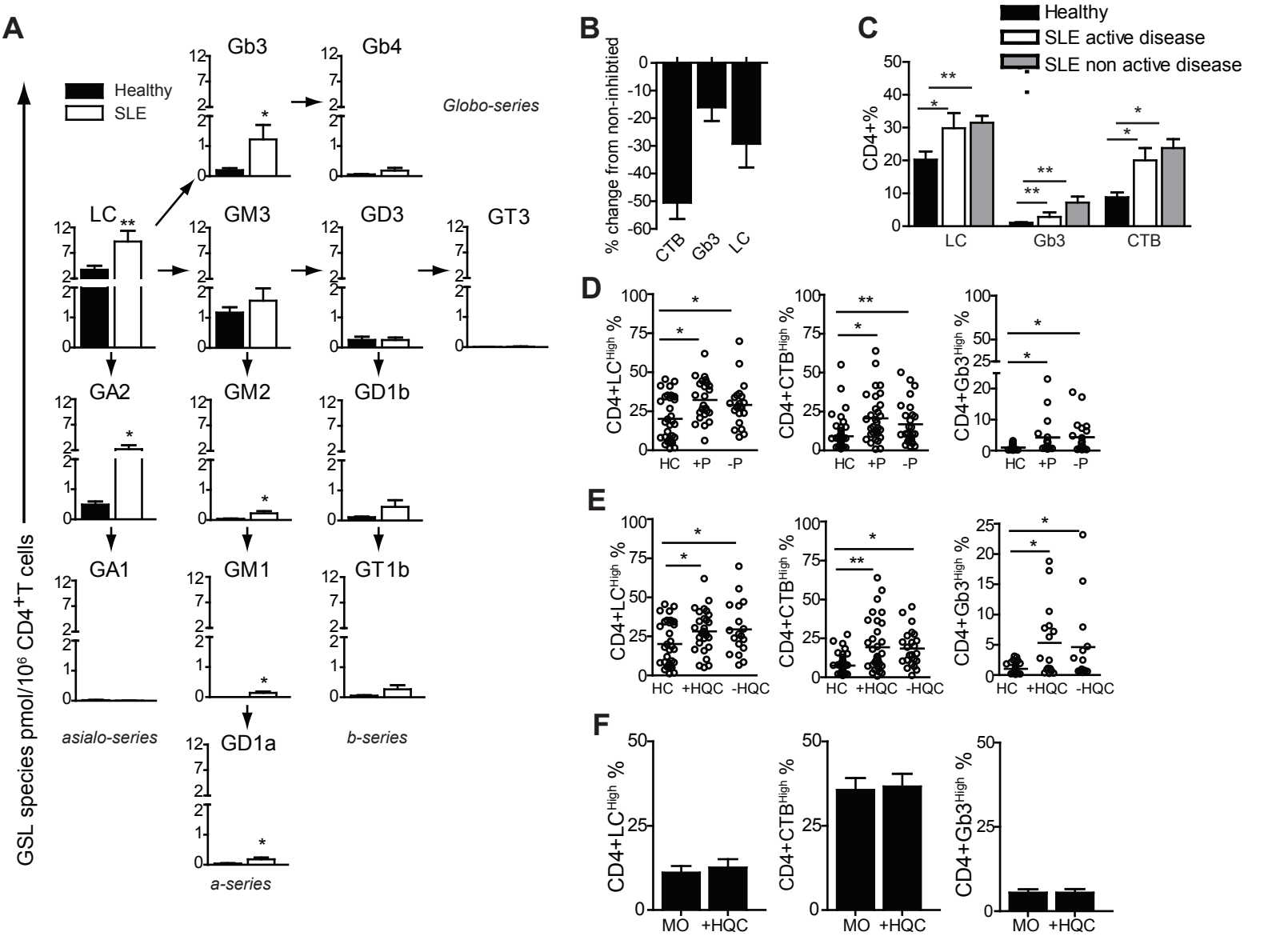
Supplemental Figure 1



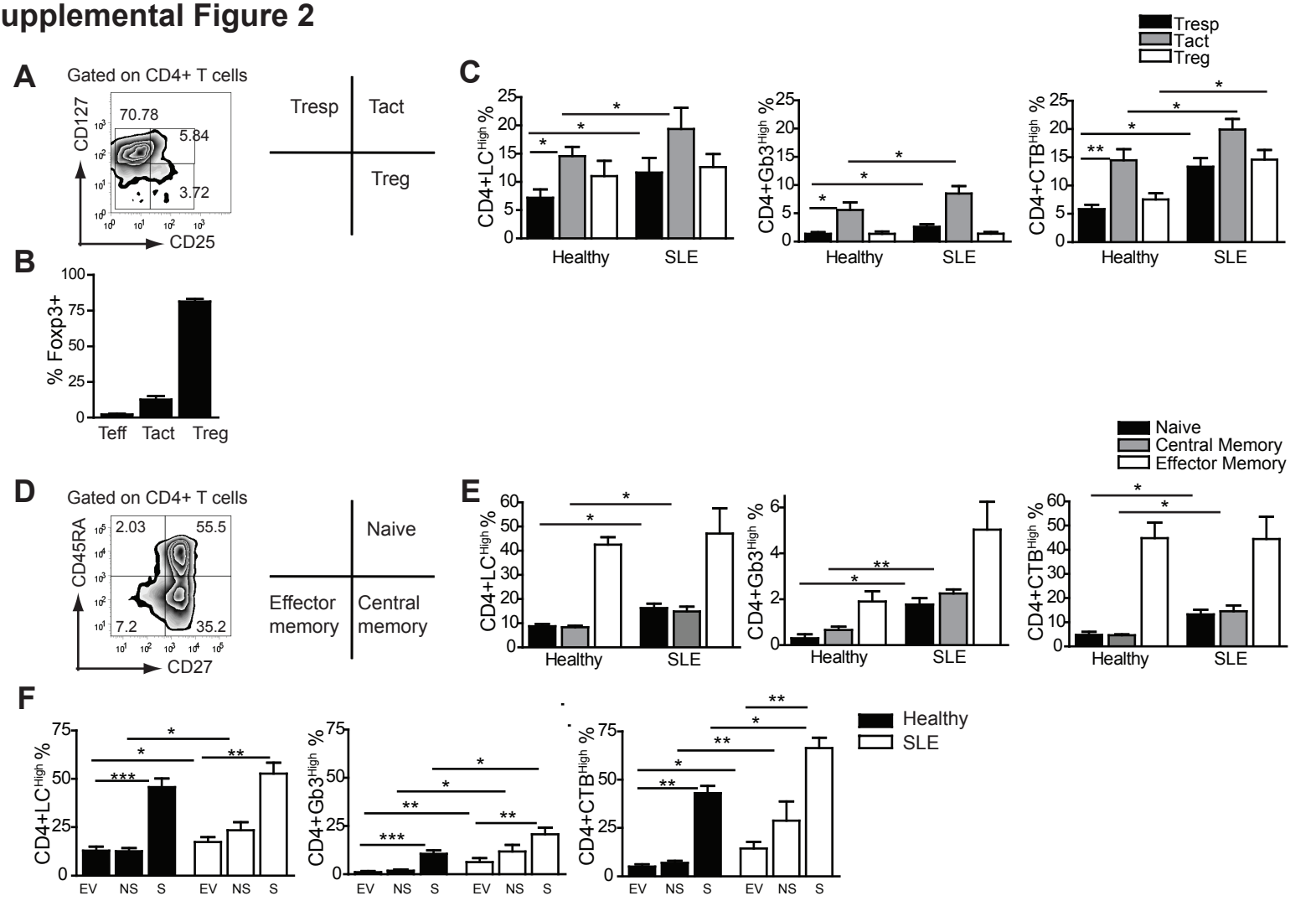
Increased expression of GSLs in T cells from patients with SLE. (**A**) The total cellular CD4⁺T cell GSL expression was detected by HPLC shown in Figure 1B and C. The results were organized according to their position in the GSL biosynthesis pathway (Figure 1A). Mean+SE, Mann Whitney two tailed test; **p=0.008; *p=<0.05.

Specificity of anti-GSL antibodies. Specificity of the anti-GSL antibodies was confirmed by culturing PBMC from 6 healthy donors for 3 days with or without D-PDMP (100μM), an inhibitor of GSL biosynthesis, together with anti-CD3/CD28 antibodies to stimulate GSL upregulation (1μg/ml). Cells were surface stained with anti-CD4-APC and relevant anti-GSL antibodies and assessed by flow cytometry. (**B**) Cumulative results showing percent change in GSL expression from T cells not treated with the inhibitor are shown. Mean+SE. LC= lactosylceramide.

