Proteomic analyses reveal that immune integrins are major targets for regulation by Membrane-Associated Ring-CH (MARCH) proteins MARCH2,3,4 and 9.

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Abstract

MARCH proteins are membrane-associated Ring-CH E3 ubiquitin ligases that dampen immune responses by down-regulating cell surface expression of major histocompatibility complexes I and II as well as immune co-stimulatory receptors. We recently showed that MARCH2,3,4 and 9 also down-regulate cell surface expression of the inflammatory cytokine receptor for interleukin-6 (IL6Rα). Here we use over-expression of these MARCH proteins in the M1 myeloid leukemia cell line and cell surface proteomic analyses to globally analyse other potential targets of these proteins. A large range of cell surface proteins regulated by more than one MARCH protein in addition to several MARCH protein-specific cell surface targets were identified most of which were down-regulated by MARCH expression. Prominent amongst these were several integrin complexes associated with immune cell

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homing, adhesion and migration. Integrin α4β1 (VLA4 or VCAM-1 receptor) was down-regulated only by MARCH2 and we showed that in MARCH2 knockout mice, Integrin α4 was up-regulated specifically in mature B-lymphocytes and this was accompanied by decreased numbers of B-cells in the spleen.

Statement of Significance

MARCH proteins are a family of membrane-associated E3 ubiquitin ligases that dampen immune responses by down-regulating cell surface expression of histocompatibility complex proteins and T and B lymphocyte co-activation receptors. Recently we showed that MARCH2,3,4 and 9 also down-regulated expression of the pro-inflammatory interleukin 6 receptor alpha chain in T lymphocytes. We now show using an unbiased global proteomics approach that expression of each of these MARCH proteins has a significant effect on the cell surface expression of a large number of integrins in both cell line and primary samples. These data add immune cell adhesion and migration to the growing list of immune functions modulated by MARCH protein expression and suggest that further study of this important phenomenon may shed light on immune cell regulation.

Introduction

MARCH proteins were first described as viral genes K3 and K5 of g-herpesviruses (including Kaposi's sarcoma-associated herpesvirus) that encoded ubiquitin ligases that down-regulated MHC class I chains on infected cells(1-3). These proteins contained two putative transmembrane domains and an N-terminal cytoplasmic RING-CH domain that mediates E3 ubiquitin ligase activity. Ubiquitinated substrates are targeted for intracellular sorting through endocytosis and late endosome routing to lysosomes where the substrate is degraded.

Eleven related proteins were subsequently described in mammalian species with most displaying the same domain structures as the viral proteins. Sequence analyses suggested that

several of the mammalian MARCH proteins occur in related pairs (MARCH1 and 8; MARCH2 and 3; MARCH4,9 and 11) while others retain the RING domain but contain larger numbers of transmembrane domains (MARCH5 and 6) or do not contain transmembrane domains (MARCH7,10). A large number of potential substrates have been described for viral and mammalian MARCH proteins including MHC-I (viral K3,K5, MARCH4,9), MHC-II (MARCH1,8), immune co-stimulatory receptors CD86, (viral K5,MARCH2,8), immune adhesion receptors PECAM1, VE-cadherin, ICAM1 (viral K3 or K5), cytokine receptors IFNGR1(viral K3, K5) and intracellular organelle sorting molecules syntaxins (viral K5, MARCH2,3) among many others(4-7).

Most of the MARCH protein targets are cell surface receptors involved in immune activation including antigen presentation, T and B lymphocyte activation, immune cell adhesion and migration. Since the almost universal effect of MARCH protein-mediated ubiquitination is loss of these receptors from the cell surface it appears that the major role of MARCH proteins is to prevent excessive responses to immune stimuli.

We recently discovered in an unbiased cDNA expression screen that MARCH2,3,4 and 9 could each prevent cells responding to the cytokine interleukin 6 (IL6) and this was exclusively due to MARCH-induced loss of the IL6 receptor alpha (IL6Rα) chain from the cell surface(8). This prompted us to explore the potential unique and overlapping targets of these MARCH proteins using over-expression and detailed proteomic analyses of loss of cell membrane proteins. We identified immune adhesion receptors (especially integrins) as major targets of these MARCH proteins and showed that MARCH2 has a unique physiological role in preventing overexpression of integrin α4 on B lymphocytes and thus regulates trafficking of B cells.

Materials and Methods

M1 cells and infection with MARCH-expressing lentivirus.

The M1 mouse myeloid leukemia cell line (ATCC cat. No. TIB-192) derived from the SL mouse strain was maintained in DME medium containing 10 % (v/v) fetal calf serum at cell density less than 1x 10^6 cells/ml in a fully humidified incubator at 37°C and 10% CO_2. M1 cells were infected individually with retrovirus expressing either mouse MARCH2, proteolytically inactive MARCH2 W97E, MARCH3, MARCH4 or MARCH9. We have previously shown that the alternate transcript MARCH3 (F8WJC4.3) used in this study was able to down-regulate cell surface expression of IL6Ra in M1 cells (8). Retroviral constructs were generated from constructs in the pCFB vector (Stratagene). All constructs contained an N-terminal FLAG sequence (MADYKDDDDK) to allow for detection by Western blot. The Uniprot accession numbers for the MARCH proteins are: MARCH2: Q99M02.1, MARCH3: F8WJC4.3, MARCH4: Q80TE3, MARCH9: Q3TZ87. These open reading frames were inserted into the *Eco*R1 and *Xho*1 sites of the vector and in addition an IRES-GFP element was inserted downstream between *Xho*1 and *Not*I sites.

Viral particles encoding these constructs were generated by transfection of 293T cells. The cells were cultured in DME medium (Life Technologies) supplemented with 10%(v/v) bovine calf serum (BCS) in a humidified atmosphere with 10% (v/v) CO_2 at 37°C. 3-4 million cells were subsequently transfected with 14.4 mg of plasmid, 4.8 mg of MDI GAG-POL plasmid and 0.6 mg of VSVg. The

plasmids were dissolved in 250 mM $CaCl_2$ and then precipitated with an equal volume of 2XHBS and added to the 293T cells pre-treated with 25 mM chloroquine (Sigma-Aldrich). Cell culture medium was replaced at 8 and 24 h post-transfection.

