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Supplemental Information

N-Glycan Branching Decouples B Cell Innate and Adaptive Immunity to Control Inflammatory Demyelination

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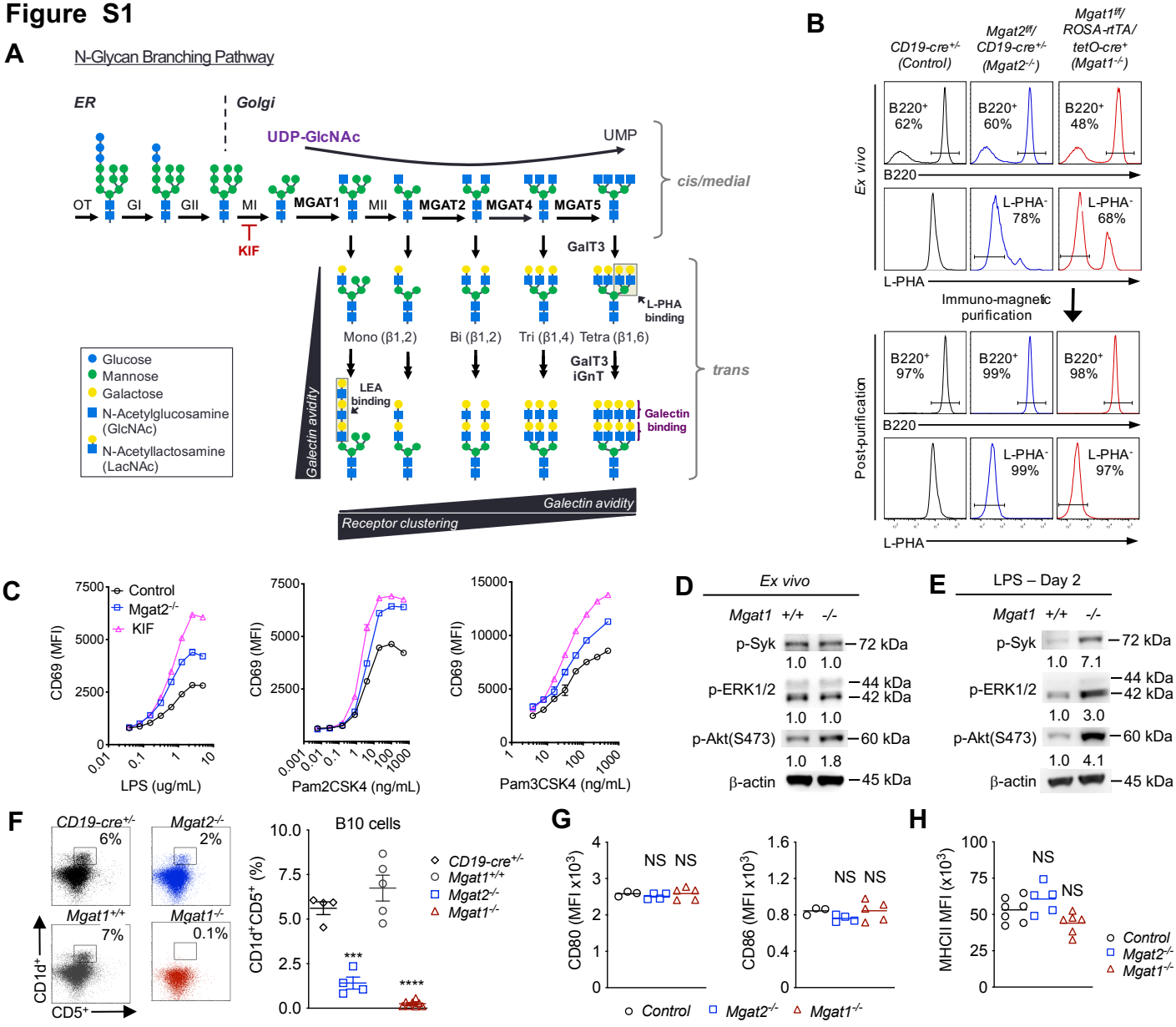
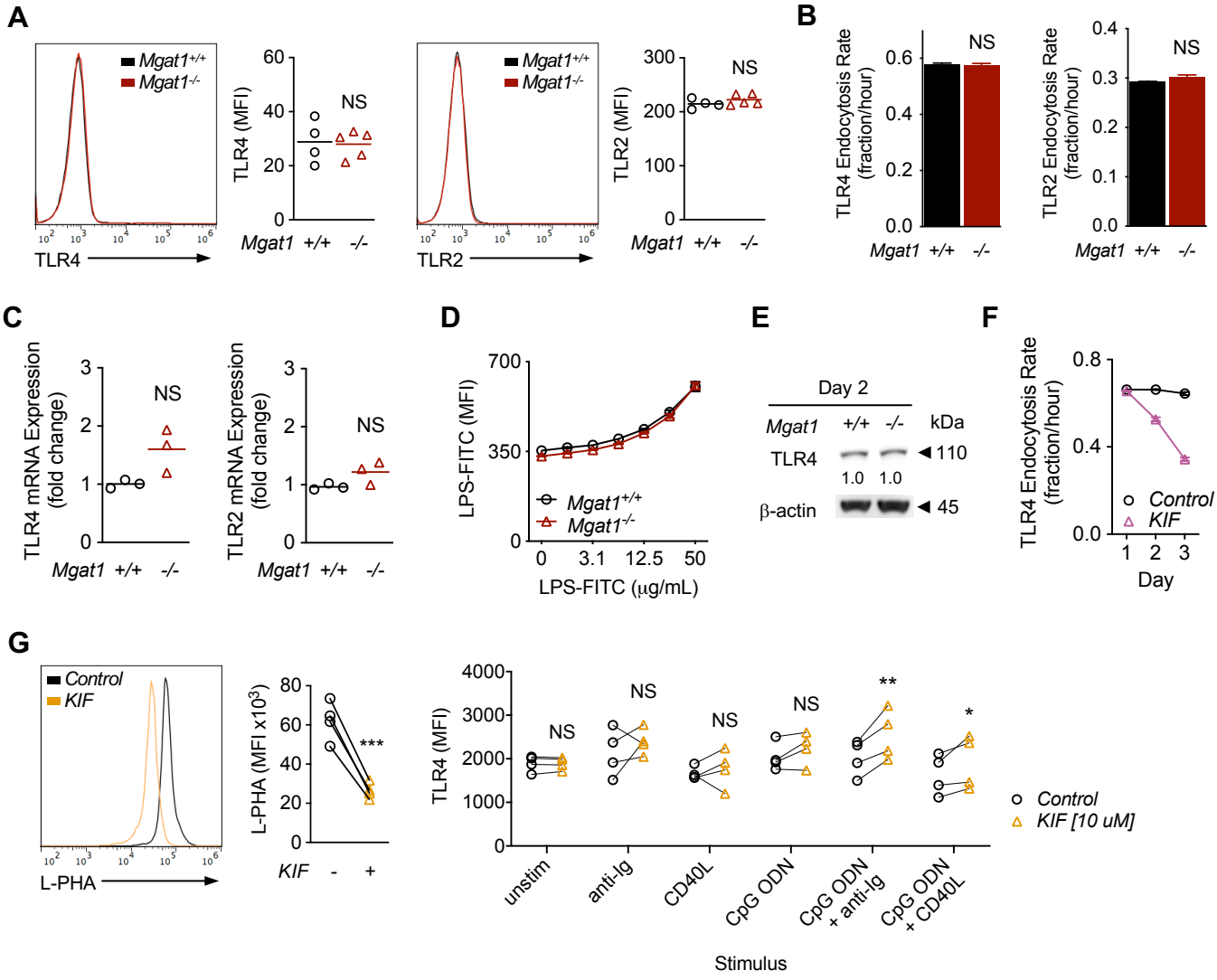
Figure S1



