Supplemental Materials for:

Bile acid metabolism is altered in multiple sclerosis and supplementation ameliorates neuroinflammation

Authors: Pavan Bhargava[1]*, Matthew D. Smith[1†], Leah Mische[1†], Emily Harrington[1], Kathryn C Fitzgerald[1], Kyle Martin[1], Sol Kim[1], Arthur Anthony Reyes[1], Jaime Gonzalez-Cardona[1], Christina Volsko[2], Ajai Tripathi[2], Sonal Singh[1], Kesava Varanasi[1], Hannah-Noelle Lord[1], Keya Meyers[1], Michelle Taylor[1], Marjan Gharagozloo[1], Elias S. Sotirchos[1], Bardia Nourbakhsh[1], Ranjan Dutta[2], Ellen M. Mowry[1], Emmanuelle Waubant[3], Peter A. Calabresi[1]

Affiliations:

[1]Department of Neurology, Johns Hopkins University, Baltimore, MD, USA.

[2]Department of Neuroscience, Cleveland Clinic Foundation, Cleveland, OH, USA.

[3]Department of Neurology, University of California San Francisco, San Francisco, CA, USA.

Supplemental Table 1. List of bile acid metabolites detected by untargeted metabolomics analyses in the discovery cohort

Metabolite
Cholic Acid (CA)
Chenodeoxycholic Acid (CDCA)
Deoxycholic Acid (DCA)
Ursodeoxycholic Acid (UDCA)
Hyocholic Acid (HCA)
Glycocholic Acid (GCA)
Glycocholic Acid Sulfate (GCAS)
Glycochenodeoxycholic Acid (GCDCA)
Glycochenodeoxycholic Acid Sulfate (GCDCAS)
Glycochenodeoxycholic Acid Glucuronide (GCDCAG)
Glycodeoxycholic Acid (GDCA)
Glycodeoxycholic Acid Sulfate (GDCAS)
Glycodeoxycholic Acid Glucuronide (GDCAG)
Glycoursodeoxycholic Acid (GUDCA)
Glycolithocholic Acid Sulfate (GLCAS)
Glycohyocholic Acid (GHCA)
Taurocholic Acid (TCA)
Taurocholic Acid Sulfate (TCAS)
Taurochenodeoxycholic Acid (TCDCA)
Taurodeoxycholic Acid (TDCA)
Taurolithocholic Acid Sulfate (TLCAS)

Supplemental Table 2. List of metabolites detected by targeted metabolomics analysis with range of detection in the targeted cohort

Analyte	Calibration Ranges (ng/mL)	
	LLOQ	ULOQ
Cholic Acid (CA)	2.50	1250
Chenodeoxycholic Acid (CDCA)	5.00	2500
Deoxycholic Acid (DCA)	5.00	2500
Lithocholic Acid (LCA)	2.50	1250
Ursodeoxycholic Acid (UDCA)	5.00	2500
Glycocholic Acid (GCA)	2.50	1250
Glycochenodeoxycholic Acid (GCDCA)	5.00	2500
Glycodeoxycholic Acid (GDCA)	2.50	1250
Glycoursodeoxycholic Acid (GUDCA)	5.00	2500
Glycolithocholic Acid (GLCA)	2.50	1250
Taurocholic Acid (TCA)	2.50	1250
Taurochenodeoxycholic Acid (TCDCA)	5.00	2500
Taurodeoxycholic Acid (TDCA)	5.00	2500
Taurolithocholic Acid (TLCA)	2.50	1250
Tauroursodeoxycholic Acid (TUDCA)	2.50	1250

Supplemental Table 3. Absolute concentrations of bile acid metabolites detected by targeted metabolomics analysis

Analyte	Healthy control		RRMS		PMS	
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Cholic Acid (CA)	9.18	(2.5, 30.3)	10.15	(2.5, 32.9)	4.26	(2.5, 20)
Chenodeoxycholic Acid (CDCA)	29.8	(13.5, 69.1)	34.2	(8.08, 112)	23.4	(6.89, 57.2)
Deoxycholic Acid (DCA)	143	(84.4, 227)	165	(54.9, 271)	135	(59.9, 225)
Glycocholic Acid (GCA)	93.9	(44.1, 159)	70.4	(29.1, 133)	48.6	(23.6, 85.9)
Glycochenodeoxycholic Acid (GCDCA)	347	(158, 673)	262	(112, 525)	192	(96.5, 319)
Glycodeoxycholic Acid (GDCA)	173	(85.5, 379)	166	(70.1, 368)	132	(60.4, 258)
Glycoursodeoxycholic Acid (GUDCA)	31.7	(16, 67.4)	31.55	(14.6, 67.5)	28.2	(9.41, 71.7)
Glycolithocholic Acid (GLCA)	10.2	(3.3, 23.3)	7.115	(2.5, 18.8)	5.19	(2.5, 12.4)
Taurocholic Acid (TCA)	13.3	(4.11. 25.3)	9.03	(3.18, 20.7)	6.47	(2.94, 13.6)
Taurochenodeoxycholic Acid (TCDCA)	45.5	(22.5, 89.7)	29	(13, 89.3)	25.6	(11.5, 48.3)
Taurodeoxycholic Acid (TDCA)	30.7	(11.8, 64.8)	21.25	(8.19, 62.6)	20.5	(8.27, 46.9)
Total Measured Bile Acids	1177.1	(653.5, 1831.2)	1054.2	(556.0, 1623.2)	802.6	(490.9, 1274.5)

*All values are in ng/ml.

Supplemental Table 4. List of bile acid metabolites detected by untargeted metabolomics analyses in the pediatric cohort

Metabolite
Cholic Acid (CA)
Chenodeoxycholic Acid (CDCA)
Deoxycholic Acid (DCA)
Ursodeoxycholic Acid (UDCA)
Hyocholic Acid (HCA)
Glycocholic Acid (GCA)
Glycocholic Acid Sulfate (GCAS)
Glycochenodeoxycholic Acid (GCDCA)
Glycochenodeoxycholic Acid Sulfate (GCDCAS)
Glycochenodeoxycholic Acid Glucuronide (GCDCAG)
Glycodeoxycholic Acid (GDCA)
Glycodeoxycholic Acid Sulfate (GDCAS)
Glycoursodeoxycholic Acid (GUDCA)
Glycolithocholic Acid Sulfate (GLCAS)
Glycohyocholic Acid (GHCA)
Glyco-alpha Muricholic Acid (GAMCA)
Glyco-beta Muricholic Acid (GBMCA)
Taurocholic Acid (TCA)
Taurocholic Acid Sulfate (TCAS)
Taurochenodeoxycholic Acid (TCDCA)
Taurodeoxycholic Acid (TDCA)
Taurolithocholic Acid Sulfate (TLCAS)
Isoursodeoxycholic Acid (IUDCA)

