Membrane-Associated RING-CH (MARCH) proteins down-regulate cell surface expression of the interleukin-6 receptor alpha chain (IL6Rα)

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Abstract

Interleukin 6 (IL6) is a cytokine that regulates a number of important immune and inflammatory pathways. We used the ability of IL6 to inhibit the clonal proliferation of the mouse M1 myeloid leukemia cell line in agar to positively screen a cDNA expression library for proteins that inhibited IL6 activity. We found three clones completely resistant to IL6 that contained the cDNA for the Membrane-Associated RING-CH E3 ubiquitin ligase MARCH2. MARCH2 is a member of a family of membrane bound E3 ubiquitin ligases that target cell-surface receptors for degradation. MARCH2 overexpressing M1 clones retained responsiveness to the related cytokines leukemia inhibitory factor and oncostatin M and we showed that its inhibitory effect was a result of selective down-regulation of the IL6 receptor alpha chain and not the shared receptor subunit, gp130 or other signalling molecules. This activity of MARCH2 was also shared with related proteins MARCH4, MARCH9 and an isoform of MARCH3. The transmembrane domains and C-terminal domains, as well as a functional RING domain, of MARCH proteins were all required for substrate recognition and down-regulation. Genetic deletion of individual MARCH proteins in mice had no or little effect on IL6Rα levels but combined deletions of MARCH2,3 and 4 displayed elevated steady-state levels of IL6Rα in selected haemopoietic cell subsets including CD8+ and CD4+ T cells. These studies extend the potential immunosuppressive roles of MARCH proteins to include down-regulation of IL6 inflammatory responses.

Abbreviations:

MARCH Membrane Associated Ring-CH, RING really Interesting New Gene, IL6 Interleukin 6, IL6Ra interleukin 6 receptor alpha chain, Gp130 glycoprotein Mr 130 (IL6 signal transducing chain), LIF Leukemia Inhibitory Factor, OSM Oncostain M, CNTF Ciliary Neurotrophic Factor, CT1 Cardiotrophin 1, CLC Cardiotrophin like cytokine, NP Neuropoietin, DLG1 Discs Large MAGUK Scaffold Protein 1, BAP31 B-cell receptor Associated Protein 31, STX syntaxin, TRAILR1 TNF-Related Apoptosis-Inducing Ligand Receptor 1, JAK JAnus Kinase, STAT Signal Transducer and Activator of Transcription, SOCS Suppressor Of Cytokine Signalling, MAPK Mitogen Activated Protein Kinase, PI3K Phosphatidyl Inositol 3 Kinase, TcPTP T cell Protein Tyrosine Phosphatase, SHP2 Src Homology region 2 containing protein tyrosine Phosphatase 2

Introduction

Membrane Associated Ring-CH (MARCH) proteins were first described as two proteins (named K3 and K5 or vMIR1 and vMIR2) encoded by Kaposi's sarcoma-associated herpesviruses (KSHV/HHV8) that enhanced the endocytosis and proteolysis of MHC class I chains and thus resulted in immune evasion of infected cells by reducing antigen presentation to T-lymphocytes (1, 2). Subsequently several mammalian orthologs of these proteins were found (MARCH1-11) with several appearing to be closely related to each other (eg MARCH1/8, MARCH2/3 and MARCH4/9). Most contain two central transmembrane domains, an intracellular N-terminal RING domain (of a subtype variously called HC or CH or v) and intracellular N and C-terminii of various lengths (3-5). The topology of these proteins is such that both the N- and C-terminal domains are intracellular whilst only the short region between the two transmembrane domains is located outside the membrane. A large number of targets of different MARCH proteins have been described and many of these are associated with immune regulation (including MHC class I and II, CD86, TRAILR1, IL1R1 and cell adhesion molecules). Interactions with molecules associated with cell organelle trafficking (DLG1, BAP31, STX4 and STX6) have also been described(6) (4, 7). In some cases it has been shown that targets are recognised at least in part by interactions of the respective transmembrane domains that include A/GXXXA/G sequences (4, 8, 9) and that this interaction then results in RING domain-mediated ubiquitination of the target protein cytoplasmic domain. Ubiquitination on lysine, cysteine or threonine residues targets cell surface receptors for endocytosis and sorting of multivesicular endosomes (through interactions with ESCRT proteins) to the lysozome where the receptor is degraded (10).

Interleukin 6 (IL6) is a pleiotropic cytokine that binds to an IL6-specific receptor subunit (IL6Rα) that then interacts with a second receptor subunit (gp130) that is the signalling

component shared by several other cytokines including interleukins 11 and 27 (IL11, IL27), Leukemia Inhibtory Factor (LIF), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-Like Cytokine (CLC), Neuropoietin (NP), Oncostatin M (OSM) and Cardiotrophin 1 (CT1). It has many pro-inflammatory activities, including induction of an acute phase response in the liver, activation of TH17 cells and supression of Treg, induction of B cell and plasma cell proliferation and maturation (11, 12) and is required for liver regeneration (13). IL6 can signal directly (classic signalling) to cells that display IL6Rα and gp130 (hepatocytes, neutrophils, macrophages and lymphoid cells) but can also signal to cells that only express gp130 (endothelial and muscle cells) by first combining with a soluble circulating form of the IL6Rα (trans-signalling) (14). Trans-signalling is thought to be exclusively pro-inflammatory while conventional signalling can have both pro- and anti-inflammatory effects (15).

Both types of signalling are mediated through gp130 and involve activation of the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway (primarily JAK1/STAT3), the Mitogen-Activated Protein kinase (MAPK) and PhosphatidylInositol-3 Kinase (PI3K) pathways among others. The pathway is also modulated by negative regulation through receptor endocytosis, Suppressor of Cytokine Signalling 3 (SOCS3), and phosphatases such as SHP2, TcPTP and PTP1B(15). Since overactivity of IL6 has been associated with a number of inflammatory diseases including Rheumatoid arthritis(16), Juvenile idiopathic arthritis, Giant Cell Ateritis(17) and Castleman's Disease(18), Tocilizumab, a neutralising antibody to the human IL6Rα, has become an approved treatment for these diseases. This suggests that negative regulatory pathways controlling IL6 activity are not only physiologically important to prevent excessive responses to infection and endogenous IL6 levels but also that their manipulation may have therapeutic benefit in a variety of human autoimmune and inflammatory diseases. In this paper we describe a new

pathway of negative regulation of IL6 activity that depends on MARCH protein-mediated loss of cell surface expression of IL6Rα.

Experimental

cDNA library screening

A mouse brain cDNA library cloned into the MMLV-based vector pCFB (ViraPort; Stratagene) (allowing for production of replication defective retrovirus) was used to infect cultures of M1 cells. The library consisted of 8.7 x 10^6 clones with an average length of 1.55 kbp. The library was subdivided into 20 pools and large scale plasmid preparations of each pool were produced using standard protocols. Amphitropic viral particles encoding the library 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) at 37°C in a humidified atmosphere with 10% (v/v) CO_2. Approximately 3-4 million cells were then transiently transfected with 14.4 μg of library plasmid (representing 50,000 cDNA clones), 4.8 μg of MD1 GAG-POL plasmid and 0.6 μg of VSVg. The plasmids were dissolved in a final concentration of 250mM $CaCl_2$ then precipitated with an equal volume of 2xHBS and added to the 293T cells pre-treated with 25μM chloroquine (Sigma-Aldrich). Cell culture medium was replaced 8 and 24 hours post-transfection and virus harvested 48 hours post-transfection, filtered and snap frozen on dry-ice.

The packaged library was then used to spin-infect M1 cells as follows; 1mL of viral supernatant was added to 500,000 M1 cells in a 12 well tissue culture plate followed by addition of polybrene (4μg/mL final concentration). Occasionally 10μl of GFP-expressing viral particles produced under identical conditions to those described above were also added to allow for estimation of infection efficiency. The plates were centrifuged at 693g for 60 minutes at 23^{o}C before being cultured at 37^{o}C in a humidified atmosphere with 10% (v/v) CO_2 in DME/10% FCS. 24 hours post-infection the cells were washed four times with 5mL

DME then transferred to 10ml DME containing 10% (v/v) BCS and cultured for 2-3 days before plating.

When IL-6 resistant clones were identified, genomic DNA was prepared using standard protocols and the cDNA cloned into the integrated retroviral vector was PCR amplified using primers 5'_pCFB (GGCTGCCGACCCCGGGGGTGG) and 3'_pCFB (CGAACCCCAGAGTCCCGCTCA) and then Sanger sequenced.

Viral MARCH constructs and infections

Open reading frames encoding murine MARCH proteins and mutants thereof were cloned by PCR into the pCFB vector (described above) using *Eco*RI and *Xho*I restriction enzyme sites. An N-terminal FLAG sequence (MADYKDDDDK) was incorporated into all constructs to allow for detection via Western Blot. To monitor transfection by flow cytometry an IRES-GFP element was inserted between the *Xho*I-*Not*I sites of the vector. The Uniprot accession numbers for the MARCH proteins studied here are as follows: MARCH1: Q6NZQ8, MARCH2: Q99M02.1, MARCH3:Q8BRX9.1, MARCH3isoformX1: Refseq XM_006526019.3, MARCH4: Q80TE3, MARCH7: Q9WV66, MARCH8: Q9DBD2, MARCH9: Q3TZ87, vMIR-1: P90495, vMIR-2: P90489.

In order to create constructs where the expression of MARCH was inducible, cDNAs encoding MARCH proteins and chimeras were also cloned into pF TRE 3G PGK Puro as described (19)) using *Bam*HI and *Nhe*I restriction sites. This lentiviral vector allows for induction of expression by the addition of doxycycline. Cells were selected in 10 μg/mL puromycin and were induced with 100 ng/mL doxycycline.

Chimeric MARCH proteins were generated by gene synthesis (Genscript, USA) and all clones were verified by sequencing. Domain boundaries (uniprot numbering) were MARCH2:NTD: 1-138, TM1: 139-160, ECD: 161-175, TM2: 176-198, CTD: 199-246,

MARCH3:NTD: 1-145, TM1: 146-167, ECD: 168-182, TM2: 183-202, CTD: 203-218, MARCH4 CTD:292-409, MARCH9 CTD: 241-348.

