Physiological Substrates and Ontogeny-Specific Expression of the Ubiquitin Ligases MARCH1 and MARCH8

Patrick Schriek[1], Haiyin Liu[1], Alan C. Ching[1], Pauline Huang[1], Nishma Gupta[1], Kayla R. Wilson[1], MinHsuang Tsai[1], Yuting Yan[2], Christophe F. Macri[1], Laura F. Dagley[3,4], Giuseppe Infusini[3,4], Andrew I. Webb[3,4], Hamish McWilliam[1,2], Satoshi Ishido[5], Justine D. Mintern[1] and Jose A. Villadangos[1,2]

[1]Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC 3010, Australia.

[2]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, VIC 3010, Australia.

[3]Advanced Technology and Biology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia.

[4]Department of Medical Biology, University of Melbourne, Parkville, VIC 3010, Australia.

[5]Department of Microbiology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 17 663-8501, Japan

Correspondence to Justine D. Mintern (jmintern@unimelb.edu.au) or
Jose A. Villadangos (j.villadangos@unimelb.edu.au)

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 *March1*$^{-/-}$ and *March8*$^{-/-}$ mice. We have also carried out quantitative proteomic comparisons of WT vs *March1*$^{-/-}$ or *March8*$^{-/-}$ 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.

98 **MATERIALS AND METHODS**

99 **Mice**

100 Wild type (WT, C57BL/6), *March1*$^{-/-}$ [31], *March8*$^{-/-}$ [9] and *I-Aα*$^{-/-}$ [32] mice were bred and
101 maintained in specific pathogen-free conditions within the Melbourne Bioresources Platform
102 at the Bio21 Molecular Science and Biotechnology Institute. Analyses were undertaken with
103 male or female mice aged between 6-14 weeks and performed in accordance with the
104 Institutional Animal Care and Use Committee guidelines of the University of Melbourne. All
105 procedures were approved by the Animal Ethics Committee at the University of Melbourne.
106

107 **Isolation of mouse primary cells and analytical flow cytometry**

108 Single cell suspensions from blood, spleen, subcutaneous lymph nodes (LN), thymus,
109 peritoneal cavity and lung were generated for analysis of B cells, T cells, DC, granulocytes,
110 macrophages, monocytes, neutrophils, eosinophils and thymic or alveolar epithelial cells.
111 Blood was collected from submandibular veins and red blood cells were lysed. Whole single
112 cell suspensions from spleen and subcutaneous LN (axillary and inguinal) were generated by
113 spleen digestion with 0.1 % DNase I (Roche) and 1 mg/ml collagenase type III (Worthington)
114 and red blood cell lysis. DCs from spleen and LN were further enriched by selection of low-
115 density cells by density gradient centrifugation in 1.077 g/cm^3 Nycodenz® (Axis shield).
116 Thymi were digested in 0.1 % DNase I (Roche) and 0.5 U/ml liberase (Roche) and thymic cDC
117 were further enriched by 1.077 g/cm^3 Nycodenz® density gradient centrifugation (Axis shield).
118 Cells from the peritoneal cavity were harvested by injection and aspiration of PBS. Lungs were
119 perfused with PBS and digested with 50 µg/ml DNase I (Roche) and 0.25 mg/ml liberase
120 (Roche) and red blood cells lysed.
121

122 For flow cytometry, cells were incubated with FcR blocking reagent (Miltenyi Biotec), prior
123 to staining with mAb detecting B220/CD45R (RA3-6B2), CD19 (6D5), CD64 (X54-5/7.1),
124 F4/80 (F4/80, Walter Eliza Hall Institute (WEHI) Antibody Facility), CD3 (KT3-1.1, WEHI
125 Antibody Facility), TCRβ (H57-597, WEHI Antibody Facility), CD4 (GK1.5), CD8
126 (YTS169.4 WEHI Antibody Facility), CD8 (53-6.7), BST-2 (927), Siglec-H (551), MHC II
127 (M5/114), CD11c (N418), CD11b (M1/70), Ly6G (1A8), Ly6C (HK1.4), NK1.1 (PK136, BD
128 Biosciences), Sirpα (P84), XCR1 (ZET), CD45 (30-F11), EpCAM (G8.8), Ly51 (6C3), UEA-
129 1 (Vector Laboratories), MerTK (2B10C42), Siglec-F (E50-2440 BD Biosciences), CD31
130 (390), CD24 (M1/69, WEHI Antibody Facility), Sca-1 (D7), CD86 (GL-1), CD40 (FGK45.5,
131 Miltenyi Biotec), CD80 (16-10A1, BD Biosciences), CD44 (IM7.81), CD71 (R17217,

132 eBiosciences), CD95 (15A7, eBiosciences), CD98 (RL388), PD-L1 (10F.9G2), PD-L2
133 (TY25), ICOS-L (HK5.3), B7-H3 (MIH35) or B7-H4 (HMH4-5G1), conjugated to
134 fluorochromes BUV395, BUV805, FITC, PE, PE-Cy7, PerCP/Cy5.5, APC, APC-Cy7, AF700,
135 BV785, BV650, BV510 or BV421 (all from BioLegend, if not stated differently). Cell viability
136 was determined with Fixable Viability Dye eFluor™ 780 (eBiosciences), propidium iodide (PI)
137 or diamidino phenylindole (DAPI). Analysis was performed using a LSRFortessa (BD
138 Biosciences) or CytoFLEX LX (Beckman Coulter) in the Melbourne Cytometry Platform
139 (University of Melbourne). Data was analyzed with FlowJo (Tree Star) and GraphPad Prism.
140 **Supplementary Figures 1 and 2** summarize gating strategies for cells from blood, spleen,
141 subcutaneous lymph nodes (LN), thymus, peritoneal cavity and lung.
142
143 **Isolation of primary immune cells for proteomic analysis.**
144 B cells were purified from spleens using Ficoll® Paque Plus (GE Healthcare) gradient
145 centrifugation and negative depletion with FITC-conjugated mAb specific for CD4 (GK1.5),
146 Ly-76 (TER119) and CD43 (S7) and magnetic anti-FITC MicroBeads (Miltenyi Biotec).
147 Preparations were approximately 95-98% pure for $CD19^+$ $B220^+$ B cells. Splenic cDC were
148 purified from mice subcutaneously injected with Flt3L-secreting melanoma cells [33], 9 days
149 before purification. cDC were purified from spleens of Flt3L-expanded mice following spleen
150 digestion with DNase I (Roche) and collagenase type III and Nycodenz® density gradient
151 centrifugation (Axis shield) with subsequent negative depletion using rat mAb specific for CD3
152 (KT3-1.1), Thy1 (T24/31.7), Ly-76 (Ter119), B220 (RA3-6B2) and Ly-6C/G (RB6-8C5) and
153 anti-rat IgG-coupled magnetic beads (Qiagen) as previously described [34]. Preparations were
154 approximately 90-95% pure for $CD11c^+$ MHC II^+ cDC.
155
156 **Preparation of subcellular fractions enriched in plasma membrane and intracellular**
157 **compartments for proteomics**
158 Subcellular fractionation was performed as previously described [35]. In brief, purified B cells
159 (4-5 x 10^7 cells, 95-98% purity) and cDC (4-5 x 10^7 cells, 90-95% purity) from spleens of WT,
160 *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice were incubated with FITC-conjugated anti-CD19 and anti-B220
161 mAb (B cells) or anti-CD11c, anti-CD45.2, anti-CD49d and anti-MHC I mAb (cDC). mAb-
162 labelled cells were homogenized in the presence of cOmplete™ protease inhibitors (Roche) by
163 mechanical disruption using a cell-cracker (HGM Laboratory equipment). Homogenized
164 preparations were centrifuged at low speed to obtain post-nuclear supernatant (PNS). Surface-
165 labelled plasma membrane (PM) microsomes were isolated by magnetic immunoaffinity using

166 anti-FITC mAb-coated magnetic beads (Miltenyi Biotec) and concentrated by
167 ultracentrifugation in thickwall polycarbonate tubes (Beckman Coulter). PNS with the PM
168 fraction removed was likewise ultracentrifuged to sediment the "intracellular compartments"
169 (IC) fraction.
170
171 **Proteomic profiling of differentially expressed PM proteins**
172 Subcellular fractions (PM and IC) were prepared for mass spectrometry analysis from three
173 independent cell preparations using FASP protein digestion (Protein Discovery) as previously
174 described [36], with the following modifications. Proteins were reduced and digested with
175 sequence-grade modified Trypsin Gold (Promega). Peptides were eluted with ammonium
176 bicarbonate and acidified peptide mixtures from each biological replicate were analyzed in
177 technical triplicates by nanoflow reverse-phase liquid chromatography tandem mass
178 spectrometry (LC-MS/MS) on a nanoAcquity system (Waters) coupled to a Q-Exactive mass
179 spectrometer equipped with a nanoelectrospray ion source for automated MS/MS (Thermo
180 Fisher Scientific). High-resolution MS/MS spectra were processed with MaxQuant (version
181 1.6.7.0) for feature detection and protein identification using the Andromeda search engine
182 [37]. Extracted peak lists were searched against the UniProtKB/Swiss-Prot Mus musculus
183 database (Oct-2019) and a separate reverse decoy database to empirically assess the false
184 discovery rate (FDR) using a strict trypsin specificity allowing up to 2 missed cleavages. The
185 minimum required peptide length was 7 amino acids. The "match between runs" option in
186 MaxQuant was used [38]. PSM and protein identifications were filtered using a target-decoy
187 approach at a FDR of 1%. LFQ quantification was performed, with a minimum ratio of 2.
188 Protein relative quantitative analysis was performed in R using MaxQuant's proteinGroups.txt
189 and LFQ intensities. Missing values were imputed using a random normal distribution of values
190 derived from the measured distribution of intensities [39] using a mean with a negative shift of
191 1.8 standard deviations and a standard deviation equal to 0.3 of the standard deviation of the
192 measured intensities. The probability of differential expression was calculated using the
193 function *lmFit* from the Bioconductor package limma [40] followed by *eBayes* using the
194 default settings [41] and false-discovery rate correction using the Benjamini–Hochberg
195 method. The output included P value, confidence interval and ratio estimate. GO-term
196 enrichment analysis was performed using the *enrichr* function in the Bioconductor
197 clusterProfiler package [42]. Enrichment was calculated separately for the proteins
198 overrepresented in each fraction, relative to all proteins identified in collected fractions across
199 all the LCMS 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 [43]. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 47(D1):D442-D450 partner repository with the dataset identifier *PXD023115*.

205 **RESULTS**

206 **MARCH1, but not MARCH8, is functional in professional APC**

207 The first objective of this study was to establish which mouse cells express MARCH1 or
208 MARCH8. Their low level of transcription combined with fast turn-over contribute to maintain
209 the two proteins at non-detectable levels in primary cells, hampering definition of their
210 expression pattern. We reasoned that MHC II and/or CD86 could be used as reporters of
211 MARCH1 and MARCH8 activity because in all primary or transformed cells analyzed so far,
212 the surface level of these two receptors decreases by expression of either ligase [44]. Cells that
213 express MHC II or CD86 and either MARCH1 or MARCH8 should therefore display higher
214 levels of the receptor(s) in *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ mice.

215

216 First, we examined professional APC (defined as cells that express detectable levels of MHC
217 II in the steady-state [45][46]) and T cells across various tissues. B cells, cDC1, cDC2, pDC
218 and macrophages from blood, spleen, subcutaneous lymph nodes (LN), thymus, peritoneal
219 cavity and lung of *Marchf1*$^{-/-}$ mice displayed elevated surface MHC II and CD86 relative to
220 WT cells, while no changes were observed in their *Marchf8*$^{-/-}$ counterparts (**Figure 1A-F**).
221 CD4^{+} and CD8^{+} T cells in spleen and LN showed no detectable surface MHC II and their CD86
222 expression [47] was not altered by MARCH1- nor MARCH8-deficiency (**Figure 1B-C**). MHC
223 II and CD86 expression in peritoneal cDC deficient in both MARCH1 and MARCH8
224 (*Marchf1*$^{-/-}$ x *Marchf8*$^{-/-}$) was not elevated above that of *Marchf1*$^{-/-}$ cells (**Supplementary**
225 **Figure 3)**. These results indicate that MARCH1 is expressed and active in all professional APC
226 across various organs/tissues whereas MARCH8 is not or, if it is, does not display enough
227 activity to compensate for the loss of MARCH1.