GSL expression in T cells stratified for disease activity and treatment. Expression of plasma membrane GSLs was assessed in ex vivo CD4⁺T cells from 36 healthy donors and 58 patients with SLE with anti-LC-FITC, Gb3-FITC and cholera toxin-B (CTB)-FITC using flow cytometry. GSL expression in T cells was stratified for disease activity (**C**) and treatment (**D and E**). Patients with active disease (BILAG score greater than 6) and inactive disease (BILAG score less than 6) were compared to healthy controls. HC=healthy donors; +P= SLE patients treated with prednisolone; -P=SLE patients not treated with prednisolone but recieving other disease modifying therapy; +HQC= patients treated with hydroxychloroquine +/- other disease modifying therapy; -HQC= patients not treated with hydroxy-chloroquine +/- other immunosuppresive therapy. Mean+SE; One-Way ANOVA, **p=<0.001; *p=<0.05.

(**F**) PBMC from 5 healthy donors were cultured for 72 hours in either complete medium (MO) alone or 0.5μM hydroxychloroquine (+HQC). Cells were surface stained for anti-CD4-APC and relevant anti-GSL antibodies and assessed by flow cytometery. Mean+SE.

Supplemental Figure 2



Differential GSL expression in functional CD4$^+$T cell subsets.

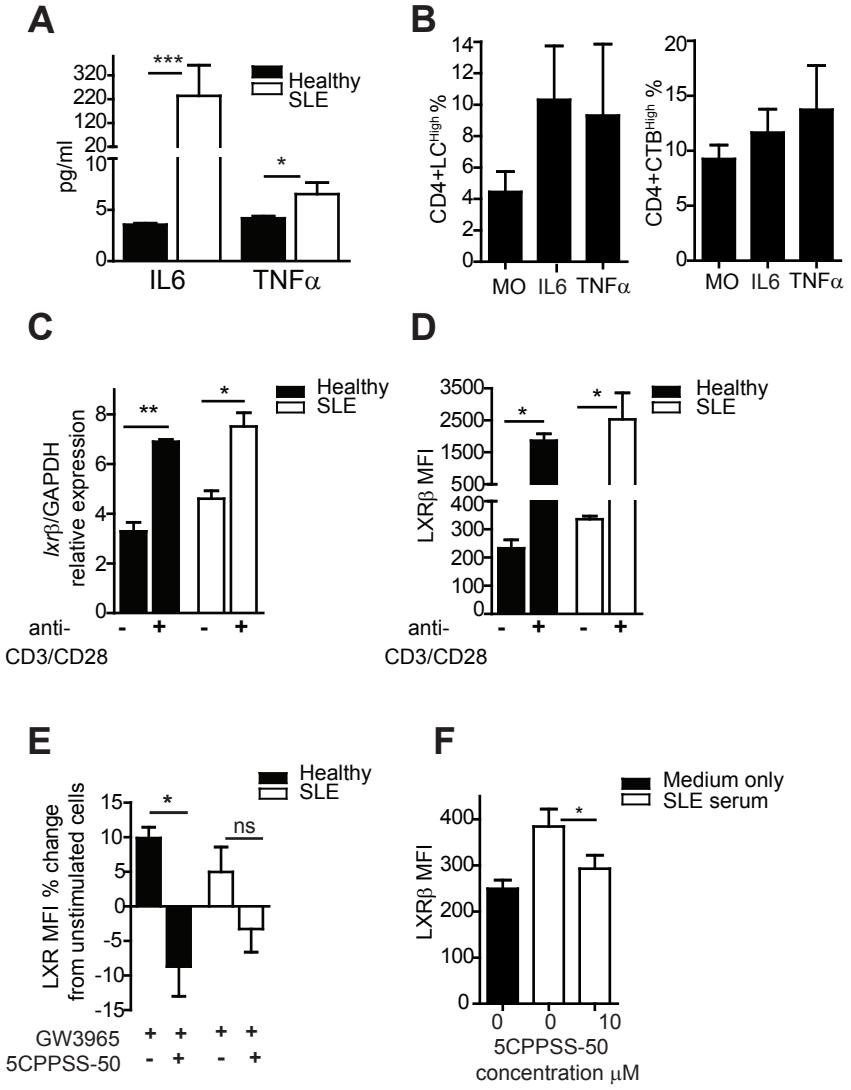
(**A**) Functional T cell subsets can be distinguished based on surface staining using fluorescently labeled antibodies to CD4-v450, CD25-PE-cy5 and CD127-PE-cy7. T cell subsets are defined as CD4+CD25-CD127+ (Responder T cells-Tresp), CD4+CD25+CD127+ (activated T cells-Tact) and CD4+CD25+CD127- (regulatory T cells-Treg). (**B**) The regulatory phenotype was confirmed by staining intracellularly for Foxp3 which is found predominantly in the regulatory subset.