Stimulation of M1 and M1-MARCH2 cells with IL-6/LIF and detection of p-Stat3, p-p44/42, gp130 and LIFR by Western blot analysis: Parental M1 or MARCH-2 transfected M1 cells were cultured in DME /10% BCS (containing IL-6 for the MARCH2 expressing clone). Prior to stimulation, culture medium was removed and cells were washed with DME and resuspended in DME containing 0.1% (v/v) BSA at 10^6 cells/mL. Five million cells in 2 mL were added to each well of a 6-well plate and incubated at 37°C for 3 h (serum and cytokine starvation), followed by the addition of 10 μL of 30 μg/mL rmIL-6 or rhLIF (diluted in DME/0.1%BSA) to each well at various time points. At the end of stimulation, plates were transferred to a tray containing ice, culture medium was removed and cells were rinsed once with 3 mL of cold mouse-tonicity PBS, followed by lysing cells in each well in 150 μL of lysis buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, freshly supplemented with 2 mM Na3VO4, 10 mM NaF, 1 mM PMSF, and complete protease inhibitor cocktail (Roche Diagnostics). Proteins were separated by SDS-PAGE using 4-12% Bis-Tris Criterion gels from Bio-Rad and transferred to PVDF-Plus membranes (GE Water & Process Technologies). PVDF membranes were blocked with 5% skim milk in PBS containing 0.1% (v/v) Tween-20 and probed with primary antibodies overnight at 4˚C and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. ECL reactions were performed with either Amersham ECL kit (GE Healthcare) or ECL reagent (Millipore) and the membranes were exposed to Amersham ECL film. Primary antibodies used in this study were rabbit-anti-gp130 (sc-656), rabbit-anti-LIFR (sc-659), and rabbit-anti-Stat3 (sc-482) from Santa Cruz, and rabbit-anti-p-Stat3 mAb (CS9145), rabbit-anti-p-p44/42 (CS910) and rabbit-anti-p44/42 (CS9102) from Cell Signalling Technology. All these antibodies were used at 1:1000

dilution. Secondary antibody was donkey-anti-rabbit-Ig-HRP from GE Healthcare. Aliquots of cell lysates were run on duplicate gels and each transferred to membranes. The first membrane was probed for p42/44 and total STAT3 while the second was probed for pSTAT3 and total p42/44. Membranes were also probed for LIFR and gp130 with sodium azide include in the blocking and primary antibody solutions to quench signals from previous probings. The high specificity of the antibodies and the different molecular weights of the signalling components allowed this to be done without fear of misinterpretation. The experiment was repeated twice.

Expression and purification of the IL-6 receptor α-chain ectodomain

A cDNA fragment encoding mature human IL-6 receptor α-chain ectodomain residues 20-325 was synthesised and codon optimised for insect cell expression (GenScript). The fragment was digested with *Kpn*I and *Xho*I and inserted into pgpHFT. Expression and purification of IL-6 receptor α-chain ectodomain was performed as described for gp130 (20). After one-step purification using the anti-FLAG M2 agarose (Sigma), the protein was further purified by size-exclusion chromatography.

Expression and purification of IL-6

Human IL-6 was expressed as an N-terminally His-tagged protein in BL21(DE3) *E. coli* cells. Briefly, a cDNA fragment encoding mature human IL-6 residues 31-212 was cloned into the pETDuet vector (Novagen) using *Bam*HI and *Asc*I sites. The expression plasmid was transformed into BL21(DE3) cells by electroporation. A colony was placed in 100 mL of 2xYT media containing 100 μg/mL ampicillin and incubated overnight at 37°C. The overnight culture was diluted 20 times in Superbroth containing 100 μg/mL ampicillin and incubated at 37°C. The cells were induced with 0.1 mM isopropyl thio-β-D-galactoside when OD_{600nm} reached approximately 1.0. The cells were harvested 3 h post induction. The cell pellets were lysed with 0.2 mg/mL lysozyme in phosphate-buffered saline containing 1%

Triton X-100, 1 mM PMSF, 30 μg/mL DNase I for 1 h on ice. The whole cell lysates were centrifuged for 15 min at 23,000 *g* at 4°C. Insoluble pellets containing IL-6 were washed twice (1 h per wash) with 1 M guanidine-HCl, 1% (v/v) Triton X-100 in 20 mM Tris-HCl, pH 8.0 at room temperature and the washed pellets were solubilised overnight at 4°C with gentle stirring in 7 M guanidine-HCl in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 10 mM imidazole, pH 8.0. The solubilised IL-6 was then purified by affinity chromatography using Ni-NTA resin (Qiagen) under denaturing conditions as per manufacturer's instructions. Fractions containing IL-6 were purified by reversed-phase HPLC using a C8 column as described (21). IL-6-containing fractions were pooled, diluted 1:25 in 20 mM HEPES, pH 5.5 and loaded onto a 1 mL HiTrap SP column (GE Healthcare). The bound proteins were eluted using a gradient from 0 to 1 M NaCl in 20 mM HEPES, pH 5.5. Fractions containing purified IL-6 were pooled and pH adjusted to 7.0 using 1 M HEPES, pH 7.5.

MARCH knockout mice and genotyping

The constructs used to generate MARCH2, MARCH3 and MARCH4 knockout mice on a C57BL6 background as well as the genotyping protocols are shown in Fig. S1. Generation of these knockout mice was as described for MARCH1 in Matsuki et al (22). MARCH2, 3 and 4 knockout mice were maintained as inbred homozygous colonies. MARCH23, 24 and 234 compound mutant mice were generated by interbreeding parental knockout mice followed by inter-crossing first generation compound heterozygotes and/or back-crossing to parental strains until knockout alleles were homozygous. The compound knockout mice were then maintained as inbred colonies. Wild-type control mice were sourced from independent C57BL/6 colonies. All experimental and mouse breeding procedures were approved by the Walter and Eliza Hall Institute Animal Ethics Committee and performed under the direction of WSA at the Walter and Eliza Hall Institute Bioservices facility.

MARCH-deficient mice were genotyped by PCR with the primers provided in Table 1. DNA was extracted from tail biopsies (~1 mm) collected at weaning and amplified with gene-specific primers using the following cycling conditions: 94oC for 30 sec, 55oC for 1 min and 72oC for 1 min for 35 cycles. PCR products were resolved by electrophoresis in 2% (w/v) agarose gels.

Flow cytometry of haemopoietic populations from wild type and MARCH knockout mice

Age and sex matched wild-type, single MARCH or multiple MARCH knockout mice were euthanased by CO_2 asphyxiation. Bone marrow cells were collected by flushing both femurs and tibias with 3 mL EDTA/BSS/2%FCS/0.02% (w/v) Na azide (EDTA/BSS/FCS) using a 21.5-gauge needle and syringe. Single cell suspensions were generated by pipetting and placed on ice. They were then passed through a 100 micron cell strainer (Falcon) that was washed with a further 5 mL of the same medium before centrifugation at 425 g for 5 min at 4oC. The cell pellet was resuspended in 1 mL of Red Cell Removal Buffer (RCRB) at 25oC then 4 mL of RCRB was added and cells centrifuged as above. The cell pellet was resuspended in 5 mL BSS and live cells counted using eosin exclusion in a haemocytometer. Spleens were removed into 3 mL BSS/FCS and placed on ice. They were then cut into small pieces using surgical scissors and the pieces transferred into 5 mL RPMI/2% (v/v) FCS medium. One mL of DNase1/Collagenase type 3 (containing 1 mg DNase and 7 mg collagenase in RPMI/2% (v/v) FCS) was then added to each tube and the tubes vigorously shaken in a mechanical shaker at 37oC for 30 min. then 700 μL of 0.5 M EDTA was added and shaking continued for a further 5 min. The cell suspension was passed through a 100 micron cell strainer using the blunt end of a 1ml syringe plunger to break up any remaining

spleen pieces and the filter washed with a further 5 mL RPMI/2% (v/v) FCS. The resulting cell suspension was then processed as described above for bone marrow cells using RCRB except that the cells were in a final total volume of 10 mL.

Spleen or bone marrow cells (5 or 10 x 10^6) were placed in Falcon 352008 tubes , centrifuged as above, resuspended in 4 ml EDTA/BSS/FCS and centrifuged again. Fc receptors were blocked by incubating cells with anti-CD16/CD32 (250 μL for 10 million and 100 μL for 5 x 10^6) on ice for 10 min, then resuspended in 5 mL EDTA/BSS/1%BSA/0.02% Na azide (BSS/BSA) before centrifugation. Cells were then incubated with primary antibody cocktails (same volumes as for Fc block) for 1 hr on ice as follows (see also Table 2).

Dendritic cell/macrophage (10 x 10^6 cells) antibody cocktail (αCD8-FITC, αF4/80-Alexa 647, αCD11c-PE, αCD3-Alexa700, αB220-Alexa700, αTer119-Alexa700, αIL6Rα(cCD126)-biotin or normal goat Ig-biotin).

B- and T-lymphocyte (5 million cells) antibody cocktail (αCD8-FITC, αCD4-Alexa594, αCD3-Alexa700, αCD19-Pacific Blue, αIgM-allophycocyanin, αIgD-PE, αIL6Rα(CD126)-biotin or normal goat Ig-biotin).

Monocyte/neutrophil (5 x 10^6 cells) antibody cocktail (αCD11b-PE, αly6C-Allophycocyanin, αLy6G-FITC, αCD3-Alexa700, αB220-Alexa700, αTer119-Alexa700, αIL6Rα(CD126)-biotin or normal goat Ig-biotin).

After incubation samples were resuspended in 4 mL BSS/BSA and centrifuged as above. Cells were then incubated with streptavidin-PE/Cy7 for 1 hr on ice (250 or 100 μL as above) then resuspended again in 4 ml BSS/BSA, centrifuged and then resuspended in the viability stain Flurogold (400 or 200 μL for 10 or 5 x 10^6 cells respectively).

Flow cytometry was performed on cells from at least two wild-type and two MARCH KO mice in each experiment and all experiments were repeated two to three times. Cells were analysed on a Becton-Dickinson LSR Fortessa operating with FACS Diva 6.2 software for

setup and FlowJo 10.4.1 software for analysis of cell subsets. The gating strategies for different cell subsets are shown in Fig S2. Briefly cells were first selected as viable (fluorogold negative) and single cells (forward scatter A/H ratios). B cells were CD3-CD19+, T cells were CD3+CD19-, Dendritic cells were lin-CD11c+F4/80-, monocyte/macrophages were lin-CD11c-F4/80+ or lin-Ly6C++Ly6G- and neutrophils were lin-Ly6C+Ly6G+, where lin = B220+, Ter119+ or CD3+).

For analysis of IL6Ra on M1 cells the same biotinylated antibody was used but the biotin was detected with streptavidin-PE and for analysis of gp130 an APC-conjugated antibody was used (see Table 2).

Semi-solid clonal cultures in agar

Viable M1 cells (grown in DME/10%BCS) were enumerated by eosin exclusion in a haemocytometer, centrifuged at 425g for 5 min at 4oC and resuspended in the same buffer at the desired cell concentration. 30% (v/v) modified double strength DME, 20% (v/v) BCS (Gibco 94999) and 50% of 0.6%(w/v) Bacto Agar (pre-warmed to 41oC) were mixed with M1 cells (final concentration 200-1000 cells/mL) and 1 mL aliquots added to 36 mm diameter Petri dishes (Greiner Bio-one) to which 0.1 mL of appropriate cytokine or saline control had been added. After setting at room temperature for 15 min, cultures were incubated at 37oC 10% (v/v) CO2 in a fully humidified incubator for 7 days after which differentiated (containing a halo of dispersed cells) and undifferentiated (tight balls of cells) colonies were separately enumerated using an Olympus dissection microscope.

