**Notch1-CD22-Dependent Immune Dysregulation in the SARS-CoV2-Associated Multisystem Inflammatory Syndrome in Children**

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**Experimental Procedures**

**Patient Cohorts Contributing to the Flow Cytometry, Transcriptomic, and Functional Studies:**

*MIS-C and Pediatric COVID-19:* Peripheral blood samples were obtained from children with COVID-19 (n=9) and MIS-C (n=23) who were prospectively recruited from Boston Children’s Hospital (BCH) as part of the Taking on COVID-19 Together Study and the Kawasaki Disease (KD) Biorepository between 5/2020 and 4/2021. In addition, blood samples were also collected from children cared for a at the Marmara University Hospital in Istanbul, Turkey (8 COVID-19, 8 MIS-C from 12/2020 to 1/2021), Istanbul University-Cerrahpaşa, Istanbul, Turkey (20 COVID-19, 2 MIS-C from 7/2020 to 1/2021), Gaslini Institute in Genoa, Italy (4 MIS-C from 12/2020 to 3/2021), and the Bambino Gesù Children’s Hospital in Rome, Italy (13 COVID-19, 12 MIS-C from 3/2020 to 4/2021). The clinical characteristics of children with MIS-C and pediatric COVID-19 are reported in Table 1.

All Patients with MIS-C met the Centers for Disease Control (CDC) Case Definition for MIS-C. Patients requiring intensive care unit (ICU) admission and/or vasopressor support or those who developed coronary artery aneurysms (z-score ≥ 2.5) were classified as having severe MIS-C.

Children with COVID-19 presented with either a fever, respiratory illness, and/or known COVID-19 exposure and were found to be SARS-CoV-2 positive by polymerase chain reaction (PCR). Moderate pediatric COVID-19 was defined by a supplemental oxygen requirement and care on the pediatric ward while severe disease required ICU admission and/or bilevel positive airway pressure (BiPAP) or mechanical ventilation. All other children with COVID-19 were defined as having mild cases.

*Adult COVID-19:* Twelve patients previous described (Harb et al, 2021) were used in this study.

*Kawasaki Disease*: Five children with KD provided peripheral blood samples through the KD Biorepository at BCH from 10/2020 to 1/2021 (Table 1). These patients fulfilled the clinical criteria for either complete or incomplete KD as outlined by the American Heart Association (AHA)(McCrindle et al., 2017). Further, all of these patients tested negative for SARS-CoV-2 by both PCR and serology and had no known close contacts with COVID-19.

*Controls*: Peripheral blood samples were obtained from pediatric control subjects recruited from Boston Children’s Hospital (n=6), Istanbul University-Cerrahpaşa (n=7), and Gaslini Institute (n=5). Clinical characteristics of the control study subjects can be found in Table 1.

*Sample Processing****:*** Peripheral blood samples were obtained at study enrollment in either sodium heparin or ethylenediamineteraacetic acid (EDTA) tubes. At each site, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen.

**Patient Cohorts Contributing to the Gene Pathway Analysis Using the Fischer and Monte-Carlo tests:**

Whole genome or whole exome sequences were obtained from patients with MIS-C and children with COVID-19. These patients were recruited from the Taking on COVID-19 Together Study at Boston Children’s Hospital (30 with MIS-C and 21 with COVID-19) as well as the Marmara University Hospital (9 with MIS-C and 3 with COVID-19).

In addition, whole exome and genome sequencing data obtained from 1,885 families (4682 samples) at the Manton Center for Orphan Disease Research were used for gene-enrichment test [Manton Center for Orphan Disease Research: https://www.mantonfoundation.org/]. Data were divided in 4 groups depending in their phenotypes: obesity (86 samples), myopathy (310 samples), autism-ADHD (1296 samples) or rare diseases (2990 samples). Rare diseases were subdivided depending on the sequencing provider, Broad Institute (1006 samples), GeneDx (715 samples), Boston Children’s Hospital (545 samples) and others (724 samples). 162 families (385 samples) with immunodeficiency, allergic dysregulation, autoimmunity, and recurrent infections obtained from Boston Children’s Hospital repositories were also included in our analysis.