Supplement Figure 1, related to Figure 1

(**A**) Shown is the N-glycan biosynthesis pathway, where the N-acetylglucosaminyltransferase (Mgat) enzymes, GalT and iGnT create N-acetyllactosamine (LacNac), the ligand for galectins. L-PHA and LEA binding sites are indicated. KIF, kifunensine. (**B**) Representative histograms of B220 and L-PHA staining on *ex vivo* splenocytes and immuno-magnetic purified B cells to demonstrate purity of (L-PHA-) B220+ B cells used for *in vitro* experiments. (**C**) TLR4 and TLR2 stimulated B cells were assessed by flow cytometry for CD69 expression after 1 day of stimulation under the indicated conditions (KIF; kifunensine). (**D,E**) Western blot analysis of phospho-Syk, phospho-ERK1/2 and phospho-Akt (Ser473) in B cells *ex vivo* (D) and stimulated for 2 days (E). (**F**) Flow cytometric analysis of *ex vivo* splenocytes gated on B220+ B cells from the indicated mouse strains. (**G,H**) Flow cytometric analysis of *ex vivo* (L-PHA-) B220$^+$ B cells for CD80/CD86 (E) and MHCII (F) surface expression. Data shown are representative of n = 3 experiments (D,E). Each symbol represents one mouse and horizontal line represents the mean (F,G,H). Unpaired two-tailed t-test. ***p<0.001, ****p<0.0001. NS, not significant. MFI, mean fluorescence intensity.