(**C**) The expression of surface GSL was determined in *ex vivo* PBMC from 23 SLE patients and 21 healthy donors. Cells were stained using fluorescently labeled antibodies to CD4-v450, CD25-PE-Cy5, CD127-PE-Cy7 and either LC-FITC, Gb3-FITC or CTB-FITC and analyzed by flow cytometry. Cumulative data showing expression of GSLs in the T cell subsets. Mean+SE; One-way Anova and Two-tailed t test; *p=<0.05; **p=0.003.

(**D**) Naive and memory T cell subsets were distinguished based on surface staining using fluorescently labeled antibodies to CD4-v450, CD27-PE-cy5 and CD45RA-APCand either LC-FITC, Gb3-FITC or CTB-FITC and analyzed by flow cytometry. T cell populations were defined as CD4+CD45RA+CD27+ (Naive), CD4+CD45RA-CD127+ (Central Memory) and CD4+CD45RA-CD27- (Effector Memory). (**E**) The expression of surface GSL was determined in ex vivo PBMC from 10 SLE patients and 10 healthy donors. Cumulative data showing expression of GSL in the T cell subsets. Mean+SE; Two-tailed t test; *p=<0.05; **p=0.003.

(**F**) CD4$^+$T cells from 11 patients with SLE and 8 healthy donors were isolated by negative selection and cultured for 3 days ± antiCD3 and CD28 (1µg/ml). Cells were stained using antibodies to CD4-v450 and LC-FITC, Gb3-FITC or CTB-FITC. The expression of LC, Gb3 and CTB in ex vivo, and cultured cells were compared. EV: Ex vivo, NS: No stimulation, S: Stimulated.
One-way ANOVA ***p=0.0001; **p=0.001; *p=0.01 and Two-tailed t test; *p=<0.05; **p=<0.009

Supplemental Figure 3



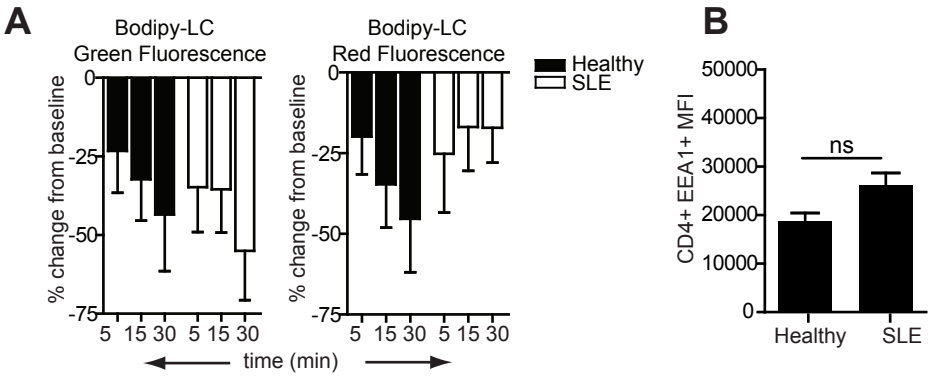
Proinflammatory cytokines do not influence GSL expression in T cells. (A) Increased levels of proinflammatory cytokines in the serum of patients with SLE. Serum from 12 patients with SLE and 8 healthy donors were analyzed by Cytokine Bead Array (CBA). (**A**) Bar chart showing cumulative results. Mean+SE; Two-tailed T test. ***p=0.0006; *p=<0.02.

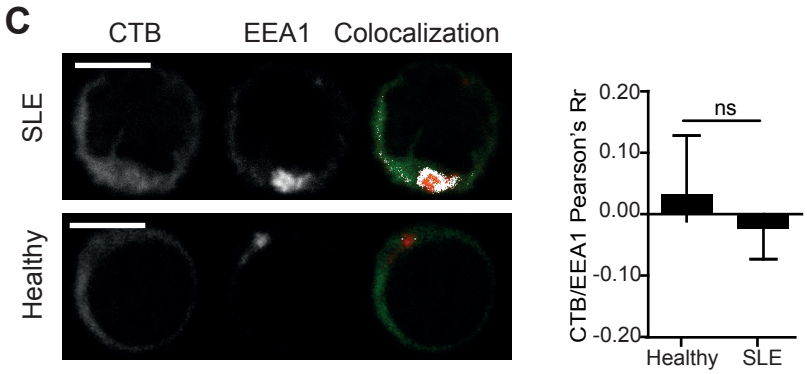
(**B**) PBMCs from six healthy donors were cultured in complete medium +/- rhIL6 (10ng/ml) and TNFα (10ng/ml) for 24hs before surface staining for CD4-v450 and CTB-FITC or LC-FITC. Bar charts showing cumulative data. Mean+SE.