**Patient Screened for Notch-related Genes:** Blood samples from patients with MIS-C enrolled in the Overcoming COVID-19 Immunobiology Study we obtained from 20 large pediatric sites in the U.S.A and were sent for whole exome sequencing (88 MIS-C from 6/2020 to 5/2021). DNA was isolated from whole blood using the Gentra Puregene Blood Kit (Qiagen) or by GeneDx using IDT xGen probes. Libraries for whole exome sequencing were prepared using Agilent Technologies SureSelectXT2 Homo Sapiens All Exon V6 Kit. Paired end sequencing was performed with an Illumina HiSeq-2000, generating 150 base reads. Sequencing alignment to the hg19/GRCh37 reference build was performed using the Burrows-Wheeler Aligner (Li and Durbin, 2009). Variant calling and candidate variant analysis were completed by utilizing the Boston Children’s Hospital Genomic Learning System, as previously described (Rockowitz et al., 2020). Minor allelic frequencies for the specified variants were identified by using the Genome Aggregation Database (Karczewski et al., 2020).

**Human Subjects:** Written informed consent (and assent when appropriate) was provided by the participants, their health care proxy and for minor children from at least one parent or legal guardian. The single center research protocols were approved by the Institutional Review Boards (IRBs) at Boston Children’s Hospital (IRB-P00035409 [Taking on COVID-19 Together], X10-01-0308, IRB-P00005723), Marmara University Hospital (224165), Istanbul University-Cerranhpasa (159066), the Gasilini Institute (egione Liguria ImmunoCOVID19" 0012337/20), and the Bambino Gesù Children’s Hospital (2083\_OPBG\_2020), Boston Children’s Hospital (P0021163, P00035489, P00035810, IRB-P00004759 and 04-09-113R). Boston Children’s Hospital serves as the single IRB for the multicenter Overcoming COVID-19 Immunobiology Study (IRB-P00033157) and all enrolling sites IRBs reviewed and approved the protocol. The Rare disease cohorts research protocols were approved by the Institutional Review Boards (IRBs) at the Manton Center samples (10-02-0053).

**Animal Studies:** All animal studies were reviewed and approved by the Boston Children’s Hospital office of Animal Care Resources and Massachusetts General Hospital office of Animal Care Resources.

**Mice**. The following mouse strains were obtained from the JAX Laboratories: *Foxp3*EGFPCre (B6129S-Tg (Foxp3-EGFP/icre)1aJbs/J), *Rosa26*N1c/N1c. *Rbpj1*fl/fl (B6.129P2-*Rbpj*tm1Hon/HonRbrc) were kind gifts of Pamela Stanley.

**Single-cell RNA sequencing sample preparation**

Cryopreserved PBMCs were thawed in plain RPMI (HyClone) pre-warmed to 37°C, washed once in PBS (HyClone) and resuspended in FACS buffer (PBS with 1.5% FBS (Genesee Scientific) and 2.5 mM EDTA (Invitrogen) for CD4 T cell enrichment through negative selection (Miltenyi Biotec). CD4 T cells were enriched from total PBMCs of 4 controls and 8 MIS-C patients (Miltenyi Biotec). Samples were studied in 2 independent experiments: experiment 1 included 3 pediatric controls, 1 pre-treatment MIS-C patient, and 4 post-treatment MIS-C patients and experiment 2 included 1 pediatric control, 2 pre-treatment MIS-C patients, and 1 post-treatment MIS-C patient.

For each experiment, isolated CD4 T cells were stained with a hashing antibody recognizing the ubiquitous markers CD298 and β2 microglobulin coupled to a unique hashtag oligonucleotide (HTO) (BioLegend TotalSeq™-C anti-human Hashtag, Clones: LNH-94 and 2M2) for subsequent sample identification. To that end, cells were spun at 500 g for 7 min at 4°C, resuspended into 75 μL Fc block (BioLegend, Cat. No. 422302, 1:20 dilution) and incubated for 10 min at 4°C. After incubation with Fc block, 75 μL of hashing antibody (6.7 μg/mL working concentration) were added and samples were incubated for 30 min at 4°C, with gentle resuspension midway through the incubation. Cells were washed three times in Hash Staining buffer (BioLegend, Cat. No. 420201) and resuspended in PBS with 0.4% BSA (Sigma, Cat. No. A7030) at a concentration of 1,000 cells per μL. Finally, 7,500 cells from each sample were pooled together for further processing (10x Genomics), thus limiting technical batch effects (Stoeckius et al., 2018). A total of 30,000 cells were loaded per 10x Genomics chip; therefore, for the first experiment, the total of 60,000 cells pooled from 8 samples were split across 2 chips, while the second experiment (30,000 cells pooled from 4 samples) was processed on one single chip. Cells were encapsulated, barcoded and lysed to enable the generation of cDNA libraries for transcriptome and HTO sequencing using the 10x Genomics technology (Zheng et al., 2017). Libraries were sequenced on an Illumina NovaSeq 6000.