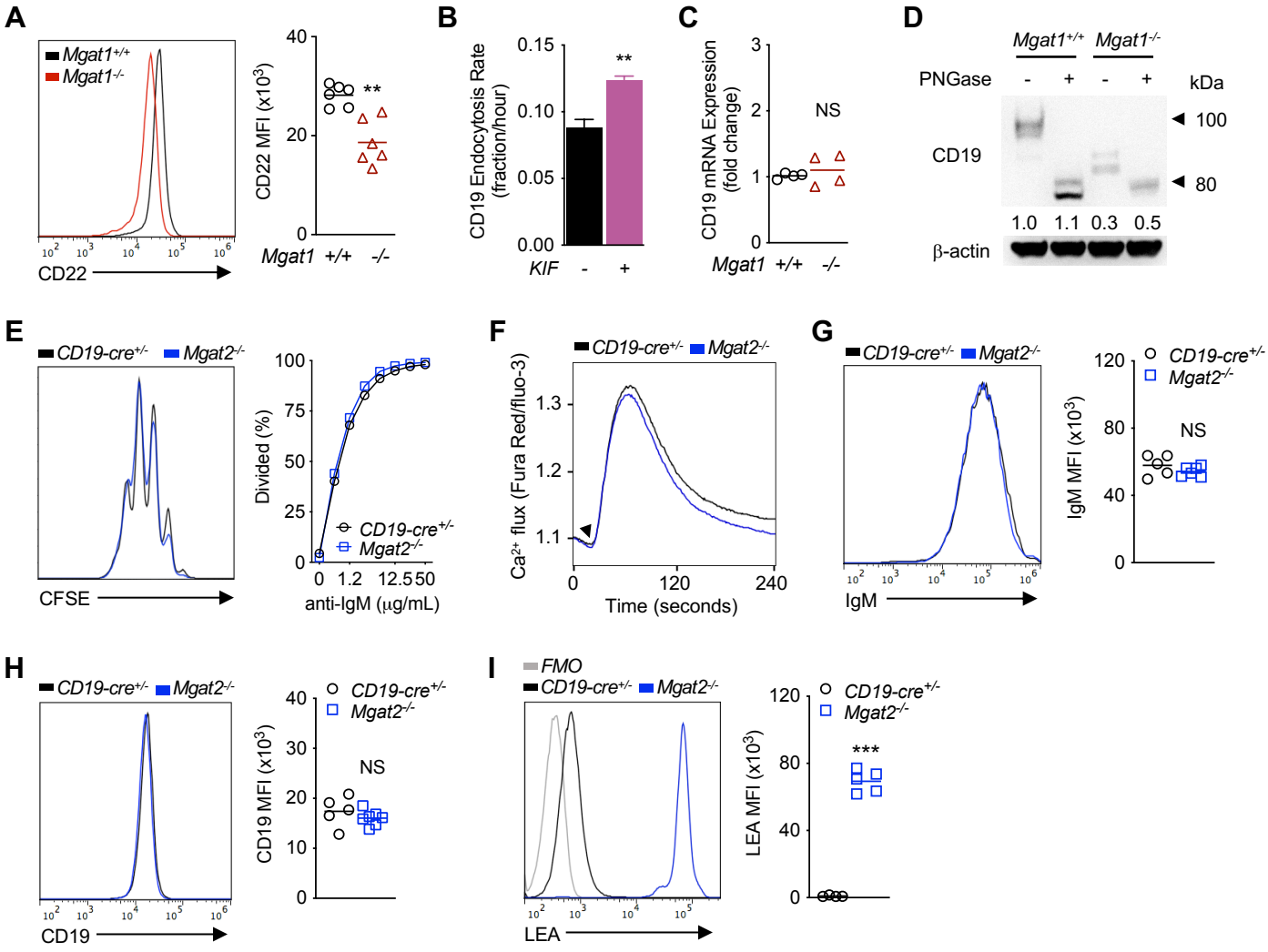
Figure S2



Supplement Figure 2, related to Figure 2

(**A-D**) Ex vivo unstimulated B cells were assessed for TLR4 and TLR2 surface levels by flow cytometry (MFIs shown are with isotype MFIs subtracted) (A), TLR4 and TLR2 endocytosis rates by flow cytometry (B), TLR4 and TLR2 mRNA expression by real-time qPCR (C), and LPS-FITC binding by flow cytometry (D). (**E**) Western blot analysis of total TLR4 levels in B cells stimulated with LPS for 2 days. (**F**) B cells ± KIF stimulated with LPS for 1-3 days and assessed for TLR4 endocytosis rate by flow cytometry. Endocytosis rate over 1.5hrs was calculated by dividing the MFI of acid-washed cells by the MFI of FACS buffer washed cells divided by 1.5hr (B,F). (**G**) Gated CD19^{+} B cells from human PBMCs (n=4 individuals) cultured for 4 days ± KIF, assessed for L-PHA binding, and then stimulated for 3 days as indicated and assessed for TLR4 surface levels (MFIs shown are with isotype MFIs subtracted) by flow cytometry. Data shown are mean ± s.e.m of cells stimulated in triplicate (B,D,F) and representative of n ≥ 3 experiments. Each symbol represents one mouse or blood donor, horizontal line represents the mean (A,C,G). Unpaired two-tailed t-tests with Welch's correction (A-C). Paired one-tailed t-test (G). NS, not significant. MFI, mean fluorescence intensity.

