Supporting Online Material for

Halofuginone Inhibits T_H17 Cell Differentiation by Activating the Amino Acid Starvation Response

Mark S. Sundrud, Sergei Koralov, Markus Feuerer, Dinis Pedro Calado, Aimee ElHed Kozhaya, Ava Rhule-Smith, Rachel E. Lefebvre, Derya Unutmaz, Ralph Mazitschek, Hanspeter Waldner, Malcolm Whitman,* Tracy Keller,* Anjana Rao*

*To whom correspondence should be addressed. E-mail: whitman@hms.harvard.edu (M.W.); tkeller@hms.harvard.edu (T.K.); arao@idi.harvard.edu (A.R.)

Published 5 June 2009, *Science* **324**, 1334 (2009)
DOI: 10.1126/science.1172638

This PDF file includes:

Supporting Online Material for

Halofuginone inhibits Th17 cell differentiation by activating the amino acid starvation response

Mark S. Sundrud, Sergei Koralov, Markus Feuerer, Dinis Pedro Calado, Aimee ElHed Kozhaya, Ava Rhule-Smith, Rachel E. Lefebvre, Derya Unutmaz, Ralph Mazitschek, Hanspeter Waldner, Malcolm Whitman*, Tracy Keller* and Anjana Rao*

* To whom correspondence should be addressed. Email: mwhitman@hms.harvard.edu, tkeller@hms.harvard.edu, arao@idi.harvard.edu

This pdf file includes

Materials and Methods

Figs. S1-S14, tables S1, S2

References

Materials and Methods

Mice

Mice were housed in specific pathogen-free barrier facilities at Harvard Medical School or Partners Research Building and were used in accordance with protocols approved by the animal care and use committees of the Immune Disease Institute and Harvard Medical School. C57B/6J mice purchased from Jackson laboratories (Bar Harbor, ME) were used for all *in vitro* experiments unless otherwise noted. $Foxp3^{gfp}$ and $Foxp3^{ko}$ T cells were isolated from organs kindly provided by Dr. Alexander Rudensky (S*1*). Adjuvant-driven EAE was induced in 8 week-old wild-type C57B/6J mice purchased from Charles River laboratories (Kingston, NY). Proteolipid protein (PLP) TCR transgenic B10.S mice used for passive EAE experiments have been described previously (S*2*).

EAE

For adjuvant-driven EAE, mice were immunized by subcutaneous injection of 250 μg MOG_{33-55} peptide emulsified in IFA (Sigma-Aldrich) supplemented with 5 mg/ml heat-killed *M. tuburculosis* (BD Biosciences) in both dorsal flanks and has been described elsewhere (S*3*). Mice were injected intraperitoneally (i.p.) daily with HF (2μg/ mouse) or vehicle control (DMSO). Analysis of adjuvant-driven EAE included PBS/CFA (n=14), MOG/CFA + DMSO (n=14), MOG/CFA + HF (n=14). Passive EAE was induced by intravenous transfer of purified $CD3^+$ splenic T cells isolated from PLP TCR transgenic B10.S mice into syngeneic RAG2-deficient mice (3×10^6 cells/mouse) (S*2*). Recipient mice were treated with HF (n = 11) or DMSO (n= 9) as above. Clinical signs of EAE were assessed daily according to the following scale: 0, asymptomatic; 1, flaccid tail; 2,

weak gait/ hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; 5, moribund. Cytokine production during EAE was determined either in peripheral T cells isolated from the spleens or paraaortic lymph nodes of mice prior to disease onset (day 6-10) or in mononuclear cells isolated from brain and spinal cord tissue of DMSO-treated mice with severe disease (clinical score $\geq$ 2), or asymptomatic HF-treated animals (clinical score = 0) between days 15-20. Briefly, splenocytes or lymph node cells were stained for cell surface antigens and intracellular cytokines following erythrocyte lysis with ammonium chloride buffer and 4 hour stimulation with PMA and ionomycin (see below). T cells were isolated from brain and spinal cord tissue of mice following perfusion with cold PBS. Minced CNS tissue was digested with liberase C1 (0.33 mg/ml) or collagenase D (10 mg/ml) (both from Roche Diagnostics) at 37°C for 30-45 minutes. Cell suspensions were passed through 70µm cell strainers (VWR) and fractionated by 70%/ 30% Percoll (Sigma-Aldrich) gradient centrifugation. Mononuclear cells were collected from the interphase, washed and used for intracellular cytokine analysis.

Cell isolation

Primary murine T and B cells were purified by cell sorting. $CD4^+$ $CD25^-$ T cells were used for all *in vitro* experiments, unless stated otherwise, and were positively selected using Dynal CD4 dynabeads and detachabeads (Invitrogen) per manufacturers instructions. $CD25^{hi}$ Treg cells were depleted using a CD25 microbead kit (Miltenyi biotech). Naïve ($CD4^+$ $CD62L^{hi}$ $CD44^{lo}$ $Foxp3^{gfp-}$ or $CD4^+$ $CD62L^{hi}$ $CD44^{lo}$ $CD25^-$) T cells were purified from $Foxp3^{gfp}$ or $Foxp3^{ko}$ mice, respectively, by FACS sorting. $CD8^+$ T cells or B cells were isolated from $CD4^-$ fractions using Dynal CD8 negative isolation kit (Invitrogen) or CD43 negative isolation kit (Miltenyi biotech), respectively. Resting human $CD4^+$ T cells were isolated from PBMC of healthy human donors using Dynal CD4 Positive Isolation Kit (Invitrogen) as previously described (S*4*). $CD4^+$ cells were further purified to obtain memory T cells by staining with PE-conjugated anti-human CD45RO-PE antibodies (BD Biosciences), and sorting on a FACSAria cytometer (BD Biosciences). $CD14^+$ monocytes were isolated from autologous PBMC using a magnetic separator (AutoMACS, Miltenyi Biotech). All cells were routinely 95-99% pure following isolation.

Cytokines, antibodies and cell culture

Purified $CD4^+$ $CD25^-$ T cells were activated in vitro as previously described (S*5*) using 0.3 μg/ml hamster anti-mouse CD3 (clone 145-2C11) (ATCC) and 0.5 μg/ml hamster anti-mouse CD28 (BD Pharmingen). Activated T cells were cultured in complete D-MEM medium and prepared in (S5) and differentiated using the following combinations of cytokines and antibodies: *iTreg* - recombinant human TGFβ1 (3 ng/ml - R&D systems), *Th17* - TGFβ1 (3 ng/ml) plus recombinant mouse IL-6 (30 ng/ml) (R&D systems). Th1 and Th2 differentiation was performed as previously described (S*5*). Human IL-2

supernatant (National Cancer Institute) was used in culture at .01 U/ml and was added at 48 hours-post activation when T cells were split into uncoated tissue culture wells, with the exception of Th17 cultures that were maintained in the absence of exogenous IL-2. $CD8^+$ T cells were activated with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 and were expanded in 0.1 U/ml IL-2 for up to 6 days. CD43-depleted B cells were activated *in vitro* by culturing with 25 μg/ml LPS (Sigma-Aldrich) for 3-4 days in the presence or absence of TGFβ. All reagents (see below) were added at the time of T cell activation and again at 48 hours post activation unless indicated otherwise. For kinetic analyses, purified T cells were labeled with either 1 μM CFSE (Invitrogen) prior to activation, or were pulse-labeled at various timepoints post TCR activation with 10 μM BrdU (BD Biosciences) for 45-60 minutes. Human T cells were activated by plating purified monocytes in a 96-well flat bottom plate at a concentration of 2×10^4 cells per well in complete medium overnight. 1×10^5 purified human memory T cells were added to monocyte cultures in the presence of soluble anti-CD3/ anti-CD28 dynabeads (Invitrogen). T cell proliferation (CFSE dilution) and CD25 upregulation were determined 48 hours after TCR-activation, Foxp3 expression was assessed after 72 hours and intracellular cytokine expression in differentiated T cells was determined on day 4 or 5 following restimulation with PMA and ionomycin (see below).

Inhibitors and amino acids

1 kg of 10% pure HF was received as a gift from Hangpoon Chemical Co. (Seoul, South Korea), which was further purified via HPLC to >99% purity and used for experiments. MAZ1310 (S*6*) was generated by chemical derivatization of halofuginone and was used at equal concentrations as a negative control. For cell culture experiments, HF and MAZ1310 were prepared as 100 mM stock solutions in DMSO and diluted to the indicated concentrations. If not indicated otherwise, HF and MAZ1310 were generally used for T cell treatment at 10 nM. 100 nM HF or MAZ1310 were used to treat human T cells. For use *in vivo*, working HF stocks were diluted in PBS from a 20 mM stock solution (~ 8 mg/ml) and 2-2.5 μg HF or an equal volume of DMSO was injected i.p. SB-431542 (S*7*) (Tocris bioscience) was prepared as a 10 mM stock solution in DMSO and was used in culture at 10 μM. Rapamycin was prepared as a 100 mM stock solution in DMSO and used for cell culture experiments at 20 nM. L-tryptophanol was prepared as a 20 mM stock solution in 0.1 M NaOH, pH 7.4 and was used at 0.2 mM. For amino acid starvation/ deprivation experiments, T cells were activated and differentiated as above in complete D-MEM medium without L-cysteine and L-methionine, or D-MEM medium without L-leucine (both from Invitrogen). Stocks containing 20 mM L-cysteine plus 10 mM L-methionine, or 400 mM L-leucine (all from Sigma-Aldrich) were prepared in filtered ddH_2O, pH 1.0 and were added to medium at the indicated concentrations. In some

experiments, HF-treated T cell cultures were supplemented with 5x or 10x combinations of essential and non-essential amino acid solutions (Invitrogen).

Retroviral transductions

MIG and MIG.RORγt retroviral cDNA were gifts from Dr. Dan Littman. pRV and pRV.FOXP3 retroviral constructs have been described previously (S*8*). Retroviral particles were generated using the phoenix-Eco system (ATCC). Supernatants were concentrated by centrifugation at 6,000 x g for 12-18 hours, resuspended in fresh retroviral supernatant and stored at -80^{o}C prior to use in culture. Retroviral supernatants were thawed in a 37^{o}C water bath and added to T cell cultures 12 hours after activation in the presence of 8 μg/ml polybrene (American bioanalytical). Infections were enhanced by centrifuging at 1,200 x g for 1 hour.