Results

The mouse M1 myeloid leukemia cell line forms tight colonies with little individual cell dispersion in agar cultures but in the presence of gp130 cytokines (particularly IL6, leukemia inhibitory factor (LIF) and oncostatin M (OSM)) the M1 cells differentiate and produce either diffuse colonies of dispersed, differentiated macrophages or, at higher doses of the cytokines,

the cells terminally differentiate without forming any colonies. We used a mouse brain cDNA library and expression screening of infected M1 cells in agar (in the presence of 100 ng/ml of IL6) to detect proteins that could suppress the capacity of IL6 to terminally differentiate M1 cells. From 0.5 million cDNAs screened we found 30 undifferentiated colonies in the presence of high dose IL6 and three of these were confirmed as true positives by showing that the viral insert encoded a full length protein that could induce at a high rate IL6-resistant colonies when M1 cells were re-infected with pure cDNA clones (30-60% of all cells, reflecting the infection efficiency). All three viral inserts were sequenced and shown to encode MARCH2.

Surprisingly, although MARCH2 over-expressing M1 cells were completely unresponsive to IL6 at all doses tested, responses to human OSM or human LIF that also use the signalling receptor chain gp130 were at most marginally effected (Fig 1A). Analyses of intracellular signalling pathways showed that the ability of IL6 to activate STAT3 (P-STAT3) and MAPK (P-p44/42) was severely reduced in M1 MARCH2 cells while LIF responses were unaffected (Fig 1B). This suggested a MARCH2 target specific for IL6 and, since most MARCH targets are membrane associated, the IL6 receptor α chain was an obvious candidate. As shown in Figure 1C flow cytometry revealed a marked loss of IL6Rα in MARCH2 overexpressing cells. Unlike IL6Rα, levels of gp130 were unaffected by MARCH2 overexpression, consistent with our earlier observation that MARCH2 did not dramatically inhibit LIF or OSM signalling. Since MARCH action is dependent on RING-mediated receptor ubiquitination we tested a MARCH2 mutant (W97E) expected by homology(23) to be defective in ubiquitination activity. In contrast to wild-type MARCH2, MARCH2^{W97E} reproducibly increased IL6Rα expression on M1 cells suggesting that it was acting in a dominant negative fashion (Fig 1C).

Unlike many other cytokine receptors, soluble IL6Rα can bind IL6 and activate cells that lack endogenous IL6Rα but express gp130. We reasoned that if the sole IL6-dependent defect in M1 MARCH2 cells was loss of endogenous IL6Rα then a combination of soluble IL6Rα and IL6 should restore responsiveness as these cells still display membrane gp130. This was indeed the case and full responsiveness was restored at IL6 /soluble IL6Rα doses known to be required for binding to gp130 (24) (Fig 1D). The higher dose of IL6 required in the complex mixture (approx. 100 ng/ml) to stimulate M1 MARCH2 cells compared to a few ng/ml to stimulate WT M1 cells is a result of the very low affinity of IL6 for the sIL6Ra and the very low proportion of IL6/sIL6Ra complexes in the mixture (approx. 5%) (25, 26)

Because MARCH proteins often share common receptor targets we tested whether other MARCH proteins are capable of down-regulating IL6Rα expression on M1 cells. Although MARCH2 expression showed the most complete inhibition of IL6 activity in M1 cells, MARCH4 and to a lesser extent MARCH9 also showed a profound reduction in IL6 responsiveness (10- to 100-fold). Surprisingly the closest homologue of MARCH2 (i.e. MARCH3) did not show any effect on IL6 responsiveness. MARCH4 and MARCH9 also significantly decreased cell surface IL6Rα levels while MARCH1, MARCH3 and MARCH8 had little effect on IL6 responsiveness or IL6Rα levels (Fig2 A,B). MARCH protein expression as measured by anti-FLAG antibody was variable but almost undetectable levels of MARCH 4 and 9 were still effective while higher levels of MARCH3 were not. Ring-inactivating mutants of MARCH2 (I66E and W97E) resulted in higher levels of MARCH protein suggesting that MARCH protein levels themselves are controlled by ubiquitination or are consumed along with their ubiquitinated targets. Despite its higher protein levels MARCH2^{W97E} showed little effect on IL6 responsiveness with perhaps a slightly increased sensitivity to IL6 (Fig 2A,C) coincident with its capacity to up-regulate IL6Rα levels. Two

different viral MARCH proteins (viral Modulators of Immune Recognition, vMIR1 and vMIR2 derived from Kaposi sarcoma herpes virus/human gammaherpesvirus 8) and known to affect antigen presentation by down-regulating MHC-I, showed no effect on IL6 responsiveness of M1 cells nor did they reduce IL6Rα levels (Fig 2D) suggesting that dampening of IL6 responses may not be an additional mechanism for their immune evasive activity. The vMIRs appear to be most closely related to MARCH4 and MARCH9 and share with these MARCH proteins the ability to down-regulate MHC-1 molecules at the cell surface(27, 28). However, vMIR1 and vMIR2 are presumably derived from human MARCH proteins and the cross-species reactivity of MARCH proteins on mouse IL6Ra is unknown. The lack of effect of MARCH3 (based on the refseq mouse DNA sequence) was a surprise given the degree of sequence identity with MARCH2. This prompted us to search the databases for alternate transcripts of MARCH3. One such alternate transcript was expressed and shown, like MARCH2, to dramatically reduce IL6 responsiveness and to dramatically reduce IL6Rα levels on M1 cells (Fig 3A,B).This MARCH3 isoform is closer in sequence to the human homologue and differs from the refseq gene primarily at the C-terminal end. It retains greater sequence similarity to MARCH2 including a putative C-terminal PDZ domain-interacting motif (29, 30)(Fig 3C).

Using chimeras of MARCH2 and the inactive form of MARCH3 we aimed to identify essential domains required for down-regulation of IL6Rα. These domain mutant swaps revealed that a combination of the second transmembrane domain and the C-terminal domain was absolutely required for MARCH2 activity (Fig 3D and Supp Fig3). Conversely, the entire N-terminal region (including RING domain) could be domain-swapped with no loss of activity. It is possible that these elements act independently to recognise IL6Rα (transmembrane domain), ubiquitinate the receptor (RING domain) and target endosomal sorting machinery (C-terminal and PDZ-interacting motif), however, it was interesting that

the MARCH2 C-terminal domain grafted onto MARCH3 backbone did not restore activity suggesting that these domains may cooperate rather than act independently.

In order to test if endogenous MARCH proteins regulate steady state levels of IL6Rα on haemopoietic cells we generated individual and multiple knockout mice for each of MARCH2, 3 and 4, the MARCH proteins that showed the largest effects on IL6Rα levels when exogenously expressed in M1 cells. Individual and multiple MARCH KO mice showed no gross abnormalities. We used flow cytometry to measure cell surface IL6Rα levels in developing and mature B-lymphocytes (CD3-CD19+IgMIgD), T-lymphocytes (CD3+CD19-CD4CD8), dendritic cells (CD11c++CD8), macrophages (CD11b+LysC++F4/80+Ly6G-) and neutrophils (CD11b+Ly6C+Ly6G+) in bone marrow and spleen of specific KO mice relative to age and sex-matched wild-type (C57BL6) mice (see Fig S2 for gating strategies). While steady state IL6Rα levels on most cell types were not affected by single or multiple MARCH KOs (eg on granulocytes, macrophages, dendritic cells) significant changes were seen in lymphoid cells. Individual MARCH KOs had no or little effect on IL6Rα levels on CD8+and CD4+ T-cells but the combination of MARCH2,3; MARCH2,4; and MARCH2,3,4 each showed a significant and reproducible increase in steady state IL6Rα levels on CD8+ T cells from spleen (Fig 4) and bone marrow and also on CD4+ T cells from both organs (Fig S4). In addition, minor increases in IL6Ra levels were seen in maturing and mature B cells (Fig S5) but no changes were seen in IL6Ra levels in myeloid cells (including CD11c+ dendritic cells, monocytes (CD11b+F4/80+ or Ly6C++), macrophages (F4/80++) or neutrophils and their precursors (CD11b+Ly6G+) for any of the MARCH genotypes (Fig S6).

Discussion

Using a cDNA expression library in the mouse M1 myeloid leukemic cell line we selected IL6-unresponsive cells that contained the cDNA for MARCH2. Related MARCH proteins

(MARCH3 -an alternate transcript form that is closest to the human homologue, MARCH4 and MARCH9) also reduced IL6 responsiveness to varying degrees. All MARCH proteins reducing IL6 responsiveness appeared to do so by a proportionate reduction in cell surface expression levels of the IL6-specific receptor subunit IL6Rα. In this regard these specific MARCH proteins act like several members of the broader MARCH family in reducing antigen presentation or other immune regulatory cell surface receptors from the cell surface of immune cells thus dampening immune responses.

In the case of MARCH1 and MARCH9 (that down-regulate MHCII antigen presenting receptors) substrate recognition appears to occur through transmembrane domain interactions possibly also including the C-terminal tail (31) and that would be consistent with our observations of required domains in MARCH2 and 3 for IL6Rα down-regulation although our experiments could not distinguish between domains required for substrate recognition and those required for intracellular vesicle sorting. Similarly, the RING domains of MARCH proteins have been shown to be required for ubiquitination of target proteins on cytoplasmic domain lysines that act as sorting signals for endosome trafficking and possibly other intracellular vesicle trafficking including from the golgi to lysosomes(32). While our results also emphasize the requirement for the RING domain to reduce IL6Rα expression and are consistent with a ubiquitination signal that leads to sorting of IL6Rα into endosomes targeted for degradation by fusion with lysosomes, we cannot exclude other mechanisms such as failure of newly synthesized IL6Rα in the ER to be expressed at the cell surface or reduced recycling of IL6Rα from endosomes to the cell surface. We were unable to find antibodies to the IL6Ra or MARCH proteins that had sufficient sensitivity and/or specificity to detect endogenous levels of these proteins in wild type M1 cells or MARCH-expressing M1 cells in order to address these questions by co-immunoprecipitation, Western blot or co-localisation by immunofluorescence.

While individual MARCH proteins were not essential for determining expression of IL6Rα on any haemopoietic cell type measured in the mouse at steady state, this is likely due to redundancy in the actions of MARCH2,3,4 and 9 since various combined knockouts did result in a significant increase in IL6Rα levels in lymphoid cells. Given the proposed roles of MARCH proteins as immune suppressors it is more likely that increased expression of particular MARCH proteins (or their post-translational activation)(6) occurs during inflammatory responses to prevent excessive responses to infection or tissue damage. It will therefore be of great interest to better understand the expression of MARCH proteins in different cell types at steady state and which stimuli induce expression or activation of MARCH proteins in these cell types. That information will better inform in what pathological states modulation of MARCH expression or function may be useful to enhance immune responses or to prevent inflammatory tissue damage.