**Single-cell RNA sequencing clustering analyses**

Sequencing data from each 10x run were processed with the CellRanger pipeline (10x Genomics) for demultiplexing and gene alignment (Zheng et al., 2017). The resulting matrices of raw features were imported in R (v4.0.2 and above) using Seurat (v4.0.3) (Hao et al., 2021). Data from all 3 runs were merged into one Seurat object. Genes detected in <1 per 10,000 cells were filtered out, leaving a transcriptomic coverage of 21,675 genes. High quality cells with >1400 unique molecular identifiers (UMIs), >700 genes, a log10(gene) to log10(UMI) ratio >0.84 and mitochondrial to nuclear gene ratio <0.08 were retained for downstream analyses. Quality control revealed no significant technical batch effect: similar distributions were observed for the key metrics mentioned above across different runs and experiments.

HTO data were normalized using centered log ratios before applying HTODemux() with the clara method and a positive quantile cutoff of 0.98. Doublets and cells with unclear HTO information were excluded at this point (Stoeckius et al., 2018). Transcriptomic data for the remaining cells were normalized using Seurat’s SCTransform() and regressing out the effects of the mitochondrial gene ratio, the number of UMIs and number of genes detected. Principal components were then calculated using the top 2000 variable features to reduce the data before mapping to a reference PBMC dataset using Azimuth (Hao et al., 2021).

Cells mapped by Azimuth to CD4 T cell subsets were retained while contaminating lymphocytes were excluded, leaving a total of 29,754 Azimuth-annotated CD4 T cells for downstream analyses. SCT normalization and PCA were repeated at this stage, to account for the top 3000 variable features after manual exclusion of *TRAV*, *TRAJ*, *TRBV* and *TRBJ* genes, thereby enabling cell clustering by transcriptomic profile independently of clonal identity. To control for inter-sample variability, the data were then harmonized by source sample using Harmony (Korsunsky et al., 2019). A uniform manifold approximation and projection (UMAP) dimensionality reduction was then computed from the first 50 components of the harmony reduction, and graph-based clustering analysis was run on the first 40 components using Seurat’s FindNeighbors() and FindClusters() functions. A low resolution of 0.6 was retained to define clusters.

For manual annotation of this Seurat-defined clusters, clusters were coarsely characterized based on the abundance of cells initially classified as naïve versus effector/memory by Azimuth. In parallel, genes significantly upregulated in each cluster compared to all others were identified with Seurat’s FindAllMarkers() using the default Wilcoxon rank sum test. Significance was here defined as a p-value of <0.05 and a log2 fold change (LFC) in gene expression >0.25. Heatmaps were generated by applying Seurat’s DoHeatmap() on scaled SCT data on a random subsample of 100 cells per cluster. To confirm upregulation of NF-κB genes, a gene signature score for the TNFα signaling via NF-κB geneset, sourced from the MSigDB Hallmark collection (Liberzon et al., 2015), was also calculated at the single-cell level using Seurat’s AddModuleScore().

**Pseudobulk differential expression analyses (DEA)**

For pseudobulk differential expression analyses (DEA), gene expression level data was aggregated at the patient level for each subset of interest, namely Tregs and activated Tconv. For the purpose of this DEA, we considered as Treg any cell assigned to Cluster 15 (*FOXP3*-expressing cells) in the Seurat graph-based clustering analysis or annotated as Treg by Azimuth, which added up to 1,925 cells across all 12 patients. Similarly, we considered as activated Tconv any cell assigned to Clusters 9 to 14 and annotated as CD4 TCM, CD4 TEM, CD4 CTL or CD4 Proliferating by Azimuth (6,674 cells). Principal component analyses of the aggregated transcriptomic data highlighted healthy control 4 as a strong outlier among both Treg and activated Tconv subsets, leading us to exclude this patient from pseudobulk DEA. Independent pairwise analyses contrasting each of the 3 patient groups (MIS-C pre-treatment, MIS-C post-treatment and control) were then run using DESeq2 (version 1.34.0, Add citation: PMID 25516281). Log2 fold change (LFC) values were corrected using the apeglm shrinkage estimator (Add citation: PMID 30395178) and used as input for gene set enrichment analyses (GSEA) against the complete MSigDB Hallmark collection, performed with clusterProfiler (version 4.2.2, Add citation: PMID 34557778). Heatmaps of gene expression for significant genes (defined as an adjusted p-value < 0.05) were generated from the centered rlog-normalized count data using pheatmap (version 1.0.12).