The virus was then used to spin-infect M1 cells. Briefly, 1 ml of viral supernatant was added to 500,000 M1 cells followed by addition of polybrene (4 mg/ml). The plates were centrifuged at 693 x g for 1 hour at 23°C before being cultured in a humidified atmosphere with 10% (v/v) CO_2 in DME/10% FCS at 37°C. After 24 hours cells were washed with 4 x 5 ml DME and transferred to 10 ml DME containing 10% (v/v) BCS and cultured for 2-3 days.

Infected cells were sorted for GFP positivity on a Becton Dickinson FACS Aria III cell sorter and expanded for 3 weeks with five replicates of 10^7 of each infected population being used for the proteomic experiments.

Enrichment and digestion of cell surface proteins

The terminal sialic acid residue on glycans attached to cell surface proteins were oxidized and labelled with biotin by addition of 1mM sodium metaperiodate, 200 µM aminooxy-biotin (Biotium, USA), 10 mM aniline, PBS pH 6.7 and incubated in the dark at 4°C with gentle agitation for 1hr. The reaction was quenched by addition of 1 mM glycerol, before washing twice with PBS pH 7.4. To determine complete cell surface labelling, a small aliquot was taken from each sample and stained with streptavidin-APC (BD biosciences, USA) before analysis by flow cytometry (Becton Dickinson). Cells were lysed (lysis buffer: 150 mM NaCl, 1% v/v Triton X-100, 50 mM Tris-HCl, pH 7.6, 5 mM iodoacetamide, supplemented with complete mini protease inhibitor tablets (Roche)) and incubated at 4°C for 30 min with end-over-end mixing followed by centrifugation at 2800 × g for 10 min at 4°C. Supernatants were retained and centrifuged at 21,000 × g for 10 min. Biotinylated proteins were enriched from lysate mixtures using high capacity Streptavidin Agarose beads (Pierce, USA) for 1hr at 4 °C with end-over-end mixing. Beads were washed with Lysis Buffer (2 times), PBS/0.5% w/v SDS (2 times) before incubation of beads with 100mM dithiothreitol, 0.5% w/v SDS, PBS pH 7.4 for 20min. Beads were transferred into snap cap columns (Pierce), washed with UC buffer (6M Urea, 100 mM Tris-HCl pH 8.5; 2 times) before alkylation of cysteine residues by incubation with 50mM iodoacetamide in UC buffer in the dark for 20min. Beads were then washed in UC buffer (10 times), PBS pH 7.4 (4 times), water (3 times) before on-bead digestion of proteins using 2µg of trypsin (modified sequencing grade, Promega) resuspended in 50mM Ammonium Bicarbonate, over-night at 37°C with gentle shaking. Digested peptides were collected by centrifugation at 1000 x g into clean LoBind tubes (Eppendorf, Germany). Beads were washed with 50mM Ammonium Bicarbonate before centrifugation again and pooling of samples followed by SpeedVac-assisted solvent removal and storage at −80 °C.

Mass Spectrometry and Proteomics analysis

Peptides were resuspended in 2% acetonitrile, 1% formic acid and injected and separated by reversed-phase liquid chromatography on a M-class UPLC system (Waters, USA) using a 250mm × 75µm column (1.6µm C18, packed emitter tip; IonOpticks, Australia) with a linear 90-min gradient at a flow rate of 400 nl/min from 98% solvent A (0.1% Formic acid in Milli-Q water) to 34% solvent B (0.1% Formic acid, 99.9% acetonitrile). The UPLC was coupled on-line to a Q-Exactive mass spectrometer (Thermo Fisher, USA). The Q-Exactive was operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the 15 most abundant peaks. Full-scans (m/z 350–1,850) were acquired with a resolution of 70,000 at 200 m/z. The 10 most intense ions were sequentially isolated with a target value of 100000 ions and an isolation width of 2 m/z and fragmented using HCD with normalized collision energy of 27. Maximum ion accumulation times were set to 50ms for full MS scan and 50ms for MS/MS. Dynamic exclusion was enabled and set to 20 seconds.

The raw files were analyzed using the MaxQuant software (version 1.5.8.3) and the database search was performed using mouse sequence obtained from Uniprot including isoforms with strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids. Carbamidomethylation of cysteine was set as a fixed modification while N-acetylation of proteins N-termini and oxidation of methionine were set as variable modifications. During the MaxQuant main search, precursor ion mass error tolerance was set to 4.5 ppm and fragment ions were allowed a mass deviation of 20 ppm. PSM and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%. The "match-between-runs" option was selected.

Further analysis was performed using a custom pipeline developed in R, which utilizes the LFQ intensity values in the MaxQuant output file proteinGroups.txt. Proteins not found in at least 50% of the replicates in one group were removed. Missing values were imputed using a random normal distribution of values with the mean set at mean of the real distribution of values minus 1.8 s.d., and a s.d. of 0.3 times the s.d. of the distribution of the measured intensities. The probability of differential protein expression between groups was calculated using the Limma R package. Probability values were corrected for multiple testing using Benjamini–Hochberg method. Protein interaction networks for lists of proteins whose expression was significantly altered by expression of individual MARCH proteins were determined by the STRING program version 11.0 (http://string-db.org/newstring_cgi/show_input_page.pl).

Flow cytometry

Bone marrow and spleen cells from MARCH2 KO (see ref (8)) or age and sex matched C57Bl6 mice were analysed by flow cytometry on a Becton Dickinson LSR Fortessa instrument with all blood cell subsets defined exactly as described in Babon et al 2019 (8). The biotinylated rat anti-mouse integrin α4 antibody was from Biolegend (Cat No 103703, 1/2000) and the biotinylated hamster anti-mouse

integrin β1 antibody was from Biolegend (Cat No 102203, 1/2000) with respective isotype controls from e-bioservices (13-4321-81) and Biolegend (400903) and these were visualised with PE-Cy7 streptavidin as described previously (8). M1 cells were also analysed as described in the same paper with the following antibodies: biotinylated goat anti-mouse IL18R1 from R&D Systems (Cat No BAF856, 1/100), biotinylated goat anti-mouse CRLF2 from R&D Systems (Cat No FAB5461P, 1/30) and were visualized with PE-streptavidin as described in (8). Respective isotype controls were from R&D Systems (Cat Nos BAF108 and Ic108P).

Results and Discussion

Global analysis of proteins whose expression is affected by MARCH2,3,4 or 9 overexpression.

