immune modulators," *PLoS Pathog.*, vol. 2, no. 10, pp. 0975–0988, 2006, doi: 10.1371/journal.ppat.0020107.

[24] M. Jabbour, E. M. Campbell, H. Fares, and L. Lybarger, "Discrete Domains of MARCH1 Mediate Its Localization, Functional Interactions, and Posttranscriptional Control of Expression," *J. Immunol.*, vol. 183, no. 10, pp. 6500–6512, Nov. 2009, doi: 10.4049/jimmunol.0901521.

[25] S. Kaul, S. K. Mittal, and P. A. Roche, "A major isoform of the E3 ubiquitin ligase March-I in antigen-presenting cells has regulatory sequences within its gene," *J. Biol. Chem.*, vol. 293, no. 12, pp. 4478–4485, Mar. 2018, doi: 10.1074/jbc.RA118.001775.

[26] J. Thibodeau *et al.*, "Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes," *Eur. J. Immunol.*, vol. 38, no. 5, pp. 1225–1230, May 2008, doi: 10.1002/eji.200737902.

[27] B. Zhao *et al.*, "Protein engineering in the ubiquitin system: Tools for discovery and beyond," *Pharmacol. Rev.*, vol. 72, no. 2, pp. 380–413, 2020, doi: 10.1124/pr.118.015651.

[28] M. J. Edelmann, B. Nicholson, and B. M. Kessler, "Pharmacological targets in the ubiquitin system offer new ways of treating cancer, neurodegenerative disorders and infectious diseases.," *Expert Rev. Mol. Med.*, vol. 13, no. November, pp. 1–17, 2011, doi: 10.1017/s1462399411002031.

[29] M.-C. Bourgeois-Daigneault and J. Thibodeau, "Autoregulation of MARCH1 Expression by Dimerization and Autoubiquitination," *J. Immunol.*, vol. 188, no. 10, pp. 4959–4970, May 2012, doi: 10.4049/jimmunol.1102708.

[30] L. Lei, J. Bandola-Simon, and P. A. Roche, "Ubiquitin-conjugating enzyme E2 D1 (Ube2D1) mediates lysine-independent ubiquitination of the E3 ubiquitin ligase March-I," *J. Biol. Chem.*, vol. 293, no. 11, pp. 3904–3912, Mar. 2018, doi: 10.1074/jbc.RA117.001322.

[31] Y. Matsuki *et al.*, "Novel regulation of MHC class II function in B cells," *EMBO J.*, vol. 26, no. 3, pp. 846–854, Feb. 2007, doi: 10.1038/sj.emboj.7601556.

[32] F. Köntgen, G. Süss, C. Stewart, M. Steinmetz, and H. Bluethmann, "Targeted disruption of the MHC class II Aa gene in C57BL/6 mice.," *Int. Immunol.*, vol. 5, no. 8, pp. 957–64, Aug. 1993, doi: 10.1093/intimm/5.8.957.

[33] N. Mach, S. Gillessen, S. B. Wilson, C. Sheehan, M. Mihm, and G. Dranoff, "Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand.," *Cancer Res.*, vol. 60, no. 12, pp. 3239–46, Jun. 2000.

[34] D. Vremec, "The Isolation of Mouse Dendritic Cells from Lymphoid Tissues and the Identification of Dendritic Cell Subtypes by Multiparameter Flow Cytometry," in *Methods in molecular biology (Clifton, N.J.)*, vol. 595, no. 11–12, 2010, pp. 205–229.

[35] E. Segura, A. L. Albiston, I. P. Wicks, S. Y. Chai, and J. A. Villadangos, "Different cross-presentation pathways in steady-state and inflammatory dendritic cells," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 48, pp. 20377–20381, 2009, doi: 10.1073/pnas.0910295106.

[36] J. R. Wiśniewski, A. Zougman, N. Nagaraj, and M. Mann, "Universal sample preparation method for proteome analysis," *Nat. Methods*, vol. 6, no. 5, pp. 359–362, May 2009, doi: 10.1038/nmeth.1322.

[37] J. Cox, N. Neuhauser, A. Michalski, R. A. Scheltema, J. V. Olsen, and M. Mann, "Andromeda: A peptide search engine integrated into the MaxQuant environment," *J. Proteome Res.*, vol. 10, no. 4, pp. 1794–1805, 2011, doi: 10.1021/pr101065j.

[38] J. Cox and M. Mann, "MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification," *Nat. Biotechnol.*, vol. 26, no. 12, pp. 1367–1372, 2008, doi: 10.1038/nbt.1511.

[39] J. Cox, M. Y. Hein, C. A. Luber, I. Paron, N. Nagaraj, and M. Mann, "Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ," *Mol. Cell. Proteomics*, vol. 13, no. 9, pp. 2513–2526, 2014, doi: 10.1074/mcp.M113.031591.

[40] M. E. Ritchie *et al.*, "Limma powers differential expression analyses for RNA-sequencing and microarray studies," *Nucleic Acids Res.*, vol. 43, no. 7, p. e47, 2015, doi: 10.1093/nar/gkv007.

[41] B. Phipson, S. Lee, I. J. Majewski, W. S. Alexander, and G. K. Smyth, "Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression," *Ann. Appl. Stat.*, vol. 10, no. 2, pp. 946–963, Jun. 2016, doi: 10.1214/16-AOAS920.

[42] G. Yu, L. G. Wang, Y. Han, and Q. Y. He, "ClusterProfiler: An R package for comparing biological themes among gene clusters," *Omi. A J. Integr. Biol.*, vol. 16, no. 5, pp. 284–287, 2012, doi: 10.1089/omi.2011.0118.

[43] Y. Perez-Riverol *et al.*, "The PRIDE database and related tools and resources in 2019: Improving support for quantification data," *Nucleic Acids Res.*, vol. 47, no. D1, pp. D442–D450, 2019, doi: 10.1093/nar/gky1106.

[44] M. Ohmura-Hoshino *et al.*, "Inhibition of MHC class II expression and immune responses by c-MIR.," *J. Immunol.*, vol. 177, no. 1, pp. 341–54, Jul. 2006, doi: 10.4049/jimmunol.177.1.341.

[45] O. Lassila, O. Vainio, and P. Matzinger, "Can B cells turn on virgin T cells?," *Nature*, vol. 334, no. 6179, pp. 253–255, 1988, doi: 10.1038/334253a0.

[46] T. Kambayashi and T. M. Laufer, "Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?," *Nat. Rev. Immunol.*, vol. 14,

no. 11, pp. 719–730, Nov. 2014, doi: 10.1038/nri3754.

[47] J. Eberlein, B. Davenport, T. T. Nguyen, F. Victorino, T. Sparwasser, and D. Homann, "Multiple Layers of CD80/86-Dependent Costimulatory Activity Regulate Primary, Memory, and Secondary Lymphocytic Choriomeningitis Virus-Specific T Cell Immunity," *J. Virol.*, vol. 86, no. 4, pp. 1955–1970, 2012, doi: 10.1128/jvi.05949-11.

[48] R. Spörri and C. Reis e Sousa, "Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function," *Nat. Immunol.*, vol. 6, no. 2, pp. 163–170, 2005, doi: 10.1038/ni1162.

[49] J. Vega-Ramos, A. Roquilly, Y. Zhan, L. J. Young, J. D. Mintern, and J. A. Villadangos, "Inflammation Conditions Mature Dendritic Cells To Retain the Capacity To Present New Antigens but with Altered Cytokine Secretion Function," *J. Immunol.*, vol. 193, no. 8, pp. 3851–3859, 2014, doi: 10.4049/jimmunol.1303215.

[50] A. De Gassart *et al.*, "MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation," *Proc. Natl. Acad. Sci.*, vol. 105, no. 9, pp. 3491–3496, Mar. 2008, doi: 10.1073/pnas.0708874105.

[51] T. Galbas and J. Thibodeau, "Cell-type specific regulation of MARCH1 E3 ubiquitin ligase by the anti-inflammatory cytokine IL-10," *Open J. Immunol.*, vol. 02, no. 04, pp. 161–167, 2012, doi: 10.4236/oji.2012.24020.

[52] J. A. Villadangos *et al.*, "MHC Class II Expression Is Regulated in Dendritic Cells Independently of Invariant Chain Degradation," *Immunity*, vol. 14, no. 6, pp. 739–749, Jun. 2001, doi: 10.1016/S1074-7613(01)00148-0.

[53] N. S. Wilson, D. El-Sukkari, and J. A. Villadangos, "Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis," *Blood*, vol. 103, no. 6, pp. 2187–2195, Mar. 2004, doi: 10.1182/blood-2003-08-2729.

[54] C. Auffray, M. H. Sieweke, and F. Geissmann, "Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells," *Annu. Rev. Immunol.*, vol. 27, no. 1, pp. 669–692, 2009, doi: 10.1146/annurev.immunol.021908.132557.

[55] C. V. Jakubzick, G. J. Randolph, and P. M. Henson, "Monocyte differentiation and antigen-presenting functions," *Nat. Rev. Immunol.*, vol. 17, no. 6, pp. 349–362, Jun. 2017, doi: 10.1038/nri.2017.28.

[56] J. Bauer, O. Bakke, and J. P. Morth, "Overview of the membrane-associated RING-CH (MARCH) E3 ligase family," *N. Biotechnol.*, vol. 38, pp. 7–15, Sep. 2017, doi: 10.1016/j.nbt.2016.12.002.

[57] E. Segura *et al.*, "Differential expression of pathogen-recognition molecules between dendritic cell subsets revealed by plasma membrane proteomic analysis," *Mol. Immunol.*, vol. 47, no. 9, pp. 1765–1773, May 2010, doi: 10.1016/j.molimm.2010.02.028.

[58] C. Stocking and C. A. Kozak, "Endogenous retroviruses," *Cell. Mol. Life Sci.*, vol. 65, no. 21, pp. 3383–3398, Nov. 2008, doi: 10.1007/s00018-008-8497-0.

[59] J. Gordon *et al.*, "Functional evidence for a single endodermal origin for the thymic epithelium," *Nat. Immunol.*, vol. 5, no. 5, pp. 546–553, 2004, doi: 10.1038/ni1064.

[60] J. E. Wosen, D. Mukhopadhyay, C. Macaubas, and E. D. Mellins, "Epithelial MHC Class II Expression and Its Role in Antigen Presentation in the Gastrointestinal and Respiratory Tracts," *Front. Immunol.*, vol. 9, no. SEP, pp. 1–14, Sep. 2018, doi: 10.3389/fimmu.2018.02144.

[61] H. Nakano, K. Nakano, and D. N. Cook, "Isolation and Purification of Epithelial and Endothelial Cells from Mouse Lung," in *Methods in Molecular Biology*, vol. 1799, R. L. Reinhardt, Ed. New York, NY: Springer New York, 2018, pp. 59–69.

[62] K. Hasegawa *et al.*, "Fraction of MHCII and EpCAM expression characterizes distal

lung epithelial cells for alveolar type 2 cell isolation," *Respir. Res.*, vol. 18, no. 1, p. 150, Dec. 2017, doi: 10.1186/s12931-017-0635-5.

[63] W. Reith and B. Mach, "The Bare Lymphocyte Syndrome and the Regulation of MHC Expression," *Annu. Rev. Immunol.*, vol. 19, no. 1, pp. 331–373, Apr. 2001, doi: 10.1146/annurev.immunol.19.1.331.

[64] J. A. Villadangos and H. L. Ploegh, "Proteolysis in MHC class II antigen presentation: Who's in charge?," *Immunity*, vol. 12, no. 3, pp. 233–239, 2000, doi: 10.1016/S1074-7613(00)80176-4.

[65] U. M. Padigel, J. J. Lee, T. J. Nolan, G. A. Schad, and D. Abraham, "Eosinophils Can Function as Antigen-Presenting Cells To Induce Primary and Secondary Immune Responses to Strongyloides stercoralis," *Infect. Immun.*, vol. 74, no. 6, pp. 3232–3238, Jun. 2006, doi: 10.1128/IAI.02067-05.