**Gene pathway analysis using the Fischer and Monte-Carlo tests:**

To identify if a pathway is relevant to MIS-C or acute-COVID-19, a comparison between MIS-C or acute-COV19 and the eight databanks described above was preformed using the following steps below. To minimize false positive and artifactual results, all samples were processed using the same pipeline, Variant Explorer (VExP) (Schmitz-Abe et al., 2019), starting with their raw data (Fastq files).

Step 1 (fastq to vcf file): Raw data were processed to obtain vcf files using the human reference assembly 19, BWA (alignment, v0.7.17), PICARD (mark/delete duplicates, v2.23.3), SAMTOOLS (variant calling, v1.10), and GATK (multi-sample variant calling, v4.1.8.1). When only bam files were available, PICARD (v2.23.3) was used to revert to fastq files. Further, ANNOVAR (2020Apr) and custom VExP scripts were used to add annotations from relevant genetic databases into each vcf file.

Step 2 (Variant filtering): Variant analysis was performed in each family based on three filtering criteria: first, include variants predicted by ANNOVAR to have a potential functional coding consequence, including stop gain or loss, splice site disruption, indel, and nonsynonymous. Second, variants are filtered based on allele frequency in control populations (gnomAD, ExAC, EVS, 1000GP, and internal data from 8114 unaffected individuals from BCH). Heterozygous/hemizygous variants were included if minor allele frequency (MAF) was <0.0005 (0.05%) in any database. In comparison, homozygous variants were included only if MAF was ≤0.00005 (0.005%) and for compound heterozygous models the MAF cutoff was ≤0.01 (1%) with no homozygous variant reported in any database. The variants were further prioritized to include those with read depth ≥10X, alternative depth ≥5X, allele balance ≥0.20, and deleterious prediction (4 or more of 23 softwares, including PolyPhen, SIFT, FATHMM, and CADD).

Step 3 (gene-enrichment): A gene-enrichment test was performed to identified rare pathogenic variants and lost/gain of function (stop-lost/gain, frameshift deletions/insertions and canonical splicing sites) using 8,626 pathways from Gene-Ontology [Gene-Ontology database: http://geneontology.org/] and KEGG [KEGG database: https://www.genome.jp/kegg/] databases (8,299 and 327 respectively). The Frequencies (families) of these rare coding pathogenic variant gene were calculated for each pathway using 3 different genetic models: a) Homozygous variants, b) Heterozygous variants and c) Homozygous and/or 2 or more heterozygous variants in the same gene with a minimum distance between them of 100 base pairs (compound heterozygous filters). P values were calculated using 2 methods: traditional Fisher test (two sided) and Monte-Carlo method. The expectation for one event (pathway) using Monte Carlo method is described by the following formula:

Where “F” represents the number of families with rare pathogenic variants in the "k" pathway (k=1:8,626 pathways) and “X” is a random control group with the same number of samples of the comparison group, for MIS-C, 39 samples and for acute-19, 24 samples. Independent samples were taken random using a uniform distribution and 4682 samples described **above.** “N” is the total number of independent simulations (10,000 in total). The use of independent samples was very important to establish fairness in our tests, so then we use only one sample per family (probands).

**Crispr-Cas9 Knock-out generation.** HEK293T cells were transfected with CRISPR/Cas9 knockout plasmids NUMB (Santa Cruz Biotechnologies) using a CalPhos Mammalian Transfection kit (Takara), following the manufacturer protocol. CRISPR positive cells were isolated by FACS and plated at very low density to induce the formation of clonal colonies. Individual clones were then isolated and tested for knockout efficiency by flow cytometry.

***In vitro* Notch1 induction:** HumanTreg cells from Healthy Donor were isolated by cell sorting (Sony Sorter, MA900) based on CD3, CD4, CD25high, CD127Low. Treg cells were seeded at 1× 104 cells in 96-well plates then stimulated with CD3/CD28 Dynabeads (ThermoFisher) alone or in presence of recombinant IL-1, IP-10, IL-6, IFN- and IFN-2 (10 μg /ml; Peprotech) for 72h. Notch1 expression on Foxp3+ Treg cells was then assessed by Flow cytometry.

**Isolation of Human peripheral blood mononuclear cells (PBMCs).** Human PBMCs were isolated from whole blood from either healthy control, mild COVID-19, moderate COVID-19 or severe COVID-19 probands via density gradient using Ficoll (GE Healthcare). PBMCs were then stored frozen in Fetal Calf Serum (FCS) (Sigma Aldrich) and 15% Dimethyl sulfoxide (DMSO) (Sigma Aldrich). The cells were later thawed for analysis of their Notch or different markers and cytokine expression by flow cytometry.