Supplemental Table 5. Comparison of bile acid metabolites altered between adult and pediatric MS patients

Metabolite	Direction and significance of change in pediatric MS	Direction and significance of change in adult MS (Discovery cohort)
Cholic Acid (CA)	Reduced	Reduced
Glycocholic Acid (GCA)	Reduced*	Reduced *** (PMS)
Taurocholic Acid (TCA)	Reduced*	Reduced* (PMS)
Taurocholic Acid Sulfate (TCAS)	Reduced	Reduced
Chenodeoxycholic Acid (CDCA)	Reduced	Reduced* (PMS)
Glycochenodeoxycholic Acid (GCDCA)	Reduced*	Reduced**(PMS)
Glycochenodeoxycholic Acid Sulfate (GCDCAS)	Reduced	Reduced* (PMS)
Glycochenodeoxycholic Acid Glucuronide (GCDCAG)	Reduced	Reduced* (RRMS & PMS)
Taurochenodeoxycholic Acid (TCDCA)	Reduced*	Reduced* (RRMS)
Ursodeoxycholic Acid (UDCA)	Reduced	Reduced
Glycoursodeoxycholic Acid (GUDCA)	Reduced	Reduced
Hyocholic Acid (HCA)	Increased	Reduced
Glycohyocholic Acid (GHCA)	Reduced*	Reduced* (RRMS)
Deoxycholic Acid (DCA)	Increased*	Reduced **
Glycodeoxycholic Acid (GDCA)	Increased	Reduced** (PMS)
Glycodeoxycholic Acid Sulfate (GDCAS)	Increased	Reduced* (RRMS)
Taurodeoxycholic Acid (TDCA)	Reduced	Reduced* (RRMS & PMS)
Glycolithocholic Acid Sulfate (GLCAS)	Increased	Reduced** (RRMS & PMS)
Taurolithocholic Acid Sulfate (TLCAS)	Reduced	Reduced

Supplemental Table 6. List of primers for murine astrocyte and microglial qPCR

Name	Forward Primer	Reverse Primer
Amigo2	GAGGCGACCATAATGTCGTT	GCATCCAACAGTCCGATTCT
Arg1	TTTTAGGGTTACGGCCGGTG	CCTCGAGGCTGTCCTTTTGA
b actin	ACCTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG
C1q	TCTGCACTGTACCCGGCTA	CCCTGGTAAATGTGACCCTTTT
C3	AGCTTCAGGGTCCCAGCTAC	GCTGGAATCTTGATGGAGACGC
Cd14	GGACTGATCTCAGCCCTCTG	GCTTCAGCCCAGTGAAAGAC
Clcf1	CTTCAATCCTCCTCGACTGG	TACGTCGGAGTTCAGCTGTG
Cp	TGTGATGGGAATGGGCAATGA	AGTGTATAGAGGATGTTCCAGGTCA
Fkbp5	TATGCTTATGGCTCGGCTGG	CAGCCTTCCAGGTGGACTTT
Gbp2	GGGGTCACTGTCTGACCACT	GGGAAACCTGGGATGAGATT
Ggta1	GTGAACAGCATGAGGGGTTT	GTTTTGTTGCCTCTGGGTGT
H2-D1	TCCGAGATTGTAAAGCGTGAAGA	ACAGGGCAGTGCAGGGATAG
H2-T23	GGACCGCGAATGACATAGC	GCACCTCAGGGTGACTTCAT
Iigp1	GGGGCAATAGCTCATTGGTA	ACCTCGAAGACATCCCCTTT
Il1a	CGCTTGAGTCGGCAAAGAAAT	CTTCCCGTTGCTTGACGTTG
NOS2	GCAAACATCACATTCAGATCCC	TCAGCCTCATGGTAAACACG
Lcn2	CCAGTTCGCCATGGTATTTT	CACACTCACCACCCATTCAG
Psmb8	CAGTCCTGAAGAGGCCTACG	CACTTTCACCCAACCGTCTT
Ptx3	AACAAGCTCTGTTGCCCATT	TCCCAAATGGAACATTGGAT
Serping1	ACAGCCCCCTCTGAATTCTT	GGATGCTCTCCAAGTTGCTC
S1pr3	AAGCCTAGCGGGAGAGAAAC	TCAGGGAACAATTGGGAGAG
SIc10a6	GCTTCGGTGGTATGATGCTT	CCACAGGCTTTTCTGGTGAT
Sgrn	GCAAGGTTATCCTGCTCGGA	TGGGAGGGCCGATGTTATTG
Steap4	CCCGAATCGTGTCTTTCCTA	GGCCTGAGTAATGGTTGCAT
Timp1	AGTGATTTCCCCGCCAACTC	GGGGCCATCATGGTATCTGC
Tm4sf1	GCCCAAGCATATTGTGGAGT	AGGGTAGGATGTGGCACAAG
Tnfa	TGTGCTCAGAGCTTTCAACAA	CTTGATGGTGGTGCATGAGA

Supplemental Table 7. List of antibodies utilized for IHC of MS and EAE tissue

EAE tissue

Antibody	Manufacturer	Catalog #	Clone	Isotype	Host	Dilution
GFAP	Dako	GA52461-2	Polyclonal	IgG	Rabbit	1:1000
GFAP	Cell Signaling Technology	3670	Mono	IgG1	Mouse	1:250
Iba-1	Wako	019-19741	Polyclonal		Rabbit	1:300
iNos	Santa Cruz Biotechnology	sc-7271	Mono; NOS2(C-11)	IgG1	Mouse	1:1000
CD3	Dako	A045201-2	Polyclonal		Rabbit	1:200
Mac-2	BioLegend	125401	Mono; M3/38	IgG2a	Rat	1:200
PSMB8	Invitrogen	MA5-15890	Mono; 1A5	IgG1	Mouse	1:200
GPBAR1	Abcam	ab72608	Polyclonal	IgG	Rabbit	1:100

MS tissue

Antibody	Manufacturer	Catalog #	Clone	Isotype	Host	Dilution
FXR	Perseus Proteomics Inc	PP-A9033A-00	Monoclonal	IgG	Mouse	1:500
GPBAR1	Thermo Fisher Scientific	PA5-27076	Polyclonal	IgG	Rabbit	1:250
GFAP	Millipore	MAB3402	Monoclonal	IgG1	Mouse	1:250
CD68	Dako	M0876	Monoclonal	IgG3	Mouse	1:250