228

229 Next, we assessed the contribution of MARCH1 to activation-dependent regulation of MHC II
230 and CD86 expression in cDC, the archetypical professional APC. Toll-like receptor (TLR)
231 ligands trigger an activation program in DC, known as DC maturation, that includes up-
232 regulation of MHC II and CD86 expression on the plasma membrane, among other receptors
233 [48]. Activation also leads to down-regulation of *Marchf1* transcription which, combined with
234 fast turn-over of MARCH1, results in negligible expression of the protein in activated DCs
235 [6][49][50][51]. It has been assumed that this change is responsible for the accumulation of
236 MHC II and CD86 on the plasma membrane during cDC activation, but this has not been
237 directly examined. If ubiquitination were the dominant mechanism controlling how much
238 MHC II and CD86 is displayed on cDC, it would be expected that the expression of these two

239 molecules would not vary during activation of *Marchf1*$^{-/-}$ cDC. However, activation of
240 *Marchf1*$^{-/-}$ cDC further increased surface expression of MHC II by ~1.5 times, and increased
241 CD86 by ~4 times, when compared than their resting counterparts (**Figure 2**). CD40, which
242 also increases in expression during activation, though it is not a MARCH1 substrate, was
243 expressed at equivalent levels in WT and *Marchf1*$^{-/-}$ cDC at both resting and activated states,
244 so up-regulation of MHC II and CD86 in *Marchf1*$^{-/-}$ cDC could not be attributed to overall
245 dysregulation of surface receptor expression (**Figure 2**). These results indicate that the main
246 contributor to MHC II and, especially, CD86 up-regulation during DC activation is not reduced
247 ubiquitination and degradation, but sustained deposition of newly synthesized molecules on
248 the cell surface [52][53]. DC lacking MARCH8 were indistinguishable from WT cDC in these
249 experiments, again indicating it has no role in resting or activated cDC (**Figure 2**).
250

251 **Granulocytes and monocytes express MHC II and CD86, but MARCH1 ubiquitination**
252 **maintains their surface expression at negligible levels**

253 Next we assessed MARCH1 and MARCH8 activity in "atypical APC", this is, immune cells
254 that are not considered professional APC but have been suggested to play antigen-presenting
255 roles under certain conditions [46]. These include neutrophils, eosinophils and "inflammatory"
256 (Ly6C^{+}) and "patrolling" (Ly6C^{-}) monocytes. While monocytes have the potential to develop
257 into macrophages or DCs in inflamed sites [54], they are not thought to perform antigen
258 presenting functions in their undifferentiated state [55]. We examined these atypical APC in
259 spleen and lung. MHC II expression in WT neutrophils, eosinophils and monocytes was barely
260 detectable by flow cytometry, staining at just above the background level observed in cells of
261 mice that do not express any surface MHC II at all (**Figure 3**). Strikingly, all four cell types
262 deficient in MARCH1 expressed MHC II at levels comparable to WT B cells or cDC (compare
263 **Figures 1B** and **F** to **Figures 3A** and **B**, respectively), though expression was higher in spleen
264 than it was in their lung counterparts (**Figure 3A** and **B)**. CD86 was also highly expressed on
265 all four MARCH1-deficient cell types, in this case both in spleen and lungs (**Figure 3**).
266 MARCH8-deficient cells did not display altered MHC II or CD86 expression, confirming this
267 member of the MARCH family is not expressed and/or active in hematopoietic cells (**Figure**
268 **3**). Of note, MARCH1-deficient T cells lacked surface MHC II and did not exhibit enriched
269 CD86 expression when deficient in MARCH1 (**Figure 1B),** so neither mutation caused ectopic
270 or increased expression of either molecule. We conclude that neutrophils, eosinophils,
271 monocytes and possibly other atypical APC types [46] produce receptors for antigen
272 presentation and T cell stimulation constitutively. While MARCH1 ubiquitination maintains

273 the surface expression of these proteins at barely detectable levels, these atypical APC might
274 be capable of $CD4^+$ T cell priming under certain conditions.
275
276 **Previously predicted MARCH1 substrates display normal expression in *March1*$^{-/-}$ mice**
277 The second objective of this study was to identify which of the receptors found to be
278 ubiquitinated by MARCH1 or MARCH8 in (transfected) cell lines are also substrates *in vivo*
279 under physiological conditions. Such receptors include CD44, CD71, CD95 and CD98
280 (reviewed in [5][56]). Carrying out this analysis also allowed us to address the possibility that,
281 contrary to our conclusions above, MARCH8 might be expressed and active in these cells but
282 dedicated to ubiquitinate these receptors rather than MHC II and CD86. This was not the case;
283 expression of CD44, CD71, and CD98 was unaltered in *March8*$^{-/-}$ cDC and B cells compared
284 to WT cells (**Figure 4A**). Furthermore, *March1*$^{-/-}$ cDC and B cells also expressed normal levels
285 of the three receptors (**Figure 4A**). We extended our analysis to other regulatory receptors of
286 T cell activation, including CD40 and members of the B7 family to which CD86 (B7.2)
287 belongs: CD80 (B7.1), CD274 (PD-L1), CD273 (PD-L2), CD275 (ICOS-L), CD276 (B7-H3)
288 and B7-H4. Expression of all these receptors on cDC1, cDC2, pDC and B cells was unaltered
289 in the absence of MARCH1 (**Figure 4B**).
290
291 **Proteomic profiling of the plasma membrane of MARCH1- and MARCH8-deficient cDC**
292 **and B cells**
293 To more comprehensively address the role of MARCH1 and MARCH8 in APC membrane
294 proteostasis, we performed an unbiased proteomic screen where we compared the proteomes
295 of subcellular microsomal fractions enriched in plasma membrane (PM) of WT versus
296 *March1*$^{-/-}$ or *March8*$^{-/-}$ cDC and B cells. We have previously shown this is a robust approach
297 to identify differentially expressed PM proteins between closely related cell populations such
298 as the two major cDC subtypes, cDC1 and cDC2 [57]. To obtain sufficient numbers of primary
299 cDC for this purpose, these cells were expanded in WT, *March1*$^{-/-}$ and *March8*$^{-/-}$ mice bearing
300 a melanoma cell line that secretes the DC growth factor, Flt3L [33]. The cDC expanded using
301 this approach are phenotypically and functionally equivalent to their counterparts in untreated
302 mice [57]. Splenic B cells were purified from untreated mice. The protein profiles of each
303 fraction were identified by semi-quantitative mass spectrometry from three biological
304 replicates, each measured in technical triplicates.
305

306 We identified 1868-3108 proteins in the PM fraction of each cell type (**Supplementary Table**
307 **1**, total number of IDed proteins regardless of any restrictions**)**. Of note, the subcellular
308 fractions are comprised of microsomes generated during mechanical homogenization of cells,
309 so their composition includes PM but also cytosolic and extracellular content 'trapped' inside
310 microsomes or tethered to the cell surface. This method enables analysis of proteins loosely
311 associated with the inner or outer leaflet of the PM. To test the efficiency of the PM-enrichment
312 method, we also sedimented and analyzed in parallel the compartments that remained in the
313 post-nuclear supernatant (PNS) of homogenized cells after retrieval of the PM fraction
314 (mitochondria, endosomes, etc, henceforth termed intracellular compartments, IC). We
315 identified 2073-3537 proteins in the IC fraction of each cell type (**Supplementary Table 2**,
316 total number of IDed proteins regardless of any restrictions). In order to assess enrichment of
317 the PM by this methodology, we compared Gene Ontology (GO) terms/annotations of the
318 proteins identified in the PM and IC fractions of each cell type. This comparison clearly
319 demonstrated enrichment of proteins known to be expressed at the cell surface in the PM
320 fractions, and enrichment of proteins known to occur in intracellular compartments in the IC
321 fractions, validating the subcellular fractionation protocol (**Figure 5A** and **Supplementary**
322 **Figure 4)**.
323
324 Comparison of the PM proteomes of WT and *March1*$^{-/-}$ cDC showed that, as expected, most
325 proteins were present at similar levels in the two preparations (1020 proteins in total,
326 **Supplementary Table 3)**. Nine proteins were differentially expressed between WT and
327 *March1*$^{-/-}$ cDC PM [$\log_2$ protein ratio >1 or <1 and -log10 adjusted *p* value >3.47 (5% FDR)]
328 (**Figure 5B** and **Supplementary Table 5**). These included MHC IIα and β chains (H2-Aa and
329 H2-Ab1), as well as CD86, confirming the validity of our approach to detect MARCH1
330 substrates. Surprisingly, the protein that appeared most significantly overexpressed in the PM
331 of *March1*$^{-/-}$ cDC was complement component 3 (C3) (**Figure 5B, Supplementary Table 5**).
332 The remaining three proteins appearing over-expressed in the *March1*$^{-/-}$ cDC PM fraction are
333 not known to be immunoreceptors expressed at the PM: Cox7a2 is a mitochondrial protein,
334 Myadm a component of the cytoskeleton and MLV-related proviral Env polyprotein, a protein
335 endogenously encoded by a retrovirus integrated in the genome of commonly used mouse
336 strains [58]. As our main goal was to identify immunoregulatory MARCH1 substrates, we did
337 not investigate further whether these were true or artifactual "hits" of the proteomic analysis.
338 Comparison of the PM fractions of WT and *March8*$^{-/-}$ cDC did not reveal any differentially

339 expressed proteins (**Figure 5B,** 922 proteins in total, **Supplementary Table 3**), supporting the
340 previous results indicating that MARCH8 is not expressed/active in cDC.
341
342 *March1*$^{-/-}$ and *March8*$^{-/-}$ B cells exhibited 45 and 40 enriched and 15 and 17 reduced proteins,
343 respectively, in their PM fractions [$\log_2$ protein ratio >1 or <1. and -log10 adjusted *p* value
344 >2.5 and >2.36 for *March1*$^{-/-}$ and *March8*$^{-/-}$, respectively (both 5% FDR)] (**Figure 5B,**
345 **Supplementary Table 6** and **Supplementary Table 7**, 1275 and 1819 proteins in total,
346 **Supplementary Table 3**). MHC IIα and β chains (H2-Aa and H2-Ab1), as well as CD86 and
347 C3 were the most significantly enriched proteins in the PM fraction of *March1*$^{-/-}$ B cells
348 (**Figure 5B** and **Supplementary Table 6**), but neither of the four were enriched in *March8*$^{-/-}$
349 B cells (**Figure 5B** and **Supplementary Table 7)**. Only 14 of the 60 proteins differentially
350 expressed in the PM fraction of *March1*$^{-/-}$ B cells, and 10 of the 57 proteins differentially
351 expressed in the PM fraction of *March8*$^{-/-}$ B cells, were immunoreceptors and/or proteins
352 known to be expressed at the plasma membrane (**Supplementary Table 6** and **Supplementary**
353 **Table 7**). They included aminopeptidase N (CD13, gene *Anpep*), antigen-presenting
354 glycoprotein CD1d, T cell differentiation antigen CD6 and the immunoglobulin epsilon Fc
355 receptor CD23 (gene *Fcer2*). However, analysis by flow cytometry did not confirm differential
356 expression in either *March1*$^{-/-}$ or *March8*$^{-/-}$ B cells (**Supplementary Figure 5).** The most
357 likely explanation for detection of these "false positives" is that they were caused by subtle
358 differences in the purity of the B cell preparations or their subcellular fractions. In conclusion,
359 MHC class II and CD86 were the only membrane proteins that we could unequivocally confirm
360 as MARCH1 substrates in B cells, and while we cannot discard the possibility that some of the
361 "hits" found in the proteomic screen of *March8*$^{-/-}$ B cells are indeed MARCH8 substrates, it is
362 more likely that MARCH8 is not active in B cells, just as it is not in DC.
363
364 **MARCH8, not MARCH1, is active in non-hematopoietic cells**
365 The only cell type in which MARCH8 activity has been demonstrated is thymic epithelial cells
366 (TEC), where it regulates MHC II surface expression but not CD86 [9][10]. Analysis of CD40,
367 CD44, CD95 and CD98 expression in WT and *March8*$^{-/-}$ medullar and cortical TEC showed
368 that neither of these receptors, which have been shown to be ubiquitinated in cell lines
369 overexpressing MARCH8, are physiological substrates (**Figure 6A**).
370
371 Although TEC constitutively present antigens via MHC II, they are not hematopoietic cells,
372 but of endodermal origin [59]. Therefore, we asked the question whether other cells