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Funding.

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Author contribution.

NAN, DJH,JJB and WSA designed and supervised the study, analysed and interpreted data. DS, LD, JGZ, AL and AC performed experiments. SI and JV provided essential materials and critical advice. NAN wrote the paper with input and approval of all authors.

Table 1. Primers used for genotyping MARCH knockout mice.

Gene	Primers	PCR products
MARCH2	M2/1212: 5'-TGA GCC ATC TTA GGT CTC TGT TT-3'	Wild-type: 178 bp
	M2/1213: 5'-CCC CAG GGA CTA AAG AAA TG-3'	Knockout: 250 bp
	M2/1214: 5'-TCA ACC TGA CAG GAG ATG GA-3'	
MARCH3	M3/1215: 5'-AGC ACT TGT GAG GTA GGG AGA-3'	Wild-type: 151 bp
	WA1216: 5'-GAC GAC TTC TAG TTC TAA GTC CCA GT-3'	Knockout: 250 bp
	WA1217: 5'-TTG ATG CTG GGT GTC ACT GT-3'	
MARCH4	WA1218: 5'-GGG GAT GGA AGC TGG ATA GT-3'	Wild-type: 169 bp
	WA1219: 5'-CCC TGA GTT CTG CCA ATC TG-3'	Knockout: 299 bp
	WA1220: 5'-CTG CCA TCT CCC ATT TTT GT-3'	

Table 2 Antibodies, conjugates and cytokines used in this work.

Antibody conjugate	clone	dilution	Cat No	Source
Flow cytometry				
CD8-FITC	53.6.7	1/400		WEHI Monoclonal Antibody Facility
F4/80-Alexa 647		1/1000		WEHI Monoclonal Antibody Facility
CD11c-PE	N418	1/100		WEHI Monoclonal Antibody Facility
IL6RA-biotin	Polyclonal Goat	1/100	BAF1830	R&D Systems
Goat Ig-biotin	Polyclonal Goat	1/100	BAF108	R&D Systems
CD3-Alexa 700	KT3-1-1	1/400		WEHI Monoclonal Antibody Facility
B220-Alexa 700	RA3-6B2	1/300		WEHI Monoclonal Antibody Facility
Ter119-Alexa 700	Ly76	1/400		WEHI Monoclonal Antibody Facility
CD4-Alexa 594	GK 1.5	1/400		WEHI Monoclonal Antibody Facility
IgM-A647	5.1	1/400		WEHI Monoclonal Antibody Facility
IgD-PE	11-26c.2a	1/800	558597	BD Pharmingen
Ly6C-APC	HK 1.4	1/1200	128016	BioLegend
CD11b-PE	M1/70	1/400		WEHI Monoclonal Antibody Facility
Ly6G-FITC	1A8	1/400		WEHI Monoclonal Antibody Facility
Streptavidin-PE-Cy7		1/750	557598	BD Pharmingen
Streptavidin-PE		1/750	405204	Biolegend
CD19 Pacific Blue	1D3	1/400		WEHI Monoclonal Antibody Facility
Fc Block (CD16/32)	24G2	1/100		WEHI Monoclonal Antibody Facility
gp130-APC	125623	1/40	Fab4681A	R&D Systems
Rat Ig-APC	R35-95	1/40	554690	BD Pharmingen
Fluorogold (Hydroxystilbamidine)		1/120	17514	AAT Bioquest
IP/Western blots				
STAT3	C20	1/1000	sc-482	Santa Cruz Biotechnology
STAT3-P	D3A7	1/1000	cs9145	Cell Signaling Technology
p42/44 MAPK		1/1000	cs9102	Cell Signaling Technology
p42/44 MAPK-P		1/1000	cs9101	Cell Signaling Technology
gp130	M20	1/1000	sc-656	Santa Cruz Biotechnology
LIFR	C19	1/1000	sc-659	Santa Cruz Biotechnology
donkey anti rabbit Ig HRP		1/10000	NA9340	GE Healthcare
Flag	9H1	0.5 ∝g/mL		WEHI Monoclonal Antibody Facility
goat anti-rat Ig HRP		1/15000	3030-05	Southern Biotechnology
actin HRP	I19	1/5000	sc-1616	Santa Cruz Biotechnology
Cytokines				
mouse IL-6				produced in-house
human LIF				produced in-house
human OSM				produced in-house

Figure Legends

Fig.1 Expression of MARCH2 in M1 cells selectively inhibits IL6 signalling by down-regulating cell surface expression of the IL6Rα chain.

A. March2 expression completely prevents IL6-induced differentiation in M1 cells without major effects on hLIF- or hOSM-induced differentiation. Titrations of IL6, LIF and hOSM on WT M1 cells and MARCH2 over-expressing M1 cells (using the MMLV-based vector pCFB) are shown. **B**. MARCH2 expression results in a lack of activation of intracellular signalling (phosphorylation of STAT3 and p44/42 MAPK) pathways by IL6 but not by hLIF. Cells were stimulated with IL6 or LIF for 0, 0.5.1.0 or 4 hrs and cell extracts were tested for expression of total and phosphorylated STAT3 and p42/44 MAPK (ERK1/2) as well as gp130 and LIFR levels using specific antibodies (IL6Ra levels were undetectable) (one of three similar experiments is shown) **C**. MARCH2 expression resulted in a dramatic loss of surface IL6Rα but not gp130 expression on M1 cells while a RING domain mutant of MARCH2 (W97E) caused an increase in IL6Rα expression. Flow cytometry results are shown for up to 4 biological replicates with WT M1 cells shown in black, MARCH2 expressing cells in red and MARCH2 (W97E) shown in green (isotype controls are in grey). **D**. hIL6 and hIL6Rα each had no effect on MARCH2-M1 cells but the combination of hIL6 and hIL6Rα completely restored differentiation induction. In WT cells the hIL6 in the hIL6/shIL6Ra mixture acted on M1 cells with the same potency as mIL6 while in MARCH2 M1 cells only the combination of hIL6 and shIL6Ra was able to induce differentiation. The higher concentration of the hIL6 required in the mixture (500 ng/ml hIL6 and 1 μg/ml hIL6Ra) reflects the very low affinity of hIL6 for the shIL6Ra. One of two biological replicates shown.

Fig.2 March2,4,9 but not viral MARCH proteins significantly reduce IL6 responsiveness and IL6Rα surface expression in M1 cells.

A. IL6 titrations on M1 cells expressing an empty GFP vector or various MARCH proteins (MARCH1,2,3,4,8.9) and a RING mutant of MARCH2 (W97E). **B**. Ability of different MARCH proteins (MARCH1,3,4 and 9) to down-regulate cell surface expression of IL6Rα by flow cytometry. **C**. Anti-FLAG Western blots of M1 cells expressing various MARCH-FLAG proteins (MARCH1,2,3,4,8,9 and two RING mutants of MARCH2 I66E and W97E). **D**. IL6 responsiveness of wild-type M1 cells (M1WT) compared to M1 cells expressing viral MARCH proteins (vMIR1 and 2).

Fig.3 Alternate transcript of mouse MARCH3 is active in down-regulating IL6 responses in M1 cells and helps define the functional sub-domains.

A. IL6 titrations on WT M1 cells or M1 cells expressing the refseq mouse MARCH3 cDNA(MARCH3) or the alternative transcript (MARCH3 alt). **B**. MARCH3 alt but not MARCH3 dramatically reduced expression of cell surface IL6Rα on M1 cells as measured by flow cytometry. **C.** Amino acid sequence alignments of mouse MARCH2 and MARCH3 or MARCH3 alt. The ring domains are boxed in red and the two transmembrane domains are boxed in grey. The main differences between MARCH3 transcripts is in the c-terminus including the putative PDZ-domain interacting sequence shown in the yellow box. **D**. Chimeras or domain swaps were constructed between MARCH2 and MARCH3 to identify domains required for MARCH inhibition of IL6 signalling in M1 cells. Chimeras on a MARCH2 backbone with swapped in MARCH3 domains were chimeras 2-1 to 2-6 while chimeras on a MARCH3 background with swapped in MARCH2 domains were chimeras 3-1 to 3-6 (see right hand side of Fig 3D for constructs and summary of effects). Only chimeras

that contained the second transmembrane and C-terminal domains of MARCH2 (2-1.2-4 and 3-6) were active as indicated by + for inhibition of IL6-induced differentiation in the IL6 column or down-regulation of IL6Rα in the IL6Ra column. One set of two biological replicates with identical results is shown.

Fig.4 Cell surface IL6Rα levels were significantly elevated in CD8+ T cells only from mice in which at least 2 of MARCH2,3 and 4 had been deleted. Single, double or triple MARCH knockout mice, age (6-12 wk) and sex matched with C57Bl6 control mice, were used to determine IL6Rα levels on spleen viable CD3+CD19-CD4-CD8+ cells (CD8+ T cells) by flow cytometry. Two mice from each group (C57Bl6 mice in black, indicated knockout mice in red and isotype controls in grey) are shown and each experiment was performed with an additional 4 mice a further 1-2 times (alternating mouse sexes) with similar results.