Supplemental Table 8. Demographics of MS patients and controls utilized for bile acid receptor staining

Case#	MS type	Age	Sex	Disease duration (Yr)	EDSS	PMI (h)	Lesion types
MS1 (25)	SPMS	56	M	32	9.5	3	Mixed Active/inactive and demyelinating
MS2 (115)	SPMS	67	M	25	8	11	Active/inactive and post-demyelinating
MS3 (160)	SPMS	35	M	21	9.5	8	Active and demyelinating lesion Mixed Active/inactive and demyelinating

Controls

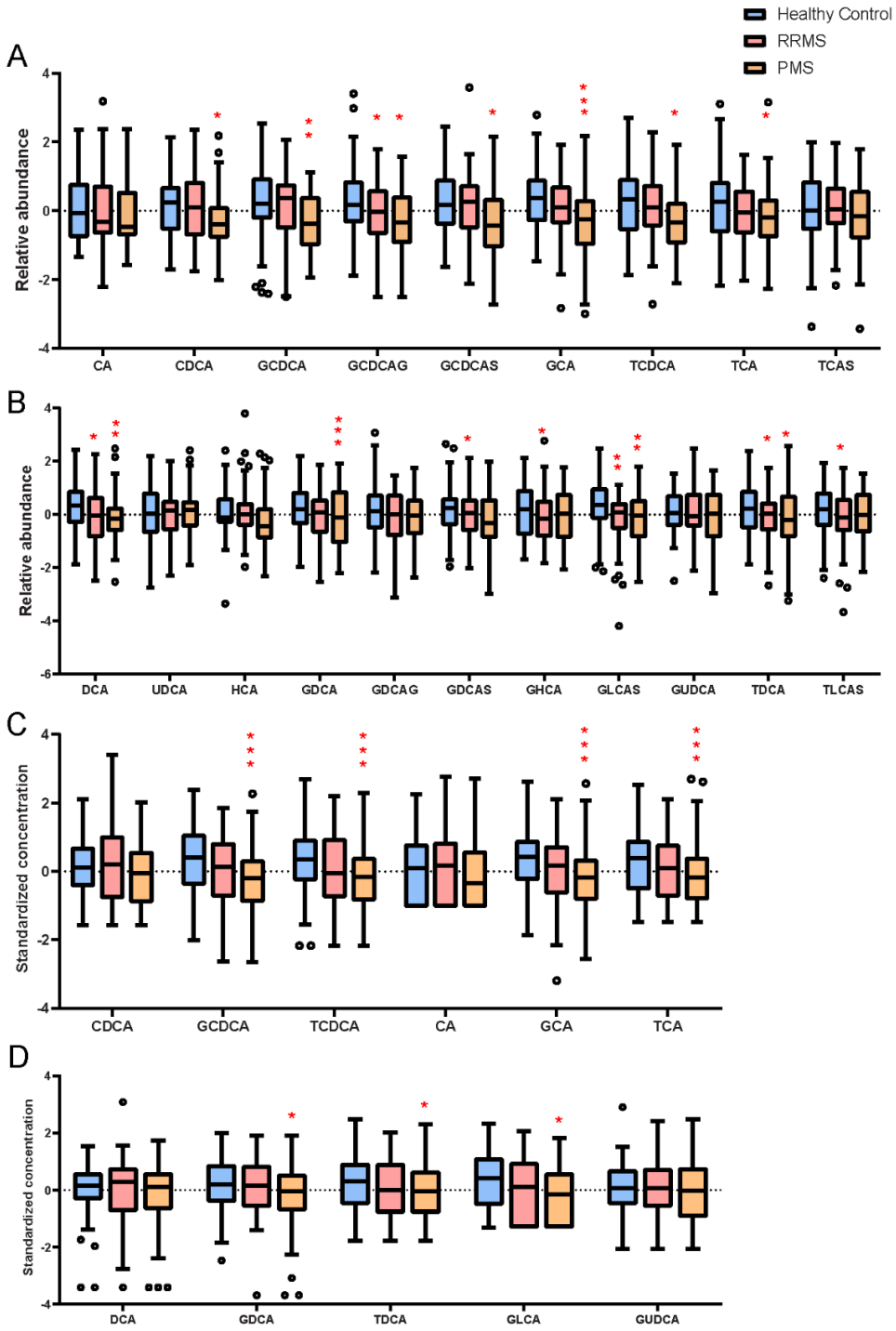
Case#	Age	Sex	PMI (h)	Cause of death
Ctr-1	53	F	36	Cardiopulmonary arrest
Ctr-2	72	M	30	Unknown
Ctr-3	96	F	9	Unknown

Supplemental Table 9: Demographics of MS patients used for qPCR

Case#	MS types	Age (yrs)	Sex	Disease duration (Yrs)	EDSS	PMI (h)	Tissue type
1	SPMS	61	F	35	9.5	10	NAWM, WML
2	PPMS	57	F	15	6.5	6	NAWM
3	SPMS	52	M	25	9.5	5	NAWM
4	SPMS	50	F	24	8	7	NAWM
5	PPMS	51	F	15	7.5	7	WML
6	SPMS	62	M	42	9.5	5	WML
7	SPMS	59	F	25	9	10	WML