Five replicates each from parental M1 cells or M1 cells infected with viral constructs expressing murine MARCH2, MARCH2 W97E, MARCH3, MARCH4 or MARCH9 were processed as described in Materials and Methods section to enrich for cell surface proteins and tryptic peptides were then analysed by quantitative LC-MS/MS. High quality data were obtained with good quantitative reproducibility observed for replicates (Supplementary Tables 1 and 2). MARCH2 W97E represents a mutant transcript that we have shown previously to lack the capacity to down-regulate IL6Rα levels on M1 cells (8) and thus serves as a negative control.

Before discussing the results and comparison with the literature it is worthwhile to consider the restrictions or limitations associated with these results and their comparison with the results of others. Firstly, we have used a mouse monocytic leukemia cell line whereas others may have used human cells or cells of different cell lineages (eg epithelial, lymphoid etc). It is possible that there are species differences in MARCH protein specificity and certainly there will be differences in the expression of different cell surface proteins in cells of different lineages. Secondly, our strategy for identifying cell surface proteins will only detect glycoproteins that also contain sialic acid moieties so some cell surface proteins will be missed in this approach. Thirdly, because our approach only measures differences in levels of surface proteins it cannot distinguish between direct targets of MARCH proteins and other proteins that may be binding partners of direct targets or whose expression is otherwise dependent on a direct target. Finally, the measured differences in protein expression levels do not inform of the mechanism(s) responsible for these changes that may include increased internalisation and degradation of receptor, decreased recycling, failure in trafficking from the endoplasmic reticulum to the cell surface or decreased rate of synthesis.

Of 66 proteins down-regulated by MARCH2 (> 2-fold change with $p<0.01$) (Fig 1) at least 30 were connected in the STRING protein-protein interaction network. The major hub proteins were integrins (integrin β1(ITGB1), integrin αM (ITGAM)), CD86 (that provides co-stimulatory signals to T cells), the Fc receptor FCGR1A and the sheddase ADAM10 (Supplementary Fig. 1). Up-regulated proteins (64) were less well connected but hub proteins again included integrins (Integrin αX (ITGAX) and integrin αL, ITGAL), T-cell co-stimulatory signals (CD28) or T cell inhibitory signals (HAVCR2) and chemokine receptor CXCR4 (Supplementary Fig. 2). Previously identified MARCH2 targets include b2-adrenergic receptor, STX6, CAL, CFTR, DLG1, TFR, CD86 and Dishevelled (4, 9) but in MARCH2-infected M1 cells these proteins were either not detected or their expression was unaffected (b2 adrenergic receptor, DLG1, TFR). CD86 down-regulation by MARCH2 was confirmed in MARCH2-M1 cells.

Fewer proteins were down-regulated by MARCH3 (19) (Fig 1) but again the main STRING hub proteins were integrins (integrin α6 (ITGA6) and integrin αL (ITGAL)) and the Fc receptor FCGR1A (Supplementary Fig. 3). A larger number was up-regulated by MARCH3 (39) with main interaction hubs being integrins (Integrin αX (ITGAX)), cell adhesion molecules (PECAM1 and CD2) and T-cell co-stimulatory signals (CD28) or T cell inhibitory signals (HAVCR2) (Supplementary Fig. 4). Of the previously identified MARCH3 targets, BAP31, STX6, FCGR2B, FOX01 and IL1R1 (4, 10) we were unable to confirm down-regulation of any in MARCH3-M1 cells although IL1R1 was down-regulated in MARCH2-M1 cells.

Of the proteins down-regulated by MARCH4 (111) (Fig 1) several different hubs could be observed in STRING including hubs centred around the receptor tyrosine phosphatases PTPRC and PTPRA (both shown to activate src-like kinases associated with integrin or lymphoid cell signalling)(11, 12), the integrins ITGAL, ITGA5 and ITGA6, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Fc receptors (FCGR2B, FCGR1A, FCER1G), IL10 and TNF receptors (Supplementary Fig. 5). The up-regulated proteins (118) showed a major hub involving several members of the spliceosome (U2SURP, SRSF4 and 11, SREK1, SRRT, SRRM2), as well as hubs for glycosylation enzymes (NAGA, MAN2B1, GLB1,GUSB) and integrins (ITGAX,PECAM1) (Supplementary Fig. 6). Previously identified targets of MARCH4 include BAP31, CD4, CD81, STX4, ALCAM, Mult1 and MHC-1 components (4, 13) but none of these were detected in M1 cells.

Smaller numbers of proteins were regulated by MARCH9 (Fig 1). Down-regulated proteins (20) showed hubs around integrins (ITGA6, ITGAL) and Fc receptors (FCGR1A) while up-regulated proteins (21) showed small hubs around the purinergic receptors (P2RX4 and 7), adhesion receptors (PECAM1 and ITGAL), cytokine receptors (IL2RG and IL18R1) and inhibitory receptors (CLEC12A and HAVCR2) (Supplementary Figs.7 and 8). Previous data including a proteomic analysis of a MARCH9 expressing B cell line (13, 14) detected decreased expression of Bap31, ALCAM, MULT1, VAMP8, TMEM2, PlexinC1, ICAM1, FCGR2b, PTPRJ, SLAM, TRAILR1, ILT, CD4, CD32B, CD86, CD150, CD155, and MHC 1 and II components. Most of these were not detected in our

analyses of MARCH9-infected M1 cells. We saw decreased expression of HLA-D1.1 but we failed to see changes in CD86, FCGR2b, ICAM1, VAMP8, TMEM2 or PTPRJ in MARCH9-M1 cells, suggesting there may be cell type-specific actions of MARCH proteins.

While MARCH2 and MARCH3 were detected as expected as up-regulated proteins in the respectively infected M1 cells MARCH4 and MARCH9 were not. It is unclear whether these latter MARCH proteins are either non-glycosylated or do not contain sialic acid residues, a requirement for detection using our protocols (see Materials and Methods section).

Proteins whose expression is uniquely affected by individual MARCH expression.