[66] D. S. Abi Abdallah, C. E. Egan, B. A. Butcher, and E. Y. Denkers, "Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation," *Int. Immunol.*, vol. 23, no. 5, pp. 317–326, May 2011, doi: 10.1093/intimm/dxr007.

[67] M. Vono, A. Lin, A. Norrby-Teglund, R. A. Koup, F. Liang, and K. Loré, "Neutrophils acquire the capacity for antigen presentation to memory CD4+ T cells in vitro and ex vivo," *Blood*, vol. 129, no. 14, pp. 1991–2001, Apr. 2017, doi: 10.1182/blood-2016-10-744441.

[68] A. Lin and K. Loré, "Granulocytes: New Members of the Antigen-Presenting Cell Family," *Front. Immunol.*, vol. 8, no. DEC, pp. 1–8, Dec. 2017, doi: 10.3389/fimmu.2017.01781.

[69] T. Tada *et al.*, "MARCH8 inhibits HIV-1 infection by reducing virion incorporation of envelope glycoproteins," *Nat. Med.*, vol. 21, no. 12, pp. 1502–1507, Dec. 2015, doi: 10.1038/nm.3956.

[70] Y. Zhang, T. Tada, S. Ozono, S. Kishigami, H. Fujita, and K. Tokunaga, "MARCH8 inhibits viral infection by two different mechanisms," *Elife*, vol. 9, pp. 1–14, Aug. 2020, doi: 10.7554/eLife.57763.

[71] S. Kumar *et al.*, "MARCH8 Ubiquitinates the Hepatitis C Virus Nonstructural 2 Protein and Mediates Viral Envelopment," *Cell Rep.*, vol. 26, no. 7, pp. 1800-1814.e5, 2019, doi: 10.1016/j.celrep.2019.01.075.

[72] K. R. Wilson *et al.*, "MARCH1-mediated ubiquitination of MHC II impacts the MHC I antigen presentation pathway," *PLoS One*, vol. 13, no. 7, p. e0200540, Jul. 2018, doi: 10.1371/journal.pone.0200540.

[73] S. E. Liyanage *et al.*, "Flow cytometric analysis of inflammatory and resident myeloid populations in mouse ocular inflammatory models.," *Exp. Eye Res.*, vol. 151, pp. 160–70, 2016, doi: 10.1016/j.exer.2016.08.007.

[74] L. Ardouin *et al.*, "Broad and Largely Concordant Molecular Changes Characterize Tolerogenic and Immunogenic Dendritic Cell Maturation in Thymus and Periphery," *Immunity*, vol. 45, no. 2, pp. 305–318, Aug. 2016, doi: 10.1016/j.immuni.2016.07.019.

[75] C. C. Bain *et al.*, "Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities," *Nat. Commun.*, vol. 7, no. 1, p. ncomms11852, Sep. 2016, doi: 10.1038/ncomms11852.

[76] F. R. Svedberg *et al.*, "The lung environment controls alveolar macrophage metabolism and responsiveness in type 2 inflammation," *Nat. Immunol.*, vol. 20, no. 5, pp. 571–580, 2019, doi: 10.1038/s41590-019-0352-y.

FIGURE LEGENDS

Figure 1. Ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in haemopoietic professional antigen presenting cells. Surface expression of MHC II and CD86 in (A) blood B cells, (B) splenic cDC1, cDC2, pDC, B cells, macrophages (MAC) and $CD4^+/CD8^+$ T cells, (C) resident and migratory cDC1 and cDC2 as well as B cells, macrophages and $CD4^+/CD8^+$ T cells in subcutaneous (axillary + inguinal) lymph nodes, (D) thymic cDC1, cDC2 and B cells, (D) peritoneal cDC1, cDC2, B cells and small/large macrophages and (E) lung cDC1, cDC2, pDC, B cells and alveolar/interstitial macrophages, all purified from WT mice or mice deficient in either MARCH1 or MARCH8. 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 (i.e. anti-CD86 or anti-MHC II mAb). 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.

Figure 2. The role of ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in DC maturation. (A) Surface expression of MHC II and CD86 in CpG-activated cDC purified from the spleen of WT mice or mice deficient in either MARCH1 or MARCH8. Purified splenic cDC ($2x10^5$ cells) were incubated for 16 hours *ex vivo* with or without 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. **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.002$, * $p < 0.03$, n.s. not significant.

Figure 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

741 one-way ANOVA followed by Sidak's multiple comparisons test. **** $p < 0.0001$, *** $p <$
742 0.0002, ** $p < 0.002$, * $p < 0.03$, n.s. not significant.
743
744 **Figure 4. Analysis of putative MARCH1 and MARCH8 substrates in haemopoietic**
745 **antigen presenting cells.** (A) Surface expression of MHC II, CD86, CD80, CD40, CD44,
746 CD71 and CD98 in splenic B cells, cDC1 and cDC2 from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice.
747 (B) Surface expression of B7 costimulatory molecules, PD-L1, PD-L2, ICOS-L, B7-H3 and
748 B7-H4, in splenic cDC1, cDC2, pDC and B cells purified from WT or *Marchf1*$^{-/-}$ mice. In all
749 cases a fluorescence-minus-one (FMO) control was included, for which cells were incubated
750 with the corresponding multi-colour staining panel, excluding the fluorescently labelled
751 antibody species of interest. Bars represent mean ± SD with each symbol representing an
752 individual mouse (n=3-6). Statistical analysis was performed using one-way ANOVA followed
753 by Sidak's multiple comparisons test. **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.002$, * $p <$
754 0.03, n.s. not significant.
755
756 **Figure 5. Proteomic analysis of differentially expressed proteins in the plasma membrane**
757 **fraction between WT and *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDC and B cells**. Proteomic analysis of
758 plasma membrane (PM)-enriched microsome fractions of splenic cDC and B cells purified
759 from WT, *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ mice. PM fractions were purified from post-nuclear
760 supernatants of mAb surface stained cDC and B cells via magnetic immunoaffinity and
761 analysed by semi-quantitative mass spectrometry from three biological replicates (in total 3x 8
762 samples; WT vs. *Marchf1*$^{-/-}$ and WT vs. *Marchf8*$^{-/-}$ cDC + WT vs. *Marchf1*$^{-/-}$ and WT vs.
763 *Marchf8*$^{-/-}$ B cells). The remaining compartments (mitochondria, endosomes, etc.) from the
764 post-nuclear supernatant of homogenized cells following PM fraction retrieval was termed
765 intracellular compartment (IC). (A) Enrichment analysis (performed using the function
766 enricher included in the Bioconductor clusterProfiler package [42]) of detected proteins via
767 MS from PM or IC fractions from cDC and B cells of WT versus *Marchf1*$^{-/-}$ and WT versus
768 *Marchf8*$^{-/-}$ mice. Annotated GO-IDs for detected proteins were grouped into categories of 'Cell
769 surface', 'Intracellular Compartment (IC)' and 'others' based on experimentally verified Gene
770 Ontology (GO) annotations. 'Cell surface' category included the GO terms 'plasma
771 membrane', 'external side of plasma membrane' and 'cell surface' among others, while the
772 categories 'Intracellular Compartment (IC)' and 'others' included GO terms such as
773 'mitochondrial membrane' and 'endoplasmic reticulum' as well as 'myelin sheath',
774 respectively. For a detailed list of all annotated GO terms of all fractions please see

Supplementary Figure 4. (B) Detection of differentially expressed proteins in the PM fraction of cDC 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. Proteins detected in both WT and *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDC/B cells were displayed in volcano plots (1020 proteins for WT vs. *Marchf1*$^{-/-}$ cDC, 922 proteins for WT vs. *Marchf8*$^{-/-}$ cDC, 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 cDC, are highlighted in green in each volcano plot.

Figure 6. Ubiquitination of MHC II, CD86 and putative substrates by MARCH1 and MARCH8 in non-haemopoietic antigen presenting cells. (A) Surface expression of MHC II, CD86, CD80, CD40, CD44, CD95 and CD98 in medullary and cortical thymic epithelial cells (mTEC and cTEC) 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 (AEC) 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. **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.002$, * $p < 0.03$, n.s. not significant.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

Representative flow cytometry gating strategies for the identification of cell populations of interest in blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung. In all cases cell doublets and dead cells were identified and excluded based on forward and side scatter (FSC and SSC) as well as staining with propidium iodide (PI), diamidino phenylindole (DAPI) or Fixable Viability Dye eFluor™780 (Viability). (A) Blood B cells were identified as $CD19^+$ $B220^+$. (B) Splenic B cells, macrophages and T cells were identified from whole splenocyte suspensions as $CD19^+$ $B220^+$, $F4/80^+$ $CD64^+$ and $TCR\beta^+$ $CD3^+$ respectively with further discrimination of $CD4^+$ and $CD8^+$ T cells. Splenic DC were identified from low-density splenocyte suspensions, with pDC identified as Siglec-H^+ BST-2^+ and cDC as $B220^-$ $CD19^-$ $CD11c^+$ MHC II^+, with further discrimination of cDC1 as $CD11b^-$ $CD8^+$ and cDC2 as $CD11b^+$ $CD8^-$. Splenic granulocytes and monocytes were identified from whole splenocyte suspensions as $B220^-$ $CD3^-$ $CD4^-$ $CD8^-$ $CD11c^{low\text{-}mid}$ $CD11b^{high}$ with neutrophils identified as $Ly6G^+$, eosinophils as $Ly6G^-$ SSC-H^{high} $Ly6C^{low\text{-}mid}$, patrolling monocytes as $Ly6G^-$ $Ly6C^{low\text{-}mid}$ SSC-H^{low} and inflammatory monocytes as $Ly6G^-$ $Ly6C^{high}$ SSC-H^{low} (as described in Liyanage *et al*. [73]). (C) cDC from subcutaneous LN were identified from low-density cell suspensions, with resident cDC identified as $CD11c^{high}$ MHC II^{mid} and migratory cDC identified as $CD11c^{mid}$ MHC II^{high} and further discrimination of cDC1 as $Sirp\alpha^-$ XCR1+ and cDC2 as $Sirp\alpha^+$ $XCR1^-$. B cells, macrophages and T cells from subcutaneous LN were identified from whole cell suspensions as $CD19^+$ $B220^+$, $F4/80^+$ MHC II^+ and $CD3^+$ respectively with further discrimination of $CD4^+$ and $CD8^+$ T cells. (D) Thymic cDC were identified from low-density cell suspensions as $B220^-$ $NK1.1^-$ $CD11c^+$ MHC II^+, with further discrimination of cDC1 as $Sirp\alpha^-$ $XCR1^+$ and cDC2 as Sirpα+ XCR1- (as described in Ardouin *et al*. [74]). Thymic epithelial cells (TECs) were identified from whole thymocyte suspensions as $CD45^-$ $EpCAM^+$, with further discrimination of cortical TECs (cTECs) as UEA-1^- $Ly51^+$ and medullary TECs (mTECs) as UEA-1^+ $Ly51^-$ (as described in Liu *et al*. [9]). (D) Peritoneal macrophages were identified as $CD11b^+$ $MerTK^+$, with further discrimination of small peritoneal macrophages as $F4/80^{low}$ MHC II^{high} and large peritoneal macrophages as $F4/80^{high}$ MHC $II^{mid\text{-}high}$ (as described in Bain *et al*. [75]). Peritoneal B cells were identified as $MerTK^-$ MHC II^+ $CD19^+$ and cDC as $MerTK^-$ $CD19^-$ $CD11c^+$ MHC II^+ with further discrimination of cDC1 as $CD11b^-$ $XCR1^+$ and cDC2 as $CD11b^+$ $XCR1^-$. (E) Haemopoietic cells in the lung were identified as $CD45^+$ with pDC as $CD11c^{low\text{-}mid}$ BST-2^+ and macrophages as $CD64^+$ $MerTK^+$,

with further discrimination of interstitial macrophages as Siglec-F^{low} CD11b^{high} and alveolar macrophages as Siglec-F^{high} CD11b^{mid} (as described in Svedberg *et al*. [76]). Lung B cells were identified as CD64^{-} MerTK^{-} CD11c^{-} MHC II^{+} CD19^{+} and cDC as CD64^{-} MerTK^{-} CD11c^{+} MHC II^{+} with further discrimination of cDC1 as CD11b^{-} XCR1^{+} and cDC2 as CD11b^{+} XCR1^{-}. Lung granulocytes and monocytes were identified as B220^{-} CD3^{-} CD4^{-} CD8^{-} CD11c$^{low-mid}$ CD11b^{high} with neutrophils identified as Ly6G^{+}, eosinophils as Ly6G^{-} SSC-H^{high} Ly6C$^{low-mid}$, patrolling monocytes as Ly6G^{-} Ly6C$^{low-mid}$ SSC-H^{low} and inflammatory monocytes as Ly6G^{-} Ly6C^{high} SSC-H^{low} (as described in Liyanage *et al*. [73]). Non-haemopoietic cells in the lung were identified as CD45^{-} with endothelial cells identified as EpCAM$^{low-mid}$ CD31^{+} Sca-1^{+} and epithelial cells as EpCAM$^{mid-high}$ CD31^{-}. Further discrimination of epithelial cells was carried out based of CD24, EpCAM and MHC II expression (as described in Nakano *et al*. [61] and Hasegawa *et al*. [62]) with bronchiolar epithelial cells identified as EpCAM^{high} CD24^{high}, type II alveolar epithelial cells (AEC) identified as EpCAM^{high} CD24^{mid} MHC II^{high} (red) and type I AEC identified as EpCAM^{mid} CD24^{low} MHC II^{low} (green).