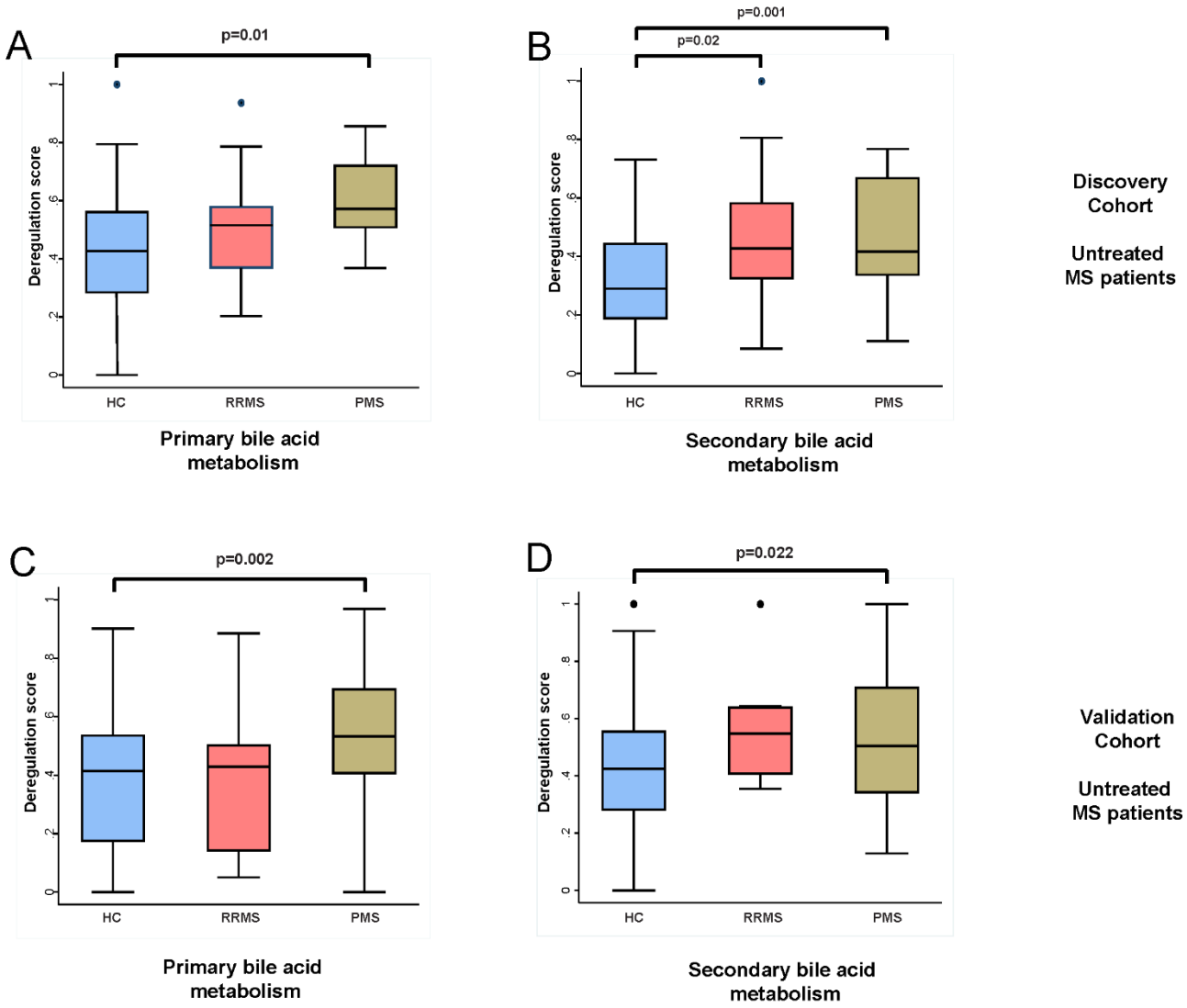
Supplemental Table 10: Antibodies utilized for flow cytometry in EAE and in vitro T cell stimulation

Product	Clone	Conjugate	Manufacturer
Viobility 405/520	N/A	N/A	Miltenyi
TruStain Ms Fc Block	93	N/A	Biolegend
CD3ε	145-2C11	APC/Fire750	Biolegend
CD25	PC61	PerCP/Cy5.5	Biolegend
CD4	GK1.5	eFluor450	ThermoFisher
CD8	53-6.7	PE/Cy7	Biolegend
FoxP3	FJK-16s	PE	ThermoFisher
IFNγ	XMG1.2	FITC	ThermoFisher
IL-17a	eBio17B7	APC	ThermoFisher
CD69	H1.2F3	FITC	BD Biosciences
CD62L	MEL-14	PE/Cy7	Biolegend
CD44	IM7	APC/eFluor780	ThermoFisher



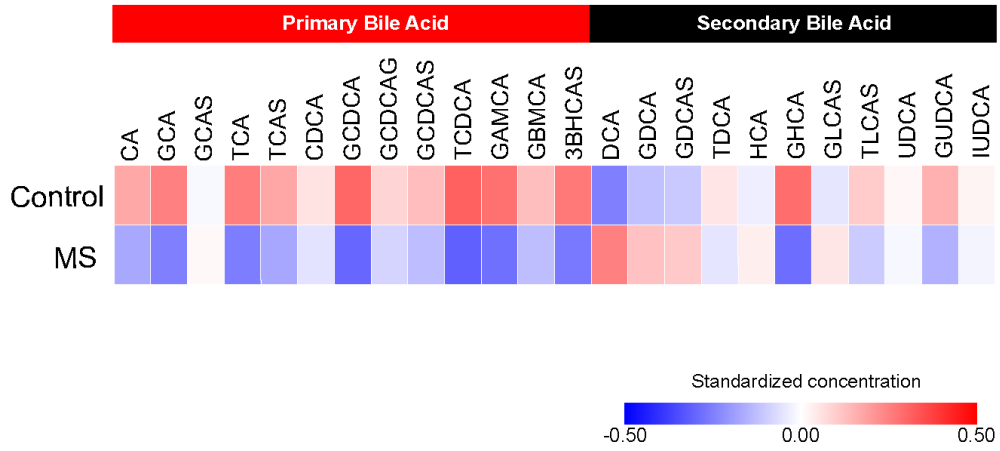
Supplemental Figure 1. Individual bile acid metabolites from adult cohorts

Box plots of primary bile acid metabolite relative abundances in the discovery cohort are shown in **(A)**, while relative abundances of secondary bile acid metabolites are shown in **(B)**. **(C)** Standardized concentrations of primary bile acids in the validation cohort, while **(D)** depicts standardized concentrations of secondary bile acids in the validation cohort. Red asterisks depict statistical significance of comparison to the control group with p values derived from multivariate regression models adjusting for age, sex and race (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). For all box plots – center line – median, box – 25th and 75th percentiles, whiskers – 1.5 x interquartile range and points – outliers.