373 ontogenically related to TEC also use MARCH8 to regulate surface MHC II expression.
374 Epithelial cells in the respiratory tract are known to express MHC II, with the highest level
375 found on type II alveolar epithelial cells (AEC) [60][61][62]. We found that MARCH8-
376 deficient type II AEC showed enriched MHC II surface expression (**Figure 6B**), but MHC II
377 levels in mutant endothelial cells, type I AEC and bronchial epithelial cells was not altered
378 (**Figure 6B**). Neither cell type displayed increased CD86 expression in the absence of
379 MARCH8, and lack of MARCH1 did not affect MHC II nor CD86 expression in any of the
380 cell types analyzed (**Figure 6B**). In conclusion, not all epithelial cells regulate MHC II
381 expression via ubiquitination, but those that do employ MARCH8.

382 **DISCUSSION**

383 Determining which cells utilize MARCH1 and MARCH8 has been hampered by their low level
384 of expression, but analysis of MHC II and CD86 as surrogate markers of activity has allowed
385 us to establish the role of MARCH1 as a master regulator of MHC II and CD86 expression in
386 all hematopoietic cells. MARCH8 plays an equivalent role in the two major types of TEC and
387 in type II AEC, where it ubiquitinates MHC II. We did not observe high CD86 expression in
388 any *March8*$^{-/-}$ cell, but this could be because these cells do not ubiquitinate CD86 or because
389 they do not express it. There are at least two precedents for ontogeny-specific differences in
390 the use of components of MHC II antigen presentation machinery. Expression of CIITA, which
391 directs transcription of the genes for MHC II and for several accessory molecules involved in
392 antigen presentation, is driven by distinct promoters in hematopoietic and non-hematopoietic
393 cells [63]. Proteolysis of the chaperone invariant chain, a critical step in the MHC II antigen
394 presentation pathway, is carried out by cathepsin S in hematopoietic cells and by and cathepsin
395 L in non-hematopoietic cells [64]. It is unclear why this dichotomy exists, which is probably
396 caused by the establishment of cell lineage-specific gene programs during embryonic
397 development.

398

399 While our finding that MARCH1 is operative in professional APC confirmed previous
400 observations, we were surprised to observe high MHC II and CD86 expression in non-
401 professional APC lacking MARCH1. This was not caused by ectopic induction or
402 overexpression of either molecule because MARCH1-deficient T cells maintained WT levels
403 of MHC II (negative) and CD86 (low) expression. As MARCH1 ubiquitinates substrates that
404 have already trafficked through the cell surface, this finding implies that atypical APC express
405 and deposit on their plasma membrane larger amounts of MHC II and CD86 than is usually
406 appreciated, but their steady-state levels are kept low by virtue of MARCH1 ubiquitination and
407 accelerated turn-over. Eosinophils are associated with inflammatory responses during allergy
408 or parasitic infections, while neutrophils are recruited in abundant numbers to sites of tissue
409 damage or infection. The role of MHC II antigen presentation by either cell type is
410 controversial. While there is evidence for both purified eosinophils and neutrophils that
411 demonstrates their capacity to present antigen via MHC II [46], it is difficult to exclude the
412 possibility of DC contamination in these assays. *In vivo* evidence of their antigen presentation
413 capacity is scarce but there are reported examples where both eosinophils [65] and neutrophils
414 [66][67][68] contribute to enhancing antigen-specific CD4^{+} T cell responses. The realization

415 that these cells regulate MHC II and CD86 via ubiquitination utilizing the same mechanism as
416 professional APC lends weight to the notion that they perform antigen presentation *in vivo*.
417
418 One of the functions attributed to MARCH8 in humans is to ubiquitinate viral proteins
419 deposited on the plasma membrane of infected cells and that will be incorporated in the envelop
420 of the virion upon budding [69][70][71]. The reduction of viral protein expression that ensues
421 inhibits spread of the infection, protecting the host. This activity has not been described in
422 mice, but our results suggest that if it occurs in this species, it is unlikely to be operative in
423 hematopoietic cells, where perhaps other members of the MARCH family replace the function
424 of MARCH8.
425
426 While several substrates have been identified for MARCH1 and MARCH8 based on studies
427 using overexpression and/or cell lines, our flow cytometry analysis rules out CD44, CD71,
428 CD95 and CD98 as bona fide MARCH1 or MARCH8 substrates in all primary cells examined.
429 This highlights that caution needs to be taken when interpreting studies that rely on E3 Ub
430 ligase overexpression. Our unbiased proteomic profiling of B cells and DC unequivocally
431 confirmed the role of MARCH1 in MHC II and CD86 ubiquitination in both cell types, but did
432 not reveal any other MARCH1 substrate that we could validate by flow cytometry with the
433 exception of complement C3. Further investigations will be required to determine if enriched
434 levels of surface C3 in these cells is a direct or indirect effect of MACRH1 deficiency, as we
435 have also shown that high MHC II expression in *March1*$^{-/-}$ cells indirectly induces higher or
436 lower expression of other surface receptors that are not direct MARCH1 substrates [72].
437 However, the magnitude of these changes is below the level of resolution afforded by high-
438 throughput, unbiased proteomic analysis of subcellular fractions. We did not observe changes
439 in expression of any protein on the plasma membrane of *March8*$^{-/-}$ DC. The "hits" detected
440 *March8*$^{-/-}$ B cell membrane could be attributed to contamination with other subcellular
441 compartments because they were not classified as plasma membrane proteins and/or could not
442 be validated as differentially expressed by flow cytometry. The proteomic analysis thus
443 confirmed that neither B cells nor DC express functional MARCH8.
444
445 In summary, MHC II is the only membrane protein unequivocally regulated by MARCH1 and
446 MARCH8 in primary mouse cells, with each ligase playing its role in haemopoietic and non-
447 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.

ACKNOWLEDGEMENTS

We thank the Antibody Services Facility and Genomics Hub (Walter and Eliza Hall Institute) and the Melbourne Cytometry Platform (The University of Melbourne) for expert assistance. JAV: NHMRC Fellowship 1058193 and 1154502, NHMRC Program 1016629 and 1113293, ARC DP160103134 and DP110101383, and Human Frontiers Science Program Grant 0064/2011.

PS: Australian Research Training Programme Scholarship provided by the Australian Commonwealth Government and the University of Melbourne.

459 **REFERENCES**

460 [1] D. Komander and M. Rape, "The Ubiquitin Code," *Annu. Rev. Biochem.*, vol. 81, no.
461 1, pp. 203–229, Jul. 2012, doi: 10.1146/annurev-biochem-060310-170328.
462 [2] H. Liu, J. D. Mintern, and J. A. Villadangos, "MARCH ligases in immunity," *Curr.*
463 *Opin. Immunol.*, vol. 58, no. Box 1, pp. 38–43, Jun. 2019, doi:
464 10.1016/j.coi.2019.03.001.
465 [3] L. Coscoy, D. J. Sanchez, and D. Ganem, "A novel class of herpesvirus-encoded
466 membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in
467 immune recognition," *J. Cell Biol.*, vol. 155, no. 7, pp. 1265–1274, Dec. 2001, doi:
468 10.1083/jcb.200111010.
469 [4] S. Ishido, C. Wang, B.-S. Lee, G. B. Cohen, and J. U. Jung, "Downregulation of Major
470 Histocompatibility Complex Class I Molecules by Kaposi's Sarcoma-Associated
471 Herpesvirus K3 and K5 Proteins," *J. Virol.*, vol. 74, no. 11, pp. 5300–5309, Jun. 2000,
472 doi: 10.1128/JVI.74.11.5300-5309.2000.
473 [5] T. Samji, S. Hong, and R. E. Means, "The Membrane Associated RING-CH Proteins:
474 A Family of E3 Ligases with Diverse Roles through the Cell," *Int. Sch. Res. Not.*, vol.
475 2014, pp. 1–23, 2014, doi: 10.1155/2014/637295.
476 [6] L. J. Young *et al.*, "Differential MHC class II synthesis and ubiquitination confers
477 distinct antigen-presenting properties on conventional and plasmacytoid dendritic
478 cells," *Nat. Immunol.*, vol. 9, no. 11, pp. 1244–1252, Nov. 2008, doi: 10.1038/ni.1665.
479 [7] K.-J. Cho, E. Walseng, S. Ishido, and P. A. Roche, "Ubiquitination by March-I
480 prevents MHC class II recycling and promotes MHC class II turnover in antigen-
481 presenting cells," *Proc. Natl. Acad. Sci.*, vol. 112, no. 33, pp. 10449–10454, Aug.
482 2015, doi: 10.1073/pnas.1507981112.
483 [8] O. Bannard *et al.*, "Ubiquitin-mediated fluctuations in MHC class II facilitate efficient
484 germinal center B cell responses," *J. Exp. Med.*, vol. 213, no. 6, pp. 993–1009, May
485 2016, doi: 10.1084/jem.20151682.
486 [9] H. Liu *et al.*, "Ubiquitin ligase MARCH 8 cooperates with CD83 to control surface
487 MHC II expression in thymic epithelium and CD4 T cell selection," *J. Exp. Med.*, vol.
488 213, no. 9, pp. 1695–1703, Aug. 2016, doi: 10.1084/jem.20160312.
489 [10] J. von Rohrscheidt *et al.*, "Thymic CD4 T cell selection requires attenuation of
490 March8-mediated MHCII turnover in cortical epithelial cells through CD83," *J. Exp.*
491 *Med.*, vol. 213, no. 9, pp. 1685–1694, Aug. 2016, doi: 10.1084/jem.20160316.
492 [11] J. Oh *et al.*, "MARCH1-mediated MHCII ubiquitination promotes dendritic cell
493 selection of natural regulatory T cells," *J. Exp. Med.*, vol. 210, no. 6, pp. 1069–1077,
494 Jun. 2013, doi: 10.1084/jem.20122695.
495 [12] R. Ishikawa, M. Kajikawa, and S. Ishido, "Loss of MHC II ubiquitination inhibits the
496 activation and differentiation of CD4 T cells," *Int. Immunol.*, vol. 26, no. 5, pp. 283–
497 289, May 2014, doi: 10.1093/intimm/dxt066.
498 [13] T. Galbas *et al.*, "MARCH1 E3 Ubiquitin Ligase Dampens the Innate Inflammatory
499 Response by Modulating Monocyte Functions in Mice," *J. Immunol.*, vol. 198, no. 2,
500 pp. 852–861, Jan. 2017, doi: 10.4049/jimmunol.1601168.
501 [14] J. Wu *et al.*, "The E3 ubiquitin ligase MARCH1 regulates antimalaria immunity
502 through interferon signaling and T cell activation," *Proc. Natl. Acad. Sci. U. S. A.*, vol.
503 117, no. 28, pp. 16567–16578, 2020, doi: 10.1073/pnas.2004332117.
504 [15] Y. Zhang *et al.*, "Membrane-associated RING-CH (MARCH) 1 and 2 are MARCH
505 family members that inhibit HIV-1 infection," *J. Biol. Chem.*, vol. 294, no. 10, pp.
506 3397–3405, Mar. 2019, doi: 10.1074/jbc.AC118.005907.
507 [16] L. Xie *et al.*, "MARCH1 encourages tumour progression of hepatocellular carcinoma
508 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,