Detection of cytokine production

Secreted cytokines were measured using the mouse Th1/ Th2 cytometric bead array (CBA - BD Pharmingen) in accordance with manufacturers instructions. Briefly, resting T cells were as described above and supernatants were collected at the indicated times and stored at -80^{o}C prior to use in the CBA assay. To detect intracellular cytokine expression in murine cells, cultured T or B cells were stimulated with 10 nM PMA and 1 mM ionomycin for 4-5 hours in the presence of 10 mM brefeldin A (all from Sigma-Aldrich). Stimulated cells were harvested, washed with PBS and fixed with PBS plus 4% paraformaldehyde at room temperature for 20 minutes. Cells were then permeabilized with PBS containing 1% BSA and 0.5% saponin (Sigma-Aldrich) at room temperature for 10 minutes before cytokine-specific antibodies were added for an additional 20 minute room temperature incubation. Human T cells were restimulated with 20 ng/ml PMA and 500 ng/ml Ionomycin (both from Sigma-Aldrich) for 6 hours in the presence of golgi plug (BD Biosciences) and intracellular staining was performed using cytofix/ cytoperm kit (BD Biosciences) per manufacturers instructions. All stained cells were stored at 4^{o}C in PBS plus 1% paraformaldehyde for FACS analyses.

FACS analyses and sorting

All cell surface staining was performed in FACS buffer (PBS/ 2% FBS/ 0.1% NaN_3) and antibodies were incubated with cells on ice for 20-30 minutes. Cells were washed with FACS buffer and fixed with FACS buffer plus 1% paraformaldehyde prior to sample acquisition. Foxp3 intracellular staining was performed using a Foxp3 intracellular staining kit (eBioscience) in accordance with manufacturers instructions. BrdU incorporation was determined using FITC or APC BrdU kits (BD Biosciences) in accordance with manufacturers instructions. Intracellular phospho-STAT3 staining was

performed on activated T cells cultured with or without TGFβ plus IL-6 for the indicated times. Cells were harvested and fixed in PBS plus 2% paraformaldehyde for 10 minutes at 37°C. Fixed cells were washed twice with staining buffer (PBS/ 1% BSA/ 0.1% NaN_3) and then permeabilized with perm buffer III (BD Pharmingen) on ice for 30 minutes. Cells were then washed twice with staining buffer and PE-conjugated anti-STAT3 ^{pY705} (BD Pharmingen) was added to cells and incubated at room temperature for 45-60 minutes. Cells were washed and stored in staining buffer prior to data acquisition. Apoptosis was determined on Jurkat T cells (ATCC) by staining with annexin V and propidium iodide (both from BD Pharmingen). Fluorohpore-conjugated antibodies purchased from BD Pharmingen were percp-Cy5.5-conjugated anti-CD4, PE-conjugated anti-CD25, PE-conjugated anti-IL-17, APC-conjugated anti-IFNγ. Fluorophore-conjugated antibodies from eBioscience included FITC-conjugated anti-CD8, APC-conjugated anti-mouse/rat Foxp3, PE-conjugated anti-IL-4, APC-conjugated anti-IFNγ, PE-conjugated anti-granzyme B, APC-conjugated streptavidin, PE-conjugated anti-IL-6 and PE-conjugated anti-human IL-17. Biotin-conjugated anti-IgA antibody was purchased from Southern biotech. All FACS data was acquired on a FACSCalibur flow cytometer (BD Pharmingen) and analyzed using FlowJo software (Treestar, Inc.). FACS sorting was performed on a FACS-Diva cytometer (BD Pharmingen).

Quantitative real-time PCR

T cells were activated as described above, collected at the indicated times and cell pellets were flash-frozen in liquid nitrogen. Total RNA was obtained by RNeasy (Quiagen) column purification per manufacturers instructions. RORγt expression was determined after reverse transcription using the message sensor kit (Ambion) per manufacturers instructions and taqman primers and probe as described elsewhere (S*9*). Sybrgreen quantitative real-time PCR was performed on RNA samples following reverse transcription via SuperScript II first-strand cDNA synthesis kit (Invitrogen). All qPCR data was collected on an iCycler thermal cycler (Bio-Rad). Primer sequences are listed below.

Asns forward: 5'-TGACTGCCTTTCCGTGCAGTGTCTGAG-3',

Asns reverse: 5'-ACAGCCAAGCGGTGAAAGCCAAAGCAGC

Gpt2 forward: 5'- TAGTCACAGCAGCGCTGCAGCCGAAGC-3'

Gpt2 reverse: 5'- TACTCCACCGCCTTCACCTGCGGGTTC-3'

eIF4Ebp1 forward: 5'- ACCAGGATTATCTATGACCGGAAATTTC-3'

eIF4Ebp1 reverse: 5'- TGGGAGGCTCATCGCTGGTAGGGCTAG-3'

Hprt forward: 5'-GGGGGCTATAAGTTCTTTGCTGACC-3

Hprt reverse: 5'-TCCAACACTTCGAGAGGTCCTTTTCAC-3'

Il17 forward: 5'-ATGAGTCCAGGGAGAGCTTCAT-3'

Il17 reverse: 5'-TTAGGCTGCCTGGCGGACAATC-3'

Il17f forward: 5'-GCACCCGTGAAACAGCCATGGTC-3'

Il17f reverse: 5'-GGCCGCTTGGTGGACAATGGGC-3'

Western blotting

Whole cell lysates were prepared from activated T cells at the indicated times. For phospho-Smad2 and phospho-STAT3 western blots, cells were harvested, washed in PBS and lysed in 50 mM Tris, pH 7.4, 0.1% SDS, 1% Triton-X100, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA supplemented with protease inhibitors tablets (Roche), 1 mM NaF and 1 mM Na_3VO_4 (Sigma-Aldrich). For all other western blot analyses, with the exception of GCN2 (see below), cells were harvested, washed in ice-cold PBS and lysed in 50 mM Tris, pH 7.4, 2% SDS, 20% glycerol and 2mM EDTA supplemented with protease and phosphatase inhibitors as above. Cytoplasmic extracts were generated to analyze GCN2 phosphorylation. Briefly, 5-8 x 10^6 T cells were resuspended in hypotonic lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT supplemented with protease and phosphatase inhibitors. After a 10 minute incubation on ice, .05% Triton X-100 was added and nuclei were pelleted by centrifugation at 4^{o}C. Lysates were cleared by centrifuging at 12,000 x g at 4^{o}C. Generally, 15-30 μg of protein was resolved by SDS-PAGE, protein was transferred to nitrocellulose membranes, blocked and blotted using specific antibodies. Antibodies used in western blots included: anti-Smad2 $^{pS465/467}$, anti-STAT3 ^{pY705}, anti-STAT3, anti-eIF2α ^{pS51}, anti-eIF2α, anti-GCN2 ^{pT898}, anti-GCN2 and anti-P70-S6K/ S6K1 ^{pT389} (all from cell signaling technology). Anti-ATF4/CREB2 and anti-β-actin were purchased from Santa Cruz biotechnology. HRP-conjugated secondary antibodies were all purchased from Sigma-Aldrich, with the exception of HRP-conjugated anti-armenian hamster antibody (Jackson Immunoresearch).

Microarrays, data analyses and statistics

RNA prepared from activated T cells treated with 10 nM HF or MAZ1310 for either 3 or 6 hours, was amplified, biotin-labeled (MessageAmp II Biotin-Enhanced kit, Ambion), and purified using the RNeasy Mini Kit (Qiagen). Resulting cRNAs were hybridized to M430 2.0 chips (Affymetrix, Inc.). Raw data were normalized using the RMA algorithm implemented in the "Expression File Creator" module from the GenePattern software package (S*10*) (www.broad.mit.edu/cancer/software/genepattern/). Data were visualized using the GenePattern "Multiplot" modules.

Statistics

Gene expression distribution analyses were performed using Chi-squared statistical tests. For all other statistical comparisons, p values were determined using one- or two-tailed student's t-test on triplicate samples.

Figure S1

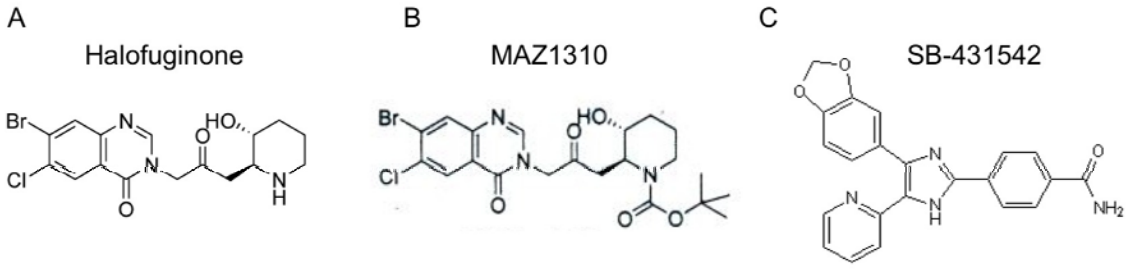


Fig. S1. Molecular structures of (A) Halofuginone, (B) MAZ1310 and (C) SB-431542.