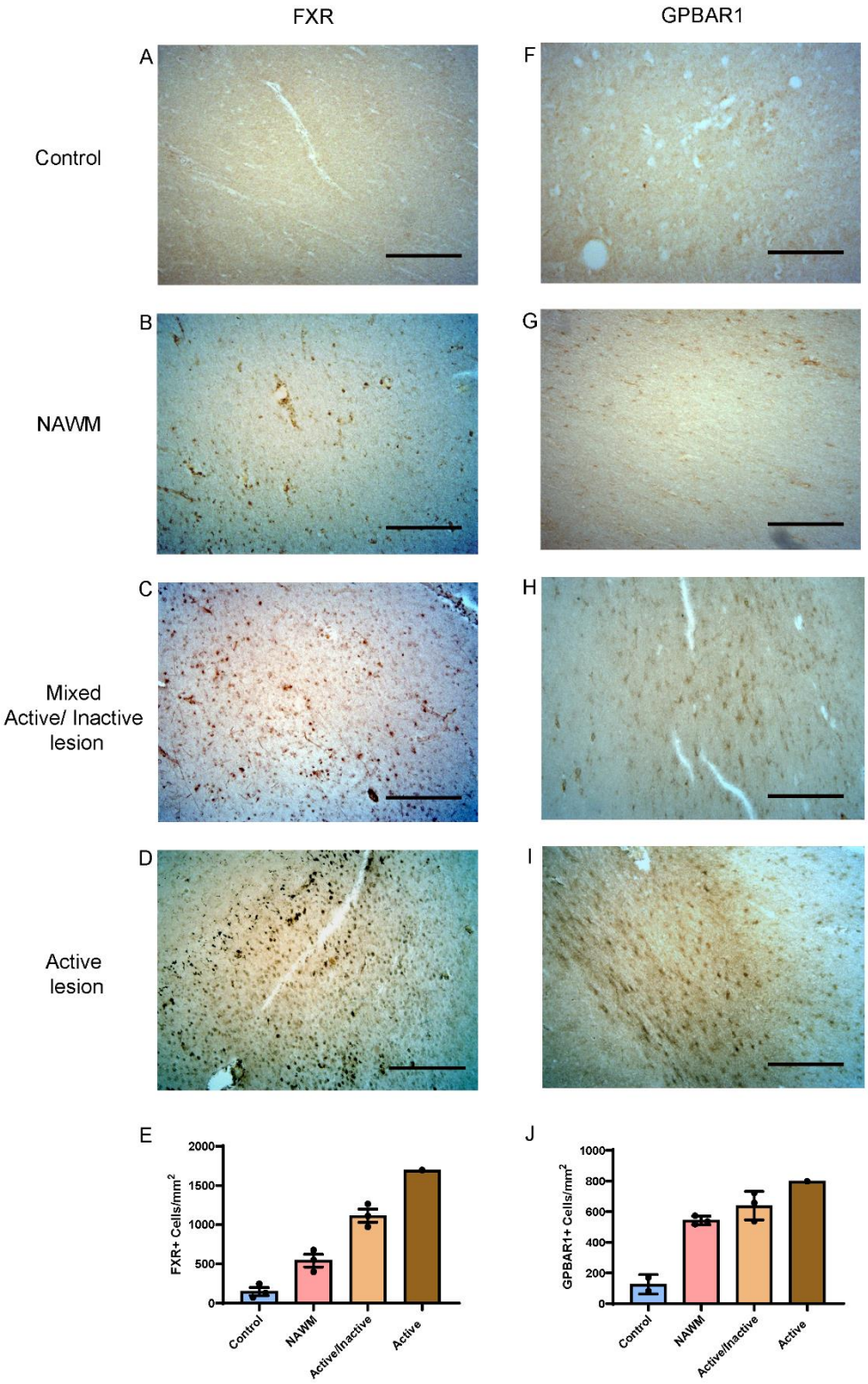
Supplemental Figure 2. Comparison of bile acid metabolism in adult cohorts restricting to untreated MS patients

Box plots of pathway deregulation scores for primary **(A)** and secondary **(B)** bile acid metabolism pathways in the discovery cohort restricting to untreated MS patients demonstrated alterations which were consistent with results from the entire cohort. Pathway deregulation scores for primary **(C)** and secondary **(D)** bile acid metabolism pathways in the validation cohort restricting to untreated MS patients were again consistent with results derived from the entire cohort. p values for A-D are derived from a one-way ANOVA. For all box plots – center line – median, box – 25th and 75th percentiles, whiskers – 1.5 x interquartile range and points – outliers.



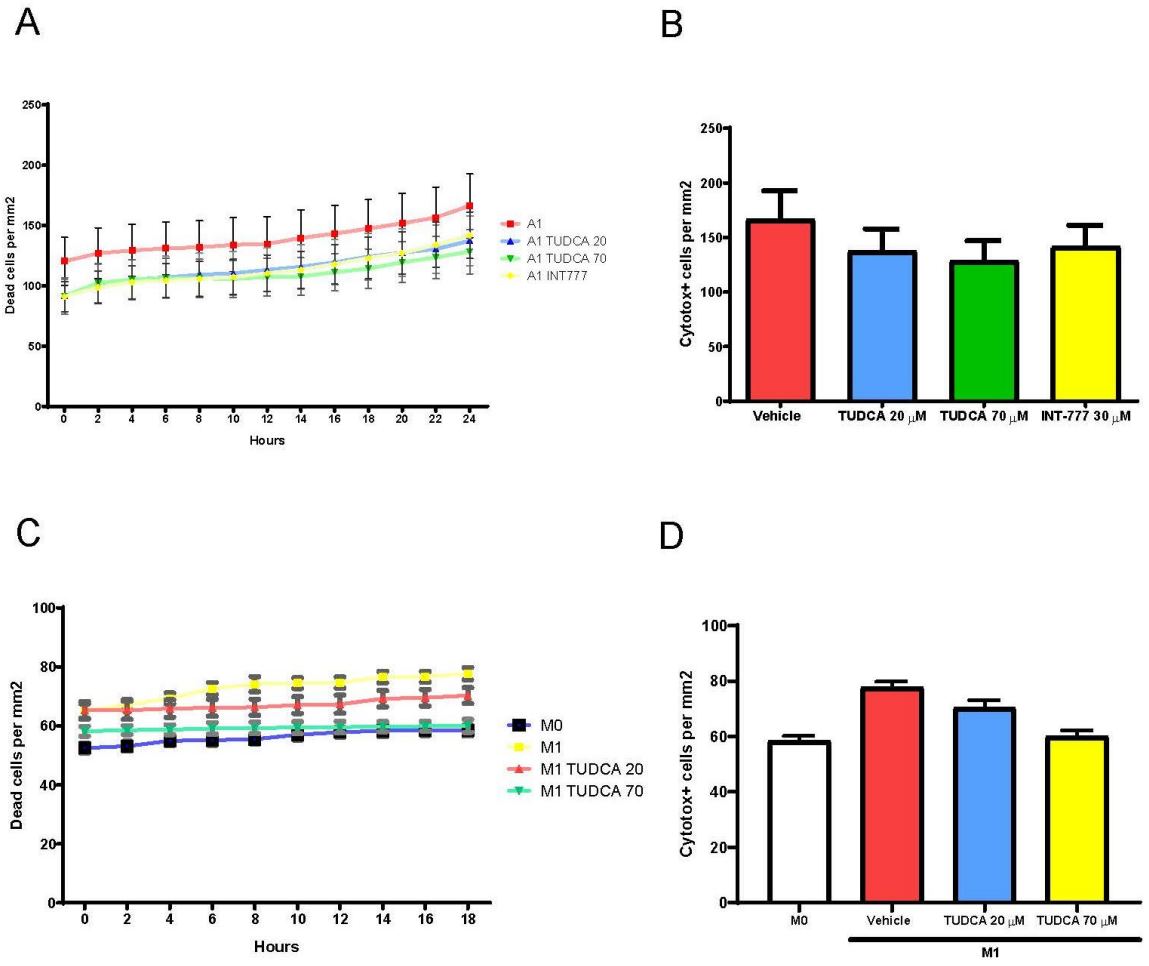
Supplemental Figure 3. Heatmap of bile acid metabolite abundance from the pediatric cohort