609 no. 11, pp. 719–730, Nov. 2014, doi: 10.1038/nri3754.
610 [47] J. Eberlein, B. Davenport, T. T. Nguyen, F. Victorino, T. Sparwasser, and D. Homann,
611 "Multiple Layers of CD80/86-Dependent Costimulatory Activity Regulate Primary,
612 Memory, and Secondary Lymphocytic Choriomeningitis Virus-Specific T Cell
613 Immunity," *J. Virol.*, vol. 86, no. 4, pp. 1955–1970, 2012, doi: 10.1128/jvi.05949-11.
614 [48] R. Spörri and C. Reis e Sousa, "Inflammatory mediators are insufficient for full
615 dendritic cell activation and promote expansion of CD4+ T cell populations lacking
616 helper function," *Nat. Immunol.*, vol. 6, no. 2, pp. 163–170, 2005, doi: 10.1038/ni1162.
617 [49] J. Vega-Ramos, A. Roquilly, Y. Zhan, L. J. Young, J. D. Mintern, and J. A.
618 Villadangos, "Inflammation Conditions Mature Dendritic Cells To Retain the Capacity
619 To Present New Antigens but with Altered Cytokine Secretion Function," *J. Immunol.*,
620 vol. 193, no. 8, pp. 3851–3859, 2014, doi: 10.4049/jimmunol.1303215.
621 [50] A. De Gassart *et al.*, "MHC class II stabilization at the surface of human dendritic cells
622 is the result of maturation-dependent MARCH I down-regulation," *Proc. Natl. Acad.*
623 *Sci.*, vol. 105, no. 9, pp. 3491–3496, Mar. 2008, doi: 10.1073/pnas.0708874105.
624 [51] T. Galbas and J. Thibodeau, "Cell-type specific regulation of MARCH1 E3 ubiquitin
625 ligase by the anti-inflammatory cytokine IL-10," *Open J. Immunol.*, vol. 02, no. 04,
626 pp. 161–167, 2012, doi: 10.4236/oji.2012.24020.
627 [52] J. A. Villadangos *et al.*, "MHC Class II Expression Is Regulated in Dendritic Cells
628 Independently of Invariant Chain Degradation," *Immunity*, vol. 14, no. 6, pp. 739–749,
629 Jun. 2001, doi: 10.1016/S1074-7613(01)00148-0.
630 [53] N. S. Wilson, D. El-Sukkari, and J. A. Villadangos, "Dendritic cells constitutively
631 present self antigens in their immature state in vivo and regulate antigen presentation
632 by controlling the rates of MHC class II synthesis and endocytosis," *Blood*, vol. 103,
633 no. 6, pp. 2187–2195, Mar. 2004, doi: 10.1182/blood-2003-08-2729.
634 [54] C. Auffray, M. H. Sieweke, and F. Geissmann, "Blood Monocytes: Development,
635 Heterogeneity, and Relationship with Dendritic Cells," *Annu. Rev. Immunol.*, vol. 27,
636 no. 1, pp. 669–692, 2009, doi: 10.1146/annurev.immunol.021908.132557.
637 [55] C. V. Jakubzick, G. J. Randolph, and P. M. Henson, "Monocyte differentiation and
638 antigen-presenting functions," *Nat. Rev. Immunol.*, vol. 17, no. 6, pp. 349–362, Jun.
639 2017, doi: 10.1038/nri.2017.28.
640 [56] J. Bauer, O. Bakke, and J. P. Morth, "Overview of the membrane-associated RING-
641 CH (MARCH) E3 ligase family," *N. Biotechnol.*, vol. 38, pp. 7–15, Sep. 2017, doi:
642 10.1016/j.nbt.2016.12.002.
643 [57] E. Segura *et al.*, "Differential expression of pathogen-recognition molecules between
644 dendritic cell subsets revealed by plasma membrane proteomic analysis," *Mol.*
645 *Immunol.*, vol. 47, no. 9, pp. 1765–1773, May 2010, doi:
646 10.1016/j.molimm.2010.02.028.
647 [58] C. Stocking and C. A. Kozak, "Endogenous retroviruses," *Cell. Mol. Life Sci.*, vol. 65,
648 no. 21, pp. 3383–3398, Nov. 2008, doi: 10.1007/s00018-008-8497-0.
649 [59] J. Gordon *et al.*, "Functional evidence for a single endodermal origin for the thymic
650 epithelium," *Nat. Immunol.*, vol. 5, no. 5, pp. 546–553, 2004, doi: 10.1038/ni1064.
651 [60] J. E. Wosen, D. Mukhopadhyay, C. Macaubas, and E. D. Mellins, "Epithelial MHC
652 Class II Expression and Its Role in Antigen Presentation in the Gastrointestinal and
653 Respiratory Tracts," *Front. Immunol.*, vol. 9, no. SEP, pp. 1–14, Sep. 2018, doi:
654 10.3389/fimmu.2018.02144.
655 [61] H. Nakano, K. Nakano, and D. N. Cook, "Isolation and Purification of Epithelial and
656 Endothelial Cells from Mouse Lung," in *Methods in Molecular Biology*, vol. 1799, R.
657 L. Reinhardt, Ed. New York, NY: Springer New York, 2018, pp. 59–69.
658 [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.

707 **FIGURE LEGENDS**

708

709 **Figure 1. Ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in**
710 **haemopoietic professional antigen presenting cells**. Surface expression of MHC II and
711 CD86 in (A) blood B cells, (B) splenic cDC1, cDC2, pDC, B cells, macrophages (MAC) and
712 $CD4^+/CD8^+$ T cells, (C) resident and migratory cDC1 and cDC2 as well as B cells,
713 macrophages and $CD4^+/CD8^+$ T cells in subcutaneous (axillary + inguinal) lymph nodes, (D)
714 thymic cDC1, cDC2 and B cells, (D) peritoneal cDC1, cDC2, B cells and small/large
715 macrophages and (E) lung cDC1, cDC2, pDC, B cells and alveolar/interstitial macrophages,
716 all purified from WT mice or mice deficient in either MARCH1 or MARCH8. In all cases a
717 fluorescence-minus-one (FMO) control was included, for which cells were incubated with the
718 corresponding multi-colour staining panel, excluding the fluorescently labelled antibody
719 species of interest (i.e. anti-CD86 or anti-MHC II mAb). Bars represent mean ± SD with each
720 symbol representing an individual mouse (n=4-5). Statistical analysis was performed using
721 one-way ANOVA followed by Sidak's multiple comparisons test. **** $p < 0.0001$, *** $p <$
722 0.0002, ** $p < 0.002$, * $p < 0.03$, n.s. not significant.

723

724 **Figure 2. The role of ubiquitination of MHC II and CD86 by MARCH1 and MARCH8**
725 **in DC maturation.** (A) Surface expression of MHC II and CD86 in CpG-activated cDC
726 purified from the spleen of WT mice or mice deficient in either MARCH1 or MARCH8.
727 Purified splenic cDC ($2x10^5$ cells) were incubated for 16 hours *ex vivo* with or without 50 nm
728 CpG in 96-well plates, then washed and analyzed by flow cytometry for MHC II and CD86
729 surface expression. A fluorescence-minus-one (FMO) control was included, for which cells
730 were incubated with the corresponding multi-colour staining panel, excluding the fluorescently
731 labelled antibody species of interest (i.e. anti-CD86 or anti-MHC II mAb). Bars represent mean
732 ± SD with each symbol representing an individual mouse (n=6). Statistical analysis was
733 performed using one-way ANOVA followed by Sidak's multiple comparisons test. **** $p <$
734 0.0001, *** $p < 0.0002$, ** $p < 0.002$, * $p < 0.03$, n.s. not significant.

735

736 **Figure 3. Ubiquitination of MHC II and CD86 by MARCH1 and MARCH8 in**
737 **granulocytes and monocytes.** Surface expression of MHC II and CD86 in neutrophils,
738 eosinophils and inflammatory and patrolling monocytes purified from (A) spleen and (B) lung
739 of WT mice or mice deficient in I-Aα, MARCH1 or MARCH8. Bars represent mean ± SD with
740 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

775 Supplementary Figure 4. (B) Detection of differentially expressed proteins in the PM fraction
776 of cDC and B cells of WT versus *Marchf1*$^{-/-}$ and WT versus *Marchf8*$^{-/-}$ mice. Equivalent
777 amounts of PM fractions (based on cell count) of three biological replicates were analyzed by
778 mass spectrometry and semi-quantitative proteomics in three technical replicates. Proteins
779 detected in both WT and *Marchf1*$^{-/-}$ or *Marchf8*$^{-/-}$ cDC/B cells were displayed in volcano plots
780 (1020 proteins for WT vs. *Marchf1*$^{-/-}$ cDC, 922 proteins for WT vs. *Marchf8*$^{-/-}$ cDC, 1275
781 proteins for WT vs. *Marchf1*$^{-/-}$ B cells and 1819 proteins for WT vs. *Marchf8*$^{-/-}$ B cells) with
782 differentially expressed proteins [red dots] identified based on two-fold ratio (log2 protein ratio
783 >1 or <1) and significance (5% FDR) across three biological replicates, each measured in
784 technical triplicates. The known MARCH1 substrates, MHC II (H2-Aa and H2-Ab1) and CD86
785 in B cells and cDC, are highlighted in green in each volcano plot.
786
787 **Figure 6. Ubiquitination of MHC II, CD86 and putative substrates by MARCH1 and**
788 **MARCH8 in non-haemopoietic antigen presenting cells.** (A) Surface expression of MHC II,
789 CD86, CD80, CD40, CD44, CD95 and CD98 in medullary and cortical thymic epithelial cells
790 (mTEC and cTEC) purified from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. (B) Surface expression
791 of MHC II and CD86 in endothelial cells, type I and type II alveolar epithelial cells (AEC) as
792 well as bronchiolar epithelial cells, purified from the lung of WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$
793 mice. In all cases a fluorescence-minus-one (FMO) control was included, for which cells were
794 incubated with the corresponding multi-colour staining panel, excluding the fluorescently
795 labelled antibody species of interest. Bars represent mean ± SD with each symbol representing
796 an individual mouse (n=5). Statistical analysis was performed using one-way ANOVA
797 followed by Sidak's multiple comparisons test. **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.002$,
798 * $p < 0.03$, n.s. not significant.