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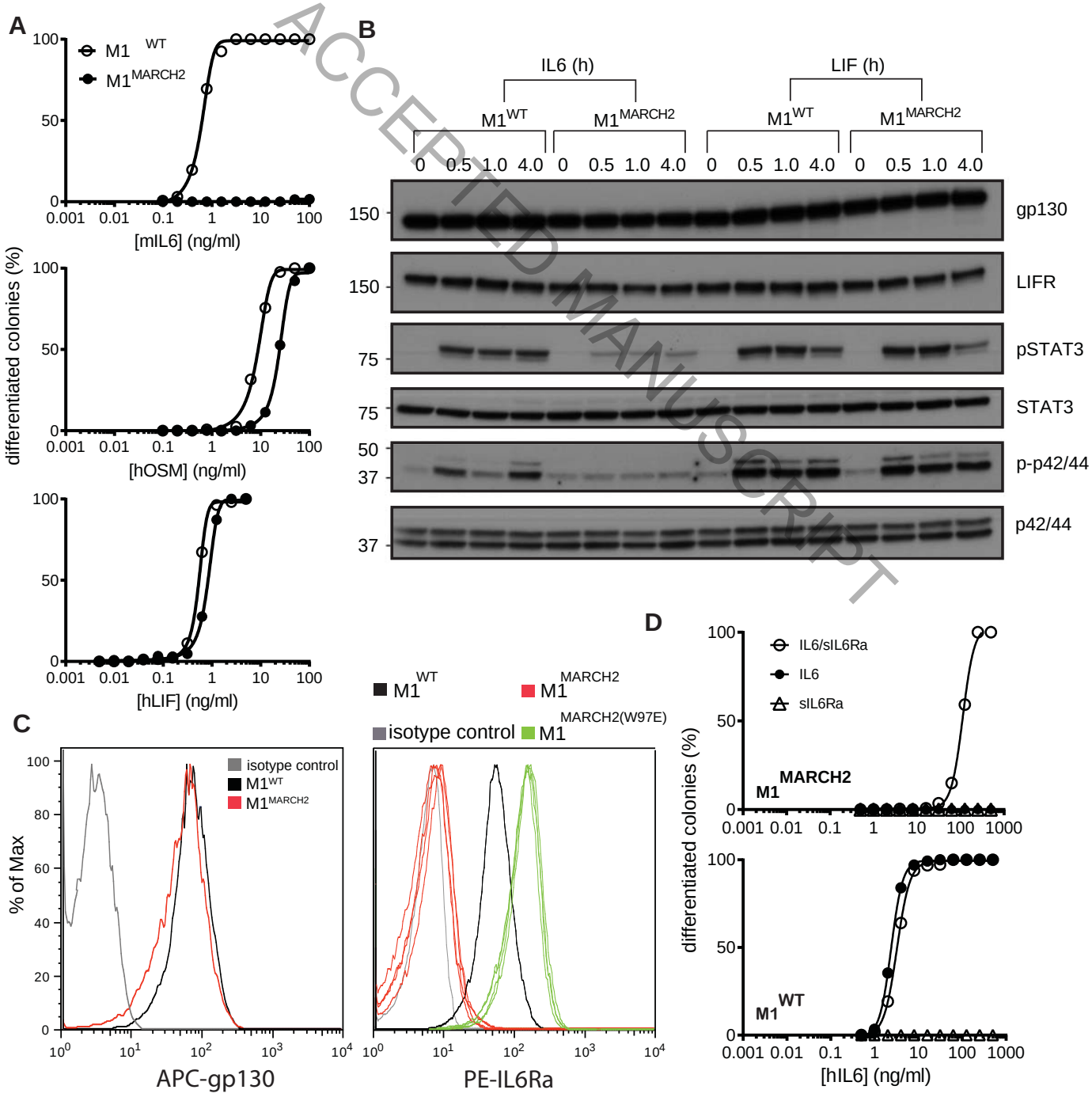
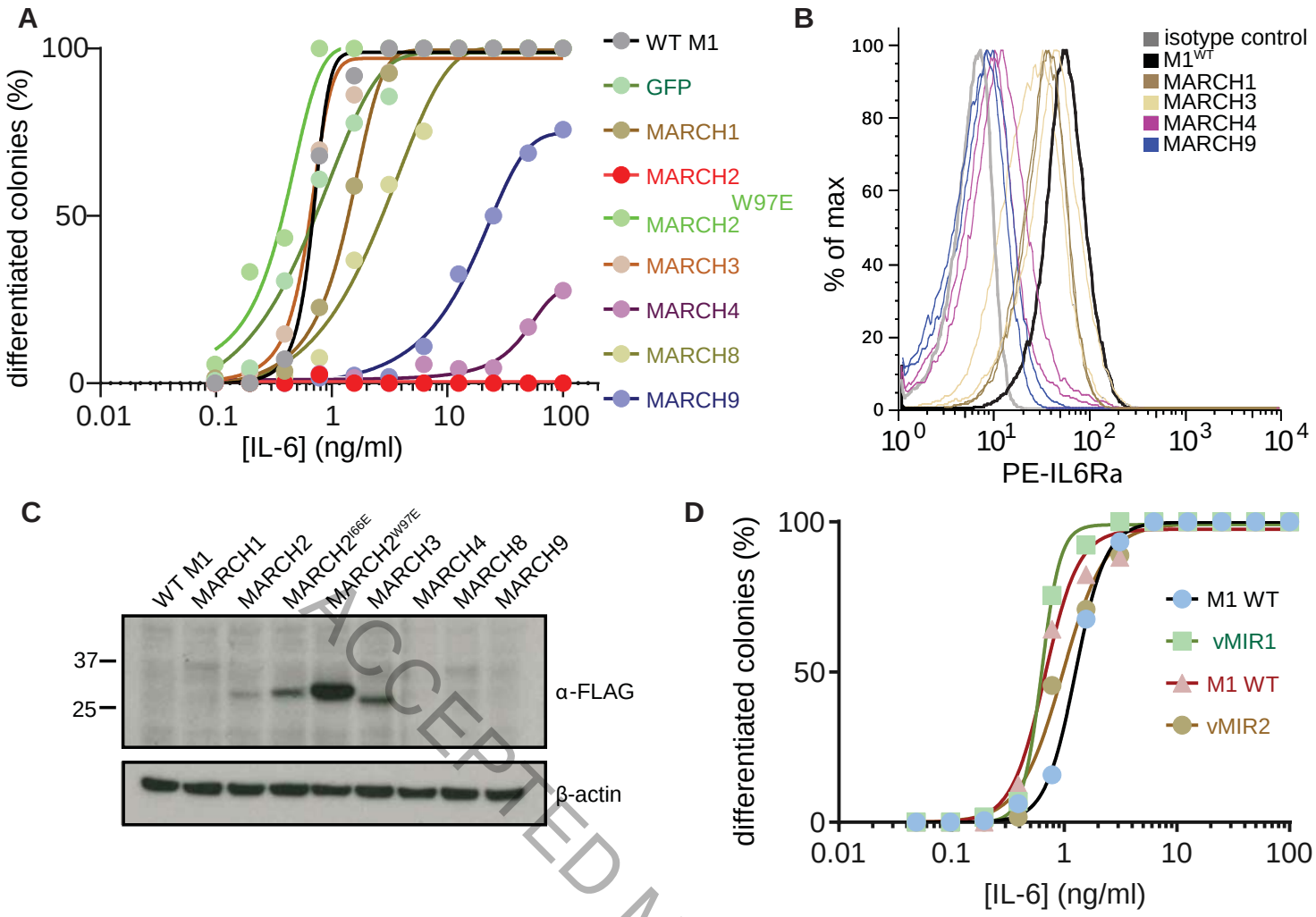
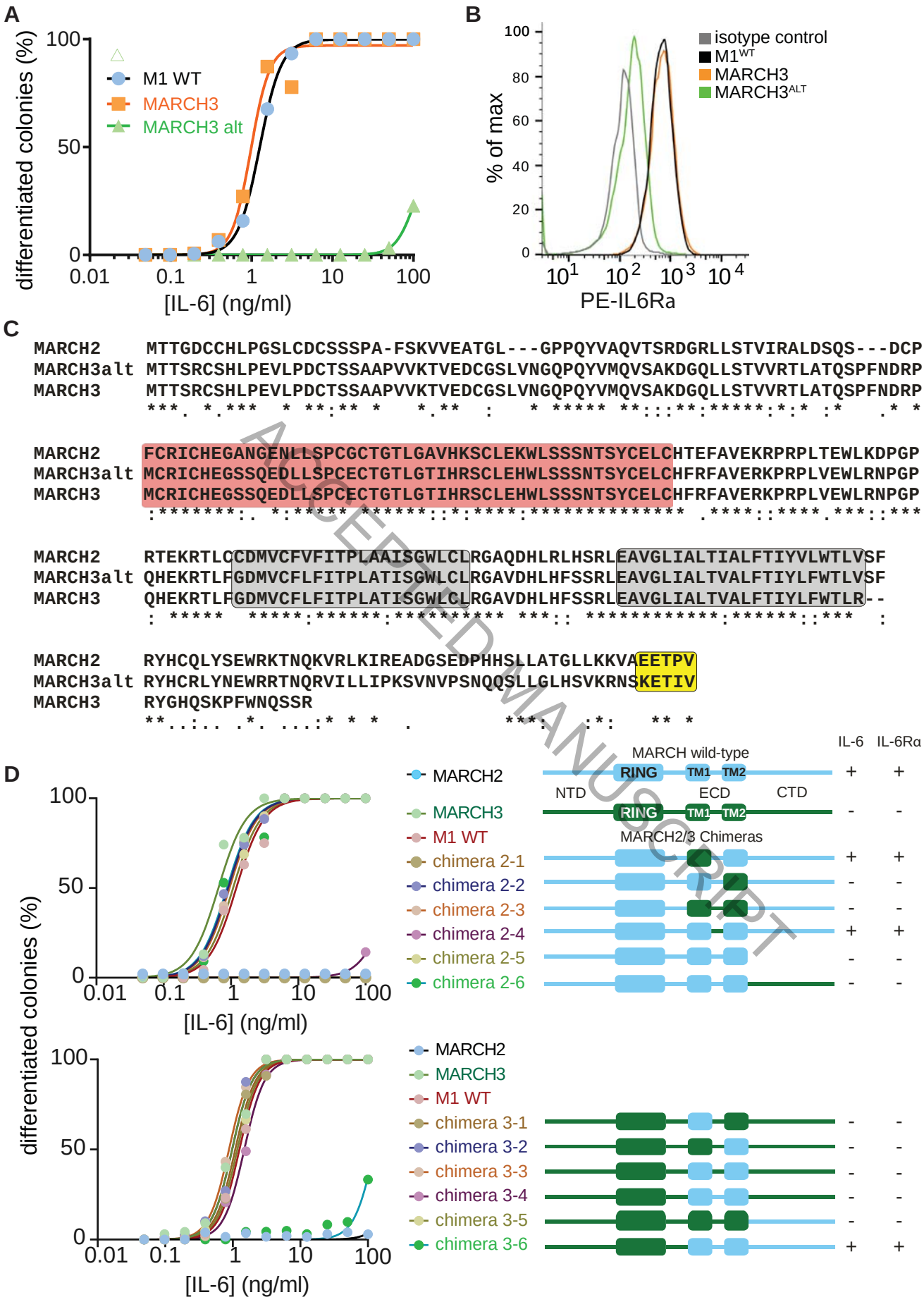
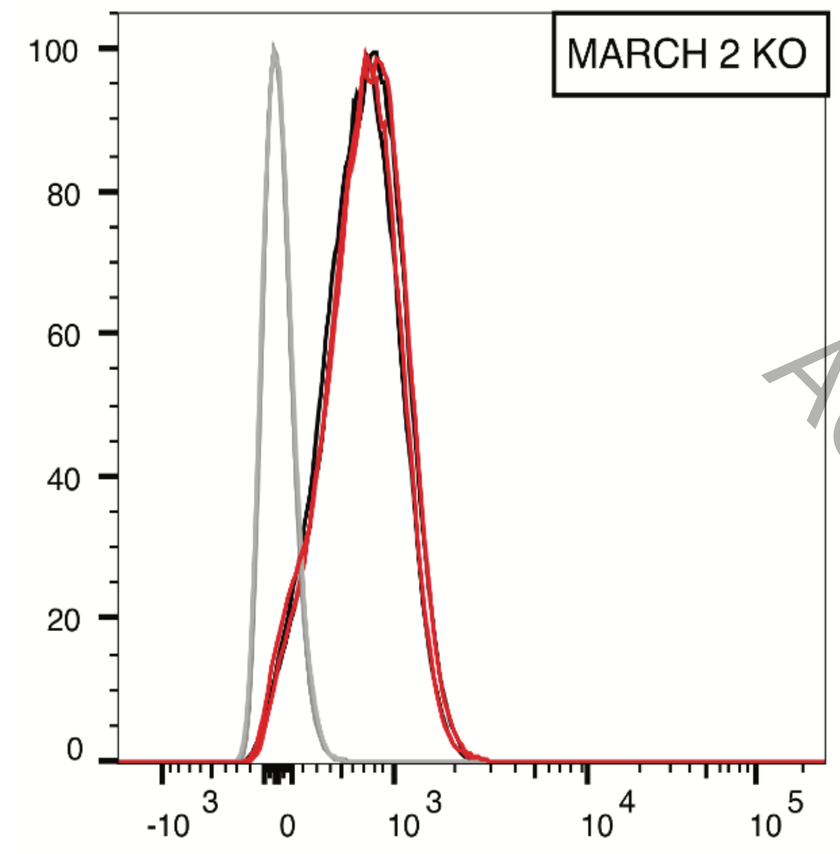
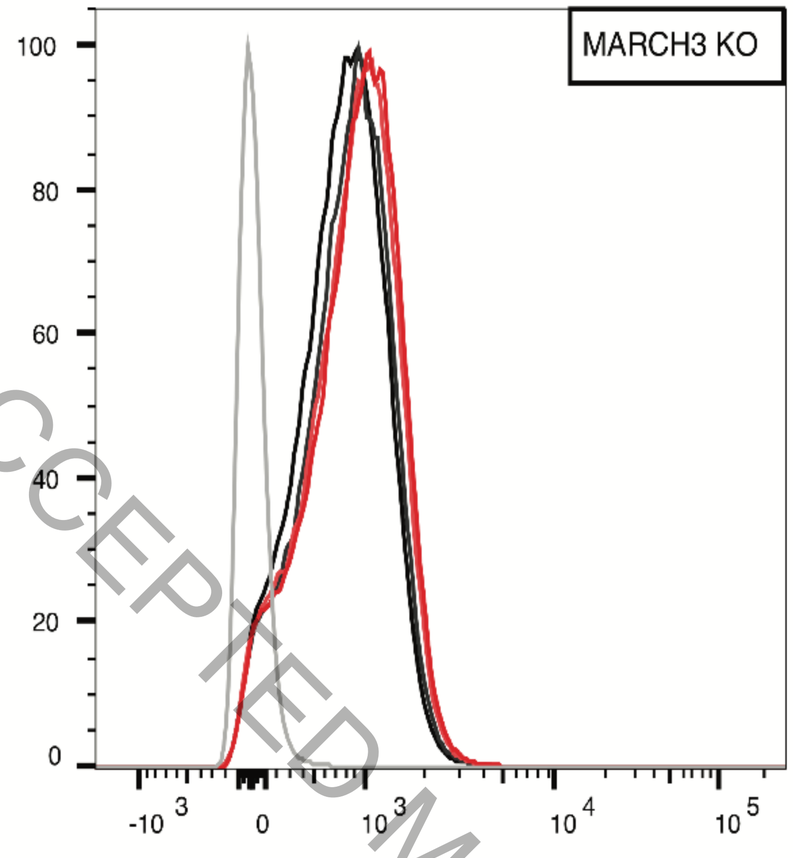