Heat map of mean standardized relative abundance of various bile acid metabolites (primary and secondary bile acid metabolites) identified in the circulation of pediatric-onset MS patients and healthy controls. CA – cholic acid, GCA – glycocholic acid, GCAS – glychocholic acid sulfate, TCA – taurocholic acid, TCAS – taurocholic acid sulfate, CDCA – chenodeoxycholic acid, GCDCA – glycochenodeoxycholic acid, GCDCAG – glycochenodeoxycholic acid glucuronide, GCDCAS – glycochenodeoxycholic acid sulfate, TCDCA – taurochenodeoxycholic acid, GAMCA – glycoalpha-muricholic acid, GBMCA – glycobeta-muricholic acid, 3BHCAS – 3-beta hydroxy-5-cholenoic acid, DCA – deoxycholic acid, GDCA – glycodeoxycholic acid, GDCAS – glycodeoxycholic acid sulfate, TDCA -taurodeoxycholic acid, HCA -hyocholic aicd, GHCA -glycohyocholic acid, GLCAS -glycolithocholic acid sulfate, TLCAS – taurolithocholic acid sulfate, UDCA – ursodeoxycholic acid, GUDCA – glycoursodeoxycholic acid, IUDCA – isoursodeoxycholic acid.



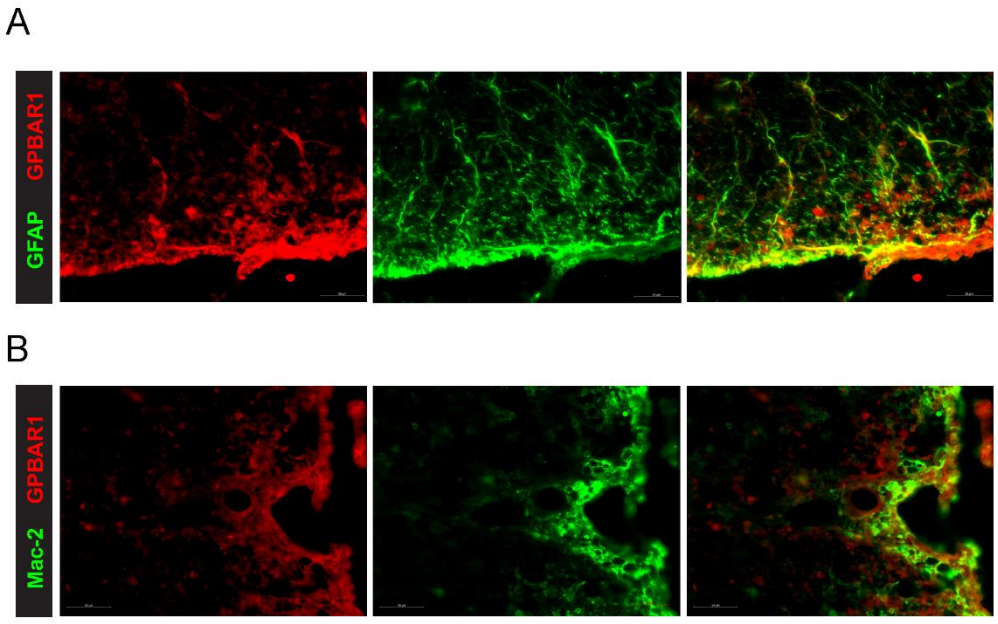
Supplemental Figure 4. Comparison of bile acid receptor expression in control and MS brain tissue

Immunohistochemistry for FXR in control **(A)**, MS normal-appearing white matter (NAWM) **(B)**, mixed active/inactive lesion **(C)** and an active lesion **(D)**. Staining is quantified in **(E)**. Immunohistochemistry for GPBAR1 in control **(F)**, NAWM **(G)**, mixed active/inactive lesion **(H)** and active lesion **(I)**. This staining is quantified in **(J)**. Scale bars in all images: A-D and F-I are 200 µm. For E and J – Bars represent mean and error-bars represent s.e.m.



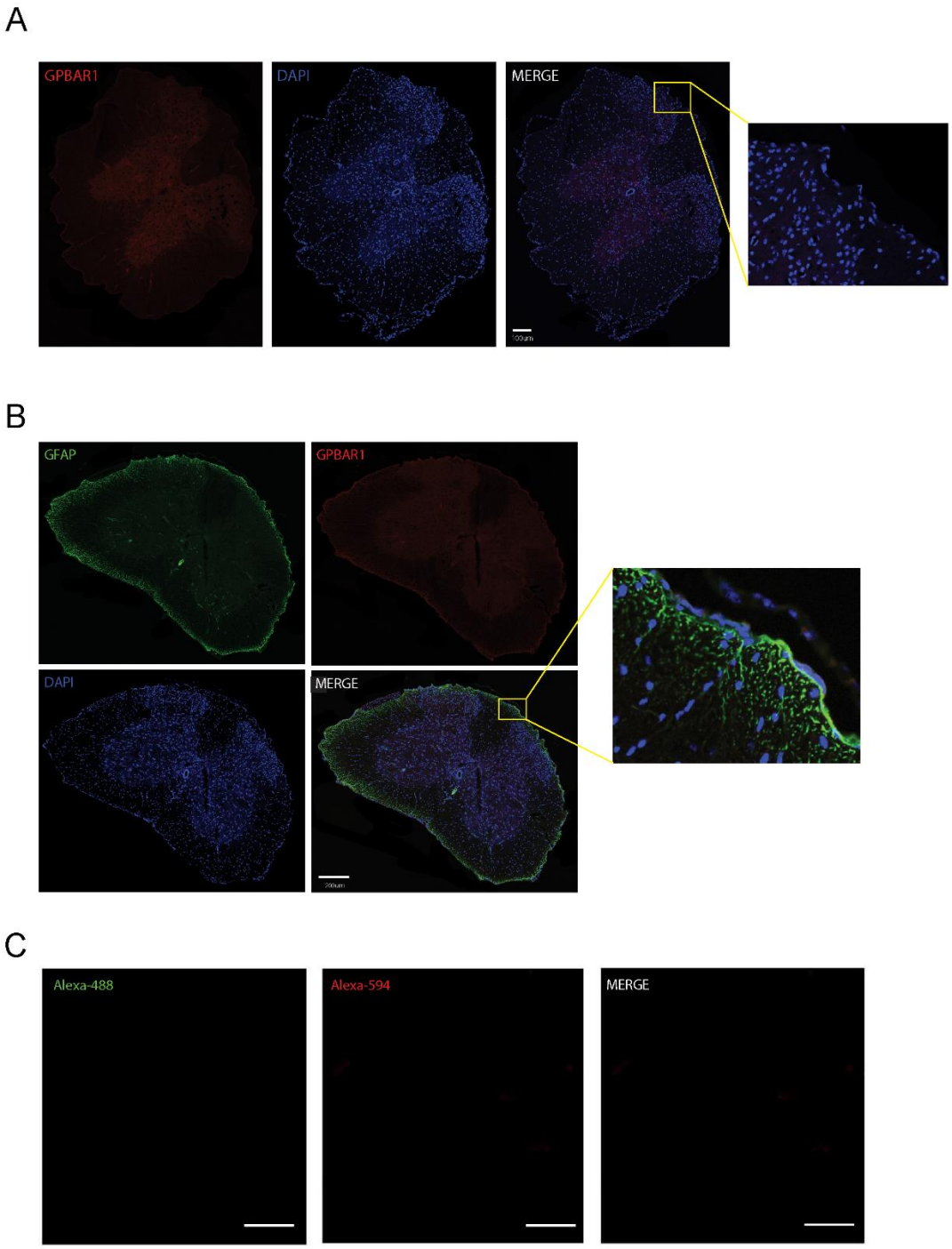
Supplemental Figure 5. TUDCA treatment does not adversely affect the viability of murine astrocytes or microglia