A comparison of the representative flow cytometry gating strategies for the identification of all cell populations of interest between WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice is shown in Supplementary Figure 2.

Supplementary Figure 2

Comparison of representative flow cytometry gating strategies for the identification of various cell populations in (A) blood, (B) spleen, (C) subcutaneous lymph node, (D) thymus, (E) peritoneal cavity and (F) lung from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. A detailed description of the gating strategies for each individual cell population of interest is presented in Supplementary Figure 1.

Supplementary Figure 3

Surface expression of MHC II and CD86 in peritoneal cDC from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice or from mice deficient in both MARCH1 and MARCH8 (*Marchf1*$^{-/-}$ x *Marchf8*$^{-/-}$). 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=4). 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.

867

868 **Supplementary Figure 4**

869 Gene ontology (GO) enrichment analysis of proteins detected in plasma membrane (PM)-
870 enriched and intracellular compartment (IC)-enriched microsome fractions of splenic cDC and
871 B cells purified from WT, *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ mice. PM fractions were purified from post-
872 nuclear supernatants of mAb surface stained cDC and B cells via magnetic immunoaffinity. IC
873 (intracellular compartments) was retrieved from the post-nuclear supernatant of homogenized
874 cells following PM fraction extraction. IC and PM fraction were analysed by semi-quantitative
875 mass spectrometry and GO term enrichment analysis was performed using the Bioconductor
876 clusterProfiler package [42] with GO-IDs grouped based on experimentally verified Gene
877 Ontology (GO) annotations.

878

879 **Supplementary Figure 5**

880 Surface expression of CD13, CD1d, CD6 and CD23 in B cells from WT, *Marchf1*$^{-/-}$ and
881 *Marchf8*$^{-/-}$ mice. A fluorescence-minus-one (FMO) control was included, for which cells were
882 incubated with the corresponding multi-colour staining panel, excluding the fluorescently
883 labelled antibody species of interest.

Figure 1

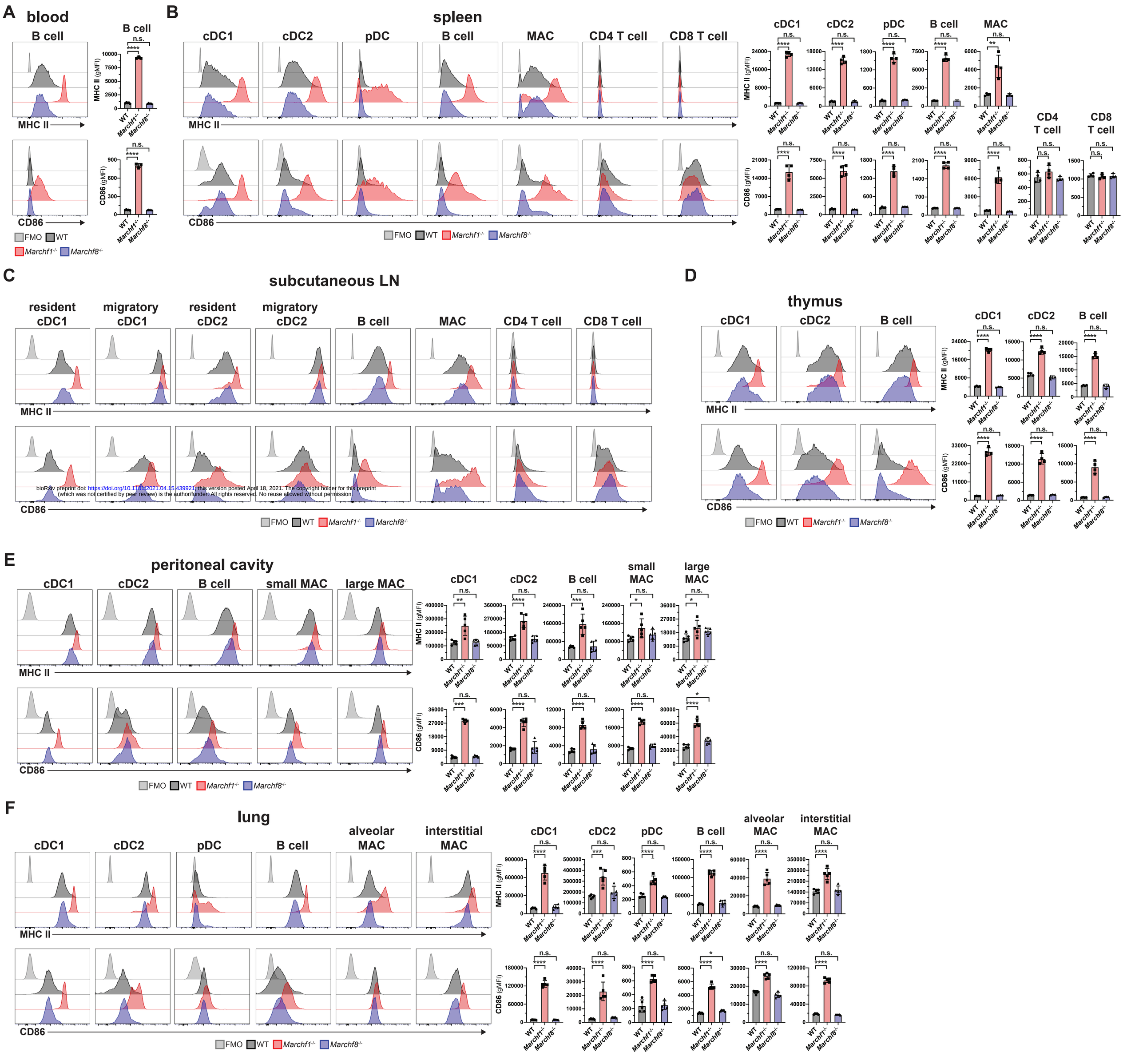
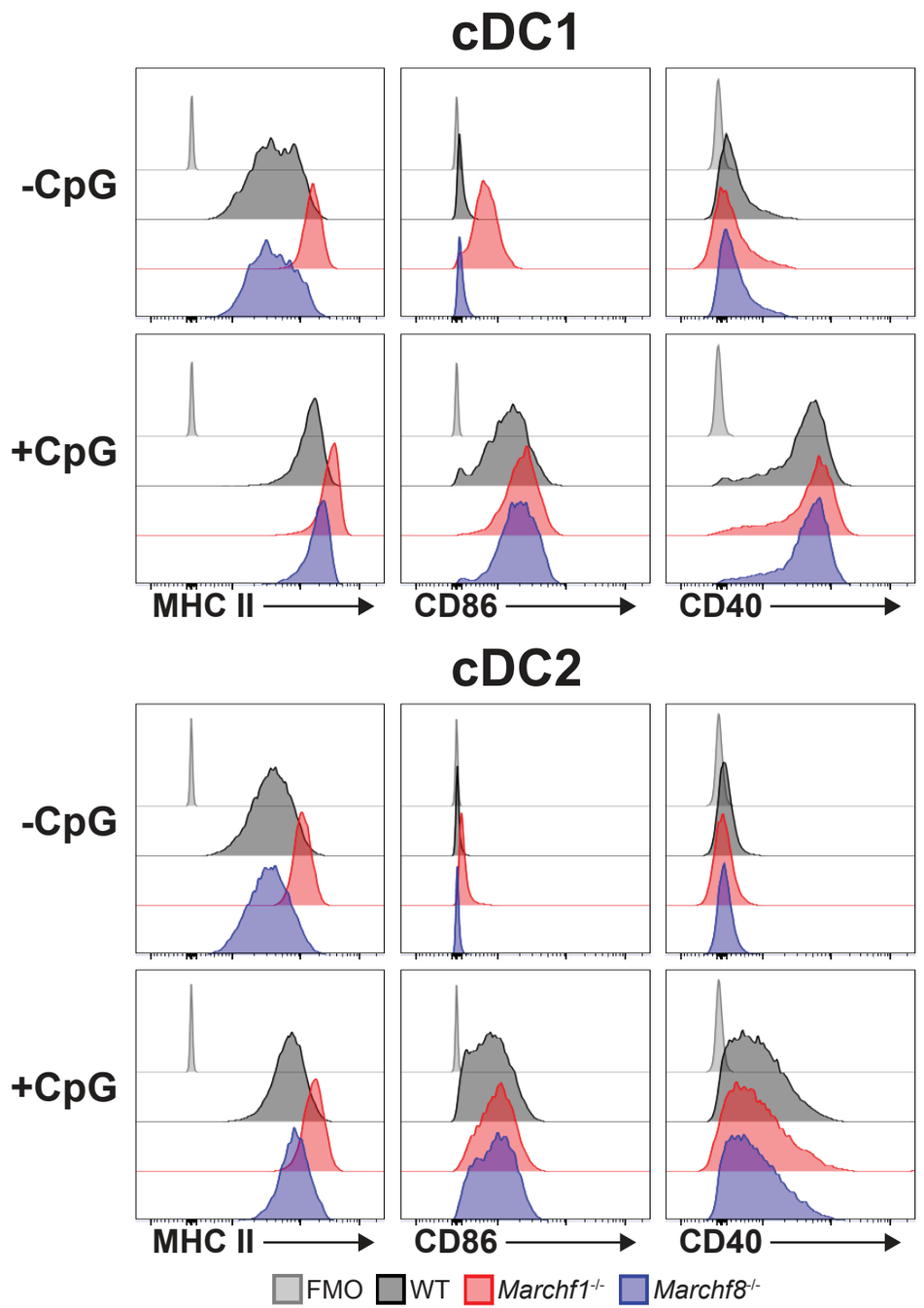


Figure 2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+CpG
MHC II
CD86
CD40
cDC2
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+CpG
MHC II
CD86
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FMO
WT
Marchf1-/-
Marchf8-/-