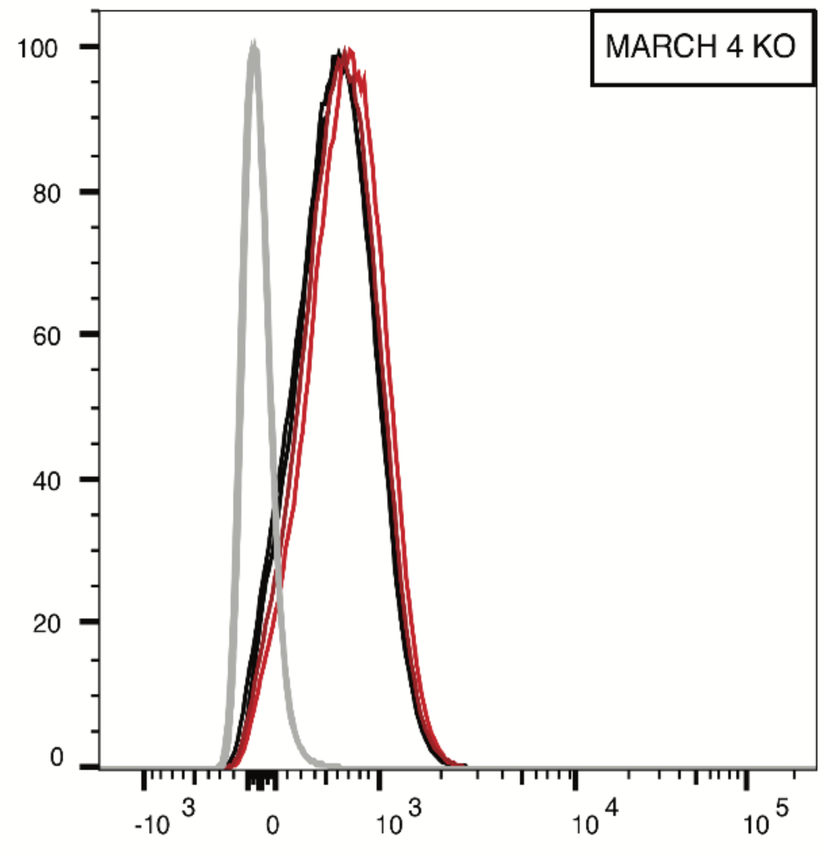


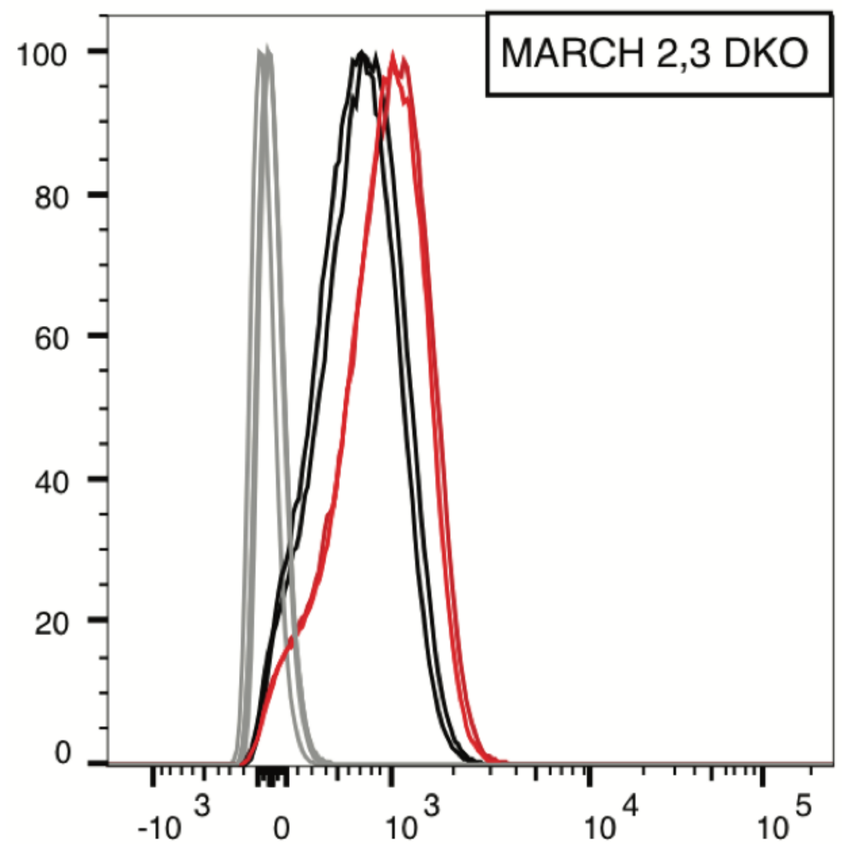


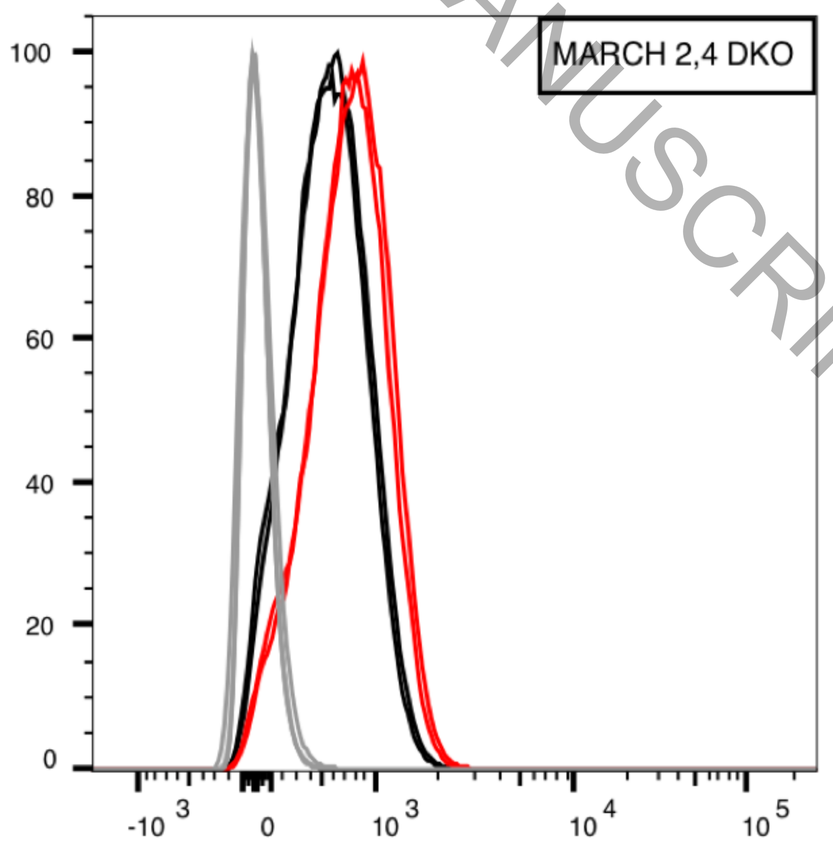
Spleen CD8+ T cells

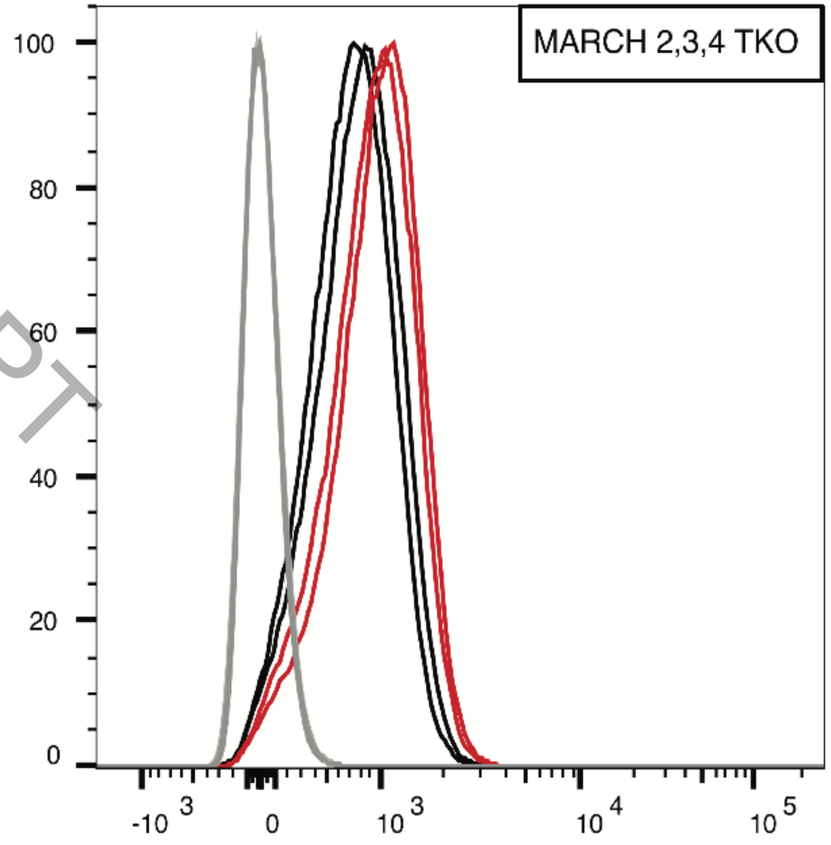












PE-Cy7-IL6Ra

Fig S1. Constructs used to generate MARCH2,3 and 4 knockout alleles. The wild type (WT) allele, the targeting construct, the floxed allele and the final mutated allele after cre-mediated recombination are shown. The deleted exons (exon3 for MARCH2, exon2 for MARCH3 and MARCH4) are numbered in blue boxes. The primers used for genotyping are in red arrows and defined in Table 1.

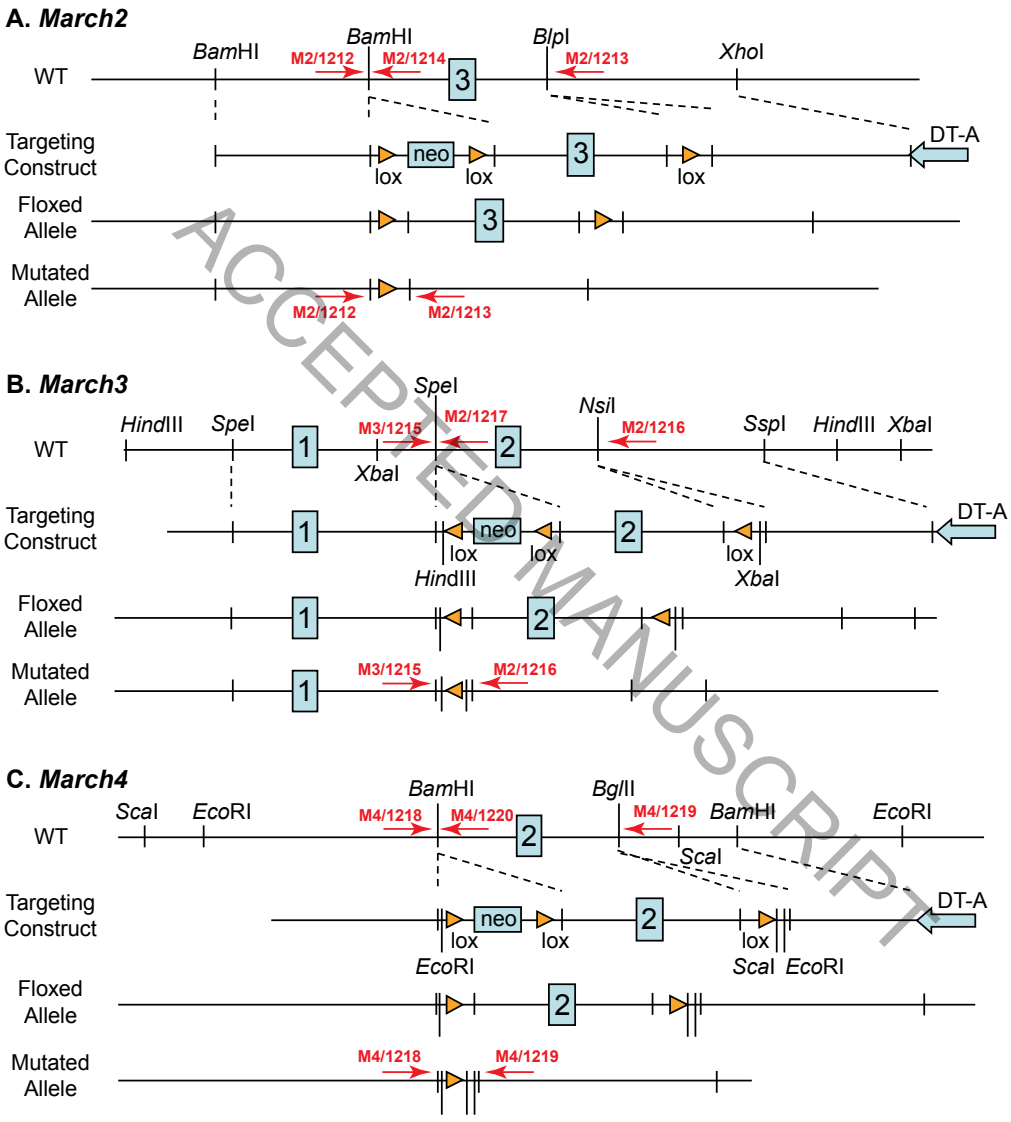