Of the integrins, integrin α4 and β1 were uniquely down-regulated by MARCH2 (Fig 2). These integrins form the complex VLA4 (very late antigen 4) that is expressed on most hemopoietic cells and binds to vascular cell adhesion molecule 1 (VCAM1) (that is itself expressed on stromal and endothelial cells) as well as the extracellular matrix component fibronectin. VLA4 is thus involved both in retaining hemopoietic cells in stromal niches as well as adhesion of hemopoietic cells to vascular endothelium and extravasation from the circulation into the tissues. Other proteins uniquely down-regulated include the ectonucleotide phosphodiesterase (Enpp1) that controls calcification and regulates insulin signalling, Toll receptor 2 (Tlr2) that recognises various bacterial lipoproteins, the deglycosidase Nagpa involved in targeting hydrolases to the lysosome, the dystroglycan Dag1 involved in cell adhesion to extracellular matrix and the anion exchanger slc4a2. Uniquely up-regulated proteins included the zinc transporter Slc39a6 and TNF superfamily 4 (Tnfsf4). Interestingly, while all the uniquely down-regulated proteins were either unaffected or slightly up-regulated in M1 cells expressing the RING-mutant form of MARCH2 (W97E), all the up-regulated proteins were also up-regulated by the RING mutant (Supplementary Fig 9). This may suggest that up-regulation of these proteins is not dependent on ubiquitination processes but other protein-protein interactions involving MARCH2.

Proteins uniquely down-regulated by MARCH3 (Fig 2) included the viral restriction factor bone marrow stromal antigen2 (Bst2,tetherin), the lysosomal enzyme Gba (glucocerebrosidase), the calcyclin binding protein (Cacybp) involved in ubiquitination, the chloride/proton exchange transporter (Clcn5), the chromatin modifier Brd4 and the cell repulsion ephrin receptor (EphA7). Uniquely up-regulated proteins include integrin α7 that is involved in lymphocyte homing to gut tissue, the G-protein coupled receptor Mgrg1, the F-actin binding protein Lsp1 that regulates hemopoietic cell adhesion and trans-endothelial migration, the vesicular protein trafficking protein (Tm2d2 or Tmed2) and receptor tyrosine phosphatase F (Ptprf) that regulates EphA2-mediated adhesion.

The most prominent proteins uniquely down-regulated by MARCH4 (Fig 2) were the receptor tyrosine phosphatases Ptpra and Ptprc, the former activating src kinases and cell adhesion and the latter also activating src kinases and antigen signalling in both B and T cells. Other proteins include the tyrosine kinase Tyro3 that signals from the extracellular matrix, the SLAM receptor Ly9 also involved in adhesion and negative regulation of immune reactions, the transmembrane glycoprotein Evi2b involved in myeloid development, transmembrane protein 119 (Tmem119) involved in osteoblast development, the immune inhibitory receptor Cd3001f, Toll receptor 4 (Tlr4) the bacterial lipopolysaccharide receptor, the insulin receptor (Insr) and the protein elongation factor Eef1g. Uniquely up-regulated proteins included the Golgi-associated glycoprotein (Glg1) that binds E-selectin, nodal modulator 1(Nomo1) an antagonist of nodal signalling, and the cell surface mucin Muc13 that may act as an immunosuppressant.

There were few proteins uniquely regulated by MARCH9 expression (Fig 2). They included down-regulation of integrin β2 (ITGB2), an essential component of the lymphocyte functions associated antigen-1 (LFA-1) that also includes integrin α1 (ITGA1) and is required for extravasation of hemopoietic cells and efficient antigen presentation, and up-regulation of the purine selective nucleobase transporter, Slc43a3.

A summary Venn diagram of overlapping and specific targets of the four MARCH proteins are shown in Fig 3. It is of interest that very few proteins were identified that were targets of all 4 MARCH proteins. Of these (ITGA6, FCGR1, CD97 and P2rx7) only CD97 was also up-regulated in M1 cells expressing inactive mutants of MARCH2 and MARCH3. It therefore remains possible that up-regulation of CD97 simply reflects viral infection per se.

MARCH proteins regulate cell surface expression of cell adhesion proteins and cytokine and growth factor receptors

The most dominant changes in protein levels induced by MARCH proteins were in immune adhesion proteins including integrins, cadherin 17 (Cdh17), platelet and endothelial cell adhesion molecule 1 (PECAM1), sialophorin (Spn), ephrins and ephrin receptors, and to a lesser extent intercellular adhesion molecules (Icams). Among the integrins, the laminin-binding integrin α6 (ITGA6) was uniformly and strongly down-regulated by all 4 MARCH proteins (but not by the MARCH2 W97E mutant) but one of its partner subunits, integrin β1 (ITGB1) was down-regulated only by MARCH2. This suggests that the β integrins are unlikely to be the direct targets of the MARCH proteins. Integrin α1 (that forms the collagen and laminin receptor when complexed with integrin β1) was down-regulated by MARCH2 and MARCH3 but up-regulated by MARCH9 and not affected by MARCH4. Integrin αL (ITGAL) (that with integrin β2 forms the intercellular adhesion molecule VLA1 that binds ICAMs) was strongly down-regulated by MARCH4 and MARCH9 but was up-regulated by MARCH2 and unaffected by MARCH3. Although Integrins αX and αM (ITGAX, ITGAM) both

form heterodimers with integrin β2 and have similar specificities for inactivated complement 3b (iC3b) and adherence to activated endothelium, ITGAX was strongly up-regulated by MARCH2, 3 and 4 while ITGAM was up-regulated by MARCH4 and down-regulated by MARCH2. Integrin α2 was mildly down-regulated by MARCH2 and MARCH9 only. Platelet endothelial cell adhesion molecule 1 (PECAM1) and the adhesion GPCR CD97 were both up-regulated by MARCH3, 4 and 9. Both are involved in binding to or attracting endothelial cells through interactions with CD177 or αvβ3 integrins, respectively. Cadherin 17 (Cdh17) mediates calcium-dependent cell-cell adhesion and was down-regulated by MARCH3,4 and 9.

The interleukin 6 receptor α chain (IL6Rα) previously shown by us to be down-regulated by expression of MARCH2,3,4 and 9 (8) showed a dramatic reduction in M1 cells expressing any of these MARCH proteins. Interleukin 1 receptor1 (IL1R1) was significantly down-regulated by all four MARCH proteins while the interleukin 10 receptor was down-regulated by MARCH2,4 and 9 but up-regulated by MARCH3. In contrast the interleukin 18 receptor 1 (IL18R1) was significantly up-regulated by MARCH2,4 and 9 but unaffected by MARCH3.

Validation of proteomic results.