**Numb and Numbl mutagenesis and cells transfection.** A plasmid pCMV6-AC-Numb-GFP encoding for human Numb (NM\_001005745) or human NumbL (NM\_004756*)* were purchased from OriGene (RG209744). The directed Numb (c.280 C>T, p.Leu 94 Phe) or NumbL (*c.236G>T, p.Ser79Ile; c.262G>A p.Val88Met)* mutagenesis plasmid were generated with standard cloning techniques by using pCMV6-AC-Numb-GFP as a template. Cell deficient Numb HEK293T (Numb-/-) were cultured in 10% FBS DMEM medium (Gibco), supplemented with 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The day before transfection, generated Numb deficient HEK293T cells were seeded at a density of 5x105 cells per well of 6 well plates. The next day, HEK293T cells were transiently transfected with 2 ug of either Numb wild type or Numb mutant plasmid using GeneJuice transfection reagent (Merck Millipore) according to the manufacturer’s instructions. After 48h of transfection, cells were collected for Flow cytometry analysis.

**Cytokine measurements.** IL-1, IL-6, IL-8, IFN, IFN, IFN, IFN, CXCL10 and TNF were measured using Legendplex (Biolegend) per manufacturer’s protocol.

**Polyinosinic-polycytidylic acid (Poly I:C) mouse model**. Mice were treated intraperiotenally with 2.5 mg/kg of Poly I:C HMW (InvivoGen) every two days for 12 consecutive days. The weight of the mice was recorded daily. Mice were euthanized and analyzed at day 13. For blockading CD22 mice were treated with an anti-CD22 antibody every two days for 12 consecutive days (InVivoMAb anti-mouse CD22; Clone: Cy34.1, Bioxcell). The antibody was given intra-peritoneally at 20 µg of antibody in PBS in a final volume of 100 µl or isotype control mAb (clone MOPC-21; Bioxcell). For the CD20 depletion experiment, the mice were given intraperitoneally 10µg of anti-CD20mAB (clone MB20-11; Bioxcell) or isotype control mAb (clone MOPC-21; Bioxcell) every 2 days, six days before the start of the experiment.

**Histopathology staining**. Paraffin-embedded lung, colon and liver sections were stained with hematoxylin and eosin (H&E) or Paraffin-acid-Schiff staining (PAS). The lung colon and liver pathology were scored by blinded operators. Inflammation was scored separately for cellular infiltration around blood vessels and airways: 0, no infiltrates; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep (Tachdjian et al., 2009). A composite score was determined by the adding the inflammatory scores for both vessels and airways.

**Flow cytometric analysis of mouse and human cells.** Antibodies against the following murine antigens were used for flow cytometric analyses: Foxp3 (FJK-16S, catalogue no: 48-5773-82 1:300, Thermofisher), IFN (XMG1.2, catalogue no: 505825 1:300, Biolegend), Helios (22F6, catalogue no: 47-9883-42 1:200, Thermofisher, CD4 (GK1.5, catalogue no: 100451, 1:500, Biolegend), CD3 (17A2, catalogue no: 100203, 1:500, Biolegend), IL-17 (TC11-18H10.1, catalogue no: 506922, 1:200, Biolegend), CD45 (30-F11, catalogue no: 103140, 1:300, Biolegend), Notch4 (HMN4-14, catalogue no: 128407 1:400, Biolegend), CD279 (J43, catalogue no:12-9985-82, 1:400 Thermofisher), CD44 (IM7, Catalogue no: 103032, 1:300 Biolegend), CD62L (MEL-14, Catalogue no: 104412, 1:300 Biolegend), N1c (mN1A, Catalogue no: 629106, 1:200 Biolegend), a4b7 ( DATK32, Catalogue no: 120606, 1:300 Biolegend), p-Erk ( 6B8B69, Catalogue no: 369506, 1:50 Biolegend),p-PLCg (A17025A, Catalogue no:612404, 1:50 Biolegend), pS6 (D57.2.2E, Cataloguie no: 5316, 1:50 , CST), p-Akts473 (M89-61, Catalogue no: 560378, 1:50 BD), p-AktT308 (J1-223.371,Catalogue no: 558375, 1:50 BD), CD22 (OX-97, Catalogue no: 126112, 1:300 Biolegend ). Antibodies against the following human antigens were used: CD3 (HIT3a, catalogue no: 300318, 1:200,Biolegend), CD4 (RPA-T4, catalogue no: 300530, 1:200,Biolegend), Foxp3 (PCH-101,catalogue no: 48-4776-42,56-4716-41, 1:100 Thermofisher), Notch1 (HMN1-519, catalogue no: 566023, 1:300, BD Pharmingen), Notch2 (HMN2-25, catalogue no: 742291, 1:300, BD Pharmingen), Notch3 (HMN3-21, catalogue no: 744828, 1:300, BD Pharmingen), Notch4 (HMN4-2, Catalogue no: 563269, 1:300, BD Pharmingen), CD25 (BC96, Catalogue no: 12-0259-42 1:300, Thermofisher), CD127 (A019D5, Catalogue no: 351320 1:300, Biolegend), IFN (4S.B3, Catalogue no: 560741 1:200, BD Biosciences), ITGB7 (FIB504, Catalogue no: 551082, 1:200 BD), CCR7 (G043H7, Catalogue no: 353208, 1:300 Biolegend), CD38 (S17015A, Catalogue no: 397114, 1;200, Biolegend), CD22 (HIB22, Catalogue no: 302516, 1;200, Biolegend), CD45RA (HI100, Catalogue no: 304134, 1:200 Biolegend), CD45RO (UCHL1, Catalogue no: 304236, 1:300, Biolegend), IL17 (BL168, Catalaogue no: 512315, 1:100, Biolegend), CD62L ( DREG-56, Catalogue no: 304810, 1:300, Biolegend), Purified anti-CD22 (HIB22, Catalogue no: 302502, Biolegend). The specificity and optimal dilution of each antibody was validated by testing on appropriate negative and positive controls or otherwise provided on the manufacturer's website. Intracellular cytokine staining was performed as previously described (Charbonnier et al., 2015). Dead cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable Viability Dye (1:1000 dilution) (Thermofisher). Stained cells were analyzed on a BD LSR Fortessa cell analyzer (BD Biosciences) and data were processed using Flowjo (Tree Star Inc.).