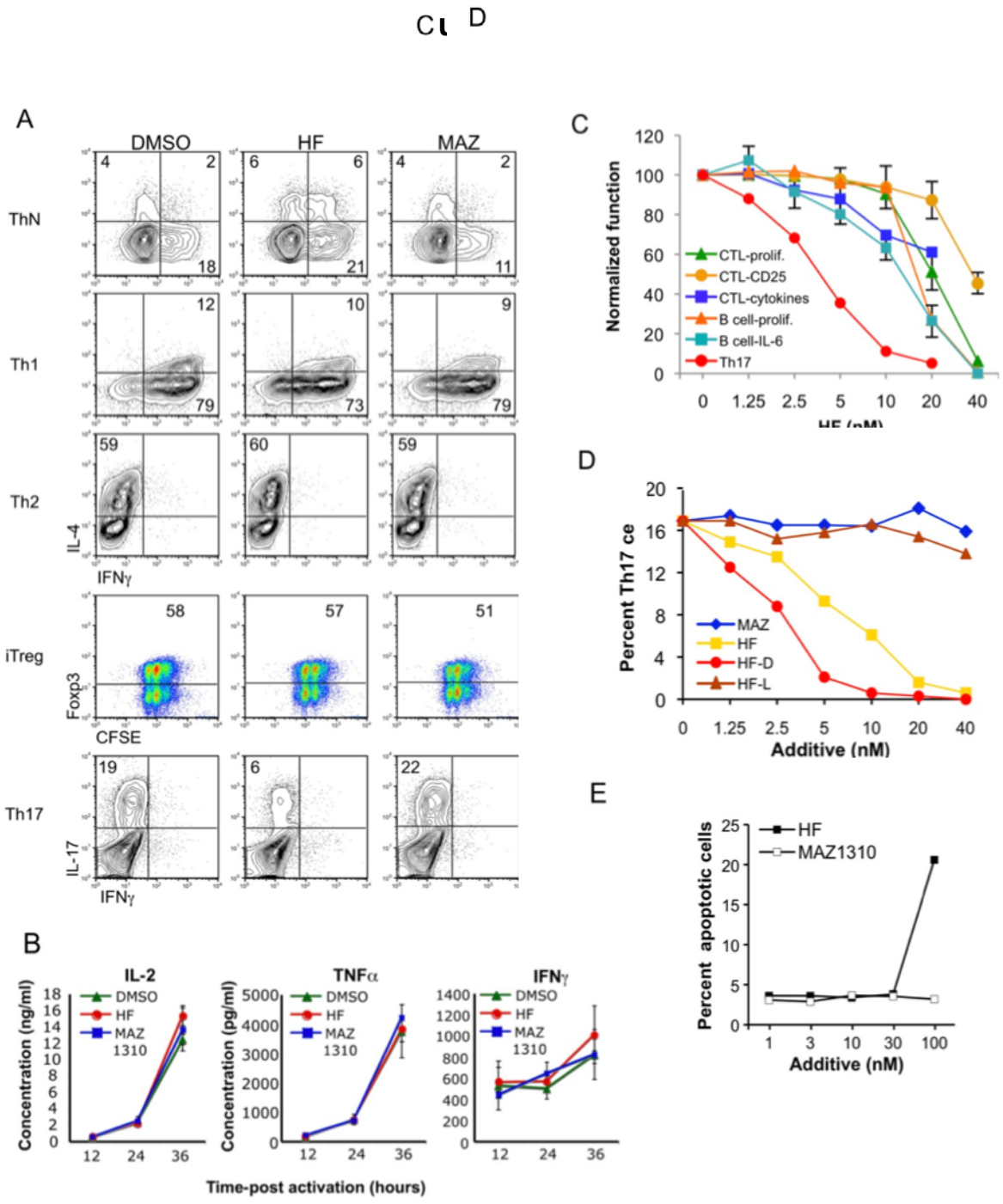


Fig. S2. HF selectively inhibits IL-17 expression. (A) CFSE-labeled T cells were differentiated in the presence of DMSO, 5 nM HF or 5 nM MAZ1310. Differentiation was determined by intracellular Foxp3 staining (day 3), or by intracellular cytokine staining following restimulation (day 4-5). Numbers in quadrants refer to percentages of cells. (B) Resting T cells were activated without exogenous cytokines and supernatants were harvested at the indicated time-points. Cytokines were quantified using a cytometric

bead array (CBA; see materials and methods), compared to protein standards and are presented as mean concentrations ± SD from duplicate samples. (C) Dose-response analyses of HF effects on $CD8^+$ T cell or B cell function. Cells were activated (see materials and methods) in the presence of DMSO, 40 nM MAZ1310 or titrating concentrations of HF as indicated. CFSE dilution, cell-surface CD25 expression and intracellular cytokine production was determined as above. CFSE dilution and percentages of $CD8^+$ T cells expressing CD25, $IFN\gamma^+$ granzyme B^+ (cytotoxic T lymphocytes) or B cells expressing IL-6 are displayed and the values are normalized to cells treated with 40 nM MAZ1310 ± SD. (D) The racemic mix of HF (HF) or HPLC-purified D- or L-enantiomers of HF (HF-D, or HF-L) were added to Th17 differentiation cultures and the percent of Th17 cells (IL-17+ IFNγ-) was determined by intracellular cytokine staining. (E) Jurkat T cells were cultured with the indicated concentrations of HF or MAZ1310. After a 20-hour incubation, cells were stained for annexin V binding and propidium iodide (PI) uptake. The percentages of apoptotic cells (Annexin V+ PI-) are shown. All data represent 2-3 similar experiments.

Figure S3

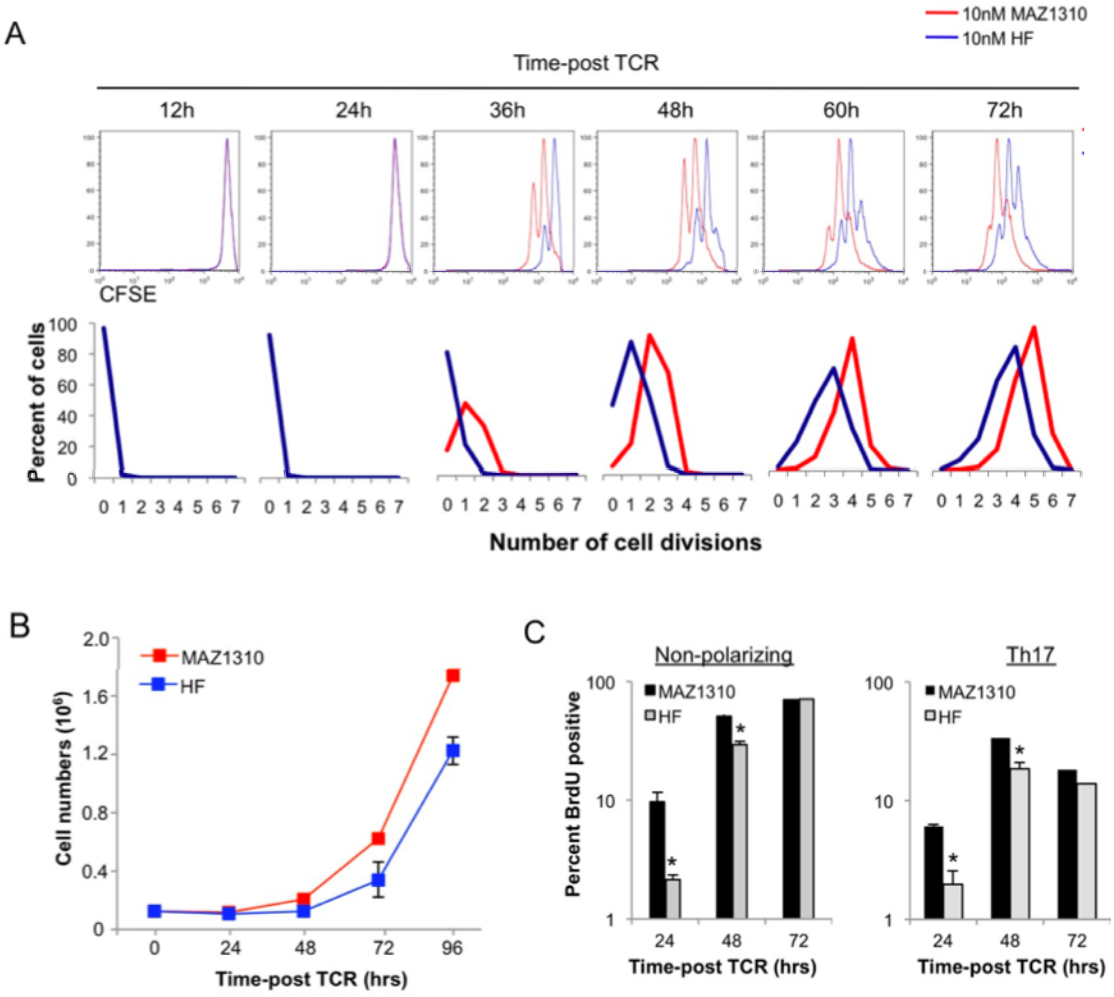


Fig. S3. HF effects on T cell proliferation kinetics. (A) *Top* – CFSE-labeled T cells were activated in the presence of HF or MAZ1310. CFSE dilution was determined at the indicated times post TCR-activation by FACS analyses. *Bottom* – CFSE dilution by MAZ1310- or HF-treated cells was quantified at each time point. Percentages of T cells completing the indicated number of cell divisions are shown. (B) 1 x 10^5 T cells were treated with HF or MAZ1310, activated through the TCR and counted at the indicated times post activation. Mean cell numbers from duplicate samples are shown $\pm$ SD. (C) T cells treated with HF or MAZ1310 were activated in either non-polarizing conditions (*left*) or in Th17-polarizing conditions (*right*). T cells were pulsed with BrdU at the indicated time points (see materials and methods) and stained to determine BrdU incorporation. The mean percentage of $BrdU^+$ cells are shown $\pm$ SD from duplicate samples. Asterisks indicate statistical significance ($p < 0.05$). All data represent 2-3 similar experiments.