(A) We assessed viability of astrocytes, over a 24-hour period, in various culture conditions – A1 polarizing conditions (IL-1α, TNF-α and C1q) plus vehicle or varying doses of TUDCA or INT-777, using labelling with a cell viability dye and noted no increase in cell death with the addition of TUDCA or INT-777. **(B)** Quantification of cell death at the 24-hour time point. **(C)** We performed similar assessments, over an 18-hour period, in microglial cultures – either M0 condition or M1 polarization (IFN-γ and LPS) either with vehicle or varying doses of TUDCA and noted no increased cell death in the TUDCA conditions compared to vehicle. **(D)** Quantification of cell death at the 18-hour time point. Points in A and C and bars in B and D represent the mean. Error bars in A-D represent standard error of the mean.



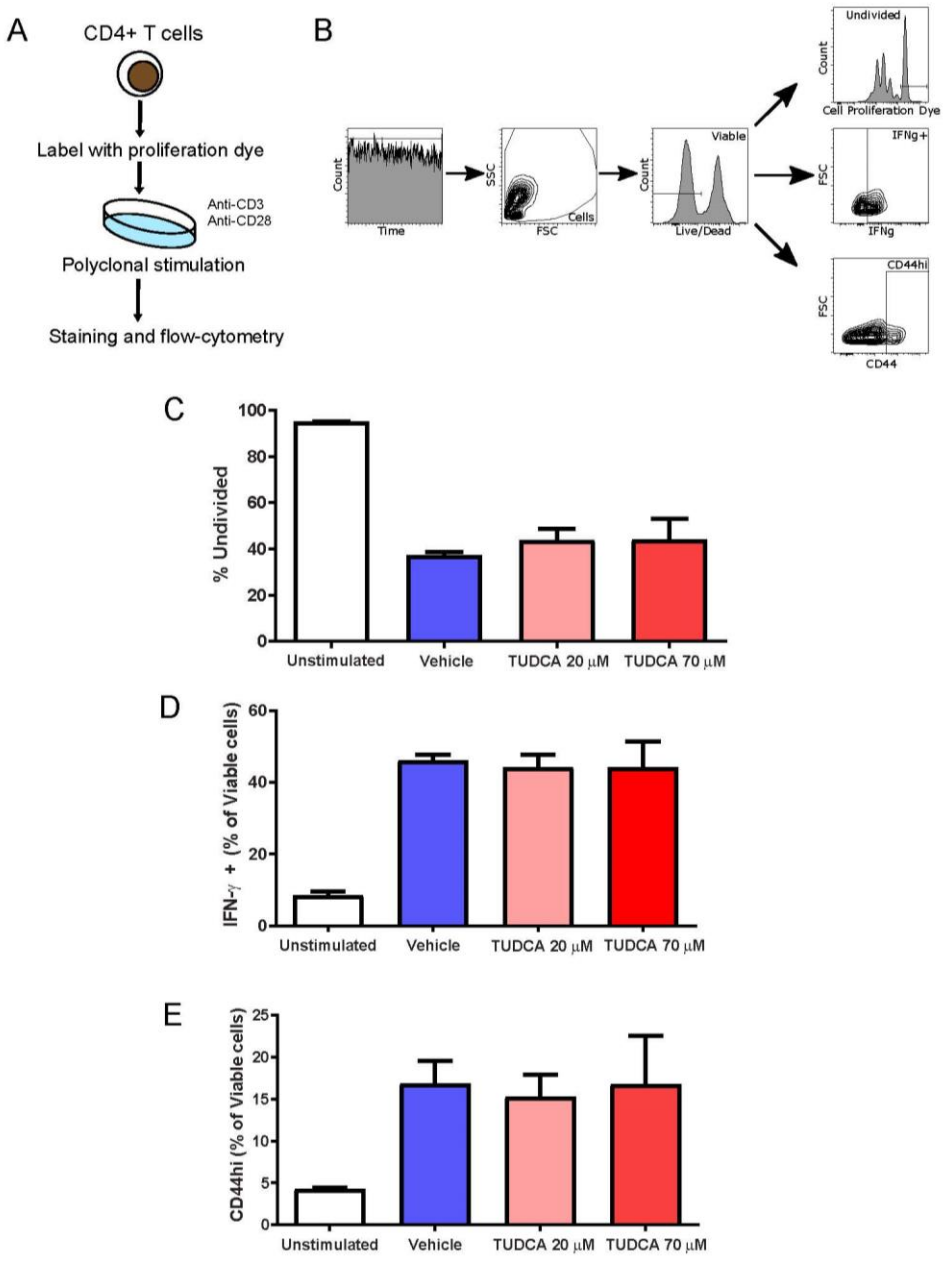
Supplemental Figure 6. Bile acid receptors are found on astrocytes and microglia/macrophages in EAE

(A) Immunohistochemistry for GFAP and GPBAR1 on spinal cord sections from mice with EAE, demonstrates the presence of several GPBAR1+ GFAP+ astrocytes. **(B)** We also performed immunohistochemistry for Mac-2 which stains myeloid cells and GPBAR-1, which revealed the presence of several GPBAR-1+ Mac-2+ cells in the spinal cord of EAE mice.