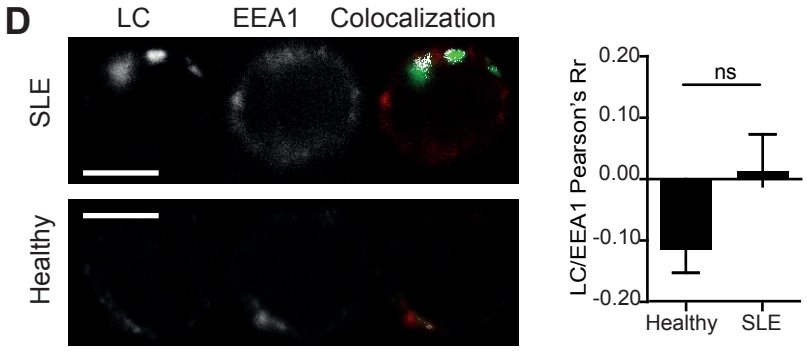
TCR stimulation upregulates LCRβ expression. Negatively isolated $CD4^+$T cells were cultured with and without anti-CD3/CD28 (1μg/ml) for 18hs. $2x10^6$ cells were lysed in Trizol and the expression of *lxr*β was assessed by qPCR. (**C**) Cumulative results are shown in relative units compared to a GAPDH control. Mean +SE. Paired T test, **p=0.005; *p=0.01.
(**D**) PBMC from five healthy donors and five SLE patients were stimulated for 18h with and without anti-CD3/CD28 (each at 1μg/ml). Cells were surface stained using anti-CD4-v450, fixed and permeabilised for 60min with Fix/Perm buffer (eBioscience) before intra-cellular staining for LXRβ using rabbit anti-human LXRβ and anti-rabbit-IgG-FITC before analysis by flow cytometry. Mean+SE, Two-tailed T test; *p=<0.01.

LXR antagonist (5C PPSS-50) inhibits LXRβ upregulation in response to synthetic oxysterol agonist GW3965 and SLE serum. (**E**) PBMCs from six healthy donors and six SLE patients were stimulated with and without GW3965 in the presence of LXR antagonist 5C PPSS-50 for 24 hs. Cells were fixed and stained as described in (**D**) and the expression of LXRβ in $CD4^+$T cells was assessed by flow cytometry. Results are expressed as % change from unstimulated cells (no GW3965). Mean+SE, Paired T test, *p=<0.01.
(**F**) Healthy PBMC were cultured with serum from five seperate different patients with SLE or medium alone for 10ds in the presence or absence of 5C PPSS-50. Cells were stained for LXRβ expression as described in (**D**). Mean+SE Paired T test, *p=0.04.