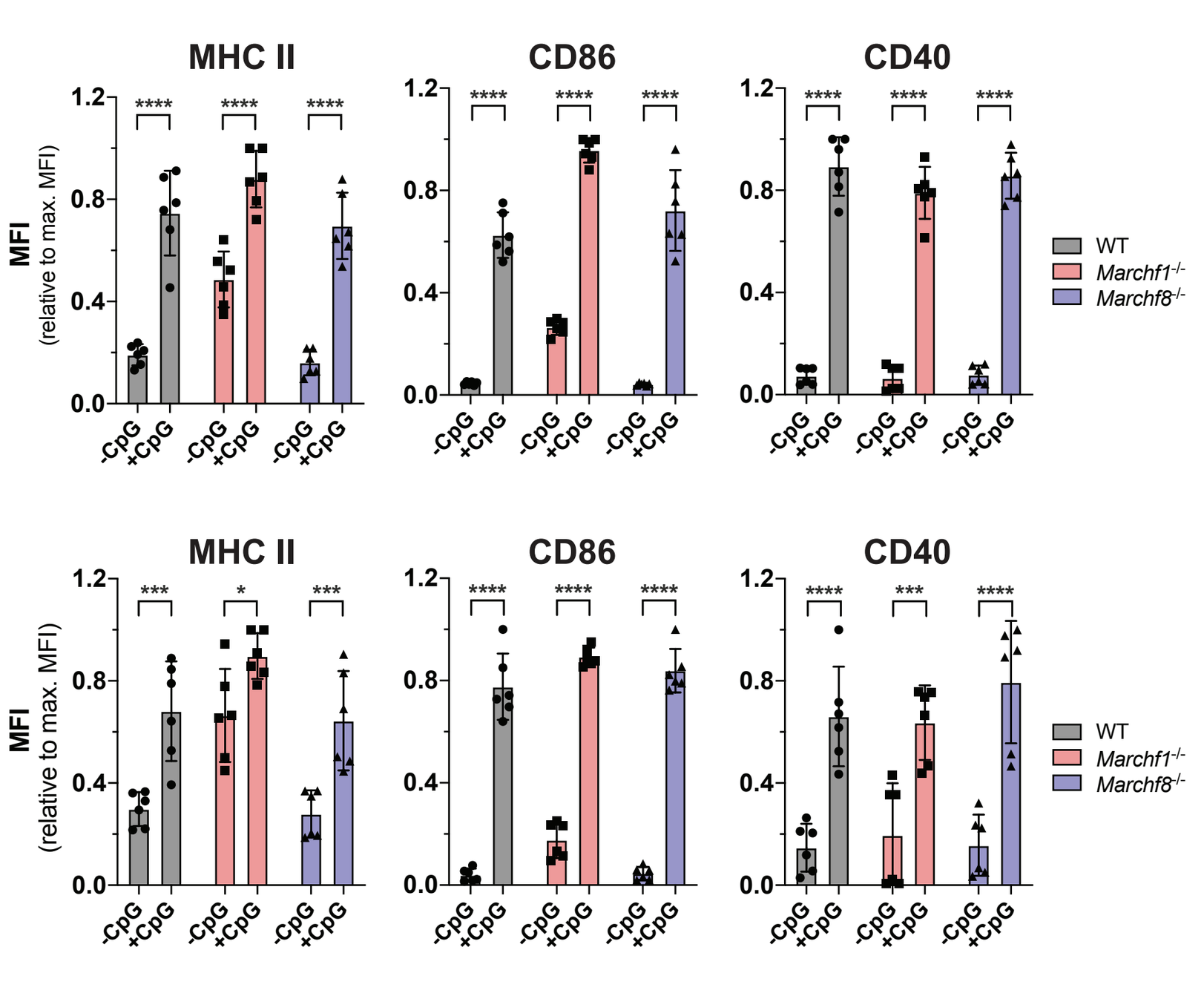

MHC II
CD86
CD40
MFI
(relative to max. MFI)
1.2
0.8
0.4
0.0
****
***
*
-CpG
+CpG
WT
Marchf1-/-
Marchf8-/-

Figure 3

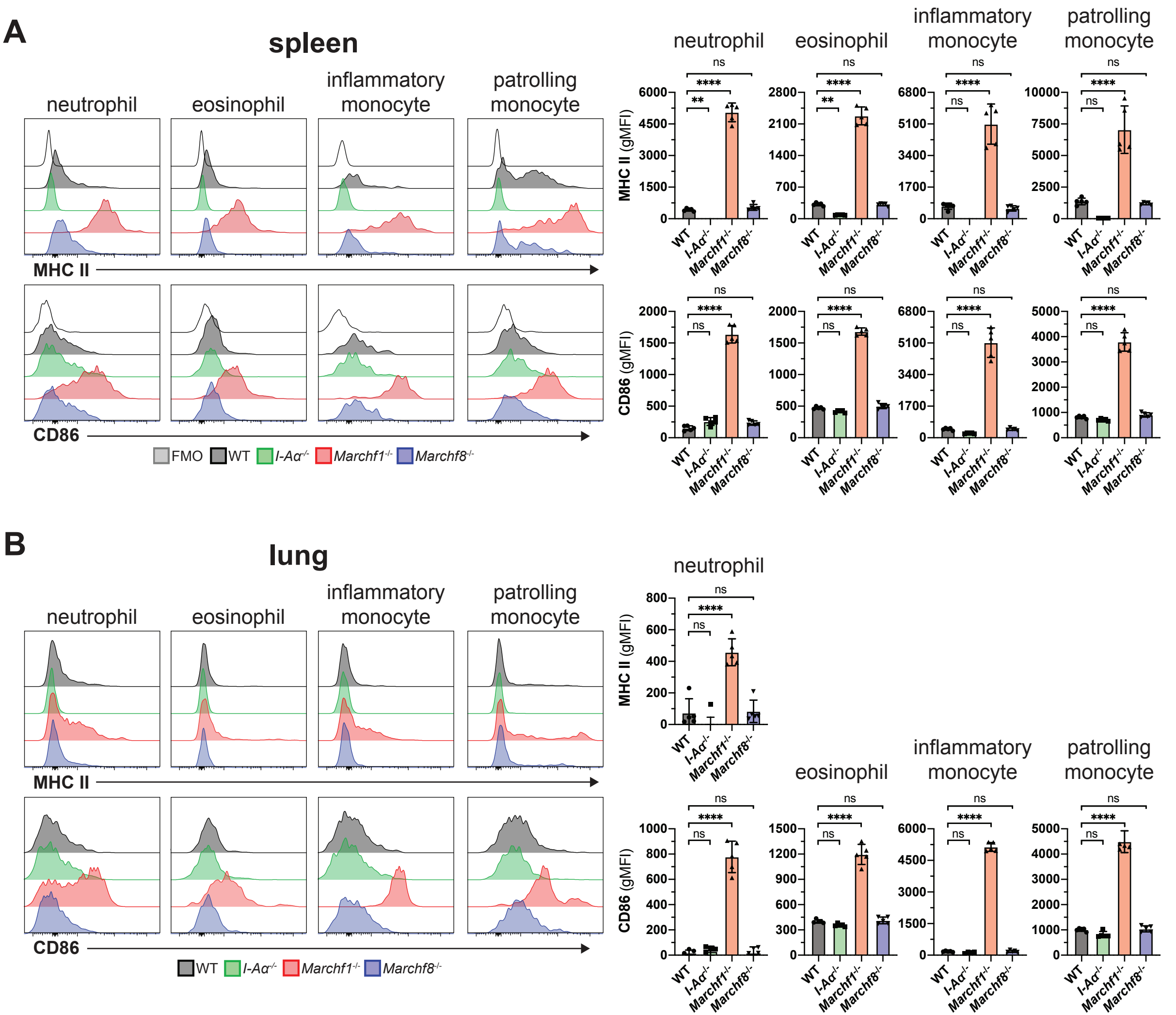
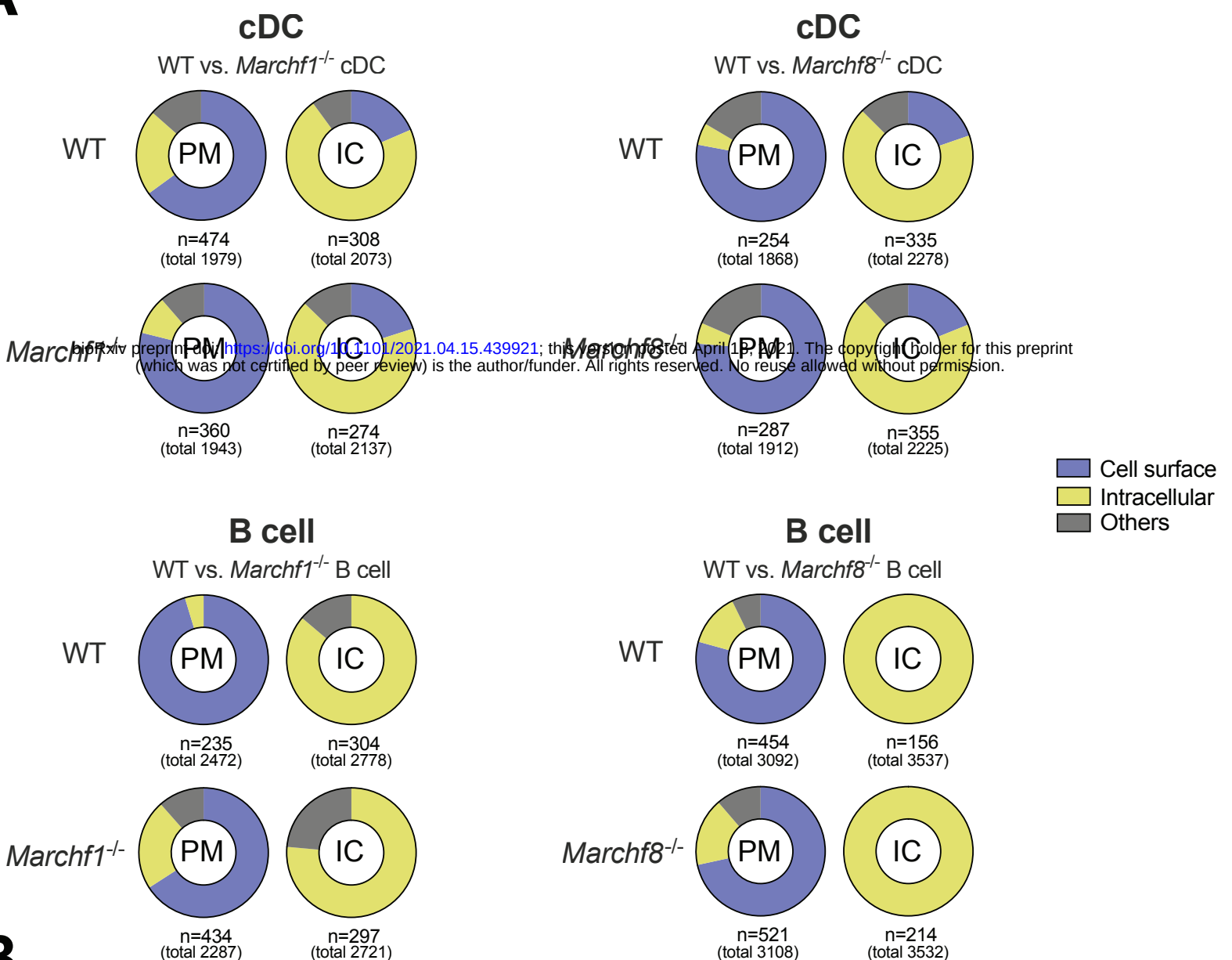