Our mass spectrometry analysis demonstrated that expression of MARCH proteins resulted in changes to cell surface protein expression. To validate some of the observed changes, a selection of the proteins was analysed by flow cytometry of M1 cells infected with viruses expressing each of the MARCH proteins. As shown in Figure 4, our results confirmed the specific reduction of integrins α4 and β1 following MARCH2 over-expression. The cytokine receptor CRLF2 was down-regulated by MARCH2,3 and 4 (but not MARCH9) and the IL18 receptor (IL18R1) was up-regulated by MARCH2,4 and 9 but not MARCH3 (Fig 4), again confirming the proteomic results. Interestingly, IL18R1 remained elevated even in M1 cells that had lost GFP (and presumably MARCH) expression suggesting that MARCH expression may have led to epigenetic changes controlling IL18R1 expression although the exact mechanism controlling the elevated expression of IL18R1 remains to be determined.

MARCH2 knockout mice show increased levels of integrin α4 specifically on mature B-cells.

Given the high specificity of MARCH2 overexpression for down-regulation of integrins α4 and β1 we were interested to see if loss of MARCH2 affected expression levels of these integrins in normal cells in vivo. Spleens and bone marrow from MARCH2 genetic knockout (KO) mice or age and sex matched C57Bl6 mice were harvested, labelled with various lineage specific antibodies and flow cytometry used to measure cell surface expression of these integrins in various hemopoietic cell

subsets. Although various hemopoietic cell subsets express integrin α4 (including macrophage, dendritic cells, B and T lymphocytes and to a lesser extent neutrophils) only mature B cells (IgM+/-IgD+) showed a clear up-regulation of integrin α4 on the cell surface while immature B cells (IgM++IgD-) showed little change in integrin α4 levels. This was very apparent for bone marrow B cells but also observed at a lower level for spleen B-cells (Fig 5 and Supp Fig. S10). Interestingly, integrin β1 was only very mildly elevated in MARCH2 KO B cells compared with the much larger elevations seen for integrin α4 (Supp Fig. S11) even though it was prominently down-regulated by over-expression of MARCH2.

Analysis of the frequency of different hemopoietic cell subsets in MARCH2 KO vs wild type bone marrow and spleen showed a dramatic and highly reproducible reduction in the mature B cell (IgM+IgD+) frequency in spleen from about 30% to 24% with inconsistent effects on the small number of B cells in the bone marrow (Fig 6 and Supp Fig. S12).

It has been reported that integrin α4β1 complex (VLA-4) is required for adhesion of immature B-cells to bone marrow stroma and its expression decreases as B cells mature in both human and mouse (15, 16). Indeed, it is thought that this interaction is required for B cell maturation in the bone marrow and that the decrease allows the release of mature B cells from the bone marrow so that they can colonise the spleen. It has also been shown that ITGA4/ITGB1 (VLA-4) is required for adhesion of human B-cells to VCAM-1 (INCAM-11) on dendritic cells in spleen germinal centres(17). Thus, alterations in integrin α4 levels on B-cells could have multiple effects on B cell trafficking to and from the spleen resulting in the observed decrease.

The differences seen between over-expression of MARCH2 in M1 cells (dramatic reduction of both integrins α4 and β1) and knockout of MARCH2 in primary cells (B cell specific and greater effect on integrin α4 than β1) might indicate that physiologically (at least in the steady state) MARCH2 only regulates integrin α4 in B cells and that the effects on integrin β1 might be indirect. Thus if most of integrin β1 in M1 cells is in integrin α4/β1 complexes its levels will be dramatically affected by down-regulation of integrin α4 but if integrin β1 is in multiple other complexes (for example with integrins α1-10) in B cells the effects of down- or up-regulation of integrin α4 may only have a minimal effect on total integrin β1 levels.

Conclusions

We identified a large number of potential protein targets of MARCH2,3,4 and 9 with a few unique and many overlapping targets. Of the proteins where a change in abundance was observed following MARCH over-expression, it was not possible to distinguish direct from indirect MARCH

ubiquitination targets, however, it was clear that the majority of affected proteins were cell surface receptors associated with immune and inflammatory responses. The most prominent amongst these were immune cell adhesion receptors and especially immune integrins. Integrin α4β1 was specifically down-regulated only by MARCH2 in M1 cells and this was confirmed by flow cytometry. A physiological role of MARCH2 in regulating expression of this integrin was confirmed in MARCH2 KO mice where integrin α4 was up-regulated in mature B-cells in the bone marrow and the levels of mature B-cells in the spleen were reduced. This may suggest a role for MARCH2 in down-regulating integrin α4 expression on B cells as they mature in the bone marrow allowing their release from the bone marrow microenvironment to traffic to the spleen while other hemopoietic cells were unaffected.

Associated Data

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019967

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Figure legends

Figure 1. Volcano plots of M1 cells over-expressing MARCH2,3,4 or 9 proteins versus wildtype M1 cells. Y-axis is the -log10 adj. P value of a difference and x-axis is the log2 fold quantitative change determined as described in Materials and Methods. The top 20 significantly differentially expressed proteins are labelled with the gene name for each MARCH protein.