799 **SUPPLEMENTARY FIGURE LEGENDS**

800

801 **Supplementary Figure 1**

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

833 with further discrimination of interstitial macrophages as Siglec-F^{low} CD11b^{high} and alveolar
834 macrophages as Siglec-F^{high} CD11b^{mid} (as described in Svedberg *et al*. [76]). Lung B cells were
835 identified as CD64^{-} MerTK^{-} CD11c^{-} MHC II^{+} CD19^{+} and cDC as CD64^{-} MerTK^{-} CD11c^{+}
836 MHC II^{+} with further discrimination of cDC1 as CD11b^{-} XCR1^{+} and cDC2 as CD11b^{+} XCR1^{-}
837 . Lung granulocytes and monocytes were identified as B220^{-} CD3^{-} CD4^{-} CD8^{-} CD11c$^{low-mid}$
838 CD11b^{high} with neutrophils identified as Ly6G^{+}, eosinophils as Ly6G^{-} SSC-H^{high} Ly6C$^{low-mid}$,
839 patrolling monocytes as Ly6G^{-} Ly6C$^{low-mid}$ SSC-H^{low} and inflammatory monocytes as Ly6G^{-}
840 Ly6C^{high} SSC-H^{low} (as described in Liyanage *et al*. [73]). Non-haemopoietic cells in the lung
841 were identified as CD45^{-} with endothelial cells identified as EpCAM$^{low-mid}$ CD31^{+} Sca-1^{+} and
842 epithelial cells as EpCAM$^{mid-high}$ CD31^{-}. Further discrimination of epithelial cells was carried
843 out based of CD24, EpCAM and MHC II expression (as described in Nakano *et al*. [61] and
844 Hasegawa *et al*. [62]) with bronchiolar epithelial cells identified as EpCAM^{high} CD24^{high}, type
845 II alveolar epithelial cells (AEC) identified as EpCAM^{high} CD24^{mid} MHC II^{high} (red) and type
846 I AEC identified as EpCAM^{mid} CD24^{low} MHC II^{low} (green).
847 A comparison of the representative flow cytometry gating strategies for the identification of all
848 cell populations of interest between WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice is shown in
849 Supplementary Figure 2.
850
851 **Supplementary Figure 2**
852 Comparison of representative flow cytometry gating strategies for the identification of various
853 cell populations in (A) blood, (B) spleen, (C) subcutaneous lymph node, (D) thymus, (E)
854 peritoneal cavity and (F) lung from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-/-}$ mice. A detailed description
855 of the gating strategies for each individual cell population of interest is presented in
856 Supplementary Figure 1.
857
858 **Supplementary Figure 3**
859 Surface expression of MHC II and CD86 in peritoneal cDC from WT, *Marchf1*$^{-/-}$ and *Marchf8*$^{-}$
860 $^{/-}$ mice or from mice deficient in both MARCH1 and MARCH8 (*Marchf1*$^{-/-}$ x *Marchf8*$^{-/-}$). A
861 fluorescence-minus-one (FMO) control was included, for which cells were incubated with the
862 corresponding multi-colour staining panel, excluding the fluorescently labelled antibody
863 species of interest (i.e. anti-CD86 or anti-MHC II mAb). Bars represent mean ± SD with each
864 symbol representing an individual mouse (n=4). Statistical analysis was performed using one-
865 way ANOVA followed by Sidak's multiple comparisons test. **** $p < 0.0001$, *** $p < 0.0002$,
866 ** $p < 0.002$, * $p < 0.03$, 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, *March1*$^{-/-}$ or *March8*$^{-/-}$ 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, *March1*$^{-/-}$ and
881 *March8*$^{-/-}$ 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

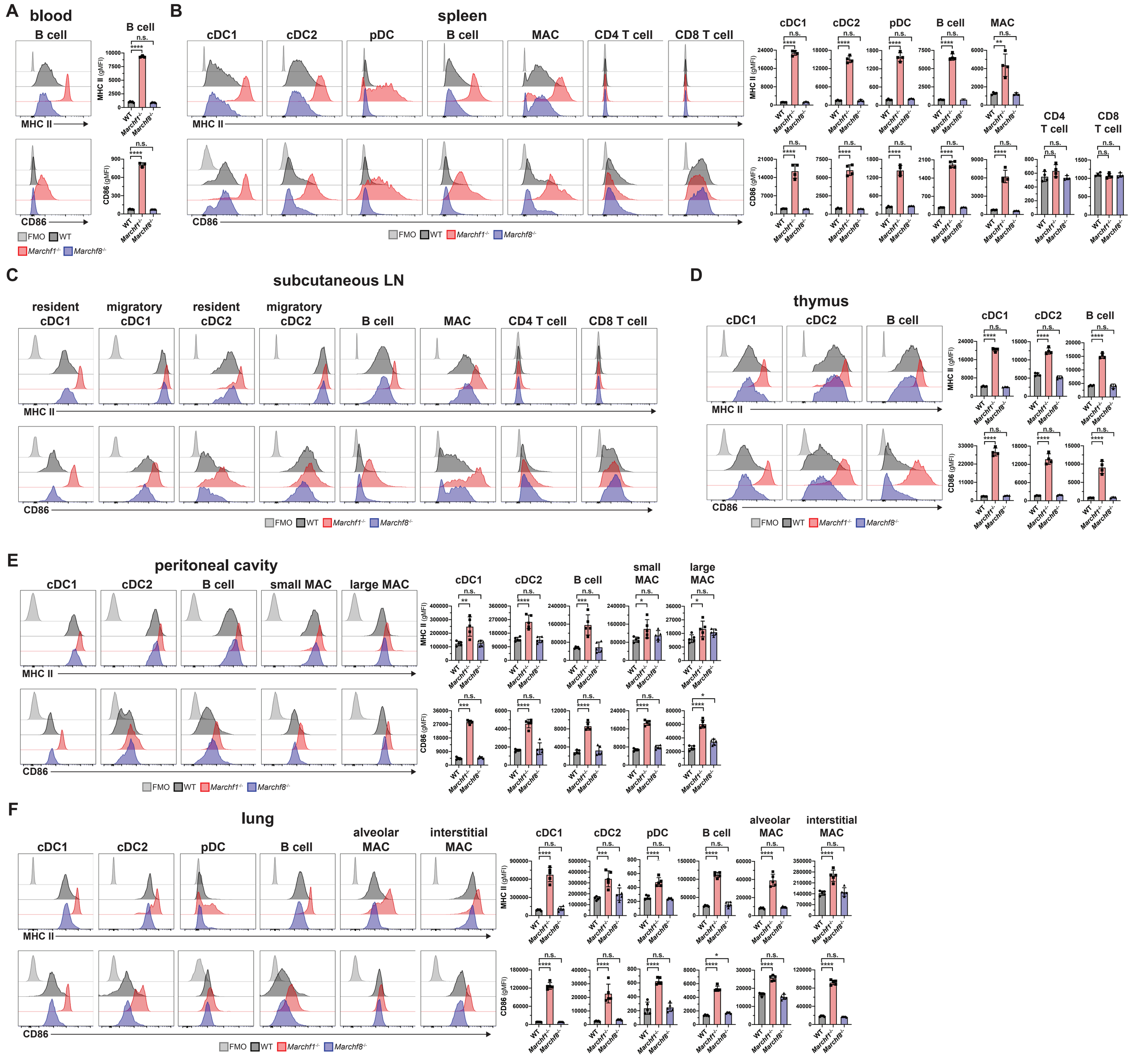

A
blood
B cell
MHC II
CD86
MHC II (gMFI)
CD86 (gMFI)
n.s.
****
WT
Marchf1-/-
Marchf8-/-
FMO
B
spleen
cDC1
cDC2
pDC
B cell
MAC
CD4 T cell
CD8 T cell
C
subcutaneous LN
resident cDC1
migratory cDC1
resident cDC2
migratory cDC2
D
thymus
E
peritoneal cavity
small MAC
large MAC
F
lung
alveolar MAC
interstitial MAC

Figure 2

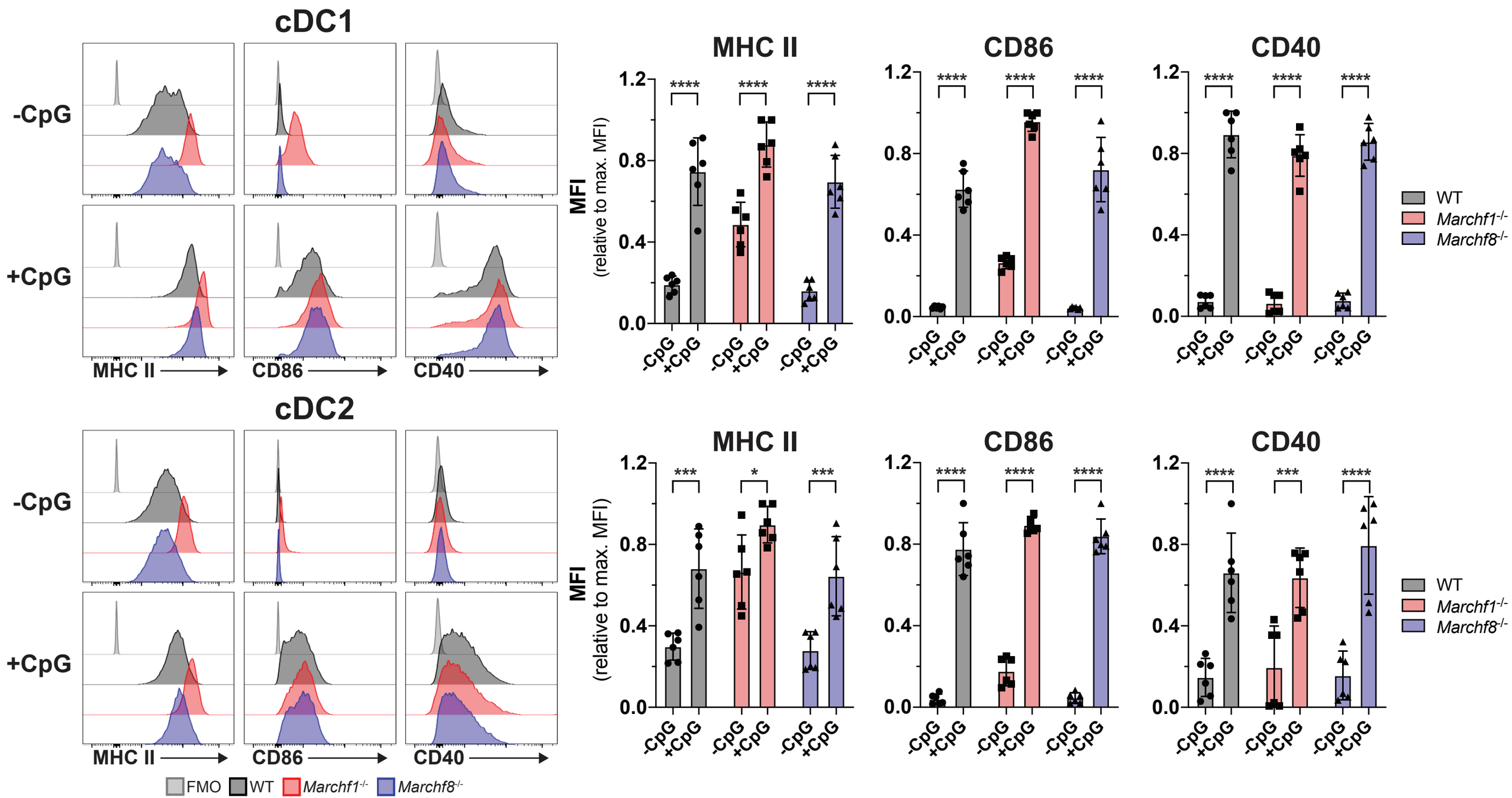

cDC1
-CpG
+CpG
MHC II
CD86
CD40
MFI
(relative to max. MFI)
1.2
0.8
0.4
0.0
****
-CpG
+CpG
WT
Marchf1-/-
Marchf8-/-
cDC2
***
*
FMO