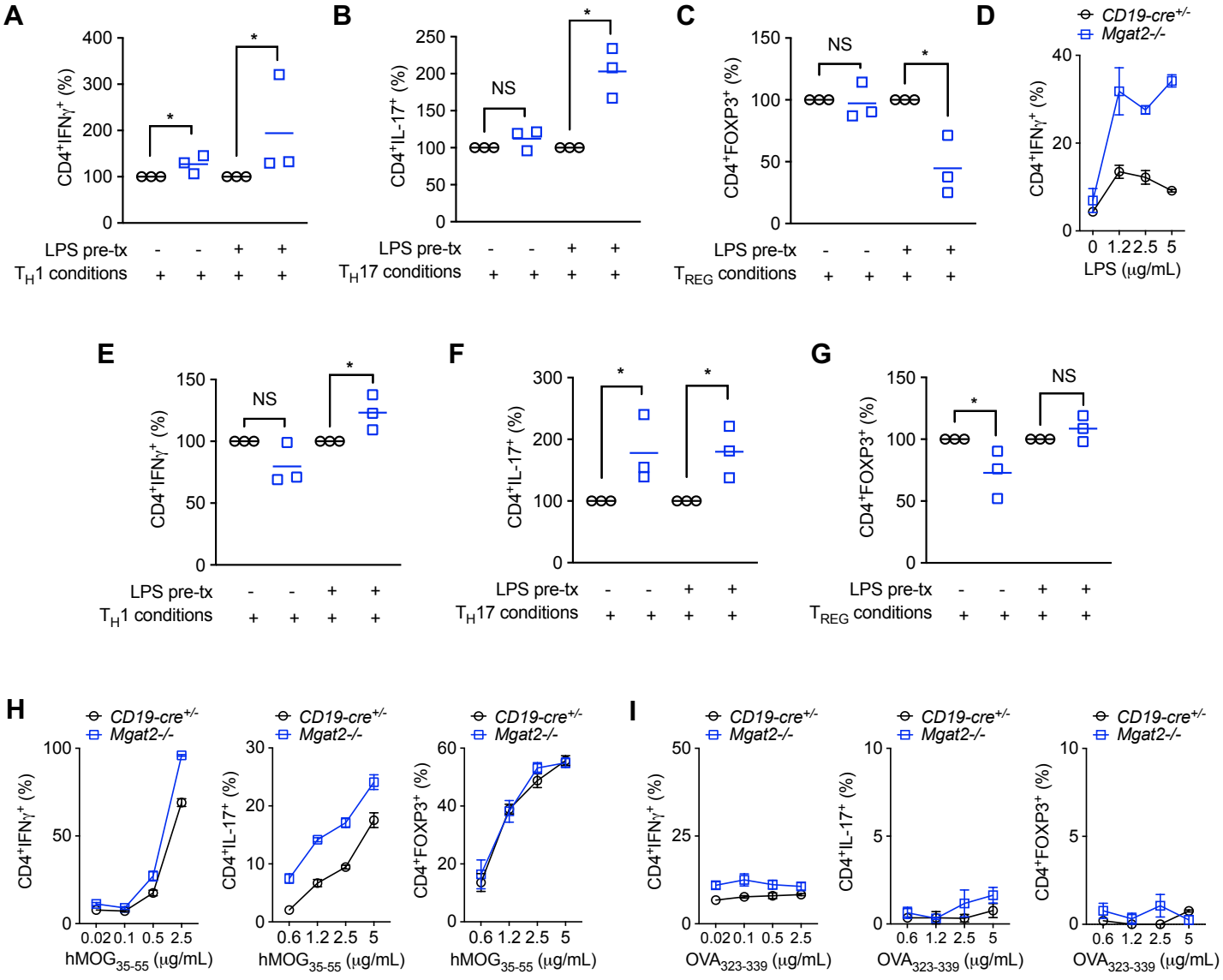
Figure S3



Supplement Figure 3, related to Figure 3.

(**A-C**,**E-I**) Flow cytometric analysis of *ex vivo* B cells for CD22 surface expression (A) and CD19 endocytosis rate ± KIF (B); anti-IgM F(ab')$_2$ induced proliferation by CFSE dilution after 2 days (E) and Ca^{2+} mobilization over 4 minutes (F); and *ex vivo* surface expression of IgM (G), CD19 (H) and LEA binding (I). Histogram in (E) represents highest anti-IgM dose. Endocytosis rate over 1.5hrs was calculated by dividing the MFI of acid-washed cells by the MFI of FACS buffer washed cells divided by 1.5hr (B). (**D**) Western blot analysis of total CD19 ± Rapid PNGase F treatment of lysates. PNGase was used to remove all N-glycans and equalize molecular weight of the protein. Data shown are mean ± s.e.m of cells stimulated in triplicate (B,E) and representative of n ≥ 3 experiments. Each symbol represents one mouse, horizontal line represents the mean (A,C,G-I). Unpaired two-tailed *t*-tests with Welch's correction. NS, not significant; **p<0.01; ***p<0.001. MFI, mean fluorescence intensity.