Supplemental Figure 4





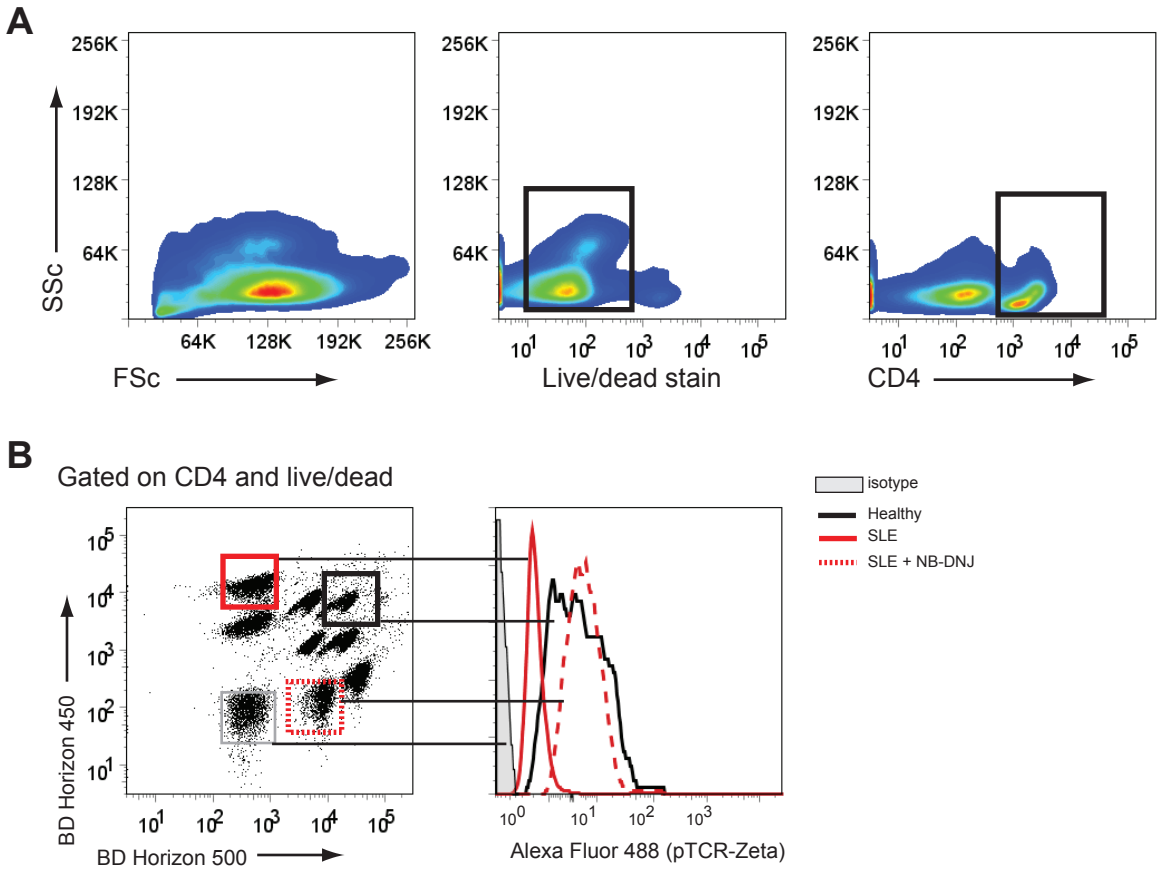


Incorporation of Bodipy-LC by CD4+T cells from SLE patients and healthy donors. Negatively isolated CD4+T cells were incubated with the fluorescently-labelled exogenous probe Bodipy-LC for 30 mins on ice before backwashing to remove unbound lipid. Cells were then cultured for 5, 15 and 30 min at 37^0C to allow endocytosis to proceed before fixing and analysis by flow cytometry. (**A**) Flow cytometry emissions at 520-560nm (green fluorescence) and >590nm (red fluorescence) expressed as percent change from baseline over the time course. Mean+SE.

Expression of early endosome marker EEA1 in T cells. (B) PBMC from three healthy donors and five SLE patients were surface stained for CD4-FITC before fixation and permeabilization. Cells were then stained with purified antibodies to early endosome antigen 1 (EEA1) followed by anti-mouse IgM-Alexa-633 secondary antibody before analysis by flow cytometry. Cumulative data showing mean+SE. Two-tailed t test p=0.0957

Colocalization of CTB and LC with early endosome marker EEA1. (**C & D**) $CD4^+$T cells were negatively isolated from six SLE patients and three healthy donors. Cells were fixed, permeabilized with 0.1% saponin and blocked using BSA and fish gelatin before staining with antibodies to EEA1 and either LC or CTB. Cells were analyzed by confocal microscopy. An average of 30 cells per sample were analyzed for (**C**) EEA1/CTB or (**D**) EEA1/LC colocalization assessed by calculating Pearson's correlation co-efficient (Rr) using ImageJ software. Representative images and cumulative colocalization data are shown. Mean +SE; two-tailed t test. Scale bar=5μM.

Supplemental Figure 5



Gating strategy for fluorescent bar-coding. (A) Whole PBMCs were labelled with Live/dead-PE-cy7 (Invitrogen) and gated on the 'live', (the negative staining population), followed by gating on $CD4^+$ T cells. (**B**) The $CD4^+$T cell population was then analysed for the expression of fluorescent bar coding reagents, BD Horizon 500 and 450 to give the 9 individual cell populations representing cells cultured under different stimulating conditions. Each cell population was individually gated and histograms prepared representing the staining for specific phosphorylated signaling molecules. All analysis was performed using FlowJo software.