Figure 3

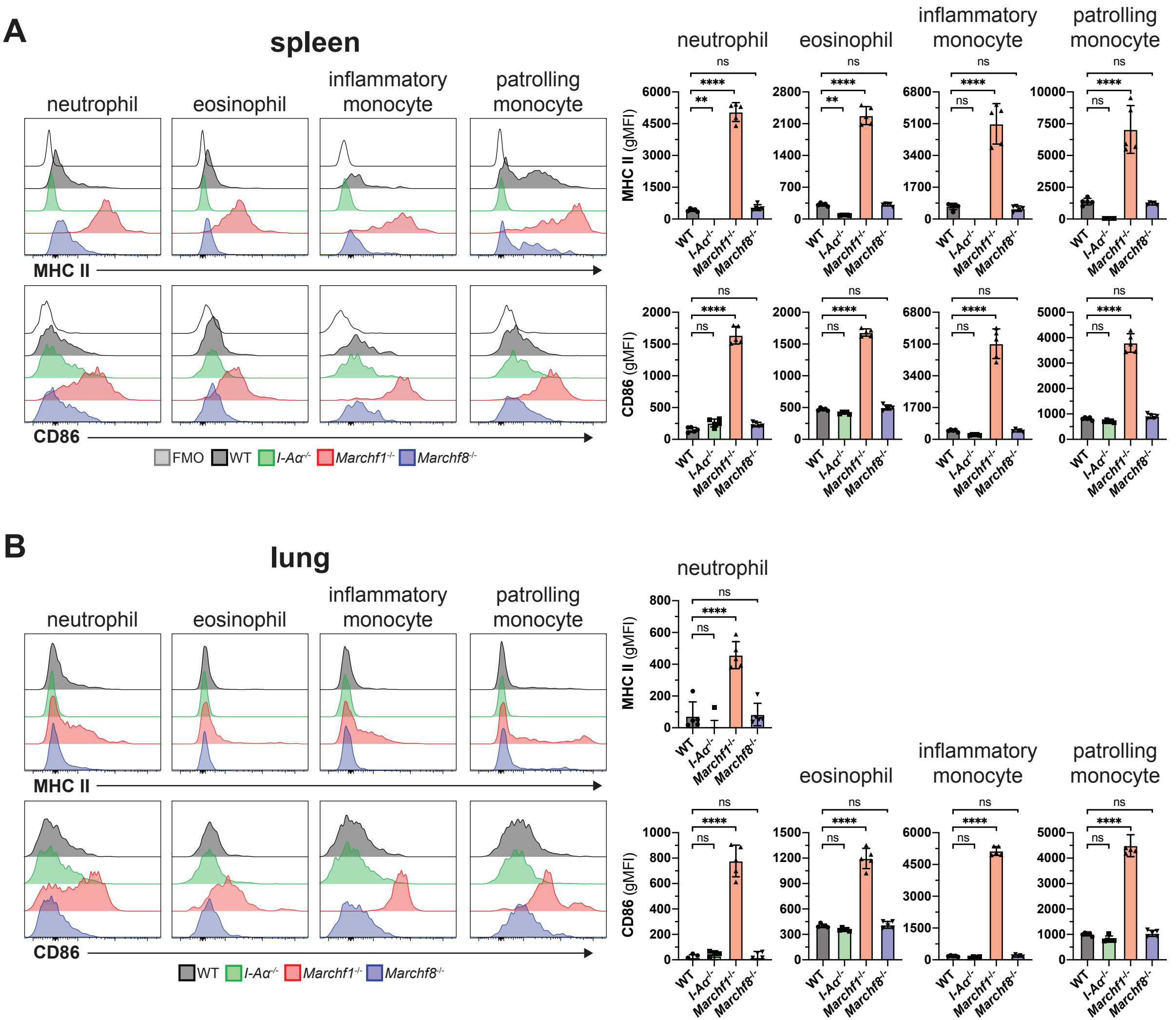

A
spleen
neutrophil
eosinophil
inflammatory monocyte
patrolling monocyte
MHC II
CD86
FMO
WT
I-Aα-/-
Marchf1-/-
Marchf8-/-
MHC II (gMFI)
CD86 (gMFI)
ns
****
**
B
lung

Figure 4

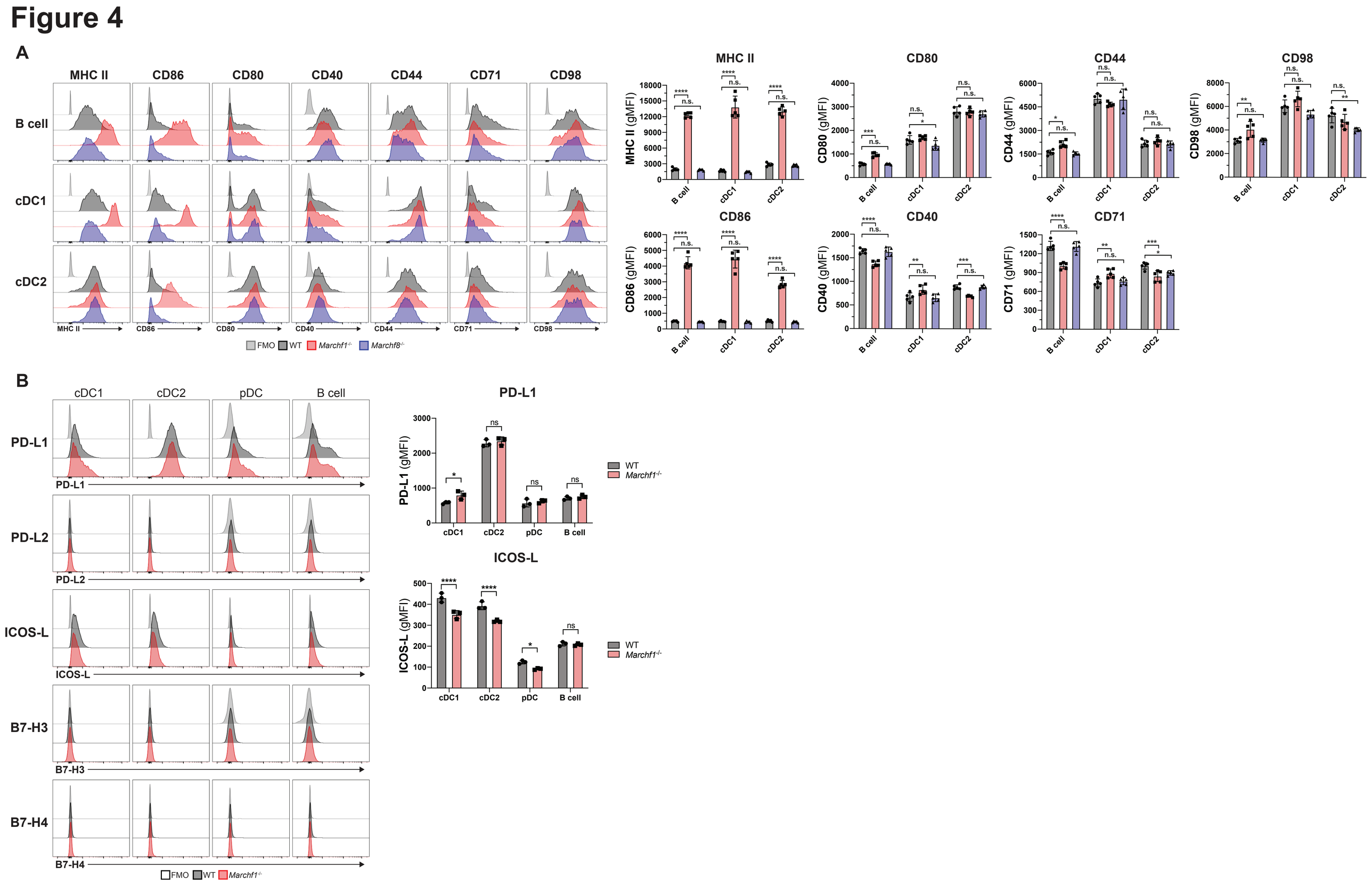

A
MHC II
CD86
CD80
CD40
CD44
CD71
CD98
B cell
cDC1
cDC2
FMO
WT
Marchf1-/-
Marchf8-/-
MHC II (gMFI)
CD80 (gMFI)
CD44 (gMFI)
CD98 (gMFI)
CD86 (gMFI)
CD40 (gMFI)
CD71 (gMFI)
B
cDC1
cDC2
pDC
B cell
PD-L1
PD-L2
ICOS-L
B7-H3
B7-H4
PD-L1 (gMFI)
ICOS-L (gMFI)
WT
Marchf1-/-

Figure 5

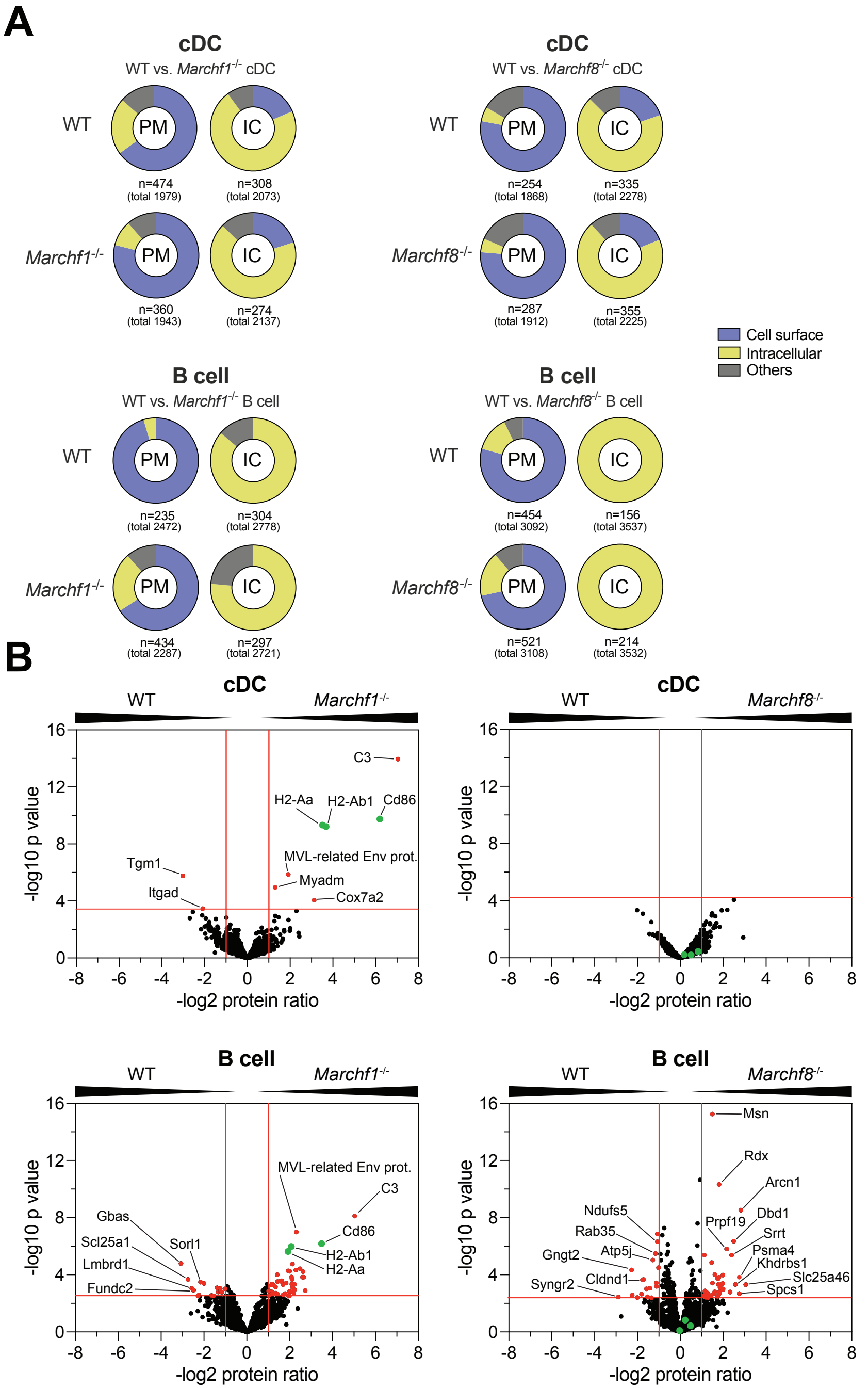
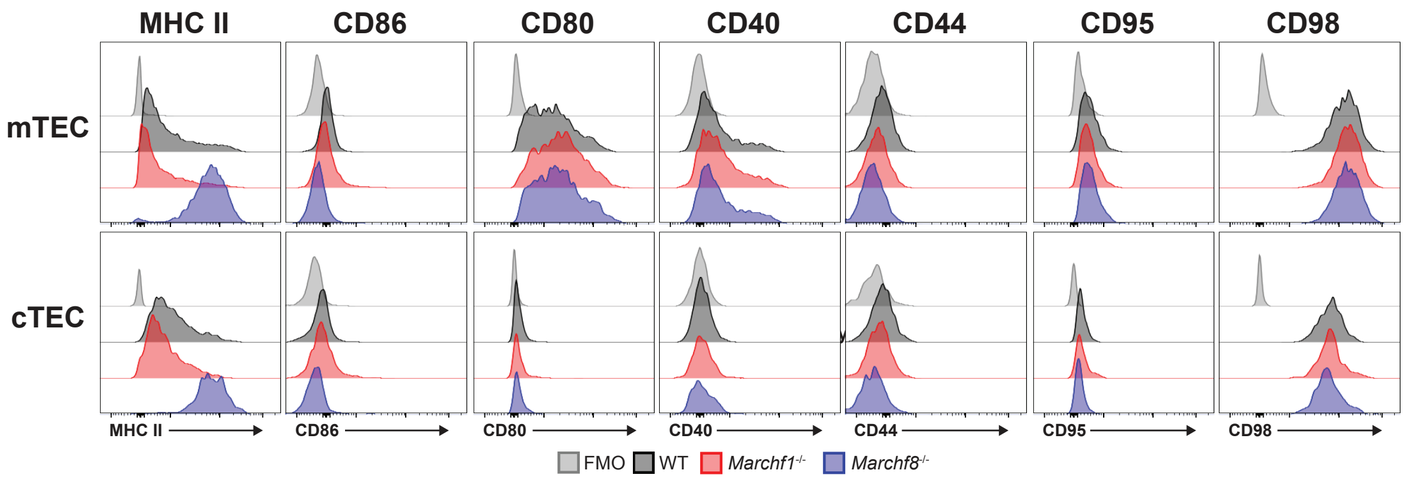
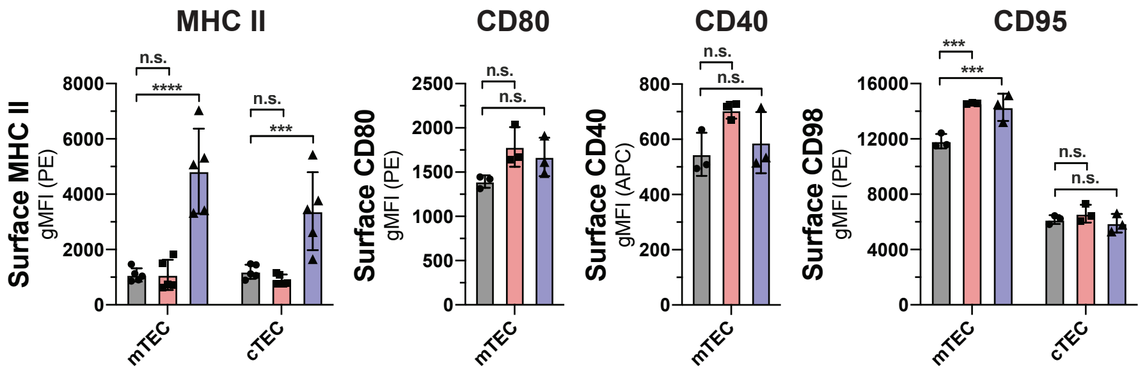