Figure S4

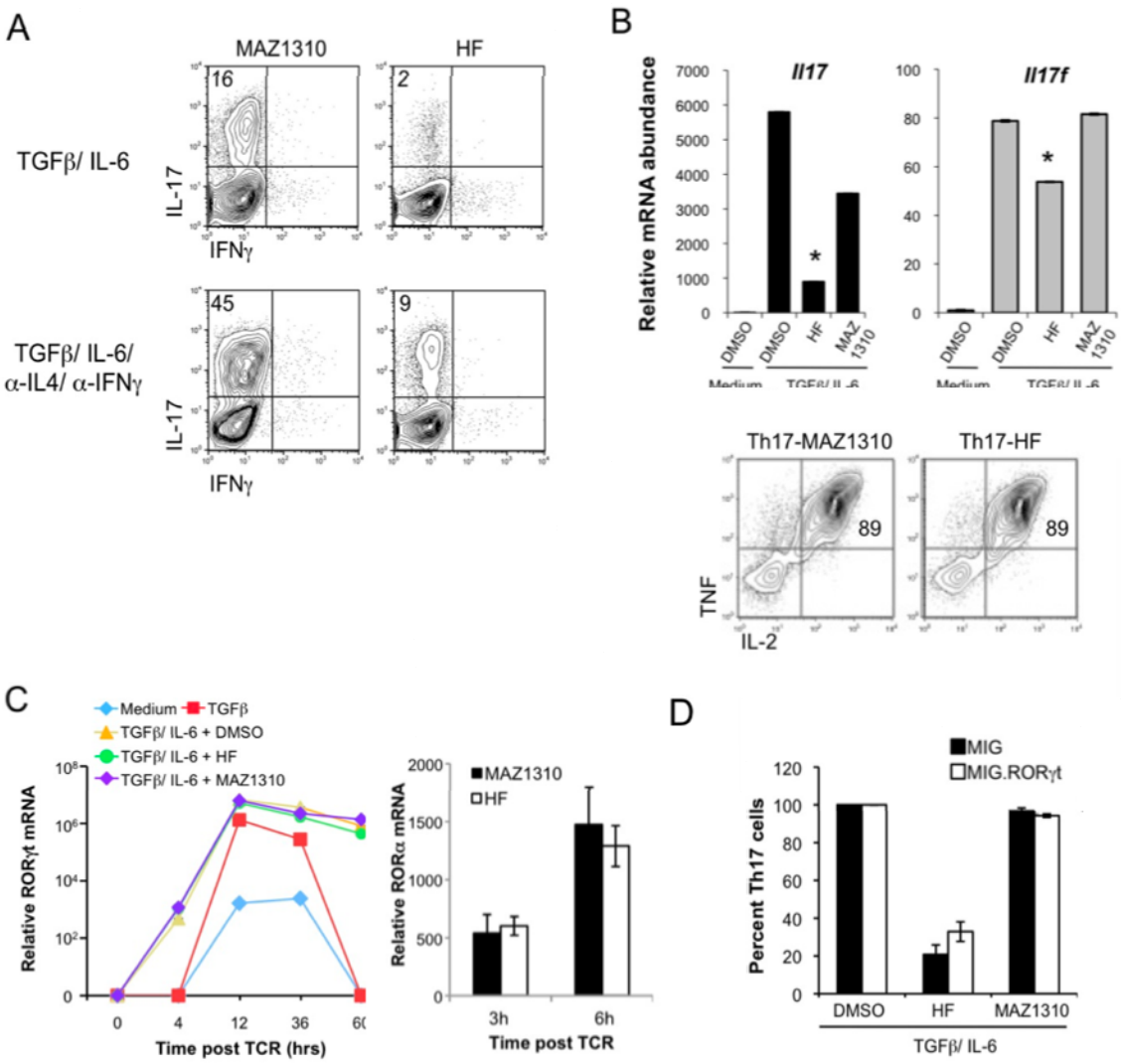


Fig. S4. Effects of HF treatment on Th17 effector function. (A) T cells were treated with HF or MAZ1310 as indicated and activated in the presence of TGFβ plus IL-6, either without (*top*) or with (*bottom*) neutralizing IL-4 and IFNγ antibodies. Intracellular cytokine expression was determined on day 4. (B) *Top* – T cells were activated in non-polarizing or Th17 polarizing cytokine conditions in the presence of DMSO, HF or MAZ1310 as indicated. After 4 days, cells were restimulated with PMA and ionomycin for 4 hours and cDNA was generated for qPCR analysis. *Il17* and *Il17f* transcript levels were normalized to *Hprt* levels and fold changes are shown as mean expression ± SD. Asterisks indicate statistical significance for *Il17* mRNA ($p < 0.001$) and *Il17f* mRNA ($p < 0.05$) in HF-treated T cells relative to MAZ1310-treated cells. *Bottom* – differentiating Th17 cells were treated with HF or MAZ1310. Cells were restimulated on day 4 and intracellular

expression of IL-2 and TNF was determined by intracellular staining and FACS analyses. (C) *left* – T cells were activated in the indicated cytokine conditions and treated with DMSO, HF or MAZ1310. RNA was isolated at the indicated timepoints following TCR activation and qPCR was performed on cDNA. *RORγt* transcript levels were normalized to *Gapdh* expression and are shown as fold changes relative to unstimulated T cells. *Right* – relative abundance of *RORα* mRNA was determined by microarray analysis of T cells activated in Th17 polarizing conditions with HF or MAZ1310 for 3- or 6-hours. Data are presented as mean normalized hybridization intensities (see materials and methods) ± SD from triplicate samples. (D) T cells were activated in Th17-polarizing conditions, treated with DMSO, HF or MAZ1310 and transduced with control (MIG) or RORγt-expressing (MIG.RORγt) retroviruses. Cells were restimulated on day 5 and stained for intracellular cytokine expression. Transduced cells were gated based on GFP fluorescence. Th17 differentiation was normalized to DMSO-treated cultures and are presented as mean values ± SD on duplicate samples. Data represent 2-3 similar experiments.

Figure S5

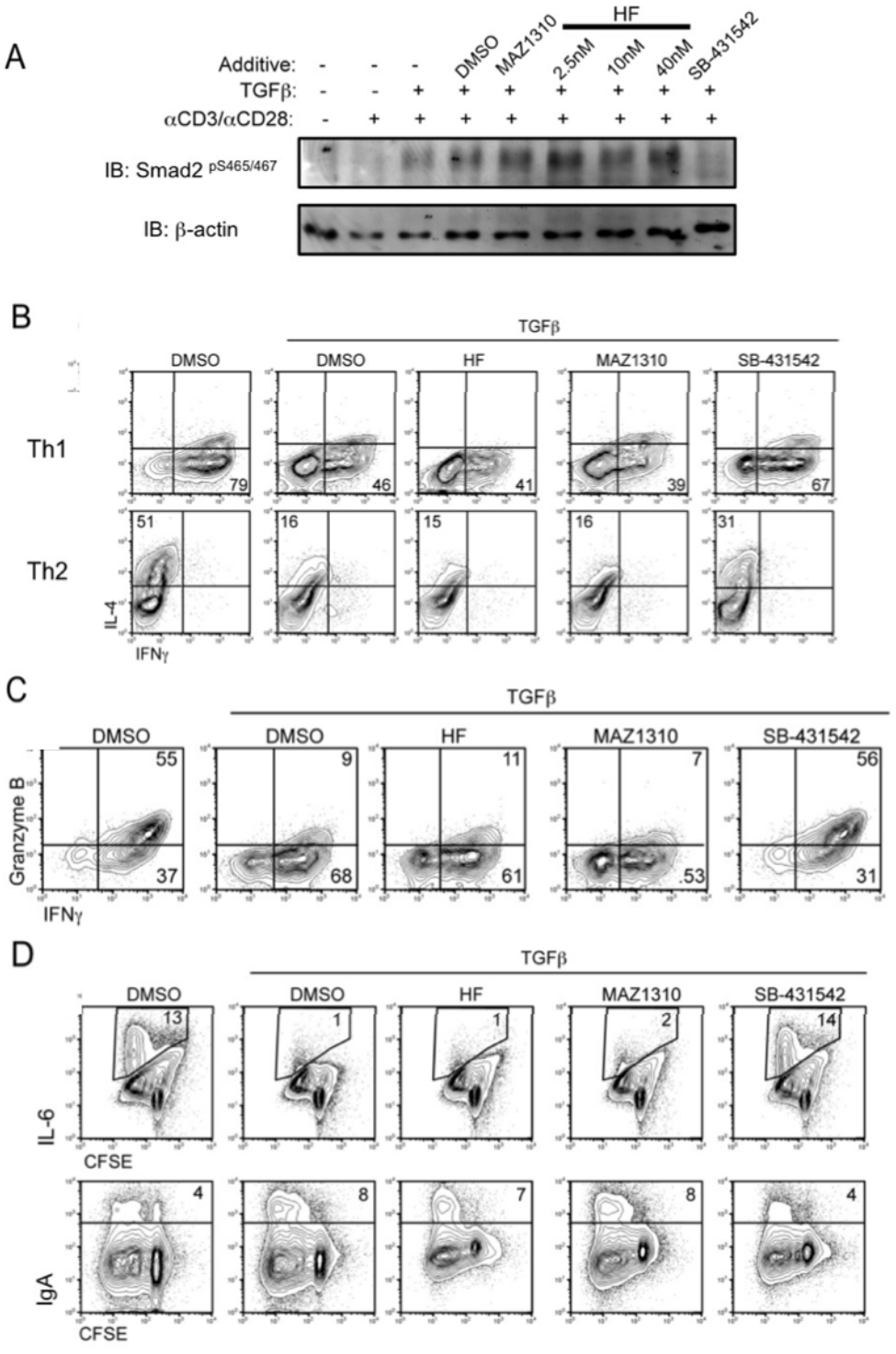


Fig. S5. HF does not regulate TGFβ signaling in T and B cells. (A) Resting T cells were activated through the TCR, treated with DMSO, 40 nM MAZ1310, titrating concentrations

of HF (2.5 – 40 nM) or SB-431542 and cultured with or without TGFβ for 30 minutes. Whole cell lysates were prepared and analyzed by western blotting. (B) T cells were activated in Th1 or Th2 polarizing conditions in the presence or absence of TGFβ. DMSO, HF, MAZ1310 or SB-431542. Intracellular cytokine expression was determined on day 5. (C) $CD8^+$ T cells were activated in the presence or absence of TGFβ and cultured with DMSO, HF, MAZ1310 or SB-431542. Expanded cells were restimulated on day 5 for intracellular cytokine staining. (D) CFSE-labeled B cells were activated by LPS stimulation (see materials and methods) in the presence or absence of TGFβ plus DMSO, HF, MAZ1310 or SB-431542. After 4 days, intracellular IL-6 production was determined after restimulation with PMA and ionomycin. Cell-surface IgA expression was also determined on day 4. These data represent 3 similar experiments.