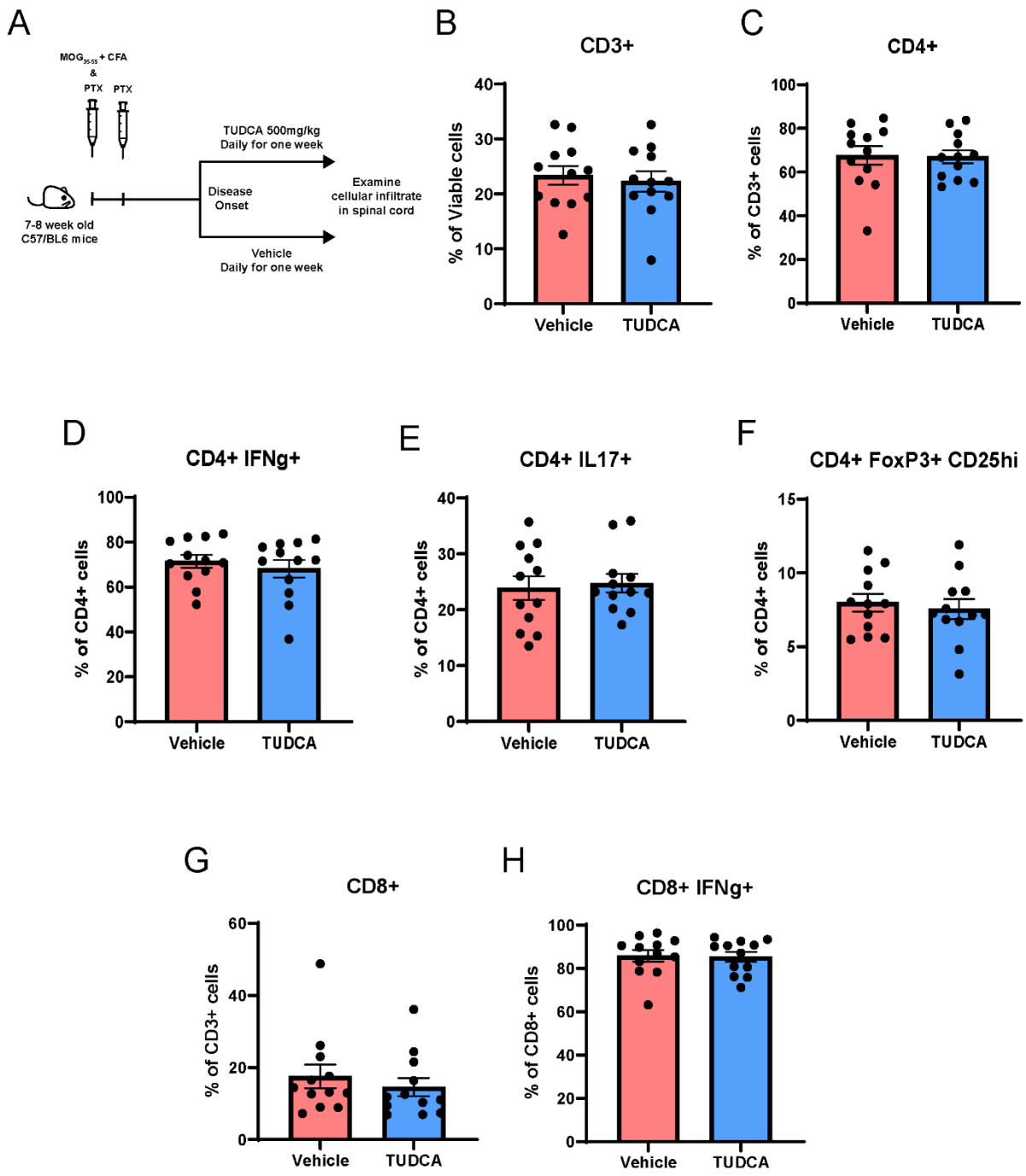
Supplemental Figure 7. Validation of Bile acid receptor staining in EAE and MS tissue

(A) Immunohistochemistry on spinal cord sections from mice with EAE in the absence of primary antibody for GPBAR1 demonstrates lack of staining for the receptor. **(B)** We also performed immunohistochemistry for GPBAR1 and GFAP in the spinal cord of GPBAR1-KO mice and noted no staining for GPBAR1. **(C)** Immunohistochemistry on a mixed active/inactive lesions from an MS patient in the absence of the primary antibody for GPBAR1 demonstrated lack of staining for the receptor.



Supplemental Figure 8. TUDCA treatment does not affect murine T cell proliferation and cytokine production

(A) Murine CD4+ T cells isolated using negative bead selection from mouse splenocytes were stained with cell proliferation dye and then cultured in c-RPMI and polyclonally stimulated with anti-CD3 and anti-CD28 antibodies, in the presence or absence of varying doses of TUDCA. Following 72 hours of stimulation, we performed flow cytometry (gating strategy depicted in **(B)** and noted no significant effect of low- or high-dose TUDCA on T cell proliferation compared to vehicle **(C)**. We also noted no significant effects of TUDCA treatment on **(D)** interferon-gamma production or **(E)** T cell activation – based on CD44 expression ($CD44^{hi}$). Data is derived from a representative experiment (one of three independent experiments). Bars in C-E represent the mean. Error bars in C-E represent standard error of the mean.



Supplemental Figure 9. TUDCA treatment in EAE does not affect CNS T cell infiltration and phenotype

(A) C57/BL6 mice with EAE were treated at disease onset with either vehicle or TUDCA 500 mg/kg for one week and CNS tissue was obtained for evaluation of cellular infiltrate in the spinal cord. There was no difference in the infiltration of T cells **(B)** or CD4+ T cells **(C)** in the spinal cord between the two groups. We noted no differences in the proportion of CD4+ T cells producing pro-inflammatory cytokines – interferon gamma (IFNg) **(D)** or interleukin-17 (IL-17) **(E)**. There was also no difference in the proportion of regulatory T cells (CD4+ FoxP3+ $CD25^{hi}$) between the groups **(F)**. There was no difference in the infiltration of CD8+ T cells between the groups **(G)** and the proportion of IFNg-producing CD8+ T cells was also similar between the groups **(H)**. In B-H bars represent mean and error bars represent s.e.m. Data is derived from two independent experiments.