SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

Human subjects

39 SLE patients fulfilling the American College of Rheumatology diagnostic criteria were studied (1). All patients were female. Age of 39 SLE patients was 42.4±1.7 years, while those of 37 HC subjects was 40.6±2.1 years. Disease activity was assessed by the SLE disease activity index (SLEDAI) scores (2), which ranged from 0 to 28 (Mean ± SEM: 6.79±0.85). Mean daily prednisone dose was 6.15±1.07 mg. Immunosuppressive drugs taken by the study subjects included hydroxychloroquine (N=39), mycophenolate mofetil (N=9), mycophenolic acid (N=2), azathioprine (N=9), methotrexate (N=2), belimumab (N=7), and anakinra (N=1). The demographics and clinical characteristics of study subjects are summarized in Table S1. In each experiment, peripheral blood was obtained from SLE patients and healthy control subjects that have been matched for age within 10 years, ethnic background, and gender and processed in parallel.

Surface and intracellular staining and culture of $CD3^+$ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll Histopaque gradient (GE Health Care Bio-Sciences). $CD3^+$ T cells were isolated by negative selection using untouched human T cell isolation kit (Life technologies). Purity of $CD3^+$ T cells was confirmed to be above 97%. Cells were stained with PE Cy7-conjugated anti-CD4 (SK3), PerCP Cy5.5-conjugated anti-CD8 (RPA-T8), and brilliant violet 510 (BV510)-conjugated anti-CD25 (M-A251: all from BD Biosciences). The cells were permeabilized as per the manufacturer's instructions and stained with AF-647-conjugated anti-FoxP3 (259D/C7: BD Biosciences) and

PE-conjugated anti-GATA-3 (TWAJ: eBioscience). The remainder of cells were cultured in RPMI culture media with 10% FCS, 1% Penicillin/Streptomycin, and 1% L-glutamine (all from Corning CellGro except for FCS, which was from Gibco) for 3 days in the presence of plate-bound anti-CD3 (anti TCR ε hybridoma from ATCC) and soluble anti-CD28 (1 μg/ml, CD28.2: BD Biosciences) with or without rapamycin (100 nM).

Treg polarization

Naive $CD4^+$ T cells were isolated from SLE and matched healthy control subjects by using Human Naive $CD4^+$ T cell Enrichment Kit (STEMCELL). The purity of naive $CD4^+$ T cells as defined by the proportion of $CD4^+CD45RA^+CD62L^+$ cells was above 99%. Cells were cultured for 24 or 72 hours in the presence of plate-bound anti-CD3, soluble anti-CD28 (1 μg/ml), TGF-β (5 ng/ml,), and IL-2 (50 IU/ml) with or without IL-6 (100 ng/ml), IL-17 (0-100 mg/ml), IL-21 (10 ng/ml), or rapamycin (100 nM). All cytokines were obtained from Peprotech. Cells were stained with FITC-conjugated anti-CD25 (M-A251: BD Biosciences), PE-conjugated anti-GATA-3, and AF-647-conjugated anti-FoxP3. Frequency of $CD4^+CD25^+FOXP3^+$ cells and GATA-3 expression in $CD4^+CD25^+FOXP3^+$ cells were determined by flow cytometry. Isotype control antibodies included PE-conjugated rat IgG2b kappa (eB149/10H5: eBioscience) and APC-conjugated mouse IgG1 (MOPC-21: BD Biosciences).

Effector and memory $CD4^+$ T cell stimulation

Magnetically isolated naïve $CD4^+$ T cells were cultured for 24 hours in the presence of anti-CD3/CD28 to induce effector $CD4^+$ T cells. Memory $CD4^+$ T cells were isolated from SLE and matched healthy control subjects by using Human Memory $CD4^+$ T cell Enrichment Kit

(STEMCELL), and stimulated in the presence of anti-CD3/CD28 for 24 hours. The purity of memory $CD4^+$ T cells as defined by the proportion of $CD4^+CD45RA^-CD45RO^+$ cells was about 80%. During effector and memory $CD4^+$ T cell stimulation, half of the cells were treated with IL-21 (10 ng/ml).

Treg suppression assay

$CD4^+CD25^-$ responder T cells (Tresp cells) and autologous $CD4^+CD25^+$ Tregs were magnetically isolated from the peripheral blood of HC and SLE donors using Complete Kit for Human $CD4^+CD25^+$ Treg cells (STEMCELL). Tresp cells were stained with 0.2 μM of CFSE (Thermo Fisher Scientific). A portion of the $CD4^+CD25^+$ Treg cells were stained with PE-conjugated anti-CTLA-4 (L3D10: BioLegend) and AF-647-conjugated anti-FoxP3 to determine CTLA-4 expression in the $CD4^+CD25^+FOXP3^+$ cells. The CFSE-stained Tresp cells were cultured for 5 days in the presence of plate-bound anti-CD3 and irradiated autologous PBMCs (1200 rad) with or without IL-21 (100 ng/ml) in the presence or absence of equal number of $CD4^+CD25^+$ Treg cells. Proliferation of Tresp cells was determined by calculating the division index of $CFSE^+$ cells using FlowJo software. Treg suppressive function was determined by calculating % suppression of division index. After 5 days of coculture, cells were stained with PE-conjugated anti-CTLA-4 or allophycocyanin-conjugated anti-CD28 (CD28.2) antibodies before permeabilization as well as PE-conjugated-GATA-3 and AF-647-conjugated-FoxP3 antibodies after permeabilization.