**Transcriptome Profiling.** Treg cells were isolated from either *Foxp3*EGFPcre or Foxp3EGFPCre *Rosa26*N1c/+ mice by cell sorting. mRNA was isolated using Qiagen RNeasy mini kit (Qiagen). RNA was then converted into double-stranded DNA (dsDNA), using SMART-Seq v4 Ultra Low Input RNA kit (Clontech). dsDNA was then fragmented to 200-300 bp size, using M220 Focused-ultrasonicator (Covaris), and utilized for construction of libraries for Illumina sequencing using KAPA Hyper Prep Kit (Kapa Biosystems). Libraries were then quantified using Qubit dsDNA HS (High Sensitivity) Assay Kit on Agilent High Sensitivity DNA Bioanalyzer.

Gene-level read counts were quantified using feature Counts and the latest Ensembl mouse annotation (GRCm38.R101). Raw data were trimmed using Trimmomatic (version 0.39, default parameters), tool for Illumina NGS data. To identify differentially expressed genes, we used 3 algorithms: DESeq2 (version 1.26.0), edgeR (version 3.28.1) and Lima (3.42.2) Bioconductor packages with default parameters. Count tables were normalized to TPM (Transcripts per Million) for visualizations and QC. Sample clustering, path analyses and integration of the results were performed using a custom-made pipeline available upon request (Variant Explorer RNAseq). Transcripts were called as differentially expressed when the adjusted p values were below 0.05, fold-changes over ±1.5 and false discovery rate (FDR) were below 0.05. For our path analyses, we tested 10,715 biological pathways from KEGG and GO annotations. We filtered the results using an adjusted p value below 0.001.

**In vitro suppression assays.** Total CD4+ T cells from either *Foxp3*EGFPcre or Foxp3EGFPCre *Rosa26*N1c/N1c mice were isolated using a CD4 negative isolation kit (Miltenyi Biotec) followed by cell sorting (Sony Sorter, MA900). Isolated Teff cells were labeled with CellTrace Violet Cell Proliferation (CTV) dye according to the manufacturer's instructions (Life Technologies) and were used as responder cells. Treg cells were isolated by cell sorting (Sony Sorter, MA900) based on CD4, YFP and/or CD22 expression and were used as suppressor cells. Responder cells were co-cultured with Treg cells, at a 1:1 ratio, and stimulated at 37ºC 5% CO2 for 3 days with 2 μg/ml of coated anti-CD3 and 1 μg/ml of soluble anti-CD28 in presence of increased dose of anti-CD22 mAb or Rapamycin in 96-well, round-bottomed plates in triplicates. The responder cells were then analyzed for CellTrace dye dilution by flow cytometry. In vitro Human suppression assays Treg cell (CD3+CD4+CD127–CD25High) were isolated from either Healthy control or MIS-C patients by cell sorting (Sony Sorter, MA900). Tconv cells were isolated by cell sorting from Healthy control and labelled with CellTrace Violet Cell Proliferation (CTV) dye according to the manufacturer's instructions (Life Technologies) and were used as responder cells. Responder cells were co-cultured with Treg cells, at a 1:1 ratio, and stimulated at 37ºC 5% CO2 for 3 days with 2 μg/ml of coated anti-CD3 and 1 μg/ml of soluble anti-CD28 in presence of 1 μg/ml anti-CD22 mAb or 1 ng/ml Rapamycin in 96-well, round-bottomed plates in triplicates. The responder cells were then analyzed for CellTrace dye dilution by flow cytometry.