A
cDC
WT vs. Marchf1-/- cDC
WT
Marchf1-/-
PM
IC
n=474 (total 1979)
n=308 (total 2073)
n=360 (total 1943)
n=274 (total 2137)
cDC
WT vs. Marchf8-/- cDC
n=254 (total 1868)
n=335 (total 2278)
n=287 (total 1912)
n=355 (total 2225)
Cell surface
Intracellular
Others
B cell
WT vs. Marchf1-/- B cell
n=235 (total 2472)
n=304 (total 2778)
n=434 (total 2287)
n=297 (total 2721)
B cell
WT vs. Marchf8-/- B cell
n=454 (total 3092)
n=156 (total 3537)
n=521 (total 3108)
n=214 (total 3532)
B
cDC
WT
Marchf1-/-
-log10 p value
-log2 protein ratio
C3
H2-Aa
H2-Ab1
Cd86
MVL-related Env prot.
Tgm1
Myadm
Itgad
Cox7a2
Marchf8-/-
B cell
Gbas
Scl25a1
Sorl1
Lmbrd1
Fundc2
Msn
Rdx
Arcn1
Ndufs5
Dbd1
Prpf19
Rab35
Srrt
Psma4
Gngt2
Atp5j
Khdrbs1
Cldnd1
Slc25a46
Syngr2
Spcs1

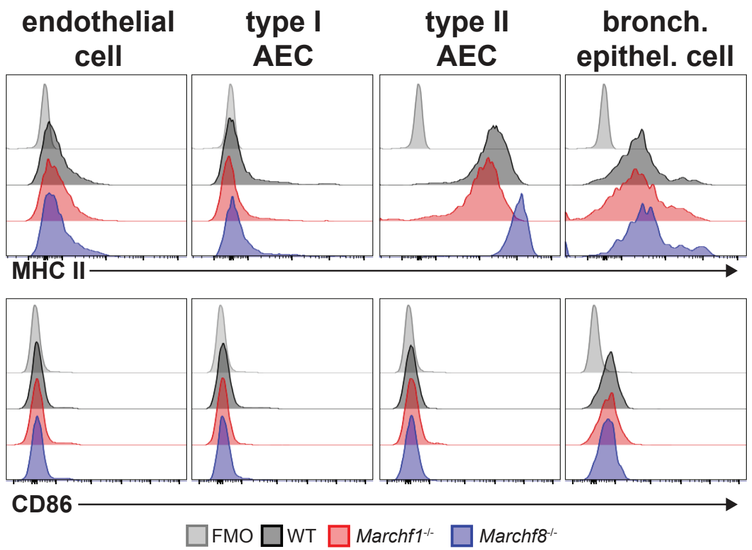
Figure 6

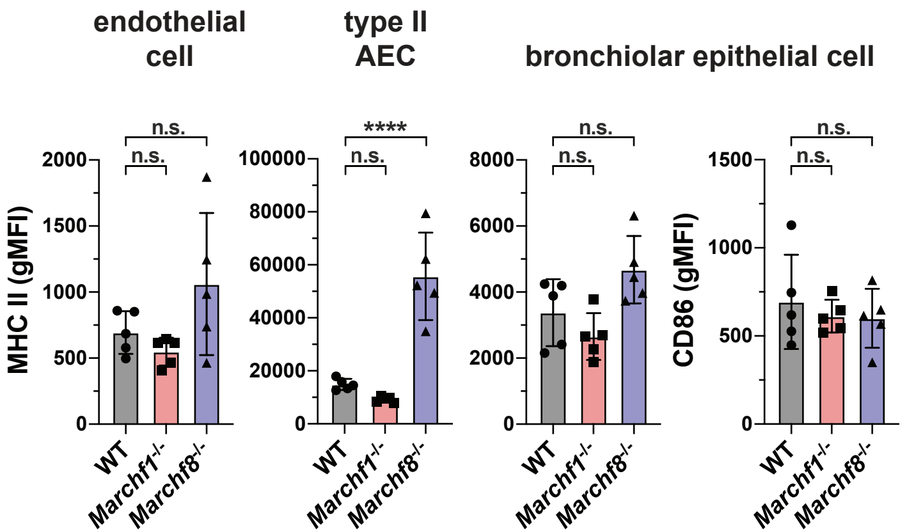
A


MHC II
CD86
CD80
CD40
CD44
CD95
CD98
mTEC
cTEC
FMO
WT
Marchf1-/-
Marchf8-/-

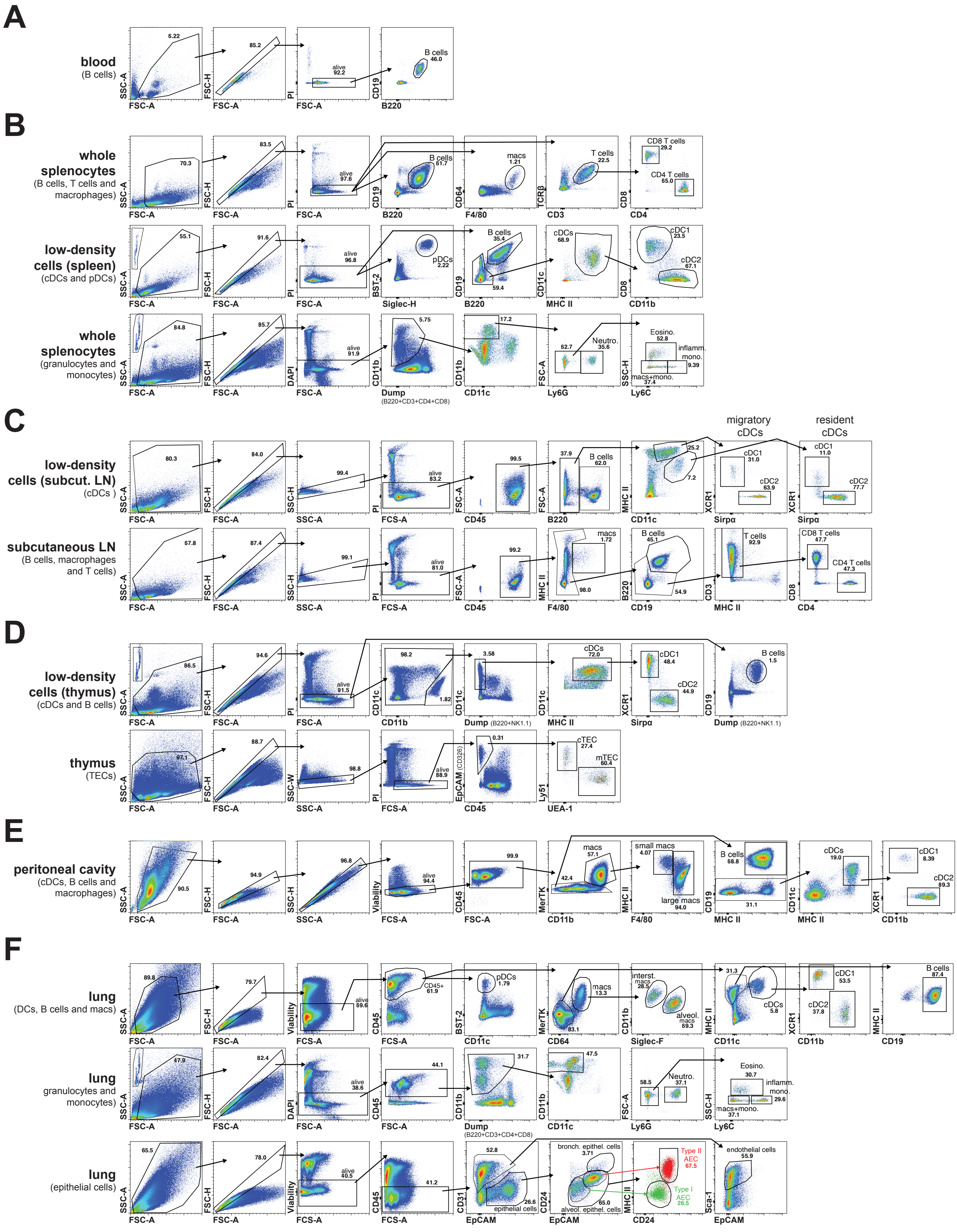

MHC II
CD80
CD40
CD95
Surface MHC II gMFI (PE)
Surface CD80 gMFI (PE)
Surface CD40 gMFI (APC)
Surface CD98 gMFI (PE)
n.s.
****
***
mTEC
cTEC