Figure 4

Figure 5

A



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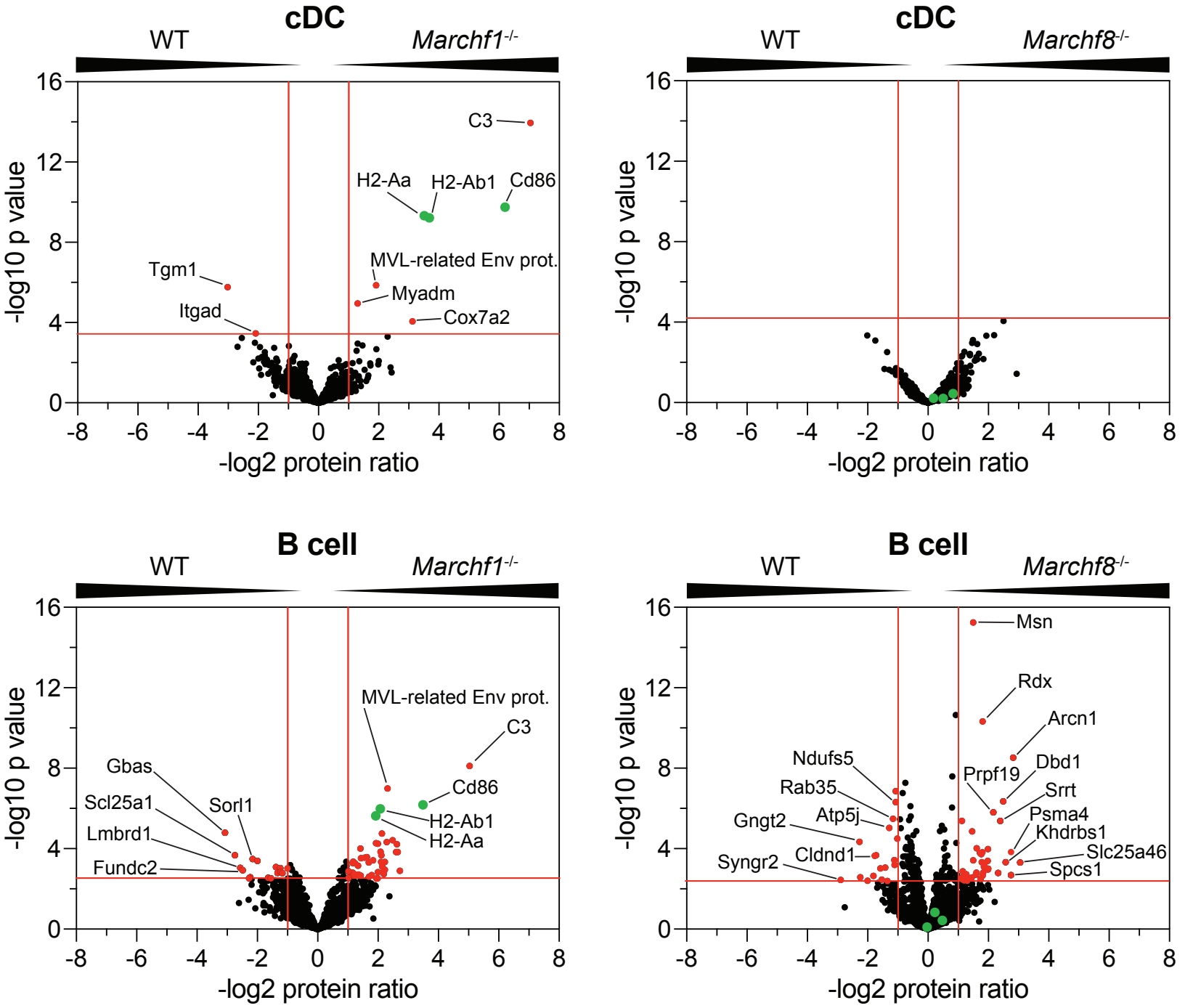
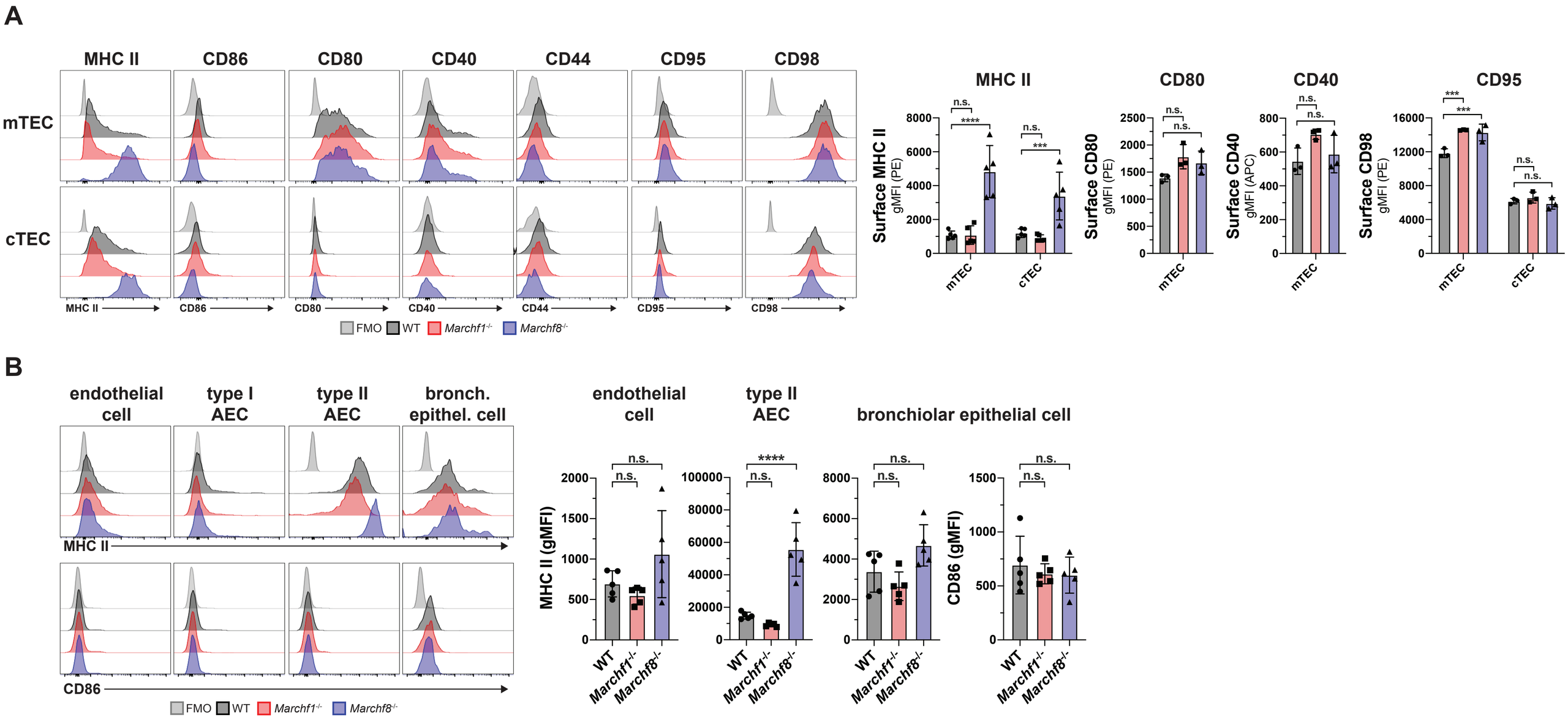
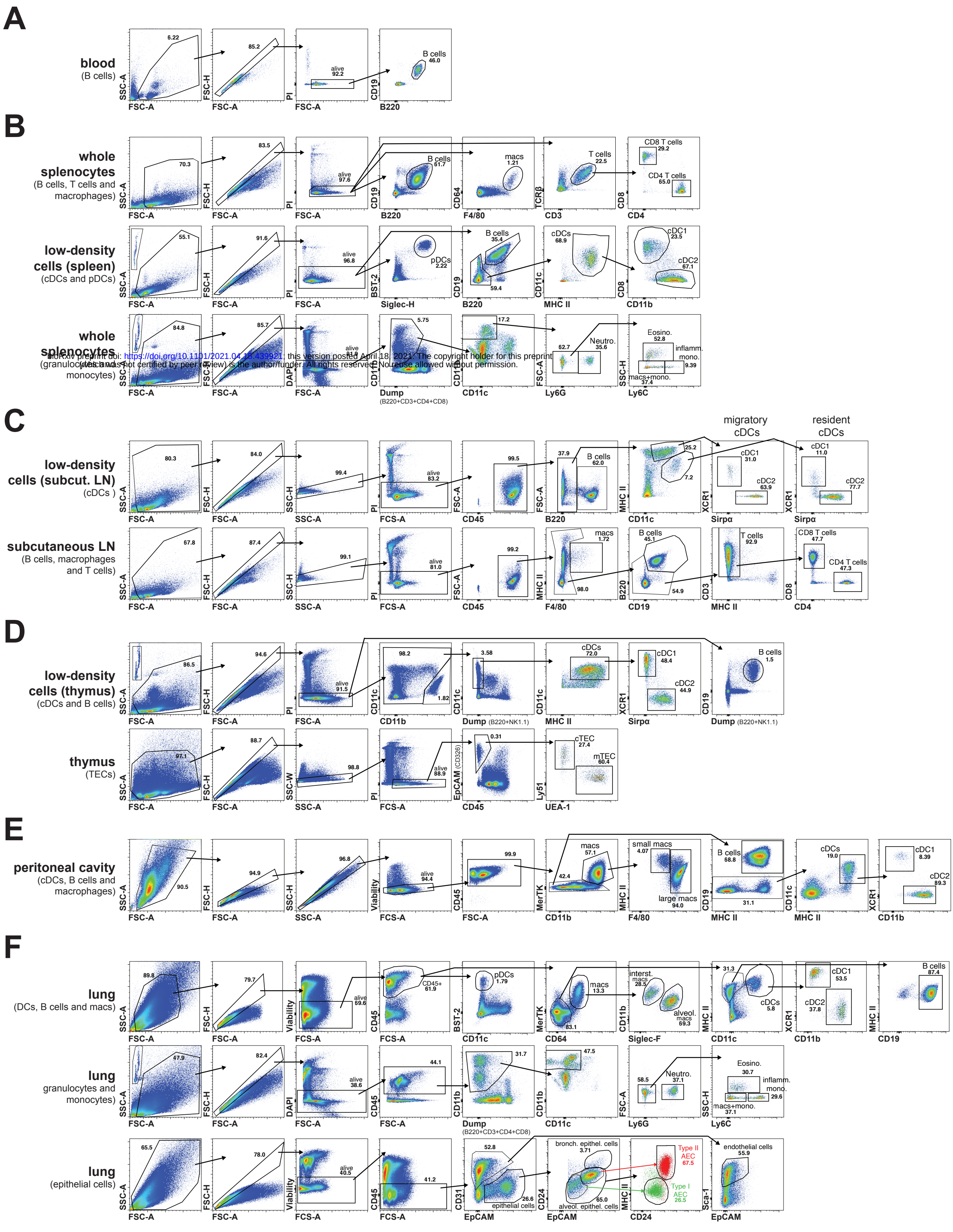