Figure S6

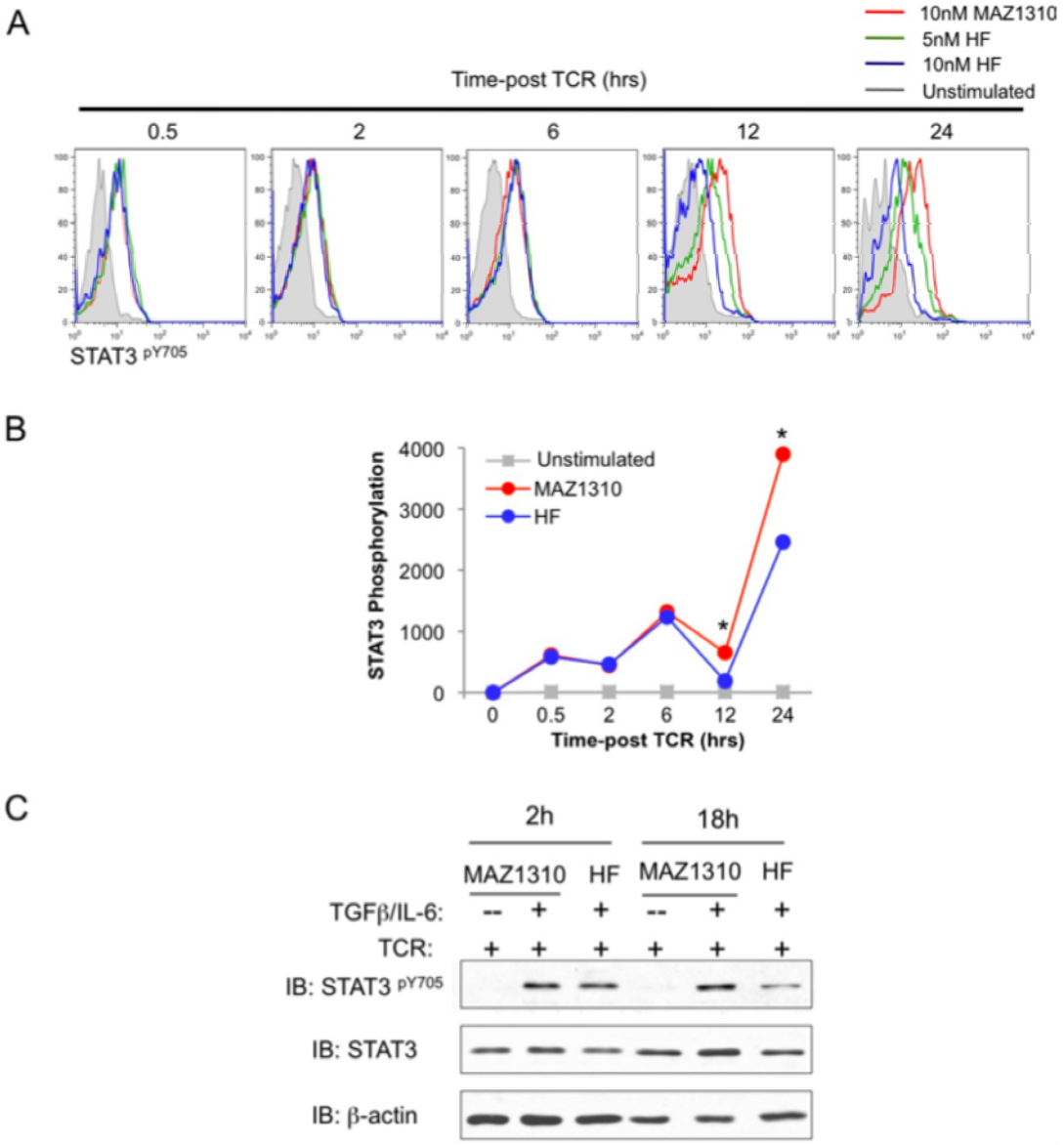


Fig. S6. HF modulates sustained STAT3 phosphorylation. (A) T cells were either left unstimulated, or were activated in the presence of TGFβ plus IL-6 and treated with HF or MAZ1310 as indicated. T cells were fixed at the indicated times post activation and stained intracellularly for STAT3 phosphorylation. (B) Intracellular phospho-STAT3 staining in HF- or control-treated cells was quantified (percentage of phospho-STAT3^{+} cells x phospho-STAT3 MFI). Data are presented as mean values $\pm$ SD on triplicate samples. Asterisks indicate statistical significance ($p < 0.05$). (CT cells were TCR-activated in the presence or absence of TGFβ plus IL-6 and treated with HF or MAZ1310 as indicated. Lysates were generated at the indicated times following activation and analyzed by western blotting.

Figure S7

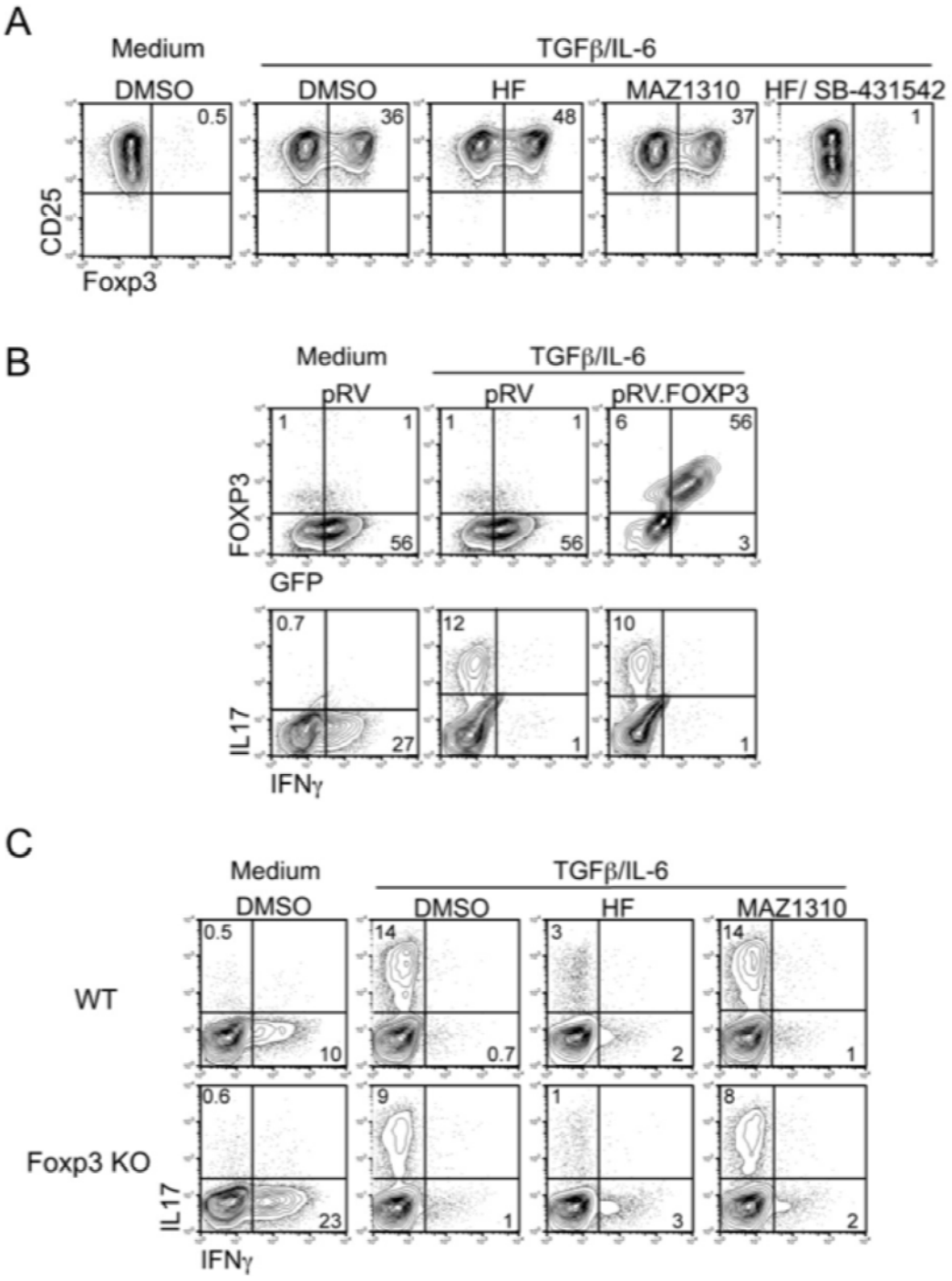


Fig. S7. HF-enforced Foxp3 expression is dispensable for inhibition of Th17 differentiation. (A) T cells activated in medium alone or TGFβ plus IL-6 were treated with DMSO, HF, MAZ1310, or HF plus SB-431542 as indicated. Intracellular Foxp3 expression was determined on day 3. (B) T cells activated in the presence or absence of

TGFβ plus IL-6 were transduced with empty (pRV) or FOXP3-expressing (pRV.FOXP3) retroviruses. Intracellular FOXP3 and cytokine expression was determined on day 4. Transduced cells were gated on based on GFP fluorescence. (C) FACS-sorted naïve T cells from wild-type (WT) or Foxp3-deficient (Foxp3 KO) male mice (see materials and methods above) were treated with DMSO, HF or MAZ1310 as indicated and activated in the absence or presence of TGFβ plus IL-6. Expanded T cells were restimulated on day 4 for intracellular cytokine staining. All data represent 2-3 independent experiments.

Figure S8

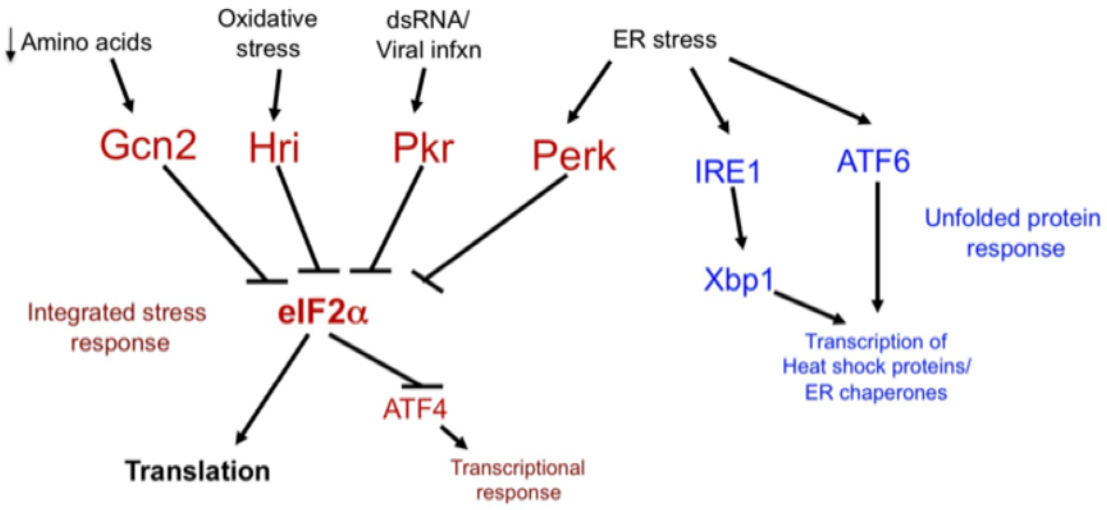


Fig. S8. Schematic diagram of stress response signaling pathways. Endogenous or environmental stresses trigger activation of the integrated stress response (red text). In addition to activating the integrated stress response, ER stress induces the activation of distinct molecules involved in the unfolded protein response (blue text).