Figure S4



Supplement Figure 4, related to Figure 4.

(**A-I**) B cells +/- LPS pre-stimulation were co-cultured with allogeneic CD4+ T cells (A-D) or congenic 2D2 TCR transgenic CD4+ T cells + 2.5 ug/mL hMOG35-55 (E-G), or indicated amounts of hMOG35-55 (H) or OVA323-339 (I), under $T_H1/T_H17/T_{REG}$ inducing conditions. CD4+ T cells were assessed by flow cytometry for intracellular staining of IFNg after 3 days (A,D, E, H, I), and IL-17A (B,F, H, I) and FOXP3 (C,G, H, I) after 4 days. Each symbol represents one mouse from 3 different experiments, horizontal line represents the mean, Unpaired one-tailed Mann-Whitney tests (A-C, E-G). Other data shown are mean ± s.e.m of cells stimulated in triplicate and representative of n =2 experiments (H, I). NS, not significant; *p<0.05.

TRANSPARENT METHODS

Mice

Mgat1$^{f/f}$ (006891), *Mgat2*$^{f/f}$ (006892), *CD19-cre* (006785), *tetO-cre* (006234), *ROSA26-rtTA* (006965), 2D2 TCR^{MOG} transgenic (006912), and PL/J (000680) mice were obtained from Jackson Laboratory. Inter-breeding generated all other mice maintained on the C57BL/6 background. Mice were selected randomly for experiments and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. For inducible deletion of Mgat1 in peripheral B cells, doxycycline was provided in 1% sucrose drinking water at 2 mg/mL to *Mgat1*$^{f/f}$*/tetO-cre/ROSA26-rtTA* mice for four weeks. For *in vivo* Mgat1 activity inhibition, intraperitoneal injections of kifunensine (KIF, GlycoSyn) at 250 μg/mL were done for 4 consecutive days.

B Cell Purification, Culture, and Stimulation

For all *in vitro* experiments, splenic B cells were immuno-magnetically purified using the EasySep^{TM} Mouse B Cell Isolation Kit (STEMCELL Technologies) according to manufacturer's instructions with resulting purity >95%; 20 μg/mL biotinylated L-PHA (Vector Labs) was supplemented to deplete L-PHA^{+} (non- Mgat1 or Mgat2 deleted) B cells. Cells were cultured in "complete" media: RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (VWR), 2 μM L-glutamine and 100 U/mL penicillin/streptomycin (Gibco), and 50 μM β-mercaptoethanol (Gibco). Stimulation conditions with TLR agonists (Invivogen) were 5 μg/mL LPS for TLR4, 500 ng/mL Pam2CSK4 for TLR2:6, and 500 ng/mL Pam3CSK4 for TLR2:1 unless indicated otherwise. B cells activated through BCR were stimulated with 10 μg/mL functional grade goat anti-mouse IgM F(ab')$_2$ (eBioscience/Thermo Fisher Scientific) unless indicated otherwise. B cells from KIF injected mice were cultured in the presence of 5 μM KIF added once at day 0. Cytokine secretion in cell culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) with ELISA MAX^{TM} Deluxe Sets (BioLegend) according to the manufacturer's instructions.

Human PBMC Culture and Stimulation

Procedures with human subjects were approved by the Institutional Review Board of the University of California, Irvine. Human PBMCs were cultured in complete media as described above, with or without 10 μM KIF for 4 days prior to stimulation. PBMCs were then stimulated with 1 μg/mL recombinant human CD40 ligand (CD40L, Enzo Life Sciences) and/or 5 μg/mL LPS, again with or without 10 μM KIF.