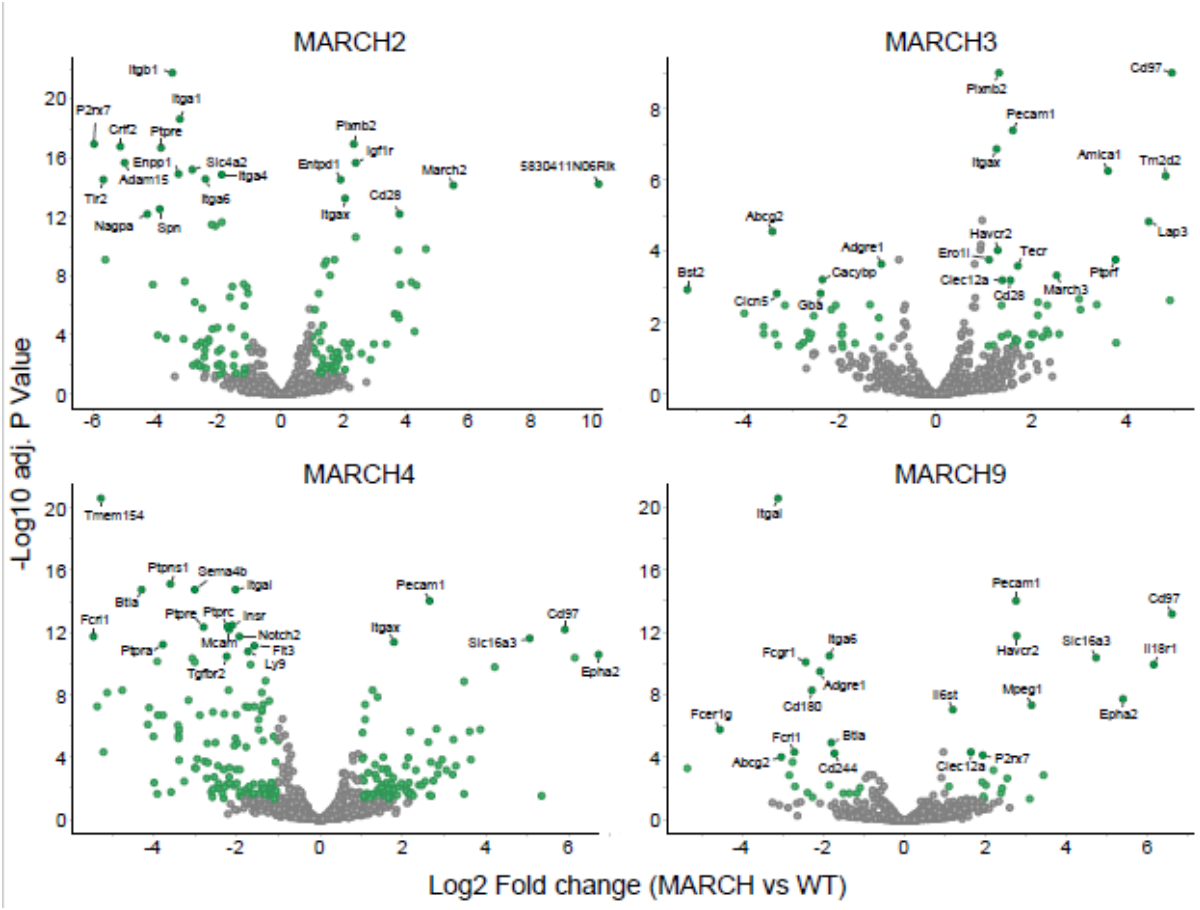


Figure 2. Comparison of -log10 adj. P values of protein changes for M1 cells expressing MARCH2,3,4 or 9 versus wildtype M1 cells. Values in each axis were made positive or negative based on a positive or negative log2 fold change for each comparison. Proteins uniquely down-regulated or up-regulated by individual MARCH proteins are labelled with the protein abbreviation. Down-regulated proteins are indicated in black and up-regulated proteins are indicated in red.

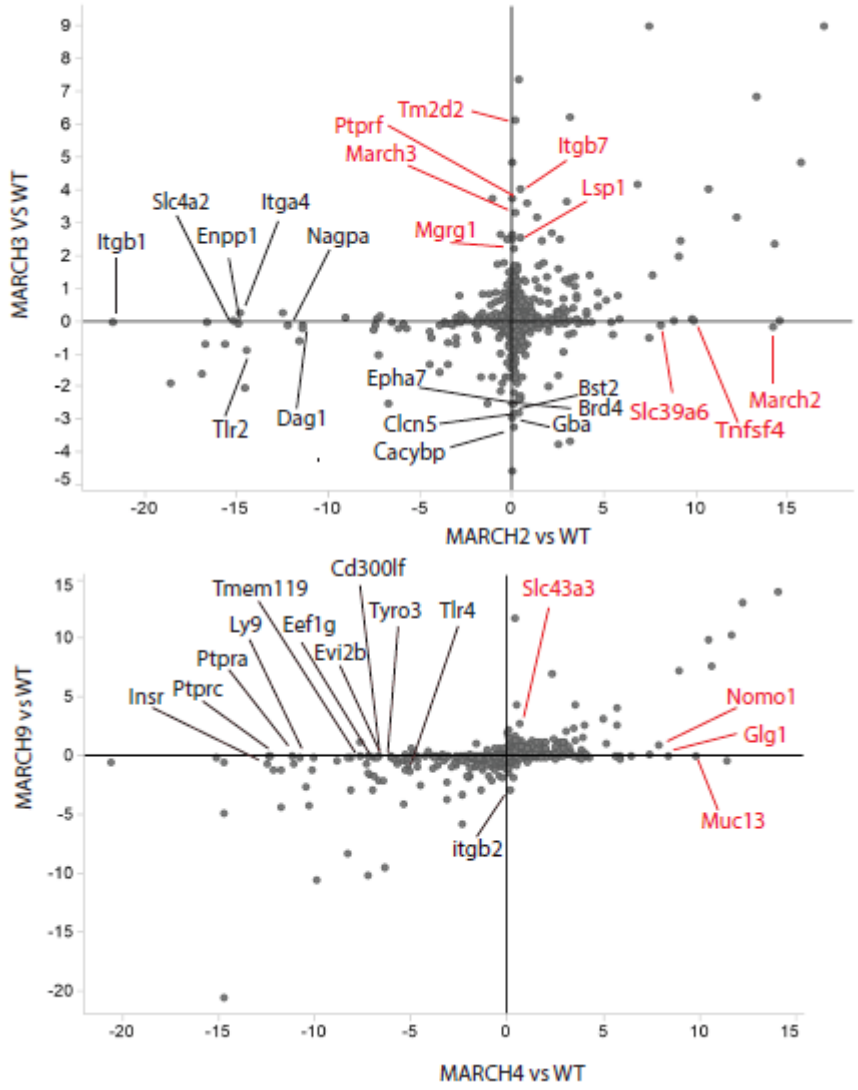


Figure 3. Venn diagram of proteins whose expression is significantly altered by the expression of MARCH2,3,4 or 9. Down-regulated proteins are indicated in black, up-regulated proteins are indicated in red and proteins that are down-regulated by some MARCH proteins but up-regulated by others are indicated in blue.