Inhibition of T cell proliferation with NB-DNJ. (C) PBMC from three healthy donors and three patients with SLE were precultured for 24h with NB-DNJ (10μM) or medium alone. Negatively isolated $CD4^+$T cells were then stimulated with and without anti-CD3 and CD28 (each 1ug/ml), with and without NB-DNJ for 48 and 72h. Cells were pulse for 16h with [3H] thymidine followed by harvesting and scintillation analysis. Mean +SE, Two tailed T test, *p=<0.05; One-way ANOVA, *p=<0.01

Supplemental Methods

Lipid Extraction, GSL Glycan labeling and HPLC analysis

Negatively isolated $CD4^+$T cells (10^7) from healthy controls and SLE patients were washed in PBS pelleted, re-suspended in distilled water and subjected to three rounds of freezing and thawing with dry ice. Chlorofom:methanol (2:1v/v) was added and mixed well for 30mins at 21°C. The lipid-containing supernatants were separated from protein precipitate by centrifugation, a 1:1 mixture of chloroform:PBS was added to the lipid extract and samples centrifuged to separate lipid layers into lower and upper fractions. The lower extract was dried in a gaseous N_2 stream, resuspended in chloroform:methanol 1:3 v/v and dried again before finally resuspending in chloroform:methanol 1:3 v/v and adding to the upper phase extract. Lipid extracts were purified from non-hydrophobic contaminants using SepPak C18 column solid-phase extraction columns (Waters). The glycan headgroups of the GSL were cleaved from the ceramide backbone using ceramide glycanase purified in house from *Hirudo medicinalis*. The released glycans were labeled with the fluorescent marker anthranilic acid and were analyzed by normal phase-HPLC as described in (25). All chromatography was controlled, and data collected and processed, using Waters Millenium or Empower software. GSLs were identified by comparing retention times of different species to external standards. The levels of GSL were quantified from the peak HPLC areas after applying an experimentally-derived response factor (1); results were expressed as pmol per 10^6 $CD4^+$T cells.

Detection of phosphorylated signaling proteins and fluorescent bar-coding

PBMCs from SLE patients and healthy donors were cultured in CM with 10μM NB-DNJ or CM for 7ds. Cells were stained with Live/Dead-PE-Cy7 (Invitrogen) for 20min at 21°C, washed and labeled with 1μg/ml anti-CD3/CD28 in PBS alone for 30min on ice. After

washing with ice-cold PBS, anti-CD3/CD28 antibodies were cross-linked using goat anti-mouse IgG $F(ab')_2$ (2µg/10^6 cells (Sigma-Aldrich)) on ice, washed and samples re-suspended in warm PBS prior to incubation at 37°C for various time points between 0 and 10min. Stimulation was stopped by adding 2× Fix/Perm buffer (eBioscience) and cells were re-suspended in bar-coding dye dilutions, prepared according to the manufacturer's instructions (BD Biosciences). After washing, bar-coded cells were combined and stained for either [CD4-APC, TCR-ζ-Alexa Fluor 488 and ERK-PE] or [CD4-APC, NF-κB-Alexa Fluor 488 and Akt-PE] (All BD Biosciences). An aliquot of bar-coded cells stained only for CD4 was also run as a fluorescence-1 control. Cells were washed, data collected on a LSRII (Beckton Dickinson) and analyzed with FlowJo (TreeStar).

Measurement of GSL trafficking

Internalization of Bodipy-LC: $CD4^+$T cells from SLE patients and healthy donors were isolated by negative selection and either labeled ex vivo or cultured overnight at 37°C, 5% CO_2 in CM or 10µM NB-DNJ. Samples were then incubated with Bodipy-LC (10µg/ml) (Invitrogen) on ice for 30min. Cells were back-washed in ice cold CM to remove free Bodipy-LC, and incubated at 37°C over a time course of 1 to 30min. Samples were fixed in 4% paraformaldehyde and analyzed by confocal microscopy and flow cytometry (excited 450-490nm, absorption maxima 505nm and emission measured at 520-560nm–green fluorescence or >590nm-red fluorescence). For inhibition of endocytosis cells were pre-incubated with either Dynasore (Invitrogen) (50µM) or src kinase inhibitor PP2 (Calbiochem) (50µM) for 30min before treatment with Bodipy-LC.

Internalization of endogenous plasma membrane GSLs (2, 3): PBMCs were stimulated with PMA (10ng/ml) to force maximal plasma membrane GSL surface expression. Cells were washed and surface stained with CTB-FITC in PBS with 1% FCS on ice. Cells were washed

again on ice then re-suspended in warm (37°C) CM and incubated at 37°C for 1, 3 or 5min to allow endocytosis of CTB-stained lipids. Cells were then washed in ice cold neutral (pH 7.0), or acidic (pH 2.0) PBS to remove remaining surface CTB conjugated lipids, fixed with 2% PFA and surface stained with CD4-v450, before analysis by flow cytometry. The rate of CTB internalisation was calculated as a ratio of CTB MFI acid wash/PBS wash x 100. Cells were pre-incubated with inhibitors of endocytosis (Dynasore and PP2, 50μM) as a control.