Treg expansion

$CD4^+CD25^+$ Tregs from SLE patients were cultured for 4 weeks in the presence of plate-bound anti-CD3, soluble anti-CD28, and IL-2 (100 IU/ml; omitted in the first week of culture) with or without rapamycin (100 nM). Culture media were changed every week. After 4 weeks of expansion, Tregs were used for suppression assay as described above.

Immunoblotting

Using lysates of naïve $CD4^+$ T cells cultured under Treg-polarizing conditions with or without IL-21 or rapamycin or SLE Tregs expanded *in vitro* in the presence or absence of rapamycin, total STAT3 (124H6, #9139) and its phosphorylation at tyrosine 705 ($pSTAT3^{Y705}$, #9131), total Akt (11E7, #4685) and its phosphorylation at Serine 473 ($pAkt^{Ser473}$, D9E, #4060), phosphorylation of S6K1 at Threonine 389 ($pS6K1^{Thr389}$, MAB8963), phosphorylation of 4E-BP1 at Threonine 37 and 46 (p4E-BP1, 236B4, #2855), FOXP3 (150D, #320001), and LC3 expression (LC3B antibody, #2775, LC3A/B antibody, #4108) were determined by immunoblotting (all from Cell Signaling Technology except for anti $pS6K1^{Thr389}$, which was from R&D systems, and anti FOXP3, which was from BioLegend). The signal intensity was normalized to that of actin (MAB1501: Millipore). For LC3 detection, lysates that went through only one cycle of freezing and thawing were used for immunoblotting.

GATA-3 overexpression

Adenoviruses that express GATA-3 isoform 2 or GFP were purchased from Vector Biolabs. Naïve $CD4^+$ T cells from SLE subjects were transfected with these viruses and cultured for 16

hours under Treg-polarizing conditions. Expression of GATA-3 (HG3-31, #sc-268, Santa Cruz Biotechnology) and FOXP3 were determined by immunoblotting.

LUMINEX assay

Using culture supernatants of $CD3^+$ T cells simulated *in vitro* for 3 days or $CD4^+CD25^+$ Tregs expanded *in vitro* for 4 weeks in the presence or absence of rapamycin, secretion of IL-2, IL-4, IL-6, IL-10, IL-17, IL-21, IFN-γ, and TGF-β were determined by LUMINEX assay (affymetrix eBioscience) as per the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed with the Student's t-test using GraphPad software (San Diego, CA). Differences were considered significant at two-tailed p values < 0.05.

RERERENCES

1. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25(11):1271-7.
2. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum. 1992;35(6):630-40.

TABLE S1. Demographics and clinical characteristics of study subjects

Number of SLE subjects	39
Age (mean ± SEM)	42.4± 1.7
Female gender	39 (100%)
Caucasian	37 (94.9%)
SLEDAI (mean ± SEM)	6.79 ± 0.85
Mean prednisone dose (mean ± SEM)	6.15 ±1.07 mg
SLE manifestations	
Neuropsychiatric	6 (15.4%)
Serositis	28 (71.8%)
Pulmonary	0
Renal	5 (12.8%)
Hematological	4 (10.3%)
Arthritis	38 (97.4%)
Mucocutaneous	36 (92.3%)
Medications	
hydroxychloroquine	39 (100%)
mycophenolate mofetil	9 (23.1%)
mycophenolic acid	2 (5.1%)
azathioprine	9 (23.1%)
methotrexate	2 (5.1%)
belimumab	7 (17.9%)
anakinra	1 (2.6%)
Number of healthy control subjects	37
Age (mean ± SEM)	40.6±2.1
Female gender	37 (100%)
Caucasian	35 (94.6%)

LEGENDS TO FIGURES S1-S7

FIGURE S1. Neither IL-6 nor IL-17 inhibits human Treg differentiation. (**A**) Naïve $CD4^+$ T cells were isolated from peripheral blood of a healthy subject and cultured for 3 days in the presence of anti-CD3/CD28, TGF-β (5 ng/ml), and IL-2 (50 IU/ml) with or without IL-6 (100 ng/ml) or IL-21 (10 ng/ml). Flow cytometry dot plots showing $CD4^+CD25^+FOXP3^+$ Tregs. Numbers below the dot plots represent the frequency of $CD4^+CD25^+FOXP3^+$ Tregs. (**B**) Naïve $CD4^+$ T cells from a healthy subject were cultured for 7 days in the presence of anti-CD3/CD28, TGF-β (5 ng/ml), IL-2 (50 IU/ml), and various concentrations of IL-17 (0-100 ng/ml). Flow cytometry dot plots showing $CD4^+CD25^+FOXP3^+$ Tregs. Numbers below the dot plots represent the frequency of $CD4^+CD25^+FOXP3^+$ Tregs. (**C**) Naïve $CD4^+$ T cells from a healthy subject were cultured for 3 days under Treg-polarizing conditions with or without IL-21 or IL-6. Expression of $pSTAT3^{Y705}$, $pAkt^{Ser473}$, and $p4E\text{-}BP1^{Thr37/46}$ were examined by immunoblotting.