Fig S2. Gating strategy used to define different haemopoietic subsets from bone marrow and spleen. Upper panel (incubation mix 1): CD3+CD19- T cells were separated according to CD4 and CD8 status while CD3-CD19+ B cells were separated according to IgM and IgD status. Middle panel (incubation mix 2): Lin – Cells were separated according to CD11c status (dendritic cells) and F4/80 status (monocytes/macrophages). Lower panel (incubation mix 3): Lin- CD11b+ myeloid cells were separated according to Ly6C only (monocyte/macrophage) or Ly6C and Ly6G (granulocytes and precursors). Details are in Experimental section of paper.

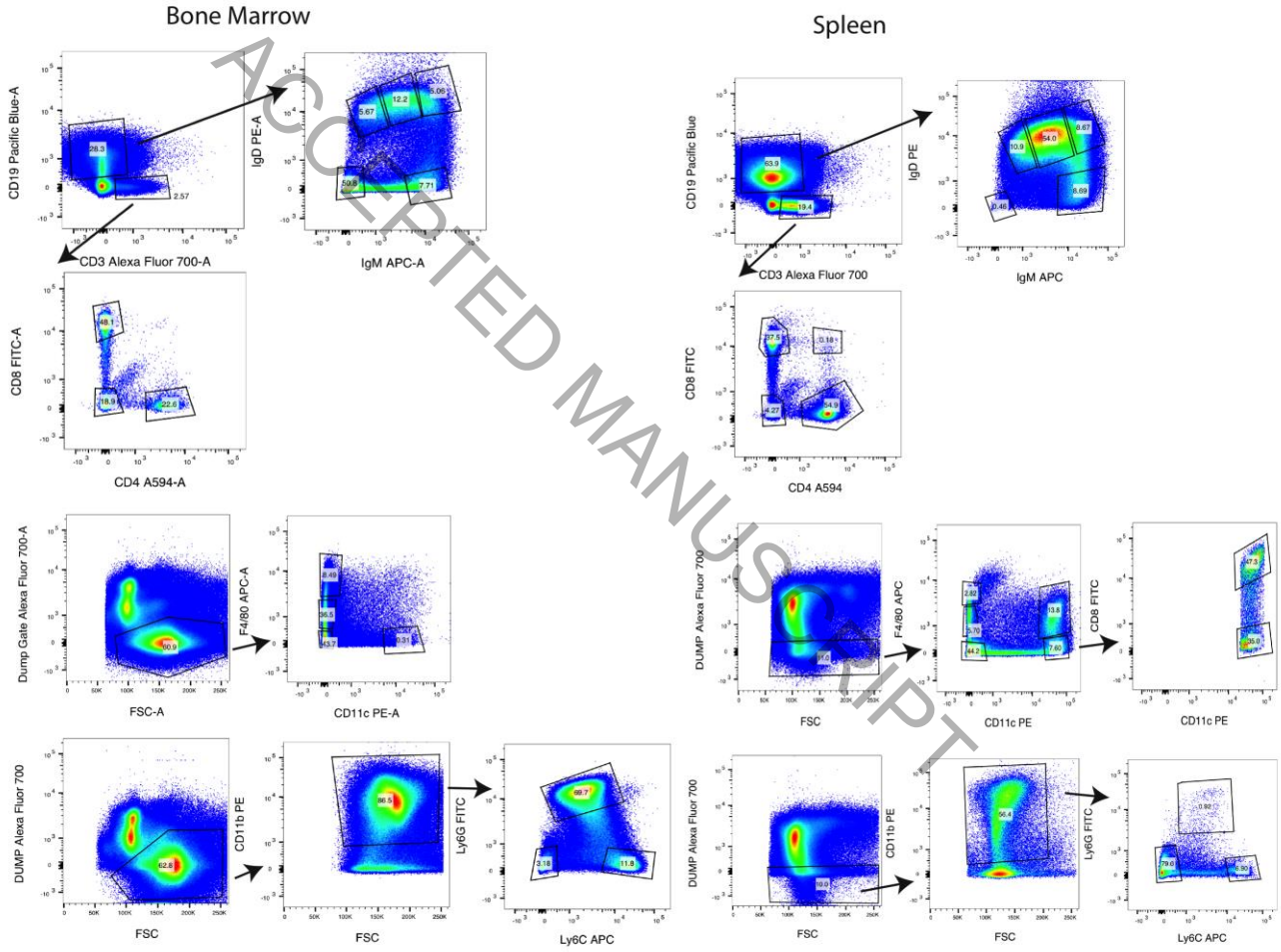
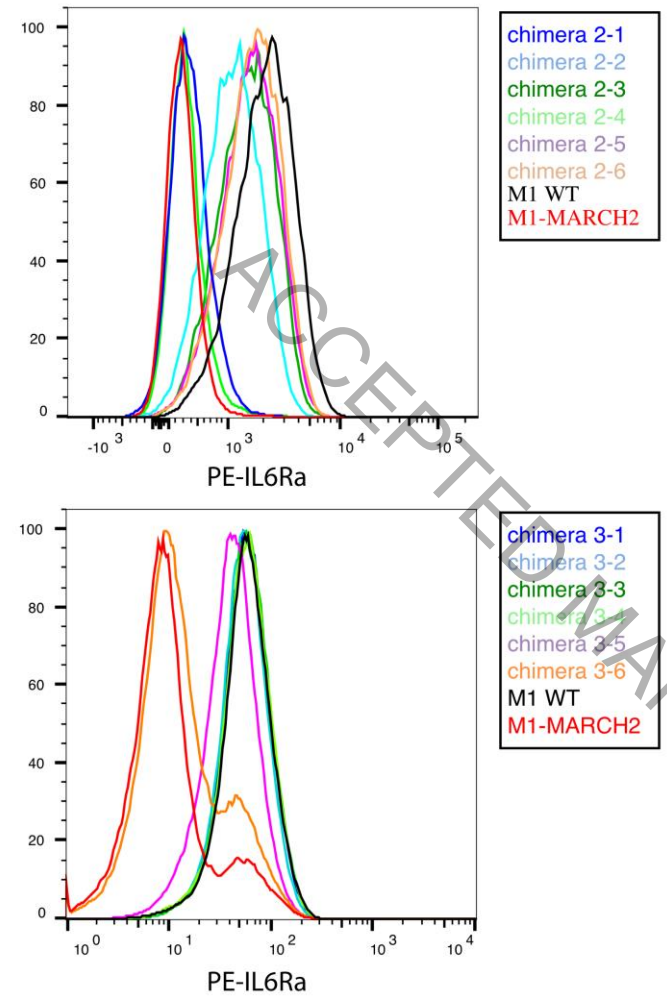