**Analysis of TCR signaling by phosphoflow**

Total spleen from either Foxp3EGFPCre or Foxp3EGFPCre Rosa26N1c/N1c mice were stimulated at 37°C in non-supplemented RPMI 1640 using pre- formed complexes of biotinylated anti-CD3 mAb (clone 145-2C11, BD, 30 μg/ml), anti-CD4 mAb (GK1.5, BD, 30 μg/ml) and streptavidin (60 μg/ml) during 1 to 5 min. Reaction was stopped and cells were permeabilized using a Foxp3/transcription factor staining buffer (eBiosciences) and Perm buffer III (BD Biosciences). Cells were stained using Alexa-Fluor488 mouse anti-p-ERK mAb (pT202/pY204, clone 20A, BD); PE mouse anti-p-Akt mAb (pS473, clone M89-61, BD), Percep-eFluor710 p-PLC-g (Tyr759, clone: 4NPRN4, Invitrogen), PE-Cy7 p-Zap70/Syk (Tyr319, Tyr352, Clone:1503310, BioLegend), PE p-AKT (T308, BD), PE p-S6 (S235/S236, Clone: cupk43k, Ebioscience). Samples were acquired on a Fortessa cytometer (BD) and data were analyzed using the FlowJo software.

**Statistical analysis**. Student’s two-tailed t-test, one- and two-way ANOVA and repeat measures two-way ANOVA with post-test analysis and log-rank test of groups were used to compare test groups, as indicated. Linear Regression was used for correlation analysis. For analysis of the human data, summary statistics were calculated using number (percentage) for binary and categorical data and mean (standard deviation) or median (interquartile range) for continuous data depending on the normality of the distribution.

**Data Availability.** The data presented in the manuscript, including de-identified patient results, will be made available to investigators following request to the corresponding author. Any data and materials to be shared will be released via a material transfer agreement. RNA sequencing datasets have been deposited in the Gene Expression Omnibus.

**Extended DATA Figure Legends**

**Extended DATA Fig. 1**. **Single-cell transcriptomic analyses of circulating CD4+ T cells from control, pre- and post-treatment MIS-C subjects. a.** Uniform manifold approximation and projection (UMAP) of normalized and harmonized dataset, split by disease group and color-coded by cluster. Clusters were delineated using Seurat. **b**. Frequencies (%) of each cluster among total CD4+ T cells for each patient. **c**, Heatmap showing expression of the top genes in each cluster, as determined using Seurat. **d**. UMAP split by disease group and color-coded by expression of *CD69*, *NFKB1* and *FOXP3* at the single-cell level. **e,** UMAP split by disease group and color-coded by single-cell score for the TNFα signaling via NF-κB gene set (MSigDB Hallmark). **f**. TNFα signaling via NF-κB gene set mean score, averaged per cluster and patient. Multiple T-test comparisons significant at an FDR of 0.05 are indicated with a star.

**Extended DATA Fig. 2**. **Characterization of circulating CD4+ Treg and Tconv cells and serum cytokines in MIS-C**. **a-f,** Flow cytometric analysis, cell frequencies and mean fluorescence intensity (MFI) of Notch2 (**a,b**) and Notch3 expression (**c, d**) in CD4+ Treg and Tconv cells of healthy control subjects, and patients with Kawasaki disease, adult subjects with severe COVID-19, pediatric subjects with mild or severe COVID-19 and MIS-C subjects (healthy controls n=18, Kawasaki disease n=5, severe adult COVID-19 n=12, mild pediatric COVID-19 n=21, severe pediatric COVID-19 n=4, and MIS-C n=29).. **e-h**. Flow cytometric analysis and cell frequencies of T cell activation state markers (CD45RA, CD45RO and CCR7) on Tconv (e,f) and Treg (g,h) cells of the respective subject groups. **i**. Heat map representation of serum cytokine concentrations in the respective subject groups **J,K.** Flow cytometric analysis and frequencies of IFN and IL-17-expressing Treg (J) and Tconv (K) cells of the respective subject groups. Each symbol represents one subject. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 by one-way ANOVA with Dunnett’s post hoc analysis (**a**, **b, e, f, g, h, j, k**).