Figure S9

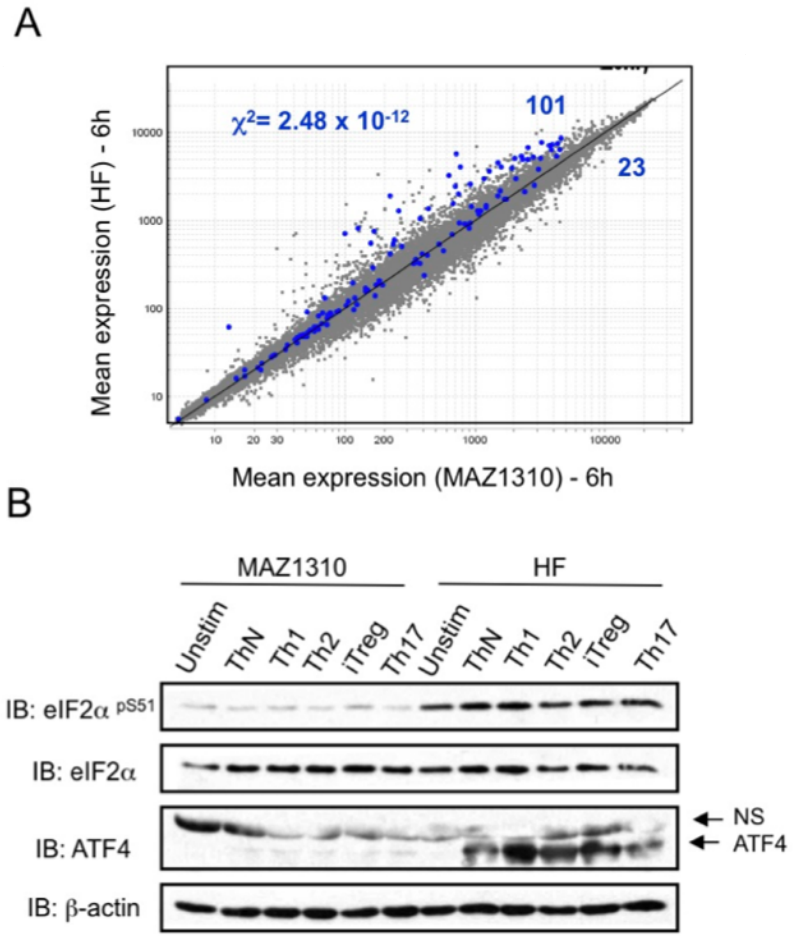


Fig. S9. HF induces transcriptional and biochemical hallmarks associated with amino acid starvation. (A) Microarray data from T cells treated with HF or MAZ1310 for 6 hours were analyzed for the expression of ATF4-regulated genes (*29*) (blue text) (Table S2) by Pearson's Chi-squared test. (B) T cells treated with HF or MAZ1310 were left unstimulated or TCR-activated in the indicated polarizing cytokine conditions (see materials and methods). Cell lysates were prepared after 4 hours and analyzed by western blotting. These data represent 2 independent experiments.

Figure S10

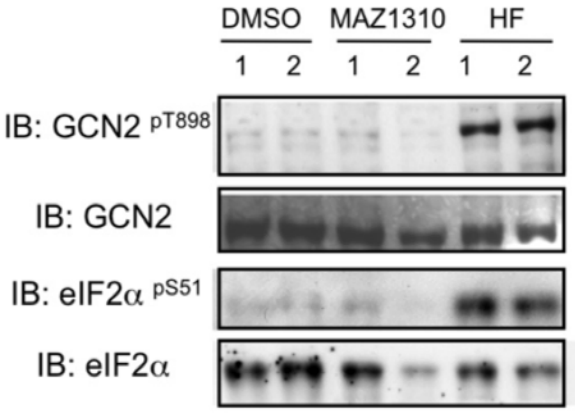


Fig. S10. HF activates the amino acid starvation response pathway in fibroblast cells. SV-MES mesangial cells were stimulated with DMSO, 20 nM MAZ1310 or 20 nM HF. Whole cell lysates were prepared after 2 hours and analyzed by western blotting. These data represent at least 2 similar experiments.

Figure S11

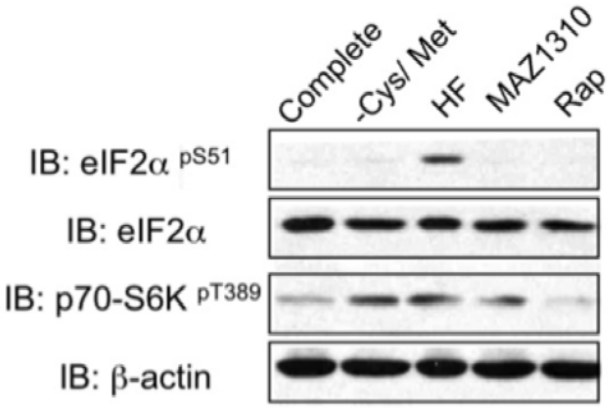


Fig. S11. HF does not directly affect signaling downstream of mTOR. T cells were activated in complete medium, medium deficient in cysteine and methionine (- Cys/ Met), or in complete medium containing HF, MAZ1310 or rapamycin (Rap; see materials and methods). After 4 hours cells were lysed and analyzed by western blotting. These data represent 2 similar experiments.

Figure S12

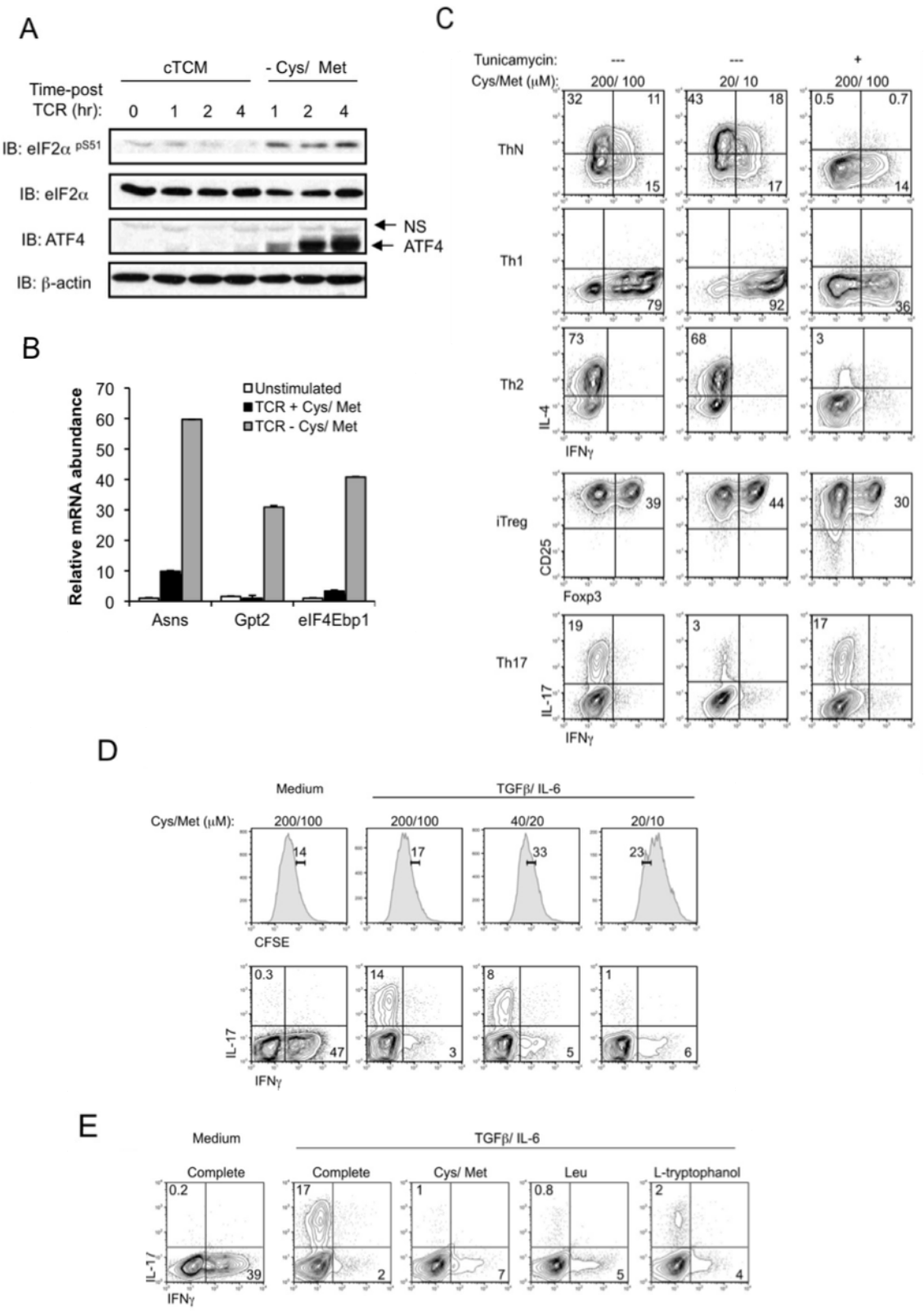
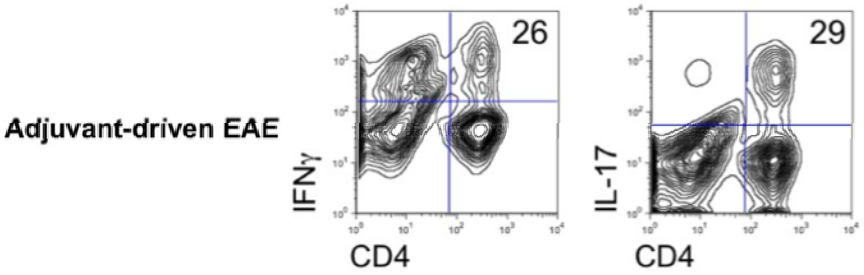

A
cTCM
- Cys/ Met
Time-post TCR (hr):
0 1 2 4 1 2 4
IB: eIF2α pS51
IB: eIF2α
IB: ATF4
IB: β-actin
NS
ATF4
B
Relative mRNA abundance
Unstimulated
TCR + Cys/ Met
TCR - Cys/ Met
Asns
Gpt2
eIF4Ebp1
C
Tunicamycin:
Cys/Met (μM):
200/ 100
20/ 10
ThN
Th1
Th2
IL-4
IFNγ
iTreg
CD25
Foxp3
Th17
IL-17
D
Medium
TGFβ/ IL-6
Cys/Met (μM):
200/100
40/20
20/10
CFSE
IL-17
IFNγ
E
Medium
TGFβ/ IL-6
Complete
Cys/ Met
Leu
L-tryptophanol
IL-17/
IFNγ