Fig S3. A. Expression of IL6Ra by flow cytometry on GFP+ M1 cells expressing various FLAG-MARCH2/3 chimeras in the pCFB IRES GFP vector. B. Flag Western blots of MARCH2, MARCH3 and chimeras 3-1 to 3-6. Chimera constructs are as shown in Fig 3D. Flag MARCH2 and series 2 chimeras were expressed at much lower levels than the 3 series and were undetectable in this set of experiments. One of two biological replicates is shown.

A.



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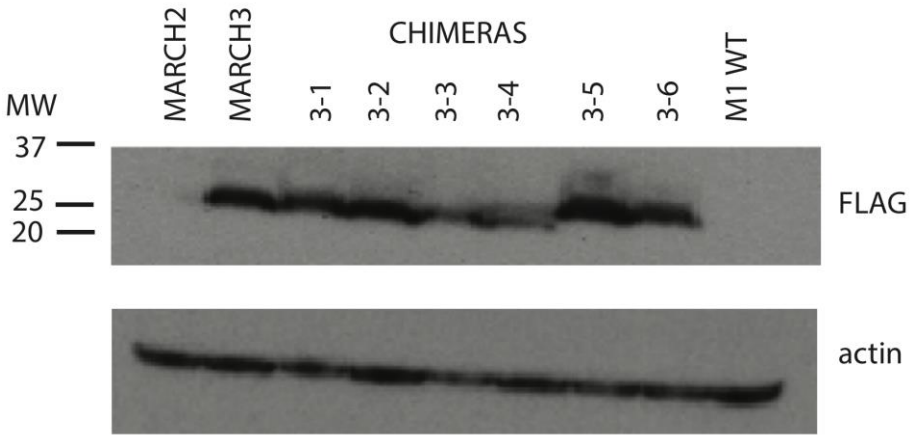

Fig S4. Spleen CD4+ T cells also show up-regulation of IL6Ra but only when more than one of MARCH2,3 or 4 are deleted.Two mice each of the indicated MARCH KO genotype (red) or age and sex matched C57 Bl6 mice (black) were used and isotype controls are in grey (all four mice). One of 2-3 biological replicates is shown. Details are in Experimental section of paper.

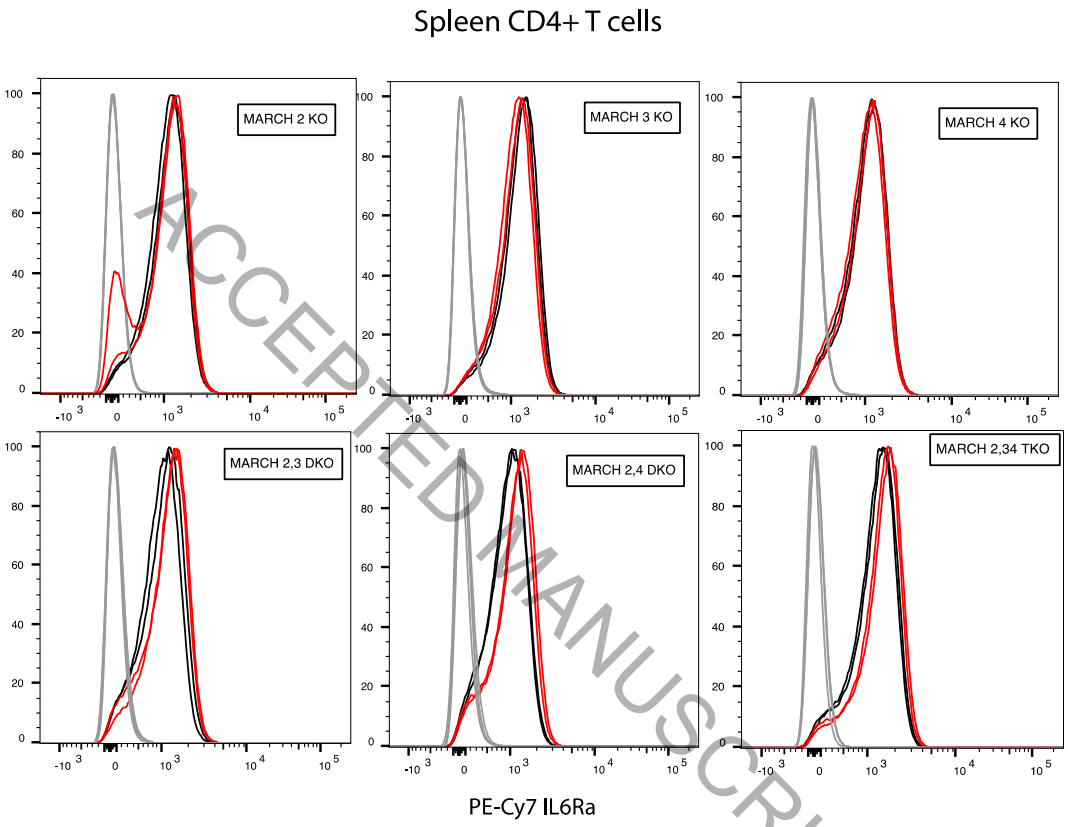

Fig S5. IL6Ra levels on IgM hi IgD+ B cell precursors in bone marrow for different MARCH knockout mice. The indicated MARCH knockouts are in red, age and sex matched C57Bl6 mice are in black and isotype controls are in grey. Two mice of each genotype are shown in all plots. One of 2-3 biological replicates is shown. Details are in Experimental section of paper.

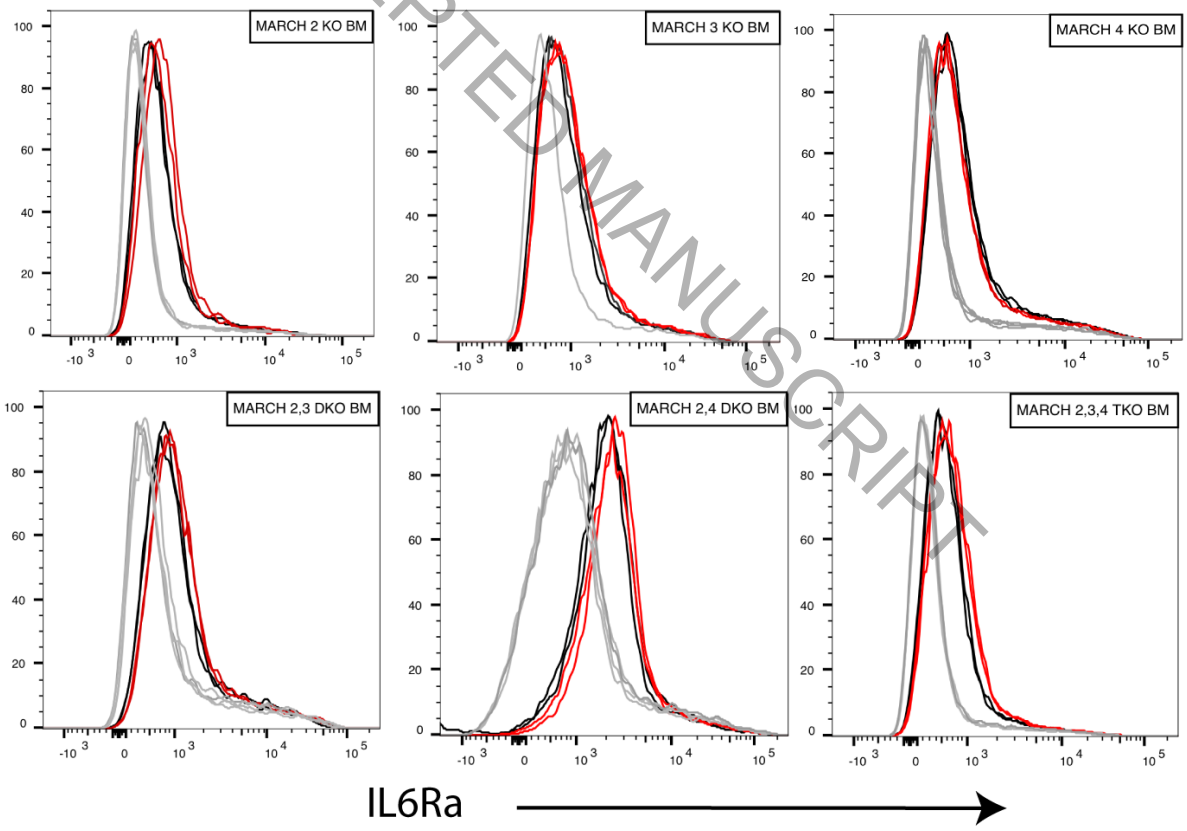

Figure S6. IL6Ra levels in myeloid cells (dendritic cells CD11c++, monocyte F4/80+ or Ly6C++, macrophage F4/80++ or neutrophils Ly6C+Ly6G+) are unaffected by single or multiple MARCH2,3,4 knockouts. Example data shown are for MARCH2,4 DKO mice (red) or age and sex matched C57 Bl6 controls (black) with isotype controls in grey. Two mice of each genotype are shown from spleen (left panels) or bone marrow (right panels). One of 2-3 biological replicates is shown. Details are in Experimental section of paper.