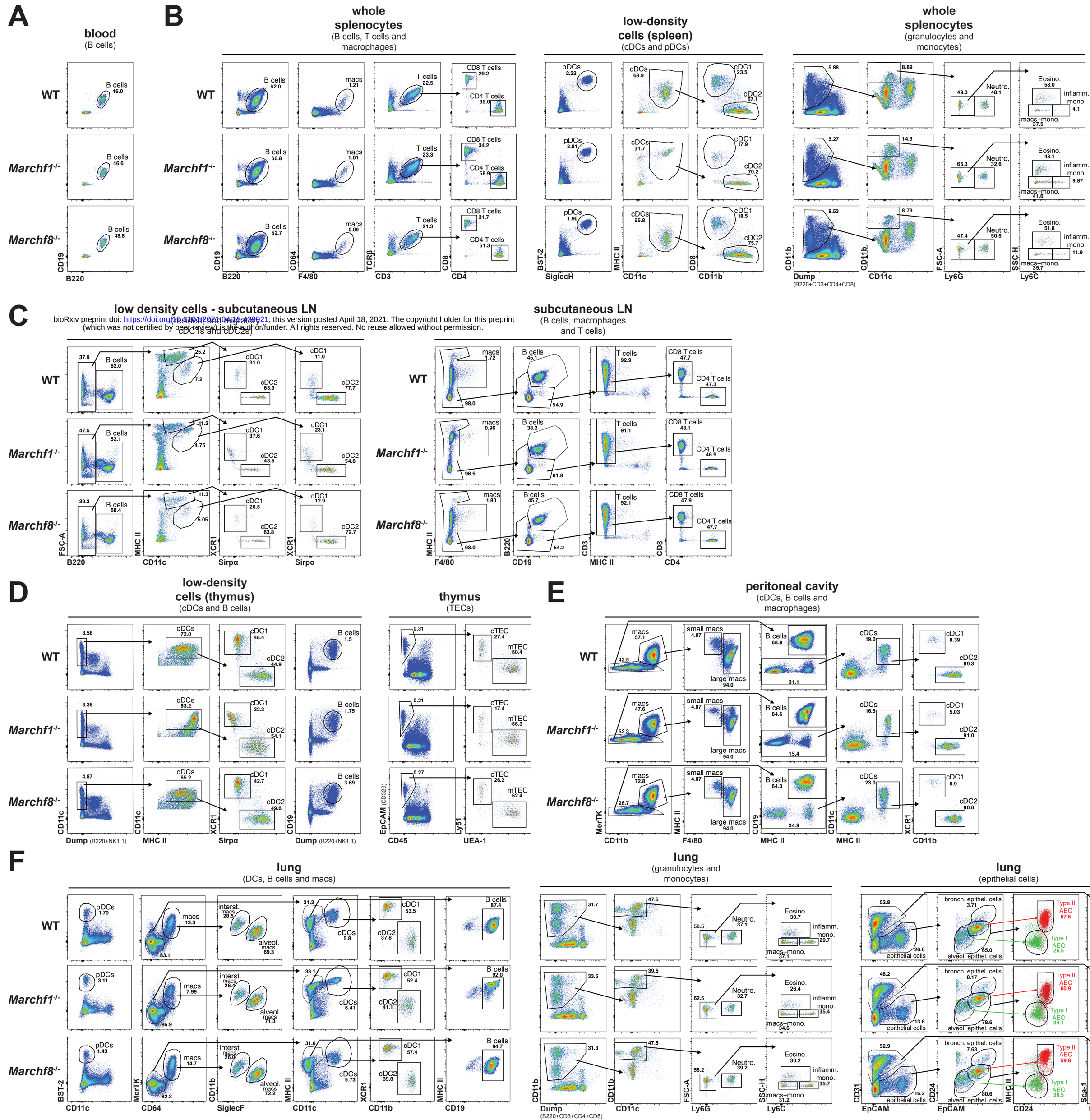
Figure 6


A
MHC II
CD86
CD80
CD40
CD44
CD95
CD98
mTEC
cTEC
FMO
WT
Marchf1-/-
Marchf8-/-
Surface MHC II gMFI (PE)
Surface CD80 gMFI (PE)
Surface CD40 gMFI (APC)
Surface CD98 gMFI (PE)
n.s.
****
***
B
endothelial cell
type I AEC
type II AEC
bronch. epithel. cell
bronchiolar epithelial cell
MHC II (gMFI)
CD86 (gMFI)
WT
Marchf1-/-
Marchf8-/-

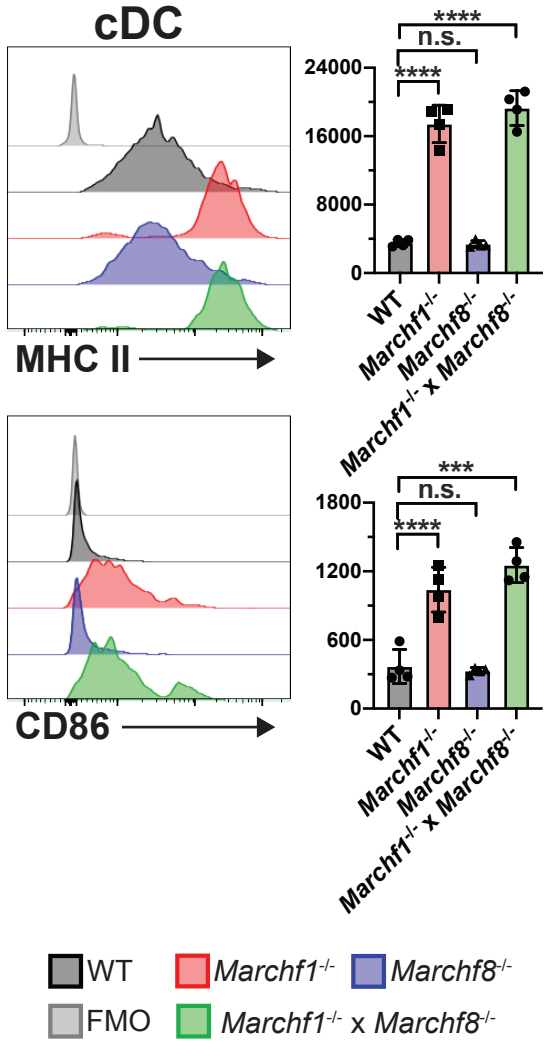
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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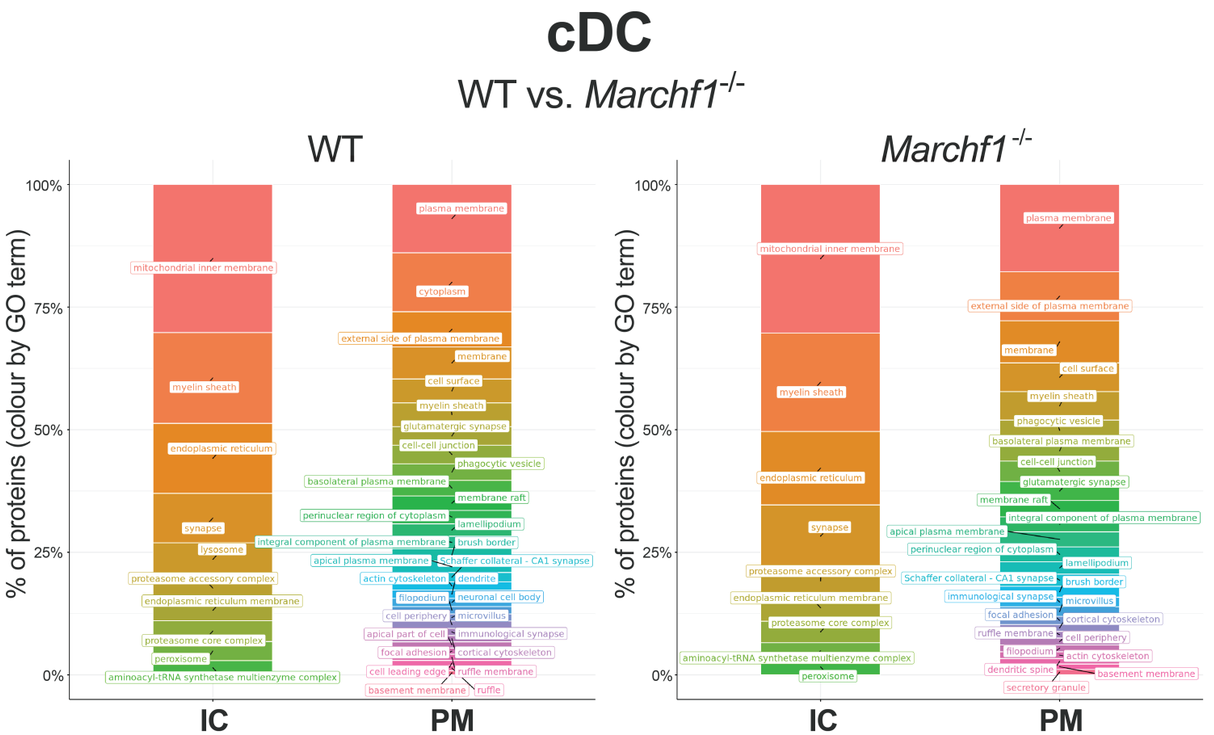
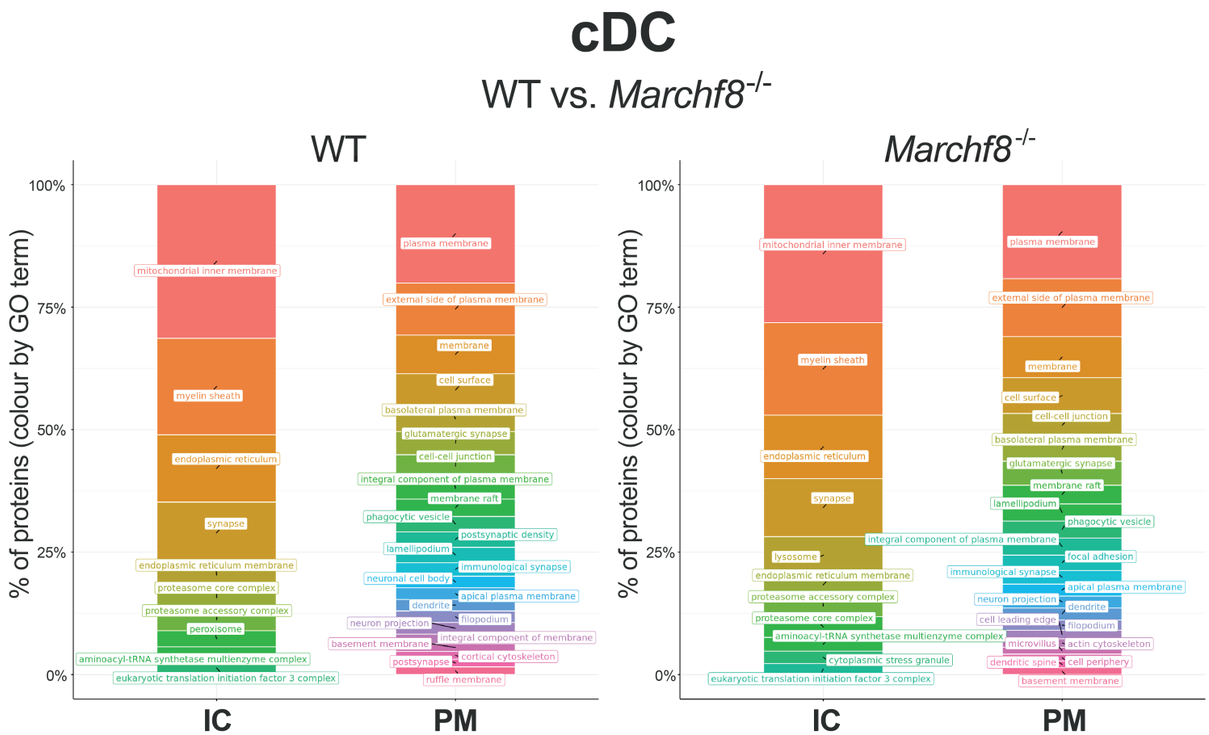
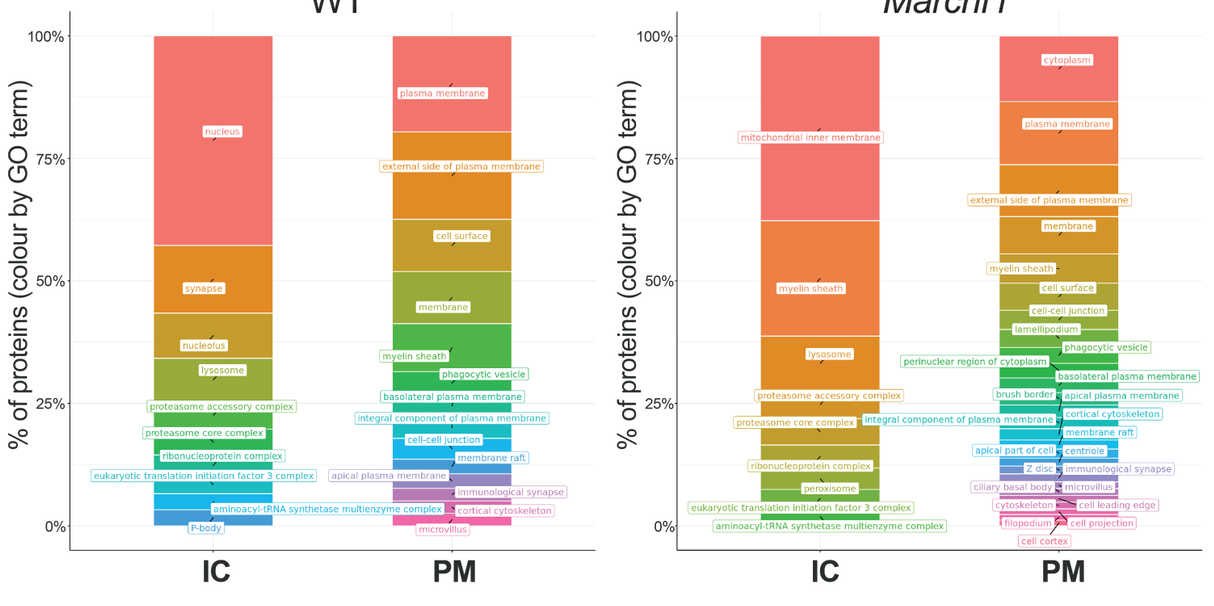
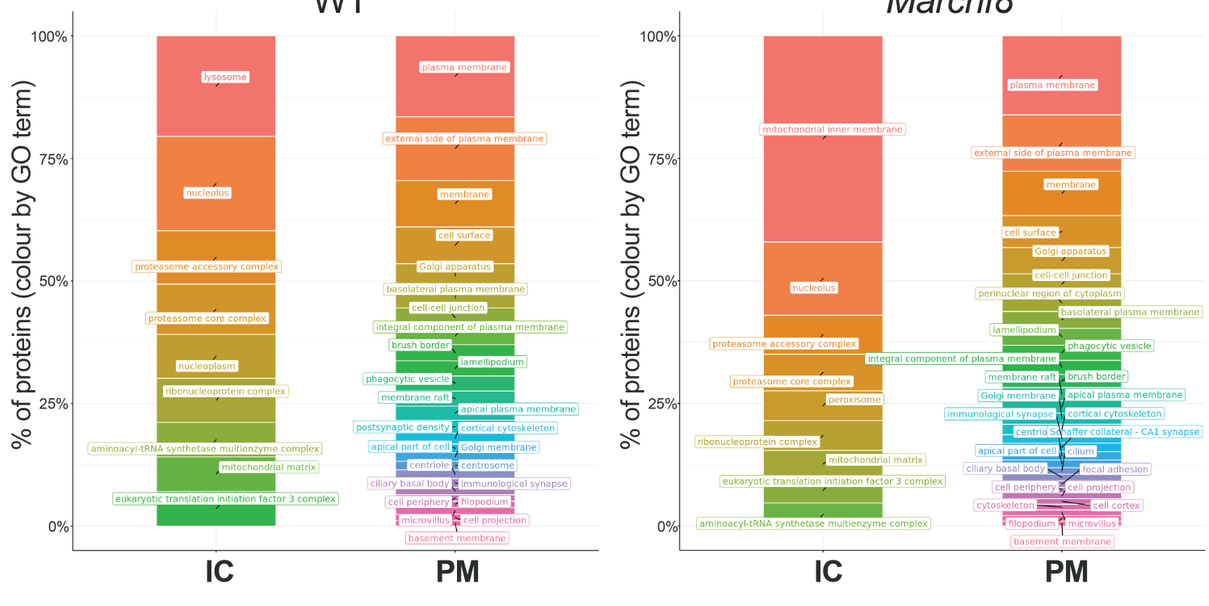
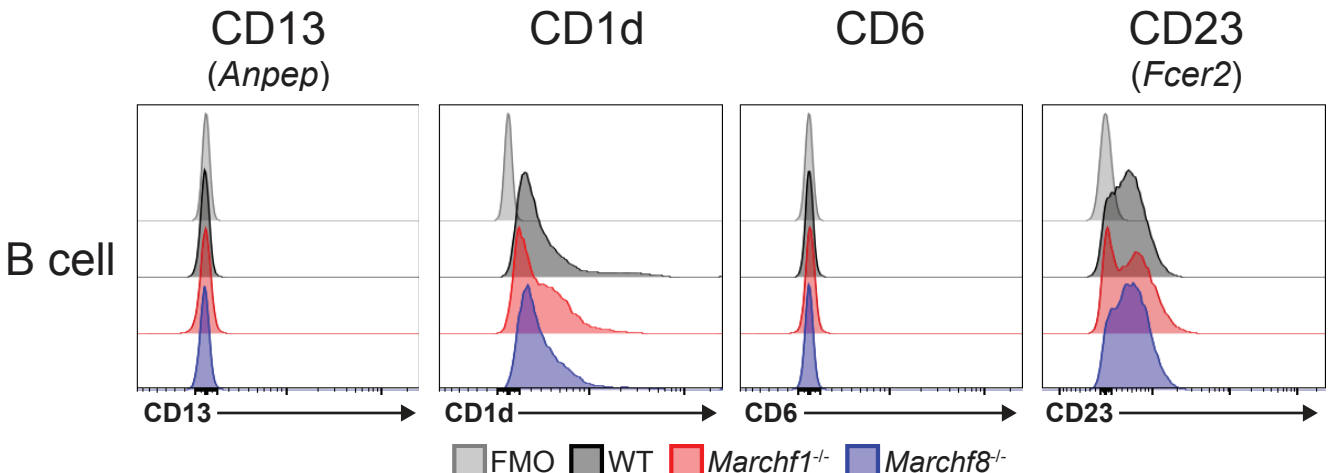