FIGURE S2. Autophagy is diminished in Tregs and memory $CD4^+$ T cells, but increased in effector $CD4^+$ T cells in SLE. (**A**) Naïve $CD4^+$ T cells were cultured under Treg-polarizing conditions or only with anti-CD3/CD28 stimulation to induce effector T cells. Magnetically isolated memory $CD4^+$ T cells were stimulated with anti-CD3/CD28. After 24 hours of culture, autophagy was examined by immunoblotting. Representative immunoblot staining of LC3 from HC and SLE subjects (left). Teff and Tmem denote effector and memory $CD4^+$ T cells, respectively. Cumulative LC3-II/LC3-I ratios from 11, 5, and 3 pairs of matched HC and SLE subjects were presented for Treg, Teff, and Tmem cells, respectively (right). (**B**) Naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 for 24 hours with or without IL-21 (10 ng/ml). Representative immunoblot staining of LC3 from HC and SLE subjects (left). Cumulative LC3-

II/LC3-I ratios from 3 pairs of matched HC and SLE subjects (right). (**C**) Memory $CD4^+$ T cells were stimulated with anti-CD3/CD28 for 24 hours with or without IL-21 (10 ng/ml). Representative immunoblot staining of LC3 from HC and SLE subjects (left). Cumulative LC3-II/LC3-I ratios from 2 HC and 1 SLE subjects (right). Data were analyzed by a paired t-test (*p<.0.05, **p<0.01, ***p<0.001).

FIGURE S3. IL-21 suppresses GATA-3 expression in Tregs cocultured with Tresp cells. CFSE-stained $CD4^+CD25^-$ Tresp cells from HC and SLE donors were cultured for 5 days with irradiated PBMCs and $CD4^+CD25^+$ Tregs in the presence or absence of IL-21 as described in **Fig 1**. Representative flow cytometry dot plots showing FOXP3 versus GATA-3 expression in $CFSE^-$ cells (upper panel). Numbers below the dot plots represent the frequency $FOXP3^+CATA\text{-}3^+$ cells. Cumulative data of frequency of $GATA\text{-}3^+$ cells, $FOXP3^+GATA\text{-}3^+$ cells, and $FOXP3^-GATA\text{-}3^+$ cells among the $CFSE^-$ cells from 8 pairs of matched HC and SLE subjects (lower panel). Data were analyzed by a paired two-tailed t-test (*, p<0.05; **, p<0.01; ***p<0.001).

FIGURE S4. IL-2 expands $FOXP3^+GATA\text{-}3^+$ and $FOXP3^+CTLA\text{-}4^+$ Tregs. CFSE-stained $CD4^+CD25^-$ Tresp cells from HC and SLE donors were cultured for 5 days with irradiated PBMCs and $CD4^+CD25^+$ Tregs in the presence or absence of IL-21 (100 ng/ml) or IL-2 (100 IU/ml). Flow cytometry dot plots showing FOXP3 versus GATA-3 expression in $CFSE^-$ cells (upper panel). The top and bottom numbers below the dot plots represent MFI of GATA-3 and the frequency of $GATA\text{-}3^+$ cells, respectively. Flow cytometry dot plots showing FOXP3 versus CTLA-4 expression in $CFSE^-$ cells (middle panel). The top and bottom numbers below the dot

plots represent MFI of CTLA-4 and the frequency of CTLA-4$^+$ cells, respectively. MFI of FOXP3 was normalized to that of IL-2-untreated cells. MFI of GATA-3 and CTLA-4 in CFSE$^-$ FOXP3$^+$ cells was normalized to that of IL-2-untreated cells. Cumulative data from 1 HC and 1 SLE subjects (lower panel). Data were analyzed by a paired two-tailed t-test (*$p<0.05$).

FIGURE S5. Selective mTORC1 blockade does not induce Treg differentiation from naïve human CD4$^+$ T cells. Naïve CD4$^+$ T cells from SLE and matched healthy control subjects were cultured for 72 hours under Treg-polarizing conditions with or without IL-21 (10 ng/ml) or rapamycin (100 nM). **(A)** Flow cytometry dot plots showing CD4$^+$CD25$^+$FOXP3$^+$ Tregs. Numbers below the dot plots represent the frequency of CD4$^+$CD25$^+$FOXP3$^+$ Tregs. **(B)** Expression of FOXP3, pAkt^{Ser473}, and p4E-BP1$^{Thr37/46}$ were determined by immunoblotting.

FIGURE S6. Rapamycin blocks IL-21 secretion, but restores diminished TGF-β secretion by SLE CD3$^+$ T cells. (A) CD3$^+$ T cells from matched HC and SLE subjects were stimulated for 3 days in the presence of anti-CD3/CD28 with or without 100 nM rapamycin. Secretion of IL-2, IL-4, IL-6, IL-10, IL-17, IL-21, IFN-γ, and TGF-β in T-cell culture supernatants from 9 pairs of HC and SLE subjects were determined by LUMINEX assay. Data were analyzed by a paired two-tailed t-test (*, $p<0.05$; **, $p<0.01$; ****$p<0.0001$). **(B)** IL-21 activates mTOR, which suppresses autophagy. In turn, autophagy induces the expression of TGF-β, which is indispensable for FOXP3 induction. FOXP3 directly controls GATA-3 and CTLA-4 expression that are critical for stable lineage commitment and function of Tregs. Taken together, IL-21-driven mTOR-dependent suppression of autophagy drives developmental and functional defects of SLE Tregs.

FIGURE S7. IL-21-mTOR axis drives developmental and functional defects of SLE Treg. In responder T (Tresp) cells, such as Th17 or Tfh cells, IL-21 elicits mTORC1 activation, which selectively phosphorylates 4E-BP1, but not S6K1. In turn, mTORC1 drives IL-21 production by Tresp cells. Treg cells suppress Tresp cells by blocking costimulatory signals to CD28 via CTLA-4-driven downregulation/transendocytosis of CD80/CD86 on antigen presenting cells (APCs). Accordingly, IL-21-mediated downregulation of CTLA-4 in Treg cells leads to reciprocal upregulation of CD28 in Tresp cells, which culminates in mTORC1 activation. Collectively, inflammatory Tresp cells expand their own lineages via IL-21->mTORC1->IL-21 auto-amplifying loop. In Treg cells, IL-21-mediated mTORC1 and mTORC2 activation results in phosphorylation of 4E-BP1 and Akt, leading to suppression of autophagy and expression of FOXP3, GATA-3, and CTLA-4. Additionally, mTORC1 suppresses TGF-β expression by Treg cells, and thereby further diminishes FOXP3 expression. As such, IL-21-mTOR axis abrogates Treg differentiation and function. Rapamycin blocks the IL-21->mTORC1->IL-21 pro-inflammatory loop in Tresp cells, and restrains their expansion. In Treg cells, rapamycin blocks mTORC1 and mTORC2, and thereby restores their autophagy, TGF-β, FOXP3, GATA-3, and CTLA-4 expression, and suppressive function. Components highlighted in red and blue indicate upregulated and downregulated in SLE, respectively.