Fig. S12. Amino acid deprivation mimics the effects of HF on T cell differentiation. (A) T cells were activated through the TCR in the presence or absence of cysteine and methionine (Cys/Met). Lysates were generated at the indicated timepoints and analyzed by western blotting. ATF4 protein is indicated by arrowhead. NS – non-specific band. (B) Unstimulated or TCR-activated cells were cultured with or without Cys/Met as in (A). Cells were harvested after 4 hours and AAR-associated gene expression was determined by qPCR analyses. *Asns*, *Gpt2* and *eIF4Ebp1* transcript levels were normalized to *Hprt* and are shown as mean expression values ± SD in duplicate samples. (C) T cells were activated and differentiated in complete medium (200 μM Cys/100 μM Met), medium containing low concentrations of Cys/ Met (0.1x – 20 μM Cys/10 μM Met) or complete medium plus 31.25 ng/ml tunicamycin. Foxp3 and CD25 expression was determined on day 3 and intracellular cytokine expression was determined following restimulation on days 4-5. (D) T cells were labeled with CFSE, cultured in medium containing the indicated concentrations of Cys/Met and activated in the absence or presence of TGFβ plus IL-6. Cells were restimulated on day 4 and CFSE dilution together with intracellular cytokine production was determined by FACS analyses. Cytokine expression is shown in T cells gated for equivalent CFSE fluorescence as shown. (E) T cells were activated and differentiated in Th17-priming conditions cultured in: complete medium (complete - 200 μM Cys/100 μM Met /4mM Leucine), medium containing 0.1x cysteine and methionine (Cys/Met), 0.1x leucine (Leu) or complete medium plus L-tryptophanol (see materials and methods). Intracellular cytokine expression was determined on day 4. All data represent at least 2-3 similar experiments.

Figure S13

A



B

	MOG + DMSO	MOG + HF
Disease frequency	13 of 14 (92.8%)	4 of 14 (28.6%)
Mean day of onset *	12.4 ± 1.04	13.3 ± 0.95
Mean max. severity *	2.3 ± 0.63	2.0 ± 1.15

C

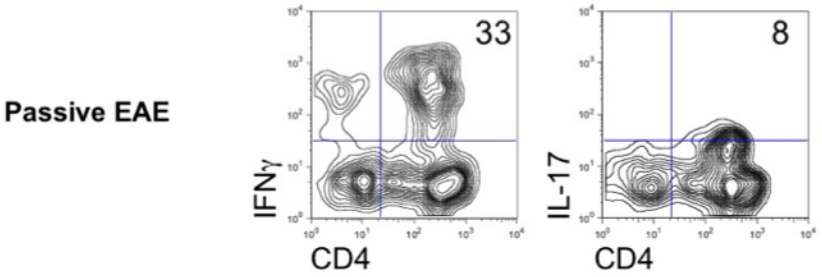


Fig. S13. Distinct effector T cell subsets are associated with adjuvant-driven versus passive EAE. (A) CNS-infiltrating mononuclear cells were isolated from MOG-immunized mice during active disease (see materials and methods). Cells were stimulated *ex vivo* (see materials and methods) and stained for intracellular cytokines. $CD4^+$ $TCR\beta^+$ T cells were gated on for analysis. (B) Statistics of adjuvant-driven EAE in MOG-immunized mice treated with DMSO or HF. Asterisks indicate that only mice which developed disease were considered for analyses. (C) Mononuclear cells were isolated from the CNS of mice showing EAE symptoms following transfer of PLP-specific T cells (see materials and methods). Cells were stimulated *ex vivo* as in (A) and stained for intracellular cytokine expression. PLP-reactive ($TCRV\beta6^+$) $CD4^+$ T cells were gated on for analysis. Data shown in (A) and (C) represent 2-3 independent experiments.

Figure S14

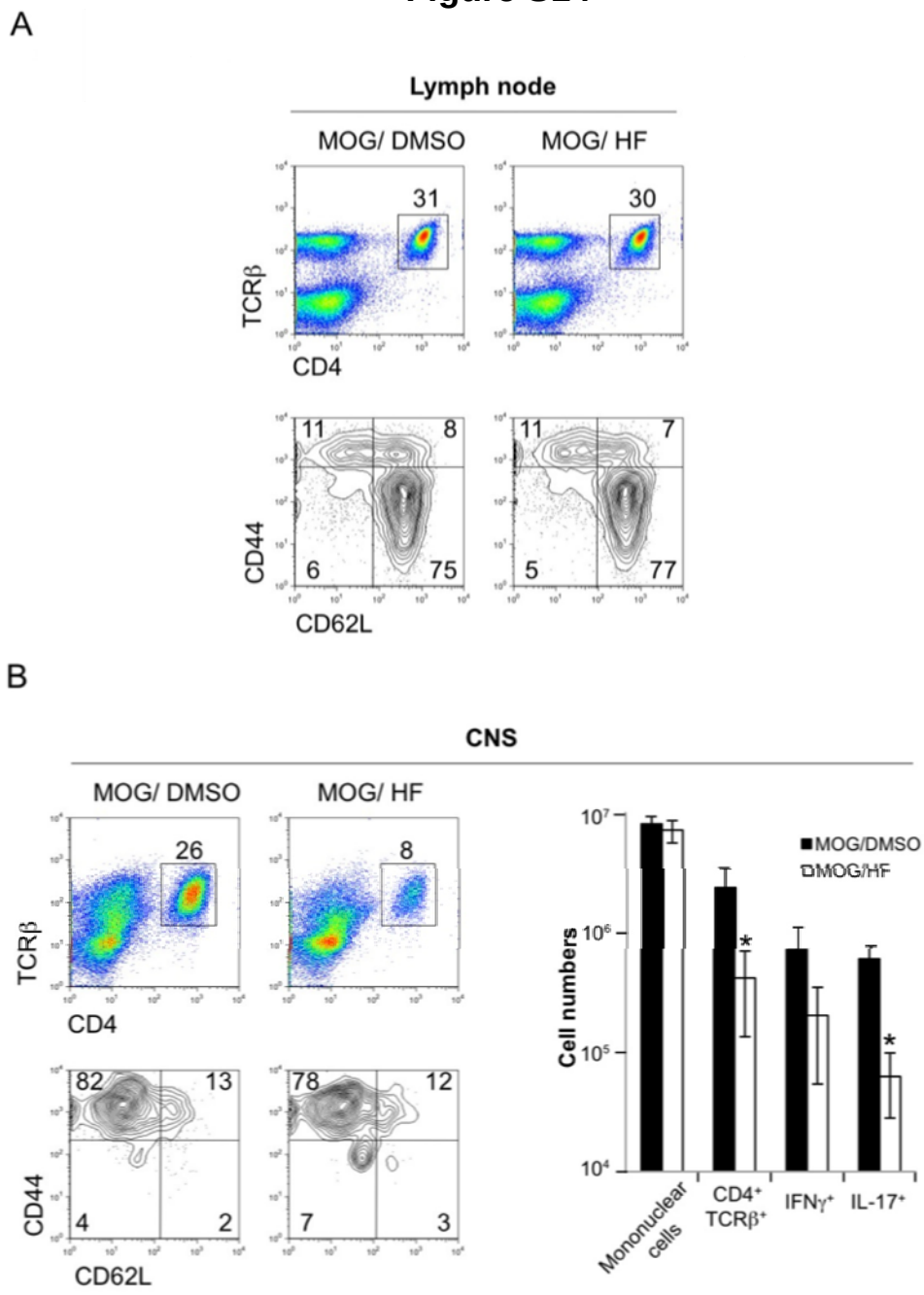


Fig. S14. Regulation of T cell differentiation by halofuginone during adjuvant-driven EAE. T cells from paraaortic lymph nodes (A) or CNS tissue (B – *left*) of control- or HF-treated MOG-immunized mice were analyzed for CD44 and CD62L expression by FACS. $CD4^+$ $TCR\beta^+$ cells were gated on as shown. (B) *Right* – CNS infiltrates in DMSO-treated mice (clinical score = 2) or HF-treated mice (clinical score = 0) were determined during active EAE disease (day 18). Total mononuclear cells, $CD4^+$ $TCR\beta^+$ T cells, Th1 cells ($IFN\gamma^+$) or Th17 cells ($IL\text{-}17^+$) present within CNS preparations were quantified following FACS analyses and are displayed as mean numbers $\pm$ SD from 3 mice.

Asterisks indicate statistical significance ($p < .05$). These data represent 3 similar experiments