Flow Cytometry and Proliferation

Fluorophore conjugated mouse specific antibodies from eBioscience/Thermo Fisher Scientific were B220 (RA3-6B2), CD19 (eBio1D3), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), MHCII (M5/114.15.2, TLR2 (6C2), TLR4 (UT41), IgM (II/41), CD4 (RM4-5), IFNγ (XMG1.2), IL-17A (eBio17B7), and FOXP3 (FJK-16s). Fluorophore conjugated antibodies from BioLegend were mouse specific CD22 (OX-97), and human specific CD19 (HIB19), and TLR4 (HTA125). For flow cytometric analysis of glycan expression, cells were stained with 2 μg/mL L-PHA-FITC or LEA-FITC (Vector Labs). To assess proliferation, cells were stained with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CellTrace^{TM} CFSE dye; Invitrogen/Thermo Fisher Scientific) and stimulated for 2 days. Samples were stained in FACS buffer (PBS with 1% BSA and 0.1% Na-azide) and acquired on the Attune NxT flow cytometer (Invitrogen/Thermo Fisher Scientific). Data analysis was performed using FlowJo software.

Endocytosis assay

Purified B cells were stained with fluorophore conjugated anti- TLR4, TLR2, or CD19, re-suspended in complete RPMI 1640 medium and incubated at 37°C for 1.5 hours. Cells were washed in FACS buffer or acidic buffer (150 mM NaCl and 20 mM HCl, pH 1.7) for 3 minutes at room temperature and then fixed in 1% PFA before analyzing by flow cytometry. The acidic buffer removes surface-bound antibody, and the MFI of acid-washed cells is divided by the MFI of FACS buffer washed cells and then by 1.hrs to determine the rate of internalized antibody.

Calcium Flux

Purified B cells were concurrently stained with 9.2 μM Fura Red AM and 4.4 μM fluo-3 AM dyes (Life Technologies/Thermo Fisher Scientific). After establishing baseline Ca^{2+} levels, anti-mouse IgM F(ab')$_2$ was added to induce Ca^{2+} flux. Samples were acquired on a BD LSR II flow cytometer, and Ca^{2+} mobilization was determined by the ratio of fluo-3 to Fura Red fluorescence intensity using the kinetics tool in FlowJo software.

Western Blot

Purified B cells were lysed in RIPA buffer with 100x Halt^{TM} protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). For total CD19 protein analysis, lysates were treated with Rapid PNGase F (New England BioLabs) to remove all N-glycans. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot antibodies to CD19 (#3574), TLR4 (D8L5W), TRAF3 (#4729), phospho-NF-κB p65 (Ser536) (93H1), phospho-Akt

(Ser473) (D9E), phospho-ERK1/2 (Thr202/Tyr204) (197G2), phospho-TBK1 (Ser172) (D52C2), phospho-CD19 (Tyr531) (#3571), phospho-Syk (Tyr525/526) (C87C1), phospho-PLCγ2 (Tyr759) (#3874), β-actin (13E5), and HRP-conjugated anti- rabbit or mouse IgG were from Cell Signaling Technology. Abundance was measured by chemiluminescence and quantified by normalization to β-actin and relative to control using ImageJ software.

Real-time qPCR

RNA from purified B cells was isolated by using RNeasy Plus kit (Qiagen) and reverse-transcribed by SuperScript III Reverse Transcriptase (Invitrogen). Real-time qPCR was conducted utilizing TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) with primer sets for mouse Tlr2 (Mm00442346_m1), Tlr4 (Mm00445273_m1), Cd19 (Mm00515420_m1), and Rn18s (Mm03928990_g1). All experiments were performed in triplicates on an Applied Biosystems 7900HT Fast Real-Time PCR System with relative mRNA expression determined by ΔΔCt method and normalized to housekeeping gene Rn18s expression.