Supplementary Figure 5



Supplementary Table 5: **Significantly up/down-regulated proteins in the PM fraction of *Marchf1*$^{-/-}$ cDCs.**

Gene	Protein Names	Log2 Fold Change	-Log10 p value	Localisation (based on GO-ID of enrichment analysis)	Localisation (based on UniProt)
C3	Complement C3	7.04	13.95	Extracellular, Cell surface	Extracellular region or secreted
Cd86	T-lymphocyte activation antigen CD86	6.20	9.76	Plasma membrane, Intracellular membrane-bounded organelle	Cell membrane, Single-pass type I membrane protein
H2-Aa	H-2 class II histocompatibility antigen, A-B alpha chain	3.51	9.33	Plasma membrane, Early endosome	Membrane, Single-pass type I membrane protein
H2-Ab1	H-2 class II histocompatibility antigen, A beta chain	3.68	9.22	Plasma membrane, Lysosome	Membrane, Single-pass type I membrane protein
-	MLV-related proviral Env polyprotein	1.91	5.85	N/A	Cell membrane, Virion membrane
Myadm	Myeloid-associated differentiation marker	1.29	4.95	N/A	Cortical actin cytoskeleton, Membrane, Multi-pass membrane protein
Cox7a2	Cytochrome c oxidase subunit 7A	3.12	4.06	Mitochondrial inner membrane	Mitochondrion inner membrane
Tgm1	Protein-glutamine gamma-glutamyltransferase K	-3.02	5.77	Adherens junction	Membrane, Lipid-anchor
Itgad	Integrin alpha-D	-2.08	3.47	N/A	Membrane, Single-pass type I membrane protein

Supplementary Table 6: **Significantly up/down-regulated proteins in the PM fraction of *Marchf1*$^{-/-}$ B cells.**

Gene	Protein Names	Log2 Fold Change	-Log10 p value	Localisation (based on GO-ID of enrichment analysis)	Localisation (based on UniProt)
C3	Complement C3	5.04	8.12	Extracellular, Cell surface	Extracellular region or secreted
-	MLV-related proviral Env polyprotein	2.32	6.99	N/A	Cell membrane, Virion membrane
Cd86	T-lymphocyte activation antigen CD86	3.49	6.18	Plasma membrane & intracellular membrane-bounded organelle	Cell membrane, Single-pass type I membrane protein
H2-Ab1	H-2 class II histocompatibility antigen, A beta chain	2.07	5.98	Plasma membrane, Lysosome	Membrane, Single-pass type I membrane protein
H2-Aa	H-2 class II histocompatibility antigen, A-B alpha chain	1.92	5.64	Plasma membrane, Early endosome	Membrane, Single-pass type I membrane protein
Stk24; Stk25; Stk26	Serine/threonine-protein kinase 24/25/26	2.13	4.76	N/A	Nucleus
Anpep	Aminopeptidase N	2.48	4.42	Plasma membrane	Cell membrane, Single-pass type II membrane protein
Ahsg	Alpha-2-HS-glycoprotein	2.29	4.32	N/A	Secreted
Lrch1	Leucine-rich repeat and calponin homology domain-containing protein 1	1.94	4.27	N/A	Cytoplasm
Ttc7a	Tetratricopeptide repeat protein 7A	1.99	4.25	N/A	Cell membrane, Cytoplasm

Rpl34	60S ribosomal protein L34	2.63	4.22	Mitochondrion	Endoplasmic reticulum, Cytosol
Coro2a	Coronin-2A	1.42	4.00	Brush border	Brush border, Transcription repressor
Sh3kbp1	SH3 domain-containing kinase-binding protein 1	2.08	3.84	Cell-cell junction, Endocytic vesicle	Cytoskeleton, Cytoplasm
Ifi30	Gamma-interferon-inducible lysosomal thiol reductase	2.60	3.83	Lysosome	Lysosome
Itgax	Integrin alpha-X	2.65	3.83	Plasma membrane	Membrane, Single-pass type I membrane protein
Hbb-b1;Hbb-b2	Hemoglobin subunit beta-1/2	2.11	3.68	Myelin sheath, Haemoglobin complex	Cytosol
Stk10	Serine/threonine-protein kinase 10	1.63	3.58	N/A	Cell membrane, Peripheral membrane
Frmd8	FERM domain-containing protein 8	1.56	3.55	N/A	Cytosol, Cell membrane
Taok3	Serine/threonine-protein kinase TAO3	1.43	3.53	N/A	Cytoplasm
Git2	ARF GTPase-activating protein GIT2	1.83	3.42	Calyx of Held	Nucleoplasm
Actr2	Actin-related protein 2	1.18	3.36	Cell cortex, Actin cap	Cytoskeleton, Nucleus
Ptprj	Receptor-type tyrosine-protein phosphatase eta	2.10	3.34	Immunol. synapse, Plasma membrane, Ruffle membrane	Cell membrane, Single-pass type I membrane protein
Ahrr	Aryl hydrocarbon receptor repressor	2.23	3.34	Nucleus	Nucleus
Fam126a	Hyccin	1.69	3.31	Neuron projection	Cytosol, Plasma membrane
Stxbp3	Syntaxin-binding protein 3	1.16	3.29	Plasma membrane, Apical plasma Membrane, Cytosol	Cell membrane, Cytosol
Gbp5	Guanylate-binding protein 5	1.24	3.28	Cytoplasmic vesicle	Golgi apparatus membrane, Cytoplasm

Stxbp2	Syntaxin-binding protein 2	1.34	3.15	Apical plasma membrane, Phagocytic vesicle, Zymogen granule membrane	Cytosol, azurophil granule, apical plasma membrane
Cct8	T-complex protein 1 subunit theta	2.13	3.12	Cell body, Zona pellucida receptor complex, Chaperonin-containing T-complex	Cytoskeleton, Cytoplasm
Ap1m1	AP-1 complex subunit mu-1	2.23	2.96	N/A	Golgi apparatus, Peripheral membrane protein
Pacsin2	Protein kinase C and casein kinase substrate in neurons protein 2	2.72	2.90	Cytoplasm, Cell-cell junction, Cytosol, Trans-Golgi network, Extrinsic component of membrane	Ruffle membrane. Peripheral membrane, Cell membrane, Early endosome, Cytoskeleton
Iqgap1	Ras GTPase-activating-like protein IQGAP1	1.00	2.89	Nucleus, Cytoplasm, Cell-cell junction, Lateral plasma membrane, Neuron projection, Cell leading edge, Ribonucleoprotein complex	Nucleus, Plasma membrane, Cytoplasm
Agfg1	Arf-GAP domain and FG repeat-containing protein 1	2.18	2.85	Cytoplasmic vesicle, Neuronal cell body, Cell projection	Nucleus, Cytoplasmic vesicle
Hba	Hemoglobin subunit alpha	2.00	2.83	Myelin sheath	Cytosol, Extracellular region or secreted, Myelin sheath
Tubgcp3	Gamma-tubulin complex component 3	1.16	2.81	N/A	Centrosome
Csk	Tyrosine-protein kinase CSK	1.16	2.77	Cell-cell junction	Plasma membrane, Cytoplasm