Table S1

Gene symbol	Gene title	HF vs. MAZ1310-3hr	HF vs. MAZ1310-6hr
Gpt2	glutamic pyruvate transaminase (alanine aminotransferase)	10.0 ± 1.2	16.7 ± 2.2
Trib3	tribbles homolog 3 (Drosophila)	7.1 ± 2.0	18.5 ± 8.5
Eif4Ebp1	eukaryotic translation initiation factor 4E binding protein 1	6.8 ± 1.8	5.3 ± 0.3
Asns	asparagine synthetase	6.1 ± 1.2	7.1 ± 0.5
Ddit3	DNA-damage inducible transcript 3	5.6 ± 1.1	5.0 ± 0.7
Pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	4.9 ± 0.8	7.4 ± 0.9
Pycr1	pyrroline-5-carboxylate reductase 1	4.6 ± 0.7	6.6 ± 0.4
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	3.9 ± 0.5	8.0 ± 0.2
Phgdh	3-phosphoglycerate dehydrogenase	3.8 ± 0.9	4.2 ± 0.3
Psph	phosphoserine phosphatase	3.5 ± 0.4	3.4 ± 0.3
Xist	inactive X specific transcripts	3.5 ± 1.7	2.1 ± 0.7
Pdcd1lg2	programmed cell death 1 ligand 2	3.2 ± 0.7	2.5 ± 0.3
Vegfa	vascular endothelial growth factor A	3.2 ± 0.2	5.8 ± 0.5
Cldn12	claudin 12	3.2 ± 0.7	4.6 ± 0.5
Slc1a4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	3.2 ± 0.9	4.6 ± 0.4
Atf3	activating transcription factor 3	3.0 ± 0.1	3.2 ± 0.5
Ncoa7	nuclear receptor coactivator 7	3.0 ± 0.3	3.2 ± 0.5
Aars	alanyl-tRNA synthetase	2.7 ± 0.4	2.6 ± 0.2
Sesn2	sestrin 2	2.6 ± 0.3	2.3 ± 0.2
Cebpg	CCAAT/enhancer binding protein (C/EBP), gamma	2.5 ± 0.5	3.1 ± 0.5
Slc6a9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	2.4 ± 0.3	5.7 ± 0.6
Herpud1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.4 ± 0.3	2.7 ± 0.1
Trim12	tripartite motif protein 12	2.4 ± 0.1	4.9 ± 0.7
Clic4	chloride intracellular channel 4 (mitochondrial)	2.4 ± 0.2	2.8 ± 0.2
Atf5	activating transcription factor 5	2.4 ± 0.1	8.9 ± 1.0
Mpa2l	macrophage activation 2 like	2.3 ± 0.3	7.3 ± 1.7
Aff1	AF4/FMR2 family, member 1	2.3 ± 0.4	2.6 ± 0.3
Lars	leucyl-tRNA synthetase	2.3 ± 0.3	2.1 ± 0.0
Cth	cystathionase (cystathionine gamma-lyase)	2.2 ± 0.7	16.0 ± 1.6
Chd2	chromodomain helicase DNA binding protein 2	2.2 ± 0.3	2.5 ± 0.5
Cars	cysteinyl-tRNA synthetase	2.2 ± 0.4	2.2 ± 0.3
Slamf7	SLAM family member 7	2.2 ± 0.4	2.1 ± 0.2
Cxcl10	chemokine (C-X-C motif) ligand 10	2.1 ± 0.3	2.1 ± 0.1
Psat1	phosphoserine aminotransferase 1	2.1 ± 0.5	2.6 ± 0.0
Aldh18a1	aldehyde dehydrogenase 18 family, member A1	2.1 ± 0.5	2.7 ± 0.2
Pycs	1-@pyrroline-5-carboxylate synthetase	2.1 ± 0.2	2.3 ± 0.1
Cd274	CD274 antigen	2.1 ± 0.2	2.0 ± 0.1
D8Ertd56e	DNA segment, Chr 8, ERATO Doi 56, expressed	2.1 ± 0.3	3.1 ± 0.8
Irf1	interferon regulatory factor 1	2.0 ± 0.3	2.6 ± 0.2
Pvr	poliovirus receptor	2.0 ± 0.3	2.0 ± 0.1
Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2.0 ± 0.3	1.9 ± 0.3
Icam1	intercellular adhesion molecule	2.0 ± 0.1	2.8 ± 0.3
Slc14a1	solute carrier family 14 (urea transporter), member 1	2.0 ± 0.1	6.6 ± 0.4
Sars1	seryl-aminoacyl-tRNA synthetase	2.0 ± 0.3	2.3 ± 0.1
Slc7a3	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	2.0 ± 0.2	6.5 ± 0.9

Table S1. Genes induced by HF treatment in T cells. Gene symbols and names of transcripts increased at least two-fold by HF treatment at both 3 and 6 hours. Mean fold increases ± SD from triplicate samples of HF- versus MAZ1310-treated T cells is shown at 3 and 6 hours.

Table S2

Affymetrix probe ID	Gene Name
1433966_x_at	asparagine synthetase
1451095_at	asparagine synthetase
1451083_s_at	alanyl-tRNA synthetase
1423685_at	alanyl-tRNA synthetase
1435154_at	similar to solute carrier family 7 (cationic amino acid transporter, y+ system), member 3
1454991_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
1454992_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
1421533_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
1421093_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 10
1420413_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
1443536_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
1419579_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 12
1422648_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
1426008_a_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
1440506_at	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
1417022_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3
1426069_s_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4
1426068_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4
1436776_x_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4
1418326_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
1460541_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 6
1433467_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 6
1417392_a_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
1447181_s_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
1417929_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
1448783_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 9
1431740_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 13
1449301_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 13
1456003_a_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
1423550_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
1423549_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
1440379_at	solute carrier family 1 (neutral amino acid transporter), member 5
1416629_at	solute carrier family 1 (neutral amino acid transporter), member 5
1422757_at	solute carrier family 5 (neutral amino acid transporters, system A), member 4b
1419253_at	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase
1419254_at	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase
1456653_a_at	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
1415917_at	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase
1415916_a_at	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase
1436704_x_at	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase
1451064_a_at	phosphoserine aminotransferase 1
1454607_s_at	phosphoserine aminotransferase 1
1415673_at	phosphoserine phosphatase
1417562_at	eukaryotic translation initiation factor 4E binding protein 1
1417563_at	eukaryotic translation initiation factor 4E binding protein 1
1434976_x_at	eukaryotic translation initiation factor 4E binding protein 1

Table S2, continued

1443672_at	leucyl-tRNA synthetase, mitochondrial
1435682_at	leucyl-tRNA synthetase, mitochondrial
1439225_at	leucyl-tRNA synthetase, mitochondrial
1425364_a_at	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
1452154_at	isoleucine-tRNA synthetase
1426705_s_at	isoleucine-tRNA synthetase
1426735_at	isoleucine-tRNA synthetase 2, mitochondrial
1441665_at	isoleucine-tRNA synthetase 2, mitochondrial
1418901_at	CCAAT/enhancer binding protein (C/EBP), beta
1427844_a_at	CCAAT/enhancer binding protein (C/EBP), beta
1425262_at	CCAAT/enhancer binding protein (C/EBP), gamma
1425261_at	CCAAT/enhancer binding protein (C/EBP), gamma
1451639_at	CCAAT/enhancer binding protein (C/EBP), gamma
1432331_a_at	paired related homeobox 2
1456903_at	pentraxin related gene
1418666_at	pentraxin related gene
1426808_at	lectin, galactose binding, soluble 3
1445626_at	Lectin, galactose binding, soluble 3 (Lgals3), mRNA
1448929_at	coagulation factor XIII, A1 subunit
1416182_at	amyloid beta (A4) precursor protein-binding, family A, member 3
1454720_at	amyloid beta (A4) precursor protein-binding, family A, member 3
1417133_at	peripheral myelin protein
1458193_at	peripheral myelin protein 2 /// similar to myelin P2 protein - mouse
1423516_a_at	nidogen 2
1454159_a_at	insulin-like growth factor binding protein 2
1418675_at	oncostatin M receptor
1418674_at	oncostatin M receptor
1459217_at	oncostatin M receptor
1426063_a_at	GTP binding protein (gene overexpressed in skeletal muscle)
1450023_at	GTP binding protein 1
1450022_at	GTP binding protein 1
1448437_a_at	GTP binding protein 2
1416691_at	GTP binding protein 2
1416690_at	GTP binding protein 2
1442305_at	GTP binding protein 2
1457975_at	GTP binding protein 3
1450980_at	GTP binding protein 3
1423143_at	GTP binding protein 4
1450873_at	GTP binding protein 4
1423142_a_at	GTP binding protein 4
1451467_s_at	GTP binding protein 5
1452636_x_at	GTP binding protein 5
1460029_at	GTP binding protein 5
1427350_a_at	GTP binding protein 6 (putative)
1424519_at	GTP binding protein 7 (putative)
1418309_at	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
1449033_at	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)

Table S2, continued

1428666_at	asparaginyl-tRNA synthetase
1452866_at	asparaginyl-tRNA synthetase
1415694_at	tryptophanyl-tRNA synthetase
1437832_x_at	tryptophanyl-tRNA synthetase
1434813_x_at	tryptophanyl-tRNA synthetase
1425106_a_at	tryptophanyl-tRNA synthetase
1430111_a_at	branched chain aminotransferase 1, cytosolic
1450871_a_at	branched chain aminotransferase 1, cytosolic
1425764_a_at	branched chain aminotransferase 2, mitochondrial
1460323_at	threonyl-tRNA synthetase
1436856_x_at	threonyl-tRNA synthetase-like 1
1431125_a_at	threonyl-tRNA synthetase-like 1
1434738_at	threonyl-tRNA synthetase-like 2
1448403_at	leucyl-tRNA synthetase
1418892_at	ras homolog gene family, member J
1448594_at	WNT1 inducible signaling pathway protein 1
1448593_at	WNT1 inducible signaling pathway protein 1
1425458_a_at	growth factor receptor bound protein 10
1425457_a_at	growth factor receptor bound protein 10
1430164_a_at	growth factor receptor bound protein 10
1440935_at	Growth factor receptor bound protein 10, mRNA (cDNA clone MGC:28740 IMAGE:4481345)
1428365_a_at	protease, serine, 15
1416168_at	serine (or cysteine) peptidase inhibitor, clade F, member 1
1453724_a_at	serine (or cysteine) peptidase inhibitor, clade F, member 1
1450196_s_at	glycogen synthase 1, muscle /// glycogen synthase 3, brain
1438606_a_at	chloride intracellular channel 4 (mitochondrial)
1423393_at	chloride intracellular channel 4 (mitochondrial)
1423392_at	chloride intracellular channel 4 (mitochondrial)
1422018_at	human immunodeficiency virus type I enhancer binding protein 2
1434904_at	Human immunodeficiency virus type I enhancer binding protein 2 (Hivep2), mRNA
1444990_at	Human immunodeficiency virus type I enhancer binding protein 2 (Hivep2), mRNA

Table S2. Probe IDs of known stress response genes. Affymetrix probe ID numbers and gene names previously identified as ATF4-responsive during tunicamycin-induced ER stress in mouse embryonic fibroblasts (S*11*).

References

S1. M. A. Gavin *et al.*, *Nature* **445**, 771 (Feb 15, 2007).
S2. H. Waldner, M. Collins, V. K. Kuchroo, *J Clin Invest* **113**, 990 (Apr, 2004).
S3. M. Veldhoen, R. J. Hocking, R. A. Flavell, B. Stockinger, *Nat Immunol* **7**, 1151 (Nov, 2006).
S4. M. S. Sundrud *et al.*, *Blood* **106**, 3440 (Nov 15, 2005).
S5. I. M. Djuretic *et al.*, *Nat Immunol* **8**, 145 (Feb, 2007).
S6. Y. Kamberov, Ph.D. Dissertation, Harvard University (2008).
S7. G. J. Inman *et al.*, *Mol Pharmacol* **62**, 65 (Jul, 2002).
S8. Y. Wu *et al.*, *Cell* **126**, 375 (Jul 28, 2006).
S9. Ivanov, II *et al.*, *Cell* **126**, 1121 (Sep 22, 2006).
S10. M. Reich *et al.*, *Nat Genet* **38**, 500 (May, 2006).
S11. H. P. Harding *et al.*, *Mol Cell* **11**, 619 (Mar, 2003).