Recycling of endogenous GSLs to the cell surface from intracellular compartments (4, 5): PBMCs were incubated with unconjugated CTB to block endogenous cell surface molecules. The cells were incubated at 37°C for 5, 10 and 15min to allow recycling of intracellular GSL molecules to the cell surface. Newly recycled GSLs were detected by surface staining with CTB-FITC; cells were stained with CD4-v450 and assessed by flow cytometry. Cells were pre-incubated with 2mM monensin (Golgi-Plug; BD Biosciences), blocked and fluorochrome stained without a 37°C incubation, or unblocked and fluorochrome stained as controls.

Quantitative immunoblots

$CD4^+$T cells (10^7) from healthy individuals and SLE patients were lyzed (Cell Signaling Technology). Lysates were run on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto a PVDF membrane (Millipore), and probed with mouse-anti-LXRβ (abcam) or anti-Actin mAb (Sigma-Aldrich). Following incubation with the appropriate HRP-conjugated secondary antibody and extensive washing, blots were developed using ECL substrate (Amersham) and exposure to high performance chemiluminescence film (Amersham) with development in a Compact X4 Automatic X-ray Film Processor (X-ograph imaging systems). Adobe Photoshop was used for quantification (Adobe Systems Incorporated, USA).

Confocal Microscopy

$CD4^+$T cells from healthy donors and SLE patients were fixed with 4% PFA, permeabilized with 0.1% saponin and blocked with 5% BSA/0.2% fish gelatin in PBS before staining for lysosomes (anti-LAMP1 IgG1, anti-IgG1-Alexa 555), early endosomes (anti-EEA1 IgG, anti-IgG-Alexa 633) and either LC (anti-IgM, anti-IgM-Alexa 488) or CTB-FITC. An average of 30 cells was imaged from each sample. Images were taken with a TCS SPE (Leica Microsystems, Wetzlar, Germany) with a 63x, 1.25NA oil immersion objective lens using 488nm argon-ion, 532nm helium, and 400nm ultraviolet laser excitation. The Pearson correlation coefficient for anti-LAMP1 and anti-LC or CTB staining was measured in composite merged images using ImageJ software (NHS, UK).

Internalization of Bodipy-LC by confocal microscopy used excitation with a 488nm argon-ion laser and emission detected at 520-560nm and >590nm.

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Supplemental Table 1. Summary of healthy donor and SLE patient clinical data

	Healthy (n=144)	SLE (n=178)
Mean Age (Range)	45.1 (20-77)	45.1 (19-77)
Sex: female:male	123:21	156:22
Sex: % female:male	85.7:14.3	(87.9:12.1)
Ethnicity: C/A/AC/O	112/17/13/2	73/32/53/20
Ethnicity: % C/A/AC/O	77.9/11.7/9.1/1.3	41.1/18.1/29.5/11.3
Mean Total Cholesterol (Range) NR: 2.5-5mmol/L	ND	4.8 (2.5-7.3)
Mean Triglycerides (Range) NR: 0.4-2.3mmol/L	ND	1.11 (0.4-2.8)
Mean HDL (Range) NR: 1.2-1.7 mmol/L	ND	1.67 (0.8-3.0)
Mean LDL (Range) NR: 0-3.5 mmol/L	ND	2.62 (0.8-4.8)
Hydroxychloroquine (%)	ND	34 (19.1)
Hcq + Disease modifying agents (%)	ND	12 (7)
Pred < 10mg/day ± hcq (%)	ND	45 (25.1)

Pred > 10mg/day ± hcq (%)	ND	13 (7.2)
Pred + Disease modifying agents (%)	ND	74 (41.6)

SLE patients fulfilling the revised classification criteria for SLE were assessed for disease activity with the British Isles Lupus Assessment Group index (BILAG)(1). The BILAG activity index distinguishes activity in 9 organs/systems (2). The component scores (from A, most active to E, never active) can be converted into a global score using the following system; A=12, B=8, C=1, D/E=0. Patients with active SLE disease (BILAG global score >6) were compared to patients without active disease (BILAG global score <6). No patients in the study were treated with intravenous immunoglobulin (IVIG), rituximab or statins. Age and sex-matched healthy controls were studied in parallel as well as age-matched Rheumatoid Arthritis and Sjögren's syndrome patients as other autoimmune disease (OAD) controls. Patients and controls were recruited after providing informed consent. The following abbreviations are used: Caucasian (C), Asian (A), African Caribbean (AC), Other (O), prednisolone (pred) and hydroxychloroquine (hcq), not determined (ND), systemic lupus erythematosus (SLE), normal range (NR).

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