Ap1b1	AP-1 complex subunit beta-1	2.19	2.74	N/A	Golgi apparatus, Peripheral membrane protein
Actr3	Actin-related protein 3	1.14	2.73	Lamellipodium, Cell-cell junction, Brush border	Cytoskeleton, Nucleus, Cell projection
Ablim1	Actin-binding LIM protein 1	1.22	2.73	Actin cytoskeleton, Postsynaptic density	Cytoskeleton, Cytoplasm
Ap2s1	AP-2 complex subunit sigma	1.35	2.71	AP-2 adaptor complex	Cell membrane, Peripheral membrane protein
Eps15	Epidermal growth factor receptor substrate 15	1.79	2.68	Plasma membrane, Clathrin-coated pit/vesicle, Ciliary membrane, AP-2 adaptor complex	Cell membrane, Peripheral membrane protein, Cytoplasm, Clathrin-coated pit
Ccm2	Cerebral cavernous malformations protein 2 homolog	1.43	2.66	Protein-containing complex	Cytoplasm
Fam65b	Protein FAM65B	1.70	2.60	Stereocilium	Cytoskeleton, Stereocilium membrane, Apical cell membrane, Cytoplasm
Arpc2	Actin-related protein 2/3 complex subunit 2	1.02	2.53	Focal adhesion, Plasma membrane, Synapse, Endosome, Cell leading edge,	Nucleus, Cytoskeleton, Cell projection
Anxa6	Annexin A6	1.99	2.52	Perinuclear region of cytoplasm, Collagen-containing extracellular matrix	Cytoplasm, Melanosome
Mpp6	MAGUK p55 subfamily member 6	1.95	2.52	Plasma membrane	Membrane, Peripheral membrane

Gbas	Protein NipSnap homolog 2	-3.07	4.79	Cytoplasm, Mitochondrion	Mitochondrion outer membrane, Cytoplasm
Slc25a1	Tricarboxylate transport protein, mitochondrial	-2.75	3.68	Mitochondrion. Mitochondrion inner membrane	Mitochondrion inner membrane
Sorl1	Sortilin-related receptor	-2.15	3.48	Nuclear envelope lumen	Cell membrane, Single-pass type I membrane protein, Endosome, Secreted, Golgi apparatus membrane, Endoplasmic reticulum membrane, Secretory vesicle membrane
-	Ig lambda-1 chain V region	-1.99	3.39	N/A	Extracellular space, Plasma membrane
Sfxn3	Sideroflexin-3	-1.38	3.08	Mitochondrion	Mitochondrion membrane
Lmbrd1	Probable lysosomal cobalamin transporter	-2.57	3.04	Plasma membrane, Lysosome, Clathrin-coated endocytic vesicle	Lysosome membrane
Arl8b	ADP-ribosylation factor-like protein 8B	-1.24	3.04	Synapse, Axon	Late endosome membrane, Cytoskeleton, Lysosome, Axon
Fundc2	FUN14 domain-containing protein 2	-2.48	2.93	N/A	Mitochondrion. Mitochondrion outer membrane
Ckap4	Cytoskeleton-associated protein 4	-1.22	2.85	Endoplasmic reticulum	Cytoskeleton, Endoplasmic reticulum membrane, Cell membrane
Mtco1	Cytochrome c oxidase subunit 1	-1.33	2.80	Mitochondrion inner membrane	Mitochondrion inner membrane

Hist1h1b	Histone H1.5	-1.17	2.78	N/A	Nucleus, Chromosome
Hccs	Cytochrome c-type heme lyase	-2.23	2.58	Mitochondrion	Mitochondrion inner membrane
Mcur1	Mitochondrial calcium uniporter regulator 1	-1.64	2.55	N/A	Mitochondrion inner membrane
Ctss	Cathepsin S	-2.27	2.53	Membrane, Lysosome	Secreted, Lysosome
Rdh11	Retinol dehydrogenase 11	-1.55	2.52	Photoreceptor inner/outer segment membrane	Endoplasmic reticulum membrane

Supplementary Table 7: **Significantly up/down-regulated proteins in the PM fraction of *Marchf8*$^{-/-}$ B cells.**

Gene	Protein Names	Log2 Fold Change	-Log10 p value	Localisation (based on GO-ID of enrichment analysis)	Localisation (based on UniProt)
Msn	Moesin	1.50	15.24	Apical plasma membrane, Basolateral plasma membrane, Apical part of cell, Microvillus, Myelin sheath	Cytoskeleton, Plasma membrane, Microvillus
Rdx	Radixin	1.81	10.32	Plasma membrane, Lamellipodium, Filopodium, Ruffle, Apical part of cell, Stereocilium, Cortical actin cytoskeleton, Microvillus, Myelin sheath	Cell membrane, Cytoskeleton, Microvillus
Arcn1	Coatomer subunit delta	2.82	8.53	Golgi apparatus, Endoplasmic reticulum, COPI-coated vesicle	Golgi apparatus membrane, peripheral membrane protein, Cytoplasm
Ddb1	DNA damage-binding protein 1	2.49	6.35	N/A	Nucleus, Cytoplasm
Prpf19	Pre-mRNA-processing factor 19	2.17	5.80	Nucleus	Nucleus, Nucleoplasm, Spindle
Srrt	Serrate RNA effector molecule homolog	2.39	5.38	Cytoplasm, Nucleoplasm	Cytoplasm, Nucleoplasm

Cd1d1;Cd1d2	Antigen-presenting glycoprotein CD1d1/2	1.13	5.38	External side of plasma membrane, Endosome, Lysosome	Cell membrane, Endosome, Lysosome
Rqcd1	Cell differentiation protein RCD1 homolog	1.46	4.86	P-body	Nucleus, P-body
Gk	Glycerol kinase	1.61	4.02	Mitochondrion	Mitochondrion out membrane
Lamp1	Lysosome-associated membrane glycoprotein 1	1.99	3.98	External side of plasma membrane, sarcolemma, cell surface, endosome, endosome, lysosome, phagocytic vesicle, cytolytic granule, synaptic vesicle, vesicle, multivesicular body, melanosome, autolysosome, phagolysosome membrane	Endosome membrane, Lysosome membrane, Cell membrane
Psma4	Proteasome subunit alpha type-4	2.79	3.82	P-body, Proteasome core complex	Nucleus, Cytoplasm
Ubtf	Nucleolar transcription factor 1	1.80	3.82	Nucleolus	Nucleus
Kars	Lysine-tRNA ligase	1.72	3.80	Mitochondrion, Aminoacyl-tRNA synthetase multienzyme complex	Mitochondrion, Cytoplasm, Nucleus, Plasma membrane
Pip4k2a	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha	1.76	3.73	N/A	Nucleus, Plasma membrane
Kpnb1	Importin subunit beta-1	1.50	3.42	Protein-containing complex, Cytoplasmic stress granule	Nucleus, Cytoplasm

Tcp1	T-complex protein 1 subunit alpha	1.98	3.39	Golgi apparatus, Microtubule organizing center, Myelin sheath, Cell body	Cytoskeleton, Cytosol, Golgi apparatus
Khdrbs1	KH domain-containing, RNA-binding, signal transduction-associated protein 1	2.57	3.33	Nucleus	Nucleus, Cytoplasm
Copz1	Coatomer subunit zeta-1	1.79	3.32	N/A	Golgi apparatus, Cytoplasm
Slc25a46	Solute carrier family 25 member 46	3.06	3.32	Mitochondrion, Mitochondrial outer membrane	Mitochondrial outer membrane
Actr1a	Alpha-centractin	1.86	3.23	Myelin sheath	Cytoskeleton, Centrosome
Diablo	Diablo homolog, mitochondrial	1.85	3.10	Cytoplasm, Mitochondrion, Cytoplasmic side of plasma membrane,	Mitochondrion
Frg1	Protein FRG1	1.99	3.01	N/A	Cajal body, Nucleolus
Psmd2	26S proteasome non-ATPase regulatory subunit 2	1.99	2.97	Proteasome accessory complex, Proteasome complex	Cytoplasm, Proteasome accessory complex,
H2afv;H2afz	Histone H2A.V; Histone H2A.Z	1.15	2.87	Nucleus	Nucleus
Fam3a	Protein FAM3A	1.83	2.80	N/A	Secreted
Cd6	T-cell differentiation antigen CD6	1.61	2.80	N/A	Cell membrane, Single-pass type I membrane protein
Srsf7	Serine/arginine-rich splicing factor 7	2.33	2.79	N/A	Nucleus, Cytoplasm
Psmd11	26S proteasome non-ATPase regulatory subunit 11	1.27	2.72	Proteasome accessory complex	Proteasome accessory complex
Arhgef6	Rho guanine nucleotide exchange factor 6	1.59	2.71	Lamellipodium, Cell-cell junction	Lamellipodium

Spcs1	Signal peptidase complex subunit 1	2.75	2.69	N/A	Microsome membrane, Multi-pass membrane, Endoplasmic reticulum
Eif5a	Eukaryotic translation initiation factor 5A-1	1.81	2.66	Nucleus, Cytoplasm	Endoplasmic reticulum, Nucleus
Golga2	Golgin subfamily A member 2	1.29	2.63	Golgi apparatus, Golgi membrane, Spindle pole, cis-Golgi network	Golgi apparatus, Spindle pole
Rpl37a	60S ribosomal protein L37a	1.15	2.57	N/A	Cytosol, Large ribosomal subunit
Hexa	Beta-hexosaminidase subunit alpha	1.14	2.56	Membrane, Lysosome	Lysosome
Pgd	6-phosphogluconate dehydrogenase, decarboxylating	1.44	2.55	N/A	Golgi apparatus, Endoplasmic reticulum, Nucleus, Secreted
Erap1	Endoplasmic reticulum aminopeptidase 1	1.09	2.52	Cytoplasm	Endoplasmic reticulum, Single-pass type II membrane protein
Pspc1	Paraspeckle component 1	1.21	2.50	Nucleoplasm, Paraspeckles	Nucleolus
Prpsap1	Phosphoribosyl pyrophosphate synthase-associated protein 1	1.74	2.49	N/A	Cytoplasm
Cd247	T-cell surface glycoprotein CD3 zeta chain	1.30	2.41	Alpha-beta T cell receptor complex	Cell membrane, Single-pass type I membrane protein
Dek	Protein DEK	1.22	2.38	Nucleus, Contractile fiber	Nucleus
-	Ig lambda-1 chain C region	-1.07	6.86		
Ndufs5	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	-1.07	6.31	Mitochondrion	Mitochondrial inner membrane

Rab35	Ras-related protein Rab-35	-1.16	5.48	Mitochondrion	Endosome, Plasma membrane
Atp5j	ATP synthase-coupling factor 6, mitochondrial	-1.29	5.03	Mitochondrion, Mitochondrial inner membrane	Mitochondrial inner membrane
Gnai3	Guanine nucleotide-binding protein G(k) subunit alpha	-1.02	4.49	Cytoplasm, Golgi apparatus	Centrosome, Plasma membrane
Gngt2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2	-2.27	4.34	N/A	Plasma membrane
Gm10881	Ig kappa chain V-V region L7	-1.72	3.67	N/A	Secreted
Cldnd1	Claudin domain-containing protein 1	-1.76	3.64	Apical plasma membrane	Multi-pass membrane protein
-	Ig kappa chain C region	-1.11	3.43		
Fcer2	Low affinity immunoglobulin epsilon Fc receptor	-1.11	3.19	External side of plasma membrane	Cell membrane, Single-pass type I membrane protein
Pon2	Serum paraoxonase/arylesterase 2	-1.42	3.06	N/A	Membrane
Ube2g2	Ubiquitin-conjugating enzyme E2 G2	-1.57	3.02	Cytosol, Endoplasmic reticulum	Cytosol, Endoplasmic reticulum
-	Ig kappa chain V-V region MOPC 149	-2.25	2.58		
-	Ig kappa chain V-III region ABPC 22/PC 9245/4050 region MOPC 63	-1.53	2.46		
Syngr2	Synaptogyrin-2	-2.90	2.44	Synaptic vesicle	Cytoplasmic vesicle membrane, Multi-pass membrane protein
Puf60	Poly(U)-binding-splicing factor PUF60	-2.00	2.41	N/A	Nucleus
Slc4a1	Band 3 anion transport protein	-1.34	2.39	Plasma membrane, basolateral plasma membrane, cortical cytoskeleton,	Cell membrane, Multi-pass membrane protein

				cytoplasmic side of plasma membrane	