**Extended DATA Fig. 3: Attributes of mucosal T cells in MIS-C and Poly I:C-treated *Foxp3*EGFPCre*R26*N1c/+ mice**. **a**. Flow cytometric analysis and graphical representation of mucosal imprinted (CD62L–CD38+) Tregs and Tconv cells in healthy control subjects, pediatric subjects with mild Covid-19 and MIS-C patients. **b**. Relative *ITGB7* gene expression in cell clusters of healthy control subjects (gray) and in MIS-C patients pre (purple) and post-treatment (blue) inferred from scRNA-seq analysis. **c**. Flow cytometric analysis and graphical representation of colonic T cell (CD3+CD4+), Tregs (CD3+CD4+Foxp3+) and activated Tconv (CD4+CD44+CD62L–) of *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice subjected to Poly I:C treatment. **d**, Flow cytometric analysis and graphical representation of colonic CD22+ Tregs and Tconv *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice subjected to Poly I:C treatment. **e**. Flow cytometric analysis and graphical representation of IFN and IL-17 expressing Tconv and Tregs cells of *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice subjected to Poly I:C treatment. Each symbol represents one human subject (a), one cell (b) or one mouse (c-e). Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett’s post hoc analysis (**a,c,d,e**)Two-way ANOVA with Sidak’s post hoc analysis (**b**)**;**. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Extended Data Fig. 4. Anti-CD22 mAb therapy of Poly I:C-induced disease in *Foxp3*EGFPCre*R26*N1c/+ mice is B cell-independent. a.** Flow cytometric analysis and frequencies of CD22 expression in Treg cells of *Foxp3*EGFPCre, *Foxp3*EGFPCre*Notch1c*+/– and *Foxp3*EGFPCre*Notch1c*+/–*RBPJ*∆/∆ mice. **b,** Weight indices of Poly I:C treated *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice co-injected with anti-CD22 mAb or with an anti-CD20mAb. **c**, Flow cytometric analysis and graphical representation of splenic B cells of *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice subjected to Poly I:C treatment. **d**. Flow cytometric analysis and graphical representation of colonic T cell (CD3+CD4+), Tregs (CD3+CD4+Foxp3+) and activated Tconv (CD4+CD44+CD62L–) of Poly I:C treated *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice co-injected with anti-CD22 mAb or with an anti-CD20mAb. **e**. Flow cytometric analysis and graphical representation of IFN and IL-17 expressing Tconv and Tregs cells of Poly I:C treated *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice co-injected with anti-CD22 mAb or with an anti-CD20mAb. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett’s post hoc analysis (**a, c-e**), Two-way ANOVA with Sidak’s post hoc analysis (**b**); \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Extended Data Fig. 5. Treg cell instability in Poly I:C-treated *Foxp3*EGFPCre*R26*N1c/+ mice.** **a-c,** Volcano plot (a), pathway analysis (b) and heat map (c) of gene transcripts of WT or CD22+ Treg cells isolated at steady state from *Foxp3*YFPCre and *Foxp3*EGFPCre*R26*N1c/+mice (n=3). **d-f**, Volcano plot (d), pathway analysis (e) and heat map (f) of gene transcripts of CD22– or CD22+ Treg cells isolated at steady state from *Foxp3*EGFPCre*R26*N1c/+mice (n=3). **g**,MFI of splenic Treg cell markers of WT or CD22+ Treg cells isolated at steady state from *Foxp3*YFPCre and *Foxp3*EGFPCre*R26*N1c/+mice. **h**. Flow cytometric analysis and MFI of p-Erk induced by anti-CD3 mAb treatment of *Foxp3*EGFPCre and CD22+ *Foxp3*EGFPCre*R26*N1c/+Treg cells. **i**, Flow cytometric analysis and MFI of splenic Treg cell markers of Poly I:C-treated *Foxp3*EGFPCre and *Foxp3*EGFPCre*R26*N1c/+ mice co-treated with isotype control mAb or anti-CD22 mAb, as indicated. **j**. Cell frequencies of Helios and NRP1 expression on splenic Treg cells in the groups shown in (g). Each symbol represents one mouse. Numbers in flow plots indicate percentages or MFI. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett’s post hoc analysis (g,i,j); Two-way ANOVA with Sidak’s post hoc analysis (h)**;** \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.

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