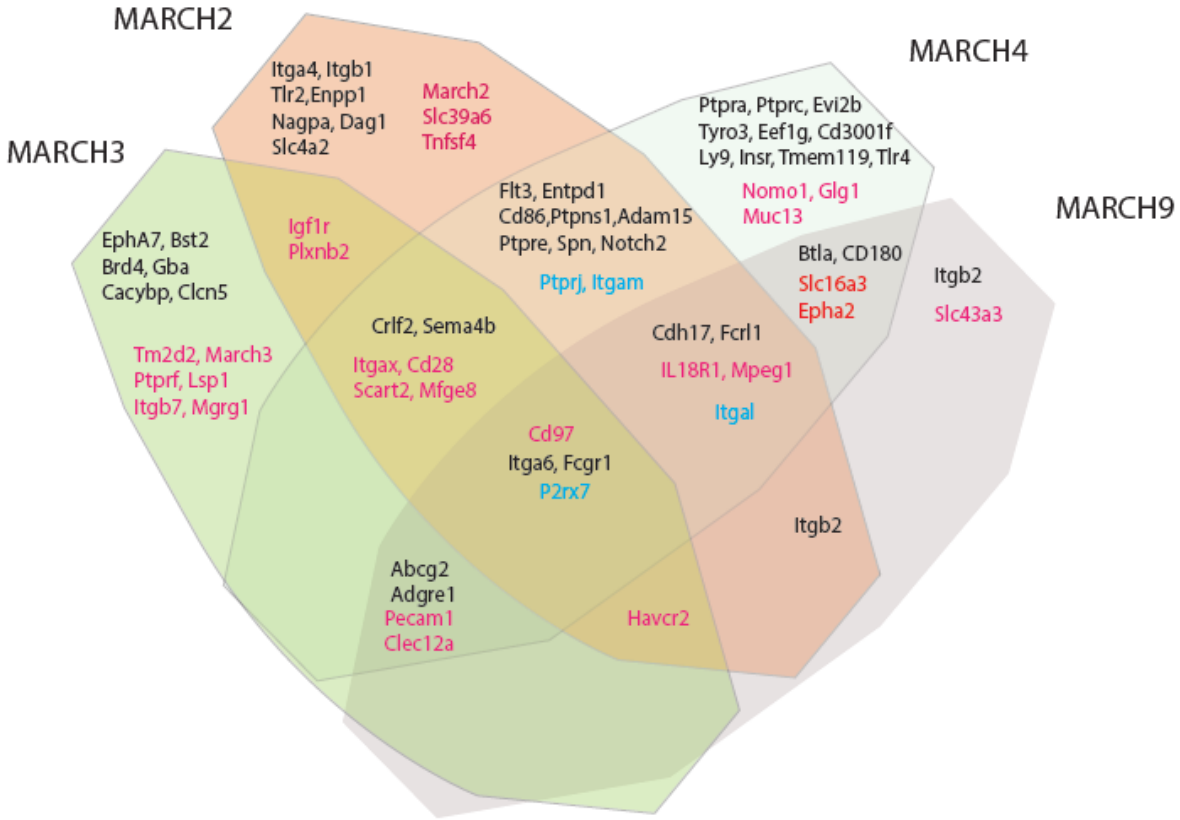


Figure 4. Dot-plot flow cytometric analyses of M1 cells infected with MARCH2,3,4, or 9 or wildtype M1 cells for cell surface expression of IL18 receptor 1 (IL18R1), CRLF2 (cytokine receptor-like factor or thymic stromal lymphopoietin receptor), CD49d (integrin α4) or CD29 (integrin β1). The y-axis represents fluorescent intensity of the PE-tagged avidin/antibody complexes and the x-axis represents fluorescent intensity due to the GFP protein co-expressed with the MARCH protein. IL18R1 expression was up-regulated by expression of MARCH2,4 and 9 and remained elevated even in M1 cells that subsequently lost GFP expression. CRLF2 expression was down-regulated by all 4 MARCH proteins while integrins α4 and β1 were down-regulated only by MARCH2.

Figure 4

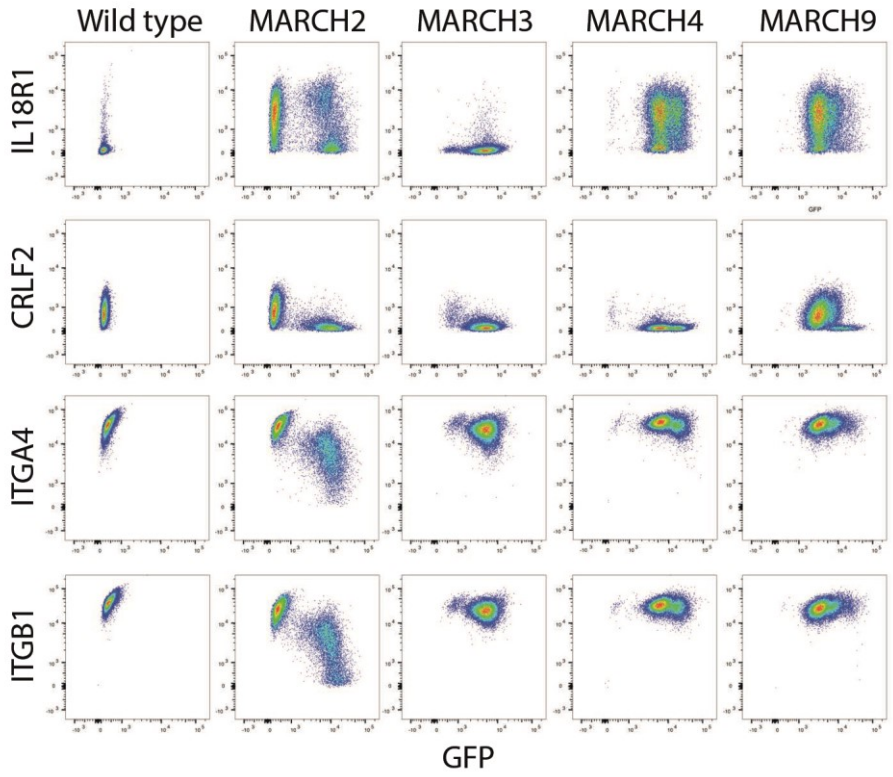


Figure 5. Flow cytometric analyses of integrin α4 (ITGA4) cell surface expression on various B cell subsets from bone marrows of age and sex-matched wildtype mice (black) or MARCH2 knockout mice (red), results from two mice of each genotype are shown with isotype controls for all 4 mice shown in grey. Y-axis is normalized cell number and x-axis is the fluorescent intensity of cells stained with the biotin-coupled antibody complexed with PE-Cy7 tagged streptavidin. The windows used to select the indicated B-cell subsets are shown in the dot-plot of IgM versus IgD cell surface expression of bone marrow cells pre-selected to be viable, CD3-CD19+ cells. This experiment was repeated twice using mice of alternating sex with similar results.