B cell and $CD4^+$ T cell Co-cultures and Intracellular Cytokine Staining

Purified B cells were stimulated with 20 μg/mL LPS for 2 hours and co-cultured at a 10:1 ratio with purified splenic $CD4^+$ T cells (EasySep™ Mouse $CD4^+$ T Cell Isolation Kit, STEMCELL Technologies) for 3-4 days in two *in vitro* co-culture systems: (1) a mixed lymphocyte reaction with allogeneic $CD4^+$ T cells from PL/J mice, and (2) with $CD4^+$ T cells from congenic 2D2 TCR^{MOG} transgenic C57BL/6 mice in the presence of human MOG_{35-55} ($hMOG_{35-55}$) or $OVA_{323-339}$ peptide (AnaSpec). Cytokine combinations for $CD4^+$ T cell differentiation conditions were as follows: 10 μg/mL of anti-IL-4 (eBioscience/Thermo Fisher Scientific) and 25ng/mL of mouse IL-12 (BioLegend) for T_H1; 10 μg/mL of anti-IL-4, 10 μg/mL of anti-IFNγ (eBioscience/Thermo Fisher Scientific), 20 ng/mL of mouse IL-23 (BioLegend), 20 ng/mL of mouse IL-6 (BioLegend), and 5 ng/mL of human TGFβ1 (eBioscience/Thermo Fisher Scientific) for T_H17; and 10 μg/mL of anti-IL-4, 10 μg/mL of anti-IFNγ, 5 ng/mL of human TGFβ1 for T_{REG}. For intracellular cytokine flow cytometric analysis, cells were re-stimulated at 37°C for 4 hours with 50 ng/mL PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) and 750 ng/mL ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (1000x, BD Biosciences) and stained using the FOXP3 Transcription Factor Fixation/Permeabilization Kit (eBioscience/Thermo Fisher Scientific) according to manufacturer's instructions.

Experimental Autoimmune Encephalomyelitis

EAE was induced in 8-12 week old male and female mice by subcutaneous immunization at day 0 with 100 μg of recombinant human MOG (rhMOG) protein (AnaSpec) emulsified in Complete Freund's Adjuvant containing 4 mg/ml heat-inactivated Mycobacterium tuberculosis (H37RA; Difco). On days 0 and 2, mice received 200 ng of pertussis toxin (List Biological Laboratories) by intraperitoneal injection. Mice were examined daily for clinical signs of EAE over the next 35 days with observer blinded to mice genotypes. Mice were scored as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, partial hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead from EAE (Miller et al., 2010). At the end of the EAE experiment (day 35), spinal cords were harvested by hydraulic extrusion (Richner et al., 2017). Briefly, mice were euthanized with CO2 only (cervical dislocation was not performed as this would damage the spinal vertebrae) and spinal columns were excised from the back and placed in a petri dish filled with cold, sterile 1x PBS. While bracing the spinal column with forceps, an 18 G needle attached to a 10 mL syringe filled with cold, sterile 1x PBS was inserted into the distal end of the spinal column until it stabilized in the cavity. Steady pressure was applied on the syringe plunger to extrude the spinal cord into a new petri dish with cold, sterile 1x PBS on ice. Mouse spinal cords were then processed into single cell suspension for flow cytometric analysis of $CD4^+$ T cell and $B220^+$ B cell infiltration. Additionally, *in vivo* cytokine production of $CD4^+$ T cells was assessed by culturing splenocytes for 4 hours in the presence of PMA+ionomycin and GolgiPlug prior to intracellular cytokine staining and flow cytometric analysis.

SUPPLEMENTAL REFERENCES

Miller, S.D., Karpus, W.J., and Davidson, T.S. (2010). Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol *Chapter 15*, Unit 15 11.

Richner, M., Jager, S.B., Siupka, P., and Vaegter, C.B. (2017). Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. J Vis Exp.