B

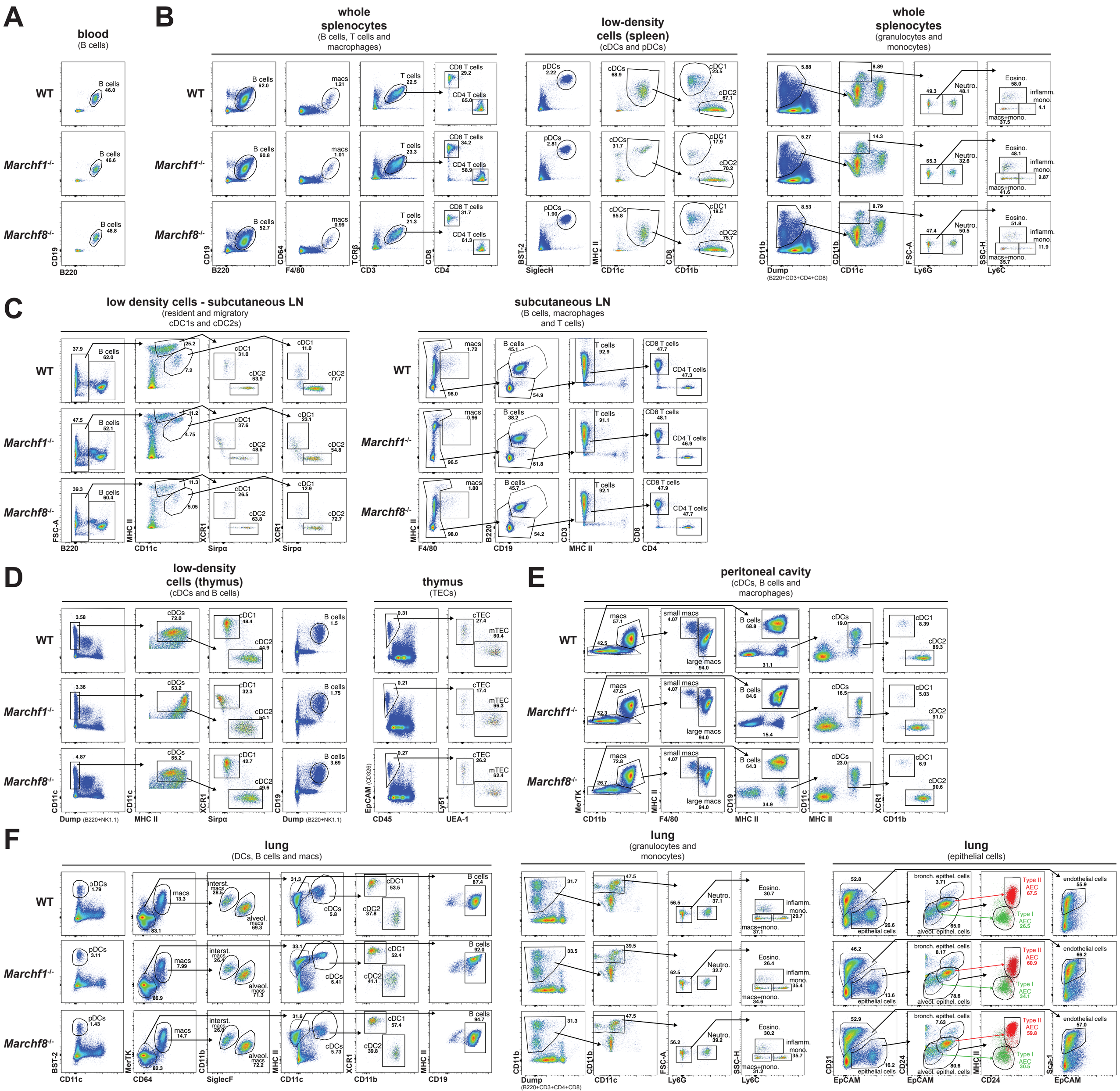

endothelial cell
type I AEC
type II AEC
bronch. epithel. cell
MHC II
CD86
FMO
WT
Marchf1-/-
Marchf8-/-


endothelial cell
type II AEC
bronchiolar epithelial cell
MHC II (gMFI)
CD86 (gMFI)
n.s.
****
WT
Marchf1-/-
Marchf8-/-

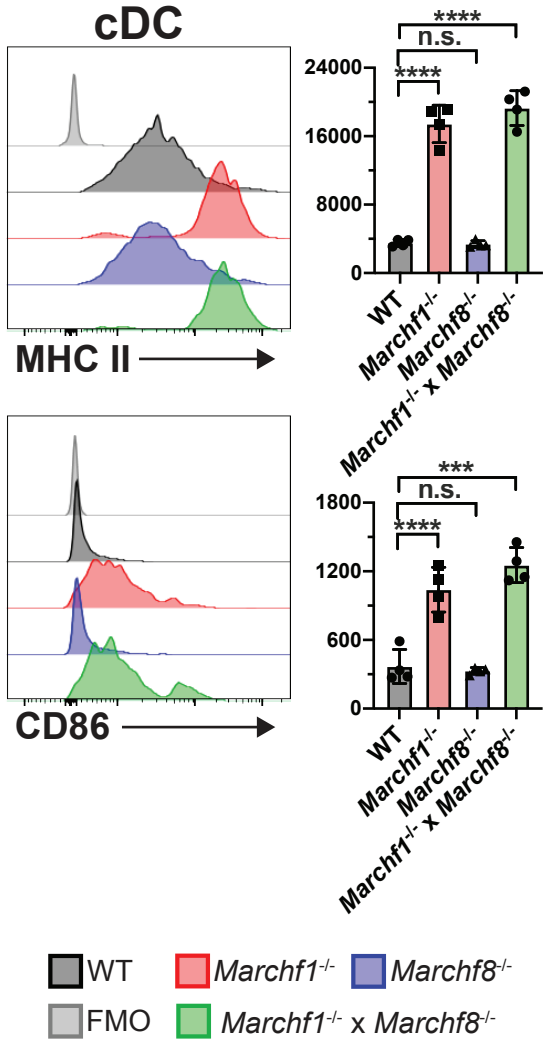
Supplementary Figure 1



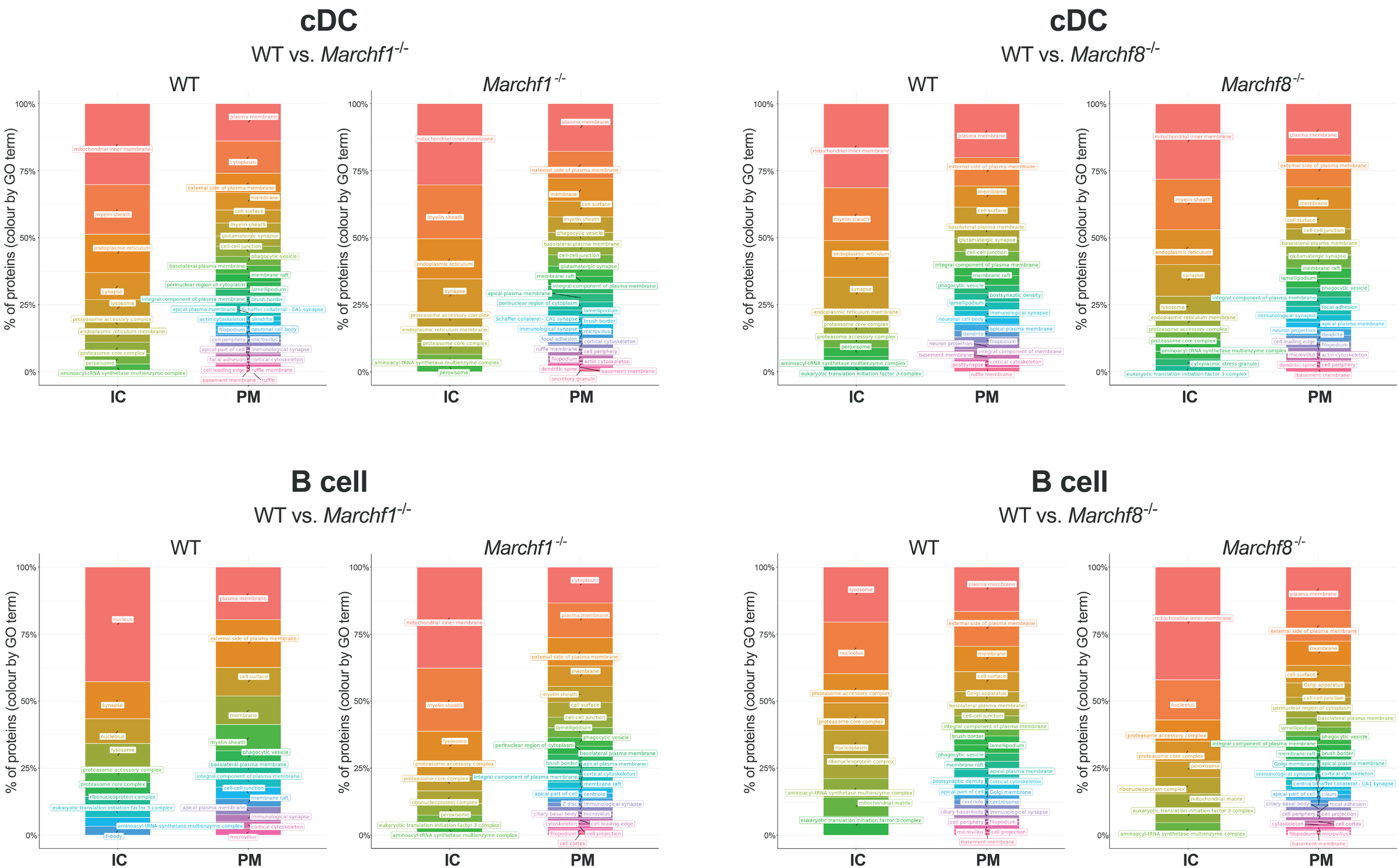
Supplementary Figure 2



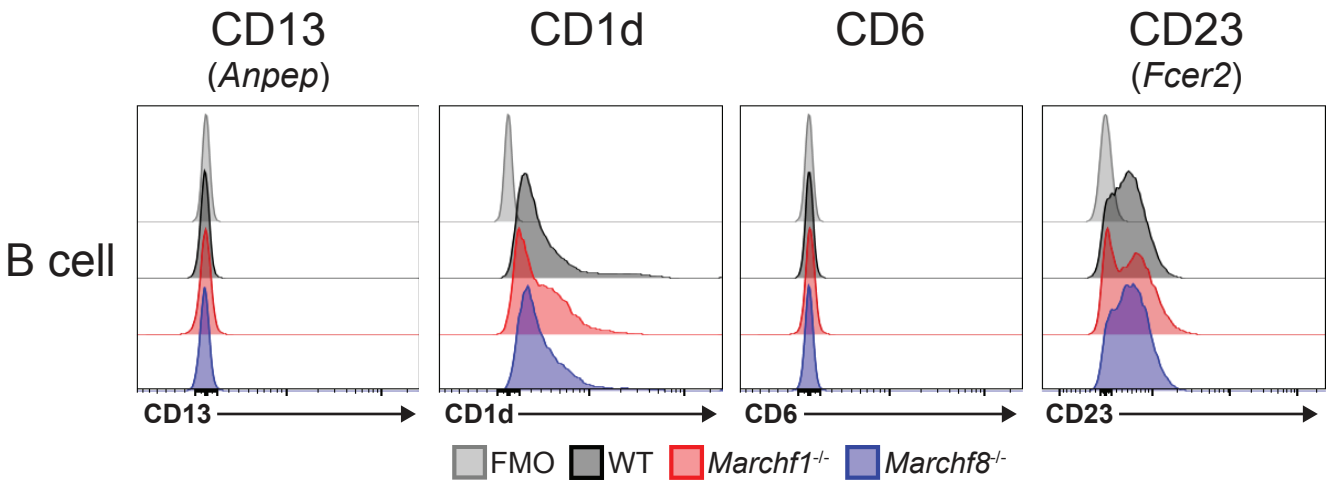
Supplementary Figure 3



Supplementary Figure 4



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	