Figure 5

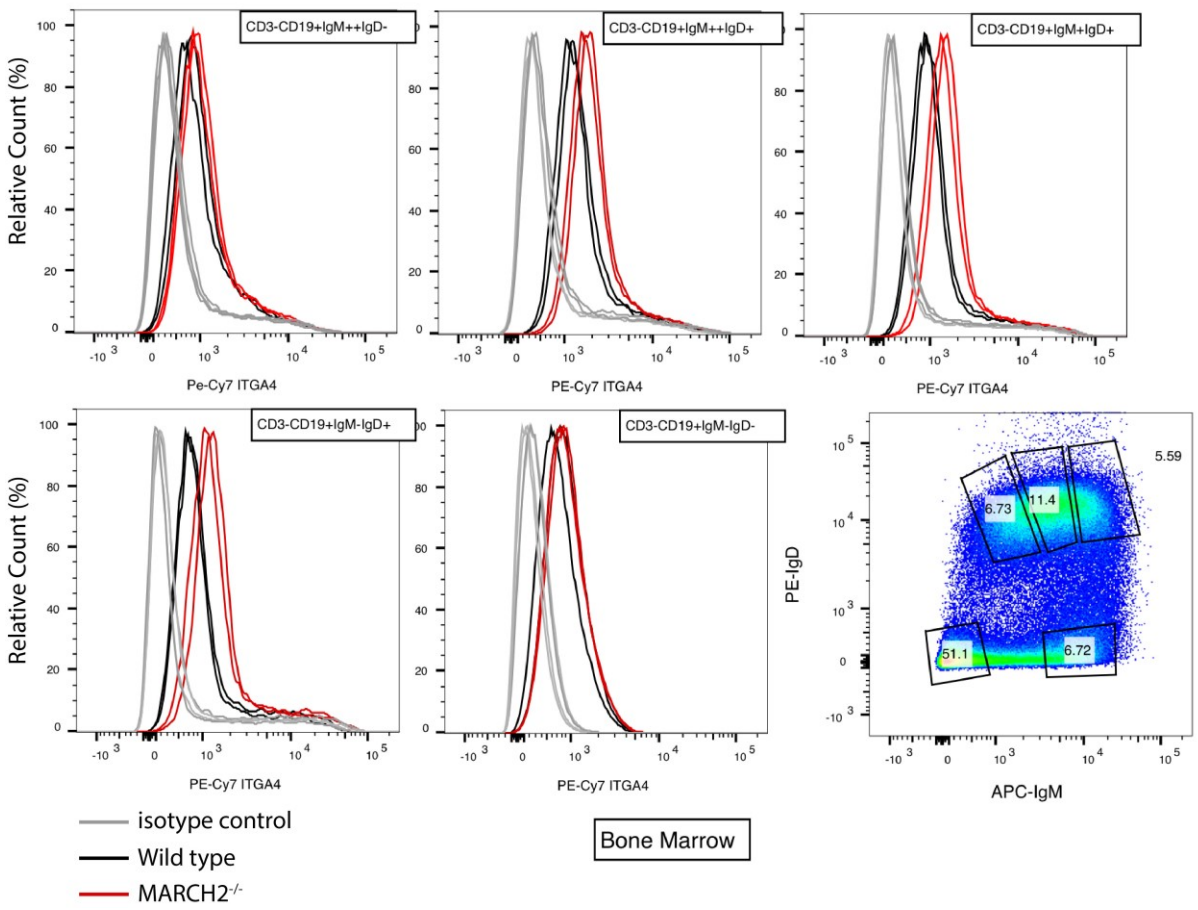
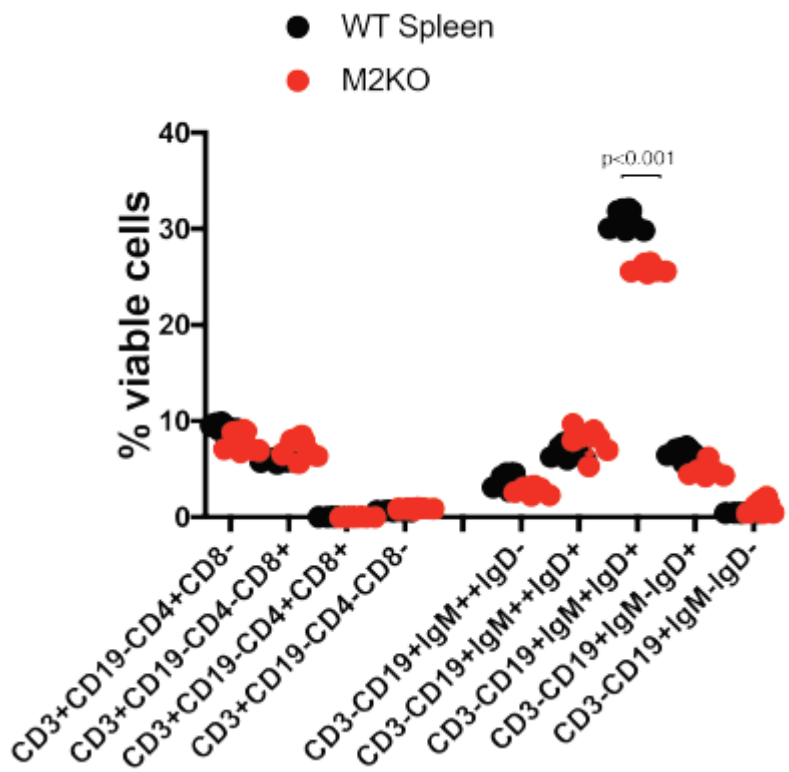


Figure 6. T cell and B cell subset frequencies in the spleens of age and sex matched C57Bl/6 wildtype or MARCH2 knockout mice. Subsets were selected by flow cytometry as described in Babon et al 2019(8) and in Fig. 5. Four mice of each genotype and both sexes from two different experiments are shown. Mature B-cells (CD3-CD19+IgM+IgD+) was the only subset showing a statistically significant reduction ($p<0.001$, paired student T test, n=4)



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Conflict of Interest statement

The authors declare no conflict of interest.