Figure S1

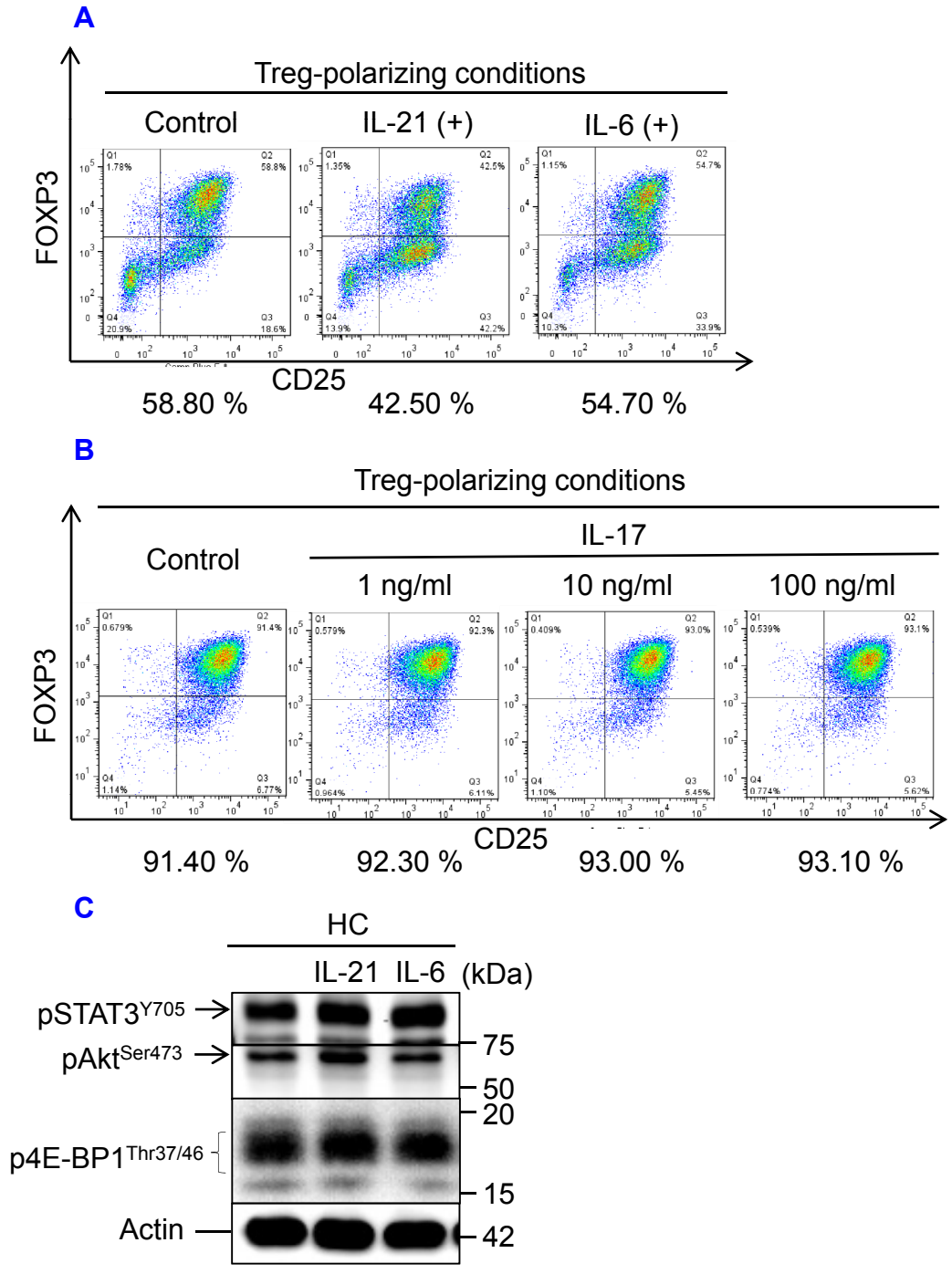

A
Treg-polarizing conditions
Control
IL-21 (+)
IL-6 (+)
FOXP3
CD25
58.80 %
42.50 %
54.70 %
B
Treg-polarizing conditions
Control
IL-17
1 ng/ml
10 ng/ml
100 ng/ml
FOXP3
CD25
91.40 %
92.30 %
93.00 %
93.10 %
C
HC
IL-21
IL-6
(kDa)
pSTAT3Y705
pAktSer473
p4E-BP1Thr37/46
Actin
75
50
20
15
42

Figure S2

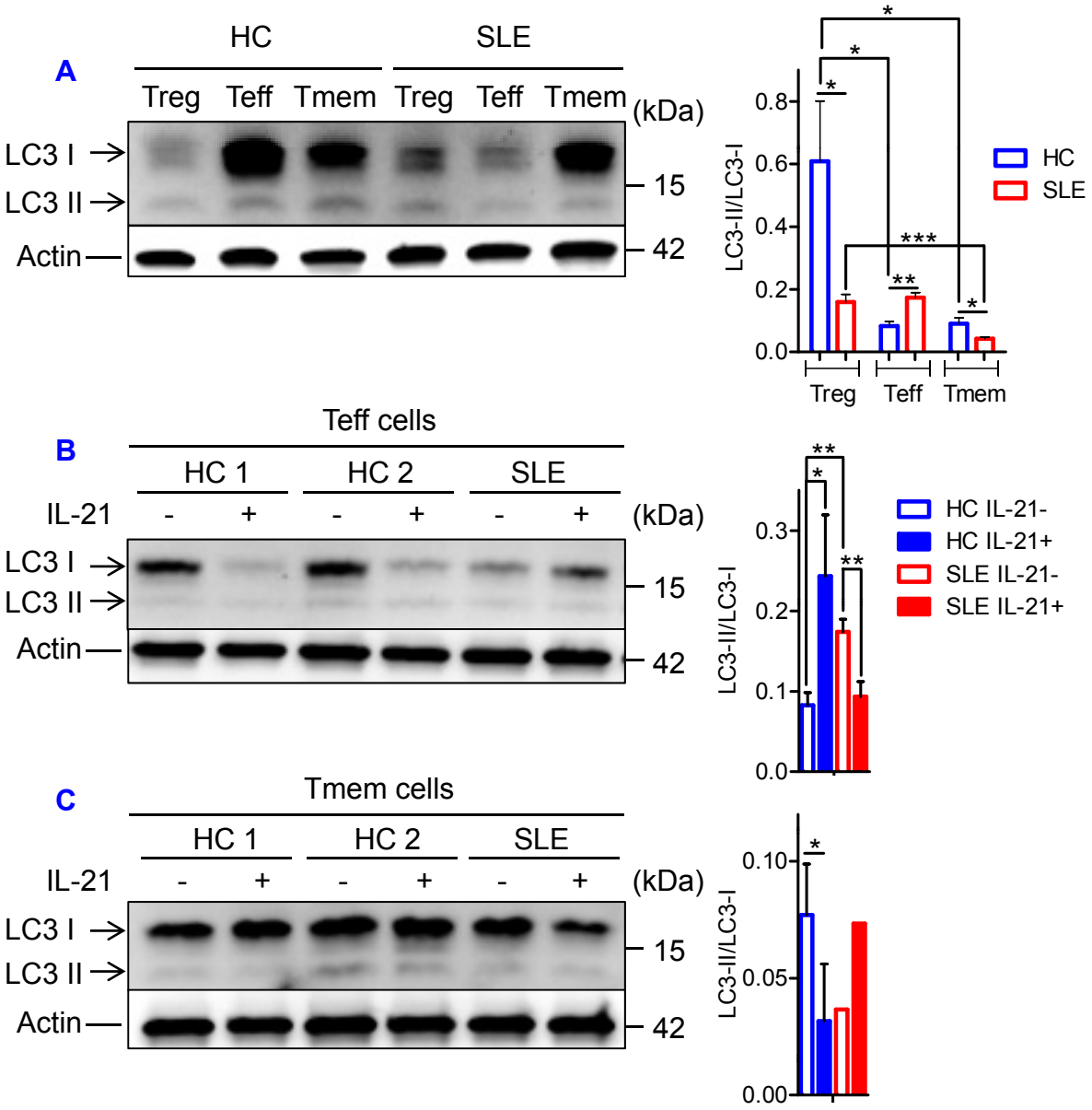

A
HC
SLE
Treg
Teff
Tmem
Treg
Teff
Tmem
(kDa)
LC3 I
LC3 II
Actin
15
42
LC3-II/LC3-I
HC
SLE
Treg
Teff
Tmem
B
Teff cells
HC 1
HC 2
SLE
IL-21
- + - + - +
(kDa)
LC3 I
LC3 II
Actin
15
42
LC3-II/LC3-I
HC IL-21-
HC IL-21+
SLE IL-21-
SLE IL-21+
C
Tmem cells
HC 1
HC 2
SLE
IL-21
- + - + - +
(kDa)
LC3 I
LC3 II
Actin
15
42
LC3-II/LC3-I

Figure S3

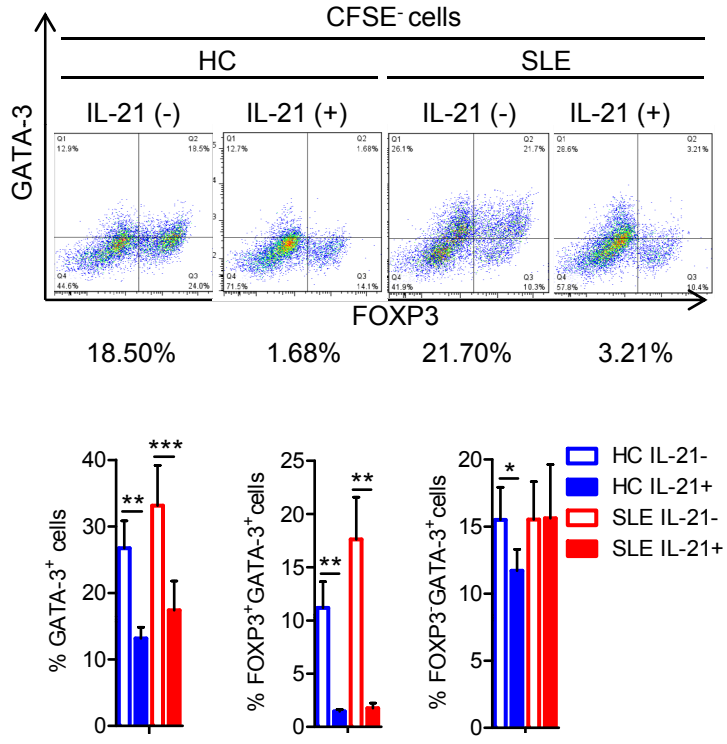
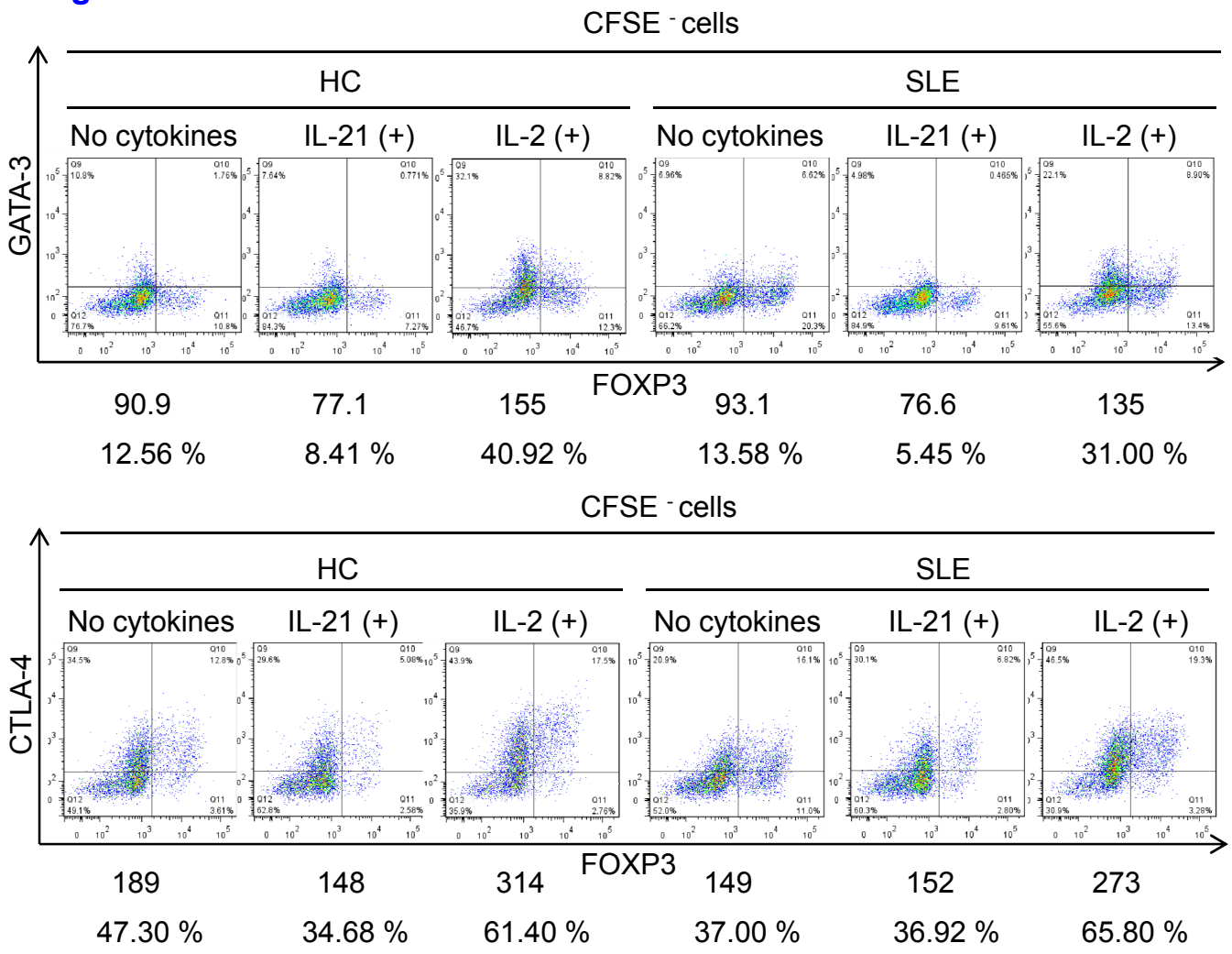

CFSE- cells
HC
SLE
IL-21 (-)
IL-21 (+)
IL-21 (-)
IL-21 (+)
GATA-3
FOXP3
18.50%
1.68%
21.70%
3.21%
% GATA-3+ cells
% FOXP3+GATA-3+cells
% FOXP3-GATA-3+cells
HC IL-21-
HC IL-21+
SLE IL-21-
SLE IL-21+

Figure S4


CFSE - cells
HC
SLE
No cytokines
IL-21 (+)
IL-2 (+)
No cytokines
IL-21 (+)
IL-2 (+)
GATA-3
FOXP3
90.9
77.1
155
93.1
76.6
135
12.56 %
8.41 %
40.92 %
13.58 %
5.45 %
31.00 %
CFSE - cells
HC
SLE
No cytokines
IL-21 (+)
IL-2 (+)
No cytokines
IL-21 (+)
IL-2 (+)
CTLA-4
FOXP3
189
148
314
149
152
273
47.30 %
34.68 %
61.40 %
37.00 %
36.92 %
65.80 %

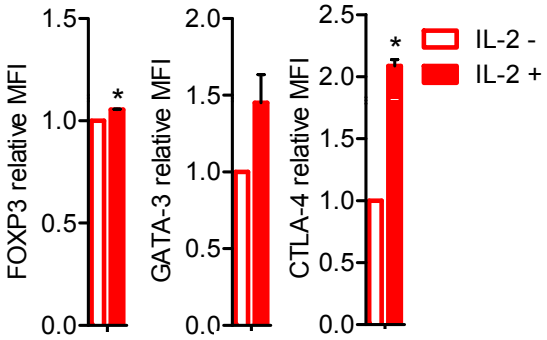
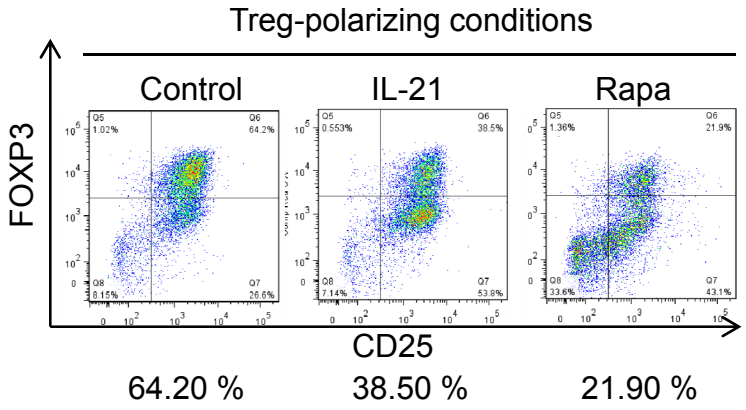

FOXP3 relative MFI
GATA-3 relative MFI
CTLA-4 relative MFI
IL-2 -
IL-2 +
*
*

Figure S5

A


Treg-polarizing conditions
Control
IL-21
Rapa
FOXP3
CD25
64.20 %
38.50 %
21.90 %

B

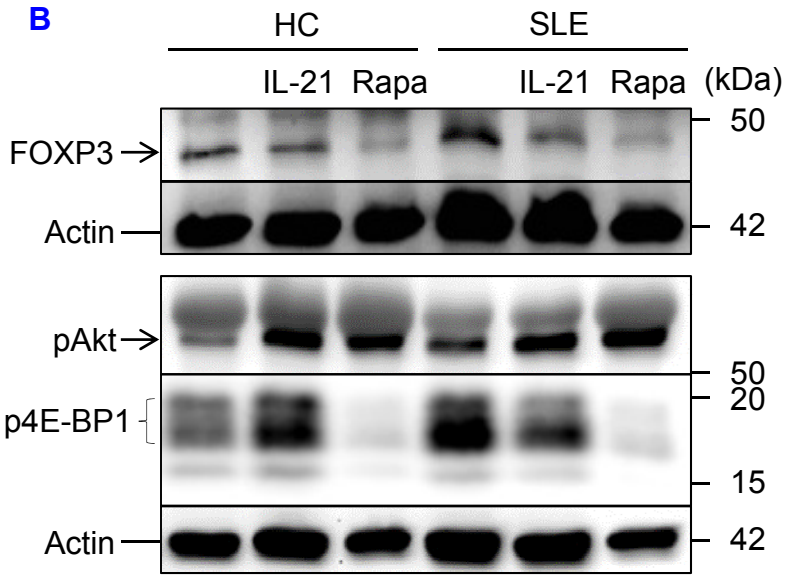

HC
SLE
IL-21
Rapa
IL-21
Rapa
(kDa)
FOXP3
Actin
pAkt
p4E-BP1
Actin
50
42
50
20
15
42

Figure S6

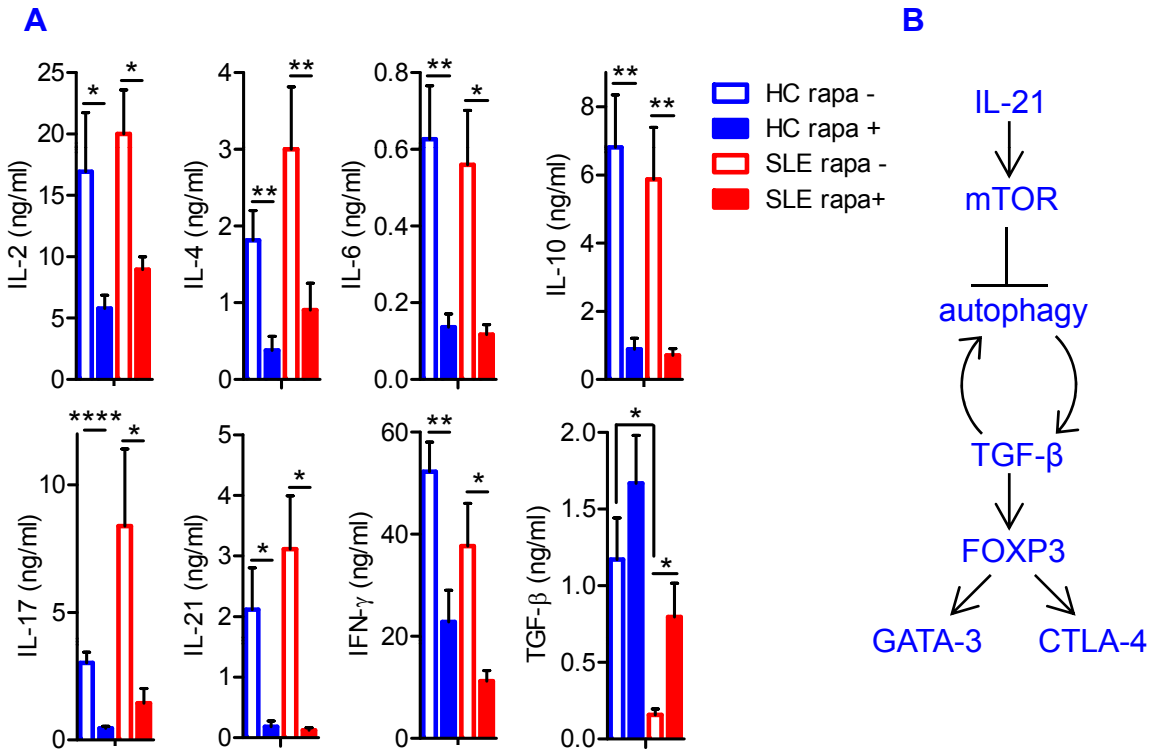
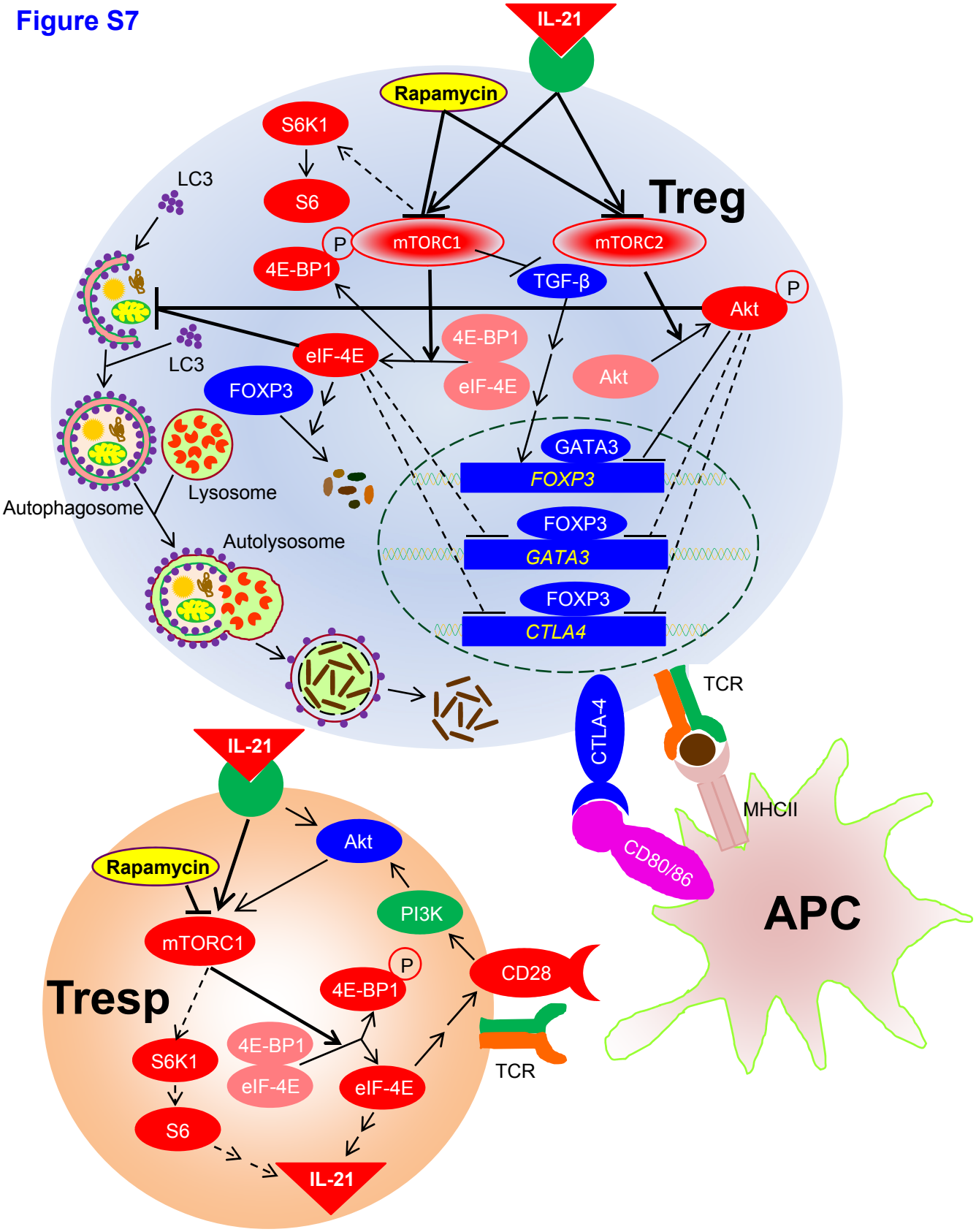

A
B
HC rapa -
HC rapa +
SLE rapa -
SLE rapa+
IL-2 (ng/ml)
IL-4 (ng/ml)
IL-6 (ng/ml)
IL-10 (ng/ml)
IL-17 (ng/ml)
IL-21 (ng/ml)
IFN-γ (ng/ml)
TGF-β (ng/ml)
IL-21
mTOR
autophagy
TGF-β
FOXP3
GATA-3
CTLA-4

Figure S7


IL-21
Rapamycin
S6K1
S6
LC3
Treg
P
mTORC1
mTORC2
4E-BP1
TGF-β
Akt
P
LC3
eIF-4E
4E-BP1
eIF-4E
Akt
FOXP3
GATA3
FOXP3
Lysosome
Autophagosome
Autolysosome
FOXP3
GATA3
FOXP3
CTLA4
TCR
CTLA-4
MHCII
CD80/86
APC
IL-21
Akt
Rapamycin
PI3K
mTORC1
P
CD28
Tresp
4E-BP1
4E-BP1
S6K1
eIF-4E
eIF-4E
TCR